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Determination of nitrofurazone in fluid milk and dairy powders. Part 2: A full international collaborative study

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ABSTRACT

An international Collaborative Study involving 13 laboratories evaluated the performance of a method for the analysis of intact nitrofurazone that is intended as a joint ISO|IDF international standard. The study found that the method was capable of detecting intact nitrofurazone in samples of UHT-treated milk and five powdered dairy products that had been deliberately spiked with nitrofurazone at levels of ≤ 1 ng g^{-1} with good within-laboratory repeatability and between-laboratory reproducibility estimates. Nitrofurazone remained stable in UHT-treated milk for 8 weeks at ambient temperature, declining by 50% after 6 months at 30 °C. Powdered dairy products showed no significant decline during 30 °C storage. When considered with the findings of the previous Pilot Study that nitrofurazone survives heat treatment exceeding pasteurisation conditions, as well as spray drying, the joint ISO|IDF international standard method will be a reliable and robust method for determining compliance against illegal nitrofurazone use in dairy farming.

1. Introduction

In a previous article (Bendall et al., 2019), an international collaborative pilot study involving five laboratories (hereafter referred to as the "Pilot Study") that investigated the performance of a method for the analysis of fluid milk and powdered dairy products for intact nitrofurazone at low ng g^{-1} levels was described. The work was carried out as part of a joint project between the International Dairy Federation (IDF) and the International Organisation for Standardisation (ISO) to create a joint ISO|IDF standard for the determination of nitrofurazone in fluid milk and dairy products. It was necessary to develop more reliable methodology to test milk and dairy products for the illegal agricultural treatment of dairy cows with nitrofurazone, because the approach of using semicarbazide as a marker metabolite has been shown to be unreliable (Abernethy, 2015; Bendall, 2009; Stadler, Verzegnassi, Seefelder, & Racault, 2015).

The Pilot Study established that: (i) nitrofurazone is stable to heat treatments in excess of pasteurisation conditions for fluid milk; (ii) nitrofurazone is stable to conditions of spray drying; (iii) nitrofurazone remains stable for up to 25 weeks. These findings are relevant to the commercial trade of dairy products across international borders. However, the method, which was based on the work of Pearson, Evans, and Bendall (2016), had practical problems in that it required large test portions, which were physically laborious to extract, and the filtration was time consuming. This meant that, overall, the method would be unappealling to contract laboratories that perform high-throughput analyses. Hence, one of the participating laboratories in the Pilot Study modified the method based on the QuEChERS approach (CEN, 2018), which significantly improved the analysis in terms of assay time, cost and environmental impact, while matching the limit of quantification of the method of Pearson et al. (2016). Therefore, given the obvious advantages of ease of sample handling, smaller sample size requirement and potential for increased laboratory throughput, the draft international standard ISO/CD 22186 | IDF 245 was revised around this QuEChERS-based method (ISO|IDF, 2019).

European regulations, which are often looked to for guidance by other regulatory authorities, have set a "minimum required

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performance limit" (MRPL) for "nitrofuran metabolites" of 1 ng g^{-1} for poultry meat and aquaculture products (EU, 2003). The actual regulatory requirement is for a "zero" amount of metabolite (EU, 1993), and an MRPL is defined as being the "minimum content of an analyte in a sample, which at least has to be detected and confirmed" (EU, 2002). During the Pilot Study, only liquid milk had been spiked to a level of 1 ng g^{-1}; the lowest spiking level for the two powdered dairy products was 2 ng g^{-1}. Therefore, achieving the EU MRPL through the draft international standard was demonstrated only for liquid milk, and still needed to be demonstrated for powdered dairy products. Furthermore, the Pilot Study did not meet the requirements of ISO Standard 5725, Part 1, for collaborative studies, i.e., a minimum of five types of product and precision parameters commonly estimated from the data of 8–15 laboratories (ISO, 1994a).

This present article describes the performance (including estimated precision parameters, repeatability and reproducibility) of the revised draft international standard method, which was examined by a full international collaborative study (hereafter referred to as the "Collaborative Study") involving 13 laboratories and six sample types that were deliberately spiked with low and sub ng g^{-1} levels of nitrofurazone to determine whether the method could reliably measure the presence of nitrofurazone at the 1 ng g^{-1} level.

2. Materials and methods

2.1. Samples and sample preparation

Six sample types were used: ultra-high-temperature (UHT)-treated fluid milk, whole milk powder (WMP), skim milk powder (SMP), buttermilk powder (BMP), whey protein concentrate 80 (WPC) and milk protein concentrate 85 (MPC). All sample types were part of commercial production by Fonterra Co-operative Group Ltd (Auckland, New Zealand) and were collected soon after manufacture.

The UHT-treatment of the fluid milk was at 140 °C for 4 s and the milk was aseptically packaged in 250 mL Tetra Brik cartons. Each 250 mL Tetra Brik carton was deliberately spiked with a solution of nitrofurazone in ethanol added via gastight micro syringe injection through the small circle of exposed aluminium foil of the straw hole, which was then covered with heavy adhesive packing tape. The final levels of nitrofurazone in the UHT-treated samples were 0.5, 1, 2 and 5 ng g^{-1}.

For the WMP, SMP, BMP and WPC samples, a "stock spike concentrate" of each sample type was first prepared by reconstituting each powder (200 g) in water (600 mL) and adding nitrofurazone (20 μL of a 0.1 mg mL^{-1} solution in ethanol, 2 μg, 10 ng g^{-1} powder basis) via a gastight micro syringe. For the MPC samples, the "stock spike concentrate" was prepared by reconstituting MPC (200 g) in water (1 L) and adding nitrofurazone (4 μL of a 0.1 mg mL^{-1} solution in ethanol, 0.4 μg, 2 ng g^{-1} powder basis) via a gastight micro syringe. The reconstituted WMP, SMP, BMP, WPC and MPC were each passed through a laboratory-scale spray drier (Büchi Mini Spray Dryer B-290, Flawil, Switzerland) with the inlet temperature set to either 175 °C for WMP, SMP, BMP and WPC or 190 °C for MPC, the aspirator set to 100% and the flow rate adjusted to provide an outlet temperature of 70–80 °C. These redried "stock spike concentrate" powders were collected from the spray drier. For individual samples, each "stock spike concentrate" was weighed carefully, along with its respective non-spiked powder, in various proportions and with 100% weight-check inspection, to generate samples (2.5 g) with final nitrofurazone concentrations of 0.5, 1, 2 and 5 ng g^{-1} on a powder basis for the WMP, SMP, BMP and WPC samples, and 0.1, 0.2, 0.4 and 1 ng g^{-1} on a powder basis for the MPC samples. The powder samples were then packed into screw-cap vials. To avoid potential problems of non-homogeneity because the spiked and blank powders had not been thoroughly mixed, laboratories were requested to analyse the entire content of each 2.5 g vial.

Each laboratory was provided with a pack containing blind duplicate samples of each product at each spiking level, viz., eight samples of each of the six product types, as well as a matrix blank of each product type to be used for preparing matrix-matched calibration standards, making a total of 48 samples and six blanks. All samples were labelled with a code containing the prefix of the product type and a numerical suffix of 1–6 allocated randomly to the different concentration levels.

For homogeneity and stability testing, a further 12 samples of each product type (2.5 g) were prepared at a single spiking level of 5 ng g^{-1} for the WMP, SMP, BMP and WPC and of 1 ng g^{-1} for the MPC. To check the sample homogeneity, 10 samples of each type were analysed soon after their preparation in a single analytical run. For stability testing, samples of each type were analysed in duplicate after accelerated aging at 30 °C for 3 months and again in duplicate after accelerated aging at 30 °C for 6 months. These results were compared against the results from the homogeneity testing, which served as a starting point.

2.2. Method of analysis

Instructions were sent to the participating laboratories, asking them to analyse the samples by closely following the revised draft international standard ISO/CD 22186 | IDF 245, to note any difficulties and to offer suggested improvements. Laboratories were asked to report their results to three significant figures, rather than two significant figures as was written in the method, to alleviate against rounding effects during the statistical analysis. For quantification, the question that needed to be addressed was whether using an external standard curve would be sufficiently robust or whether the matrix-matched calibration curve approach would be required. To resolve that issue, the participating laboratories were asked to quantify nitrofurazone in the samples using both approaches and to provide two lists of results, which could then be statistically compared for differences.

2.2.1. Principle

The method is based on the QuEChERS protocol of EN 15662:2018 (CEN, 2018) with some modifications as described by Bendall et al. (2019). In brief, the liquid milk sample or reconstituted dairy powder sample is spiked with an isotopically labelled internal standard of $^{13}C^{15}N_2$-nitrofurazone and extracted with acetonitrile. Liquid–liquid partitioning is then performed using a mixture of magnesium sulphate and sodium chloride. After centrifugation, the resulting supernatant is cleaned by dispersive solid phase extraction using a mixture of $MgSO_4$/PSA/C18 sorbents. An aliquot of the extract is evaporated to dryness and reconstituted in methanol before liquid chromatography–tandem mass spectrometry analysis in scheduled multiple reaction monitoring mode using negative electrospray ionisation. Positive identification of nitrofurazone in the sample is conducted according to the confirmation criteria defined in EU Commission Decision 2002/657/EC (EU, 2002). Quantification can be performed either by an external calibration curve using the isotope dilution approach or from a matrix-matched calibration curve.

2.3. Statistical design and analysis

The statistical design and the analysis were based upon ISO Standard 5725, Part 2 (ISO, 1994b).

3. Results and discussion

3.1. Statistical analysis of laboratory data

3.1.1. Evaluation of the validity of the data

The Collaborative Study involved 13 laboratories from Switzerland, Singapore (2), USA (2), China (2), Germany, United Kingdom, New Zealand (2), Italy and Belgium. With the exception of two laboratories, the results of which were excluded because of equipment or instrument problems, 11 of the laboratories were able to measure nitrofurazone at trace levels in the fluid milk samples and the five powdered dairy product samples. The raw data are given (Supplementary material, Tables S1–S6), and the results from laboratory I in Fig. 1 provide an illustrative example.

The laboratories were able to provide results that responded linearly with the spiked nitrofurazone concentration. The spiking levels for the Collaborative Study covered a lower range (0.5–5 ng g^{-1} for UHT-treated milk, WMP, SMP, BMP and WPC) than that used in the earlier Pilot Study, so that the ability of the method to meet the EU MRPL of 1 ng g^{-1} could be examined

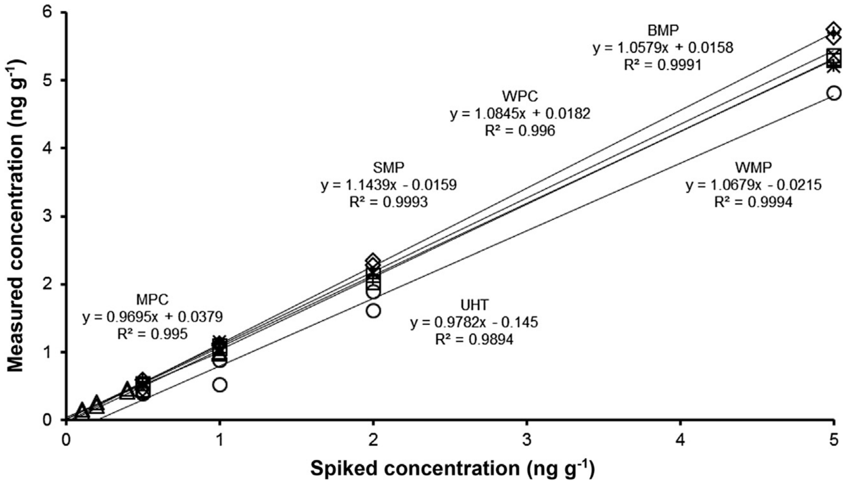

Fig. 1. Measurements of nitrofurazone in spiked samples of UHT-treated milk (○), WMP (□), SMP (◇), BMP (×), WPC (+) and MPC (△) from laboratory I as an illustrative example.

Table 1
Statistical results from Collaborative Study of nitrofurazone determination in UHT-treated milk.

Parameter	External calibration				Matrix-matched calibration			
	Spiking level (ng g^{-1})				Spiking level (ng g^{-1})			
	0.5	1	2	5	0.5	1	2	5
N (number of laboratories with valid data)	8	8	10	6	8	9	9	6
n (number of replicates)	2	2	2	2	2	2	2	2
M (average) (ng g^{-1})	0.39	0.75	1.61	3.88	0.41	0.77	1.63	4.04
s_r (within-laboratory repeatability, SD)	0.13	0.16	0.18	0.20	0.03	0.17	0.10	0.20
r (within-laboratory repeatability, 95%)	0.37	0.45	0.50	0.57	0.07	0.47	0.28	0.56
RSD$_r$ (within-laboratory repeatability, relative SD) (%)	34	21	11	5	6	22	6	5
s_R (between-laboratory reproducibility, SD)	0.13	0.16	0.20	0.45	0.04	0.17	0.12	0.56
R (between-laboratory reproducibility, 95%)	0.37	0.45	0.55	1.27	0.11	0.47	0.34	1.56
RSD$_R$ (between-laboratory reproducibility, relative SD) (%)	34	21	12	12	10	22	7	14

Table 2
Statistical results from Collaborative Study of nitrofurazone determination in WMP.

Parameter	External calibration				Matrix-matched calibration			
	Spiking level (ng g^{-1})				Spiking level (ng g^{-1})			
	0.5	1	2	5	0.5	1	2	5
N (number of laboratories with valid data)	10	10	10	10	11	10	11	11
n (number of replicates)	2	2	2	2	2	2	2	2
M (average) (ng g^{-1})	0.51	1.02	1.99	5.01	0.47	1.00	1.93	4.81
s_r (within-laboratory repeatability, SD)	0.06	0.05	0.14	0.25	0.06	0.06	0.14	0.28
r (within-laboratory repeatability, 95%)	0.16	0.15	0.39	0.71	0.16	0.15	0.39	0.79
RSD$_r$ (within-laboratory repeatability, relative SD) (%)	11	5	7	5	12	6	7	6
s_R (between-laboratory reproducibility, SD)	0.09	0.11	0.18	0.49	0.21	0.15	0.29	0.69
R (between-laboratory reproducibility, 95%)	0.26	0.30	0.50	1.38	0.58	0.42	0.81	1.92
RSD$_R$ (between-laboratory reproducibility, relative SD) (%)	18	10	9	10	44	15	15	14

Table 3
Statistical results from Collaborative Study of nitrofurazone determination in SMP.

Parameter	External calibration				Matrix-matched calibration			
	Spiking level (ng g^{-1})				Spiking level (ng g^{-1})			
	0.5	1	2	5	0.5	1	2	5
N (number of laboratories with valid data)	10	10	10	10	10	11	11	11
n (number of replicates)	2	2	2	2	2	2	2	2
M (average) (ng g^{-1})	0.53	1.00	1.92	4.79	0.43	0.94	1.96	5.02
s_r (within-laboratory repeatability, SD)	0.06	0.12	0.13	0.21	0.05	0.14	0.13	0.36
r (within-laboratory repeatability, 95%)	0.17	0.32	0.36	0.58	0.14	0.38	0.35	1.02
RSD$_r$ (within-laboratory repeatability, relative SD) (%)	11	12	7	4	11	14	6	7
s_R (between-laboratory reproducibility, SD)	10	10	10	10	10	11	11	11
R (between-laboratory reproducibility, 95%)	2	2	2	2	2	2	2	2
RSD$_R$ (between-laboratory reproducibility, relative SD) (%)	0.53	1.00	1.92	4.79	0.43	0.94	1.96	5.02

Table 4
Statistical results from Collaborative Study of nitrofurazone determination in BMP.

Parameter	External calibration				Matrix-matched calibration			
	Spiking level (ng g^{-1})				Spiking level (ng g^{-1})			
	0.5	1	2	5	0.5	1	2	5
N (number of laboratories with valid data)	10	10	10	10	11	10	10	11
n (number of replicates)	2	2	2	2	2	2	2	2
M (average) (ng g^{-1})	0.47	1.00	1.95	4.78	0.51	0.99	1.94	4.94
s_r (within-laboratory repeatability, SD)	0.04	0.07	0.11	0.37	0.05	0.08	0.14	0.43
r (within-laboratory repeatability, 95%)	0.10	0.21	0.31	1.05	0.14	0.22	0.38	1.21
RSD$_r$ (within-laboratory repeatability, relative SD) (%)	8	7	6	8	10	8	7	9
s_R (between-laboratory reproducibility, SD)	0.07	0.13	0.23	0.65	0.07	0.11	0.16	0.52
R (between-laboratory reproducibility, 95%)	0.20	0.37	0.64	1.82	0.20	0.30	0.43	1.45
RSD$_R$ (between-laboratory reproducibility, relative SD) (%)	15	13	12	14	14	11	8	10

Table 5
Statistical results from Collaborative Study of nitrofurazone determination in WPC.

Parameter	External calibration				Matrix-matched calibration			
	Spiking level (ng g^{-1})				Spiking level (ng g^{-1})			
	0.5	1	2	5	0.5	1	2	5
N (number of laboratories with valid data)	10	10	10	10	10	11	11	11
n (number of replicates)	2	2	2	2	2	2	2	2
M (average) (ng g^{-1})	0.55	1.03	1.93	5.03	0.51	0.99	1.91	5.03
s_r (within-laboratory repeatability, SD)	0.03	0.07	0.12	0.26	0.03	0.07	0.13	0.24
r (within-laboratory repeatability, 95%)	0.09	0.20	0.35	0.73	0.09	0.19	0.37	0.66
RSD$_r$ (within-laboratory repeatability, relative SD) (%)	6	7	6	5	6	7	7	5
s_R (between-laboratory reproducibility, SD)	0.08	0.16	0.19	0.51	0.12	0.17	0.19	0.42
R (between-laboratory reproducibility, 95%)	0.22	0.44	0.52	1.42	0.34	0.47	0.53	1.18
RSD$_R$ (between-laboratory reproducibility, relative SD) (%)	15	15	10	10	24	17	10	8

Table 6
Statistical results from Collaborative Study of nitrofurazone determination in MPC.

Parameter	External calibration				Matrix-matched calibration			
	Spiking level (ng g^{-1})				Spiking level (ng g^{-1})			
	0.1	0.2	0.4	1	0.1	0.2	0.4	1
N (number of laboratories with valid data)	7	8	10	10	8	8	10	11
n (number of replicates)	2	2	2	2	2	2	2	2
M (average) (ng g^{-1})	0.17	0.23	0.40	0.93	0.15	0.23	0.38	0.90
s_r (within-laboratory repeatability, SD)	0.02	0.03	0.04	0.07	0.02	0.04	0.04	0.06
r (within-laboratory repeatability, 95%)	0.06	0.09	0.12	0.19	0.06	0.11	0.11	0.17
RSD$_r$ (within-laboratory repeatability, relative SD) (%)	13	14	11	7	13	16	10	7
s_R (between-laboratory reproducibility, SD)	0.07	0.07	0.08	0.14	0.07	0.08	0.08	0.12
R (between-laboratory reproducibility, 95%)	0.19	0.21	0.21	0.40	0.21	0.21	0.23	0.35
RSD$_R$ (between-laboratory reproducibility, relative SD) (%)	40	32	19	15	49	32	21	14

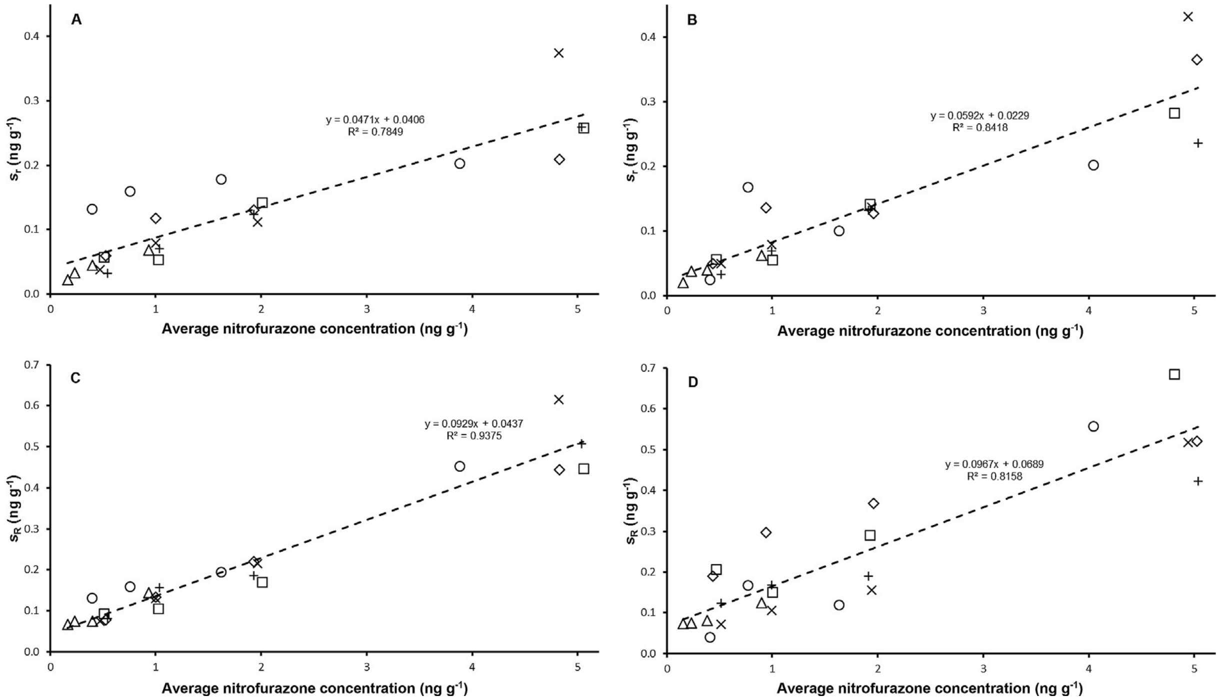

Fig. 2. Standard deviation of repeatability, s_r, versus average measured nitrofurazone concentration by (A) the external calibration approach and (B) the matrix-matched calibration approach and standard deviation of reproducibility, s_R, by (C) the external calibration approach and (D) the matrix-matched calibration approach for UHT-treated milk (○), WMP (□), SMP (◇), BMP (×), WPC (+) and MPC (△).

robustly. The most difficult sample type to work with was MPC, which has a propensity to form a sticky, viscous paste at the point at which water is added and the sample is shaken to disperse it. An additional challenge of analysing the MPC samples was that the spiking levels were in the range 0.1–1 ng g^{-1}, i.e., significantly lower than those used for the other sample types. Eight of the 11 laboratories were still able to report results for MPC at the 0.1 and 0.2 ng g^{-1} spiking levels, and all 11 laboratories reported results for the 0.4 and 1 ng g^{-1} spiking levels.

For the UHT-treated milk samples packaged in 250 mL Tetra Brik cartons, four laboratories each reported that one of their UHT-treated samples had deteriorated and curdled upon arrival. The results from those samples were excluded from the statistical analysis. Whilst care had been taken during the dosing process to use aseptic handling conditions, a few cases of compromised seal integrity had inadvertently occurred.

The participating laboratories were asked to calculate their results by two approaches: (i) by an external standard curve; (ii) by the matrix-matched calibration curve approach. In this way, any superiority of one approach over the other could be assessed. Laboratory G provided results using only the matrix-matched quantification approach, which meant that data from 10 laboratories were available for the external calibration approach.

Laboratory J reported that it obtained higher and more variable results for its WMP, SMP, WPC and MPC samples when measured by the external calibration approach because of an interfering compound for the 197 → 80 transition that it was using. It also noted that its results from the matrix-matched approach were variable with that transition, although without the upwards bias. However, by the simple step of re-measuring using a different 197 → 82 transition, its results from both the external calibration approach and the matrix-matched approach were greatly improved and the variation was diminished.

For laboratory H, some data were rejected on technical grounds because the ion ratio deviated by more than 20%.

Cochran's and Grubbs' outliers were identified and were removed from the evaluation. Cochran's and Grubbs' stragglers were identified but retained.

3.1.2. Precision of analytical method

The average nitrofurazone concentrations and other statistical parameters were calculated for each pair of blind duplicate samples, and are shown in Tables 1–6. As expected, the results between laboratories (reproducibility, R) showed greater differences than results within laboratories (repeatability, r).

The estimated standard deviation of repeatability, s_r, is shown plotted against the average measured values obtained for nitrofurazone with each spiking level and sample type, using the external calibration approach (Fig. 2A) and the matrix-matched calibration approach (Fig. 2B). The estimated standard deviation of reproducibility, s_R, is shown plotted against the average measured values obtained for nitrofurazone with each spiking level and sample type, using the external calibration approach (Fig. 2C) and the matrix-matched calibration approach (Fig. 2D).

These figures demonstrate that the external calibration approach and the matrix-matched calibration approach give similarly precise results, and that both s_r and s_R increase with the nitrofurazone concentration. Consequently, the draft international standard ISO/CD 22186 | IDF 245 treats the two approaches as equally acceptable ways to achieve calibration, and allows laboratories the choice of which approach they wish to use.

Using the linear fit to the data, the relationship can be described, irrespective of the matrices, by the equations:

$s_r = 0.047 \times$ nitrofurazone concentration by the external calibration approach

$s_r = 0.059 \times$ nitrofurazone concentration by the matrix-matched calibration approach

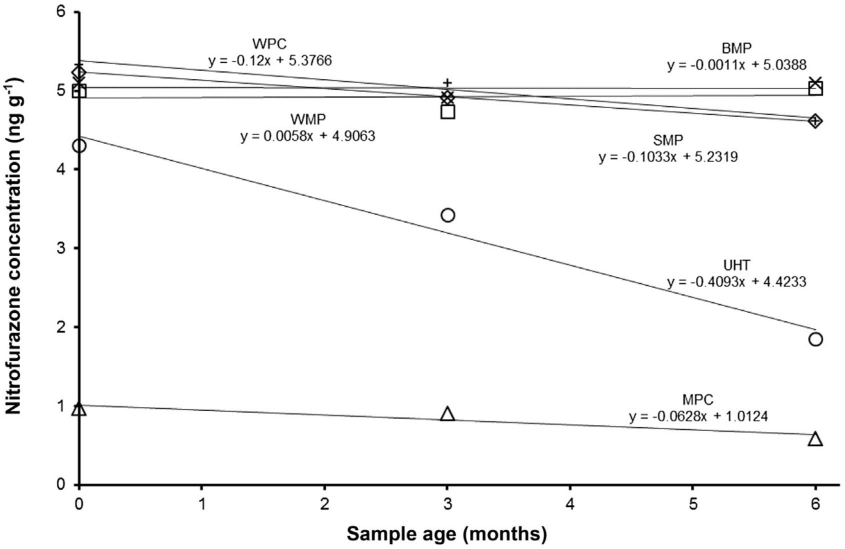

Fig. 3. Change in nitrofurazone concentration of spiked samples of UHT-treated milk (○), WMP (□), SMP (◇), BMP (×), WPC (+) and MPC (△) during accelerated storage at 30 °C.

$s_R = 0.093 \times$ nitrofurazone concentration by the external calibration approach

$s_R = 0.097 \times$ nitrofurazone concentration by the matrix-matched calibration approach

This means that, for the range 0.1–5 ng g^{-1} of nitrofurazone, it is possible to express repeatability as: "on 95% of occasions, duplicate analyses are expected to agree within 13% and 16% of their average by the external calibration approach and the matrix-matched calibration approach respectively". And reproducibility can be expressed as: "on 95% of occasions, replicate analyses of the same sample are expected to agree within 26% and 27% of their average by the external calibration approach and the matrix-matched calibration approach respectively".

The majority of the measured values for the relative standard deviation of reproducibility (RSD_R), shown in Tables 1–6, were less than half the value that was expected from the Horwitz ratio (Horwitz & Albert, 2006). This may have been due to the use of an isotopically labelled internal standard for quantification in the draft international standard ISO/CD 22186 | IDF 245, as well as to the competency of the participating laboratories.

3.2. Homogeneity

To ensure that any differences in results between the participating laboratories were due solely to inter-laboratory variation, it is necessary to check the homogeneity of the spiked samples that are used during an inter-laboratory comparison. To this end, 10 samples each of UHT-treated milk, WMP, SMP, BMP and WPC deliberately spiked with 5 ng g^{-1} of nitrofurazone and 10 samples of MPC deliberately spiked with 1 ng g^{-1} of nitrofurazone were analysed. The standard deviation between the 10 results for each sample type was then compared against the repeatability standard deviation for that sample type from the main trial. Any significant inhomogeneity would have appeared as excessive between-sample variation, which would have been shown by the calculation of *F*-tests, which, in turn, were used to calculate *p*-values for those measurements (Supplementary material, Table S7). Because none of the *p*-values were <0.05, the null hypothesis that the samples are homogeneous could not be rejected, and therefore it was concluded that the samples were homogeneous.

3.3. Stability

Four additional samples each of UHT-treated milk, WMP, SMP, BMP and WPC that were deliberately spiked with 5 ng g^{-1} of nitrofurazone and a further four samples of MPC that were deliberately spiked with 1 ng g^{-1} of nitrofurazone were prepared. These samples were subjected to accelerated storage at 30 °C. Two samples of each product type were analysed after 3 months of storage, and the remaining samples were analysed after 6 months of storage. Duplicate results were then averaged and compared against the average levels from the homogeneity testing done soon after sample preparation. For WMP and BMP, there was no apparent decline in the nitrofurazone levels. For SMP, WPC and MPC, there was a slight decline in the nitrofurazone levels. For the UHT-treated milk samples, there was a decline of 50% of the initially measured nitrofurazone levels after 6 months (Fig. 3). This is a better result than was found during the Pilot Study (Bendall et al., 2019).

As was seen during the Pilot Study, the accelerated storage experiment at 30 °C for UHT-treated milk showed a greater extent of decline than was shown by the results during the main trial, for which there were delays in shipping, with international border control and with laboratory scheduling, which unavoidably caused the samples to be kept stored at ambient temperature for prolonged periods. The participating laboratories A–K completed their analyses at times of 4, 4, 5, 6, 6, 6, 6, 6, 7, 7 and 8 weeks, respectively, after sample preparation. In contrast to the accelerated storage trial at 30 °C, the results for the UHT-treated milk spiked with nitrofurazone showed no discernible trend of a decline for any of the four spiking levels over the length of time for which each participating laboratory took to complete their analyses.

This stability of intact nitrofurazone in UHT-treated milk, without preservatives and at an elevated storage temperature for 6 months, is quite remarkable and is superior to the reported stability of semicarbazide in meat. And it was the stability of semicarbazide in meat that was the original motivation for its use as a marker metabolite for nitrofurazone. When pigs were given feed containing nitrofurazone in a medication dose of 400 μg g^{-1} for 10 days, and then slaughtered at intervals over the following 6-week period, the semicarbazide levels in the meat were found to follow an exponential decay, with a 7-day half-life in the liver and kidneys and with a 15-day half-life in muscle (Cooper et al., 2005).

3.4. Practicality of the analytical method

The revised method for the analysis of intact nitrofurazone, based on the QuEChERS approach, was considered by the participating laboratories to be faster and more practical than the previous draft method used during the Pilot Study. Importantly, it was considered to be suitable for use amongst contract laboratories that perform high-throughput analyses.

4. Conclusions

The performance of an analytical method to test for intact nitrofurazone in UHT-treated fluid milk and five types of powdered dairy products, based on the QuEChERS approach, was evaluated against several criteria by 13 laboratories in a Collaborative Study. Both the within-laboratory repeatability and the between-laboratory reproducibility were lower than the expected values from the Horwitz ratio for the majority of dairy product samples containing deliberately spiked nitrofurazone at low and sub ng g^{-1} levels. Therefore, the method can be performed by other laboratories to detect nitrofurazone when it is present at or even below 1 ng g^{-1}, being the MRPL required by EU regulations.

This study has demonstrated that, for UHT-treated fluid milk, WMP, SMP, BMP, WPC and MPC containing nitrofurazone at a 1 ng g^{-1} level, such presence can be “detected and confirmed” using this method, thus meeting the needs of the EU MRPL (EU, 2002). As a substance with a so-called “zero tolerance” under EU regulations, any detection of nitrofurazone is a compliance breach (EU, 2003); therefore, reliable detection at the 1 ng g^{-1} level is more important than accurate discernment between whether either 0.9 or 1.1 ng g^{-1} might be present in a hypothetical sample if the 1 ng g^{-1} was a maximum residue limit rather than an MRPL. Therefore, whether replicate analyses of the same sample, performed with the draft international standard ISO/CD 22186 | IDF 245, agree within 26 or 27% on 95% of occasions is of no practical significance for regulatory compliance purposes.

Additionally, this study has demonstrated that nitrofurazone remains stable in powdered dairy products for 6 months of accelerated storage at 30 °C and that, for UHT-treated milk, there is a 50% decline in nitrofurazone levels after 6 months of storage at 30 °C, but that there is no apparent decline at ambient temperatures for up to 8 weeks when the products crossed international borders such as could occur during commercial trade.

When combined with the findings from the previous Pilot Study, that nitrofurazone is stable to heat treatments in excess of pasteurisation conditions for fluid milk, and that nitrofurazone is stable to conditions of spray drying, the draft international standard ISO/CD 22186 | IDF 245 has been demonstrated to be a practical, robust and reliable method for determining the compliance status of dairy products against the illegal practice of nitrofurazone in the agricultural production of milk and dairy products. Hence, the method may now proceed to final publication as a joint international ISO|IDF standard.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2018.11.014.

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