International Dairy Journal 91 (2019) 185–192

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Determination of nitrofurazone in fluid milk and dairy powders. Part 1: An international pilot study

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ARTICLE INFO

Article history:
Received 24 September 2018
Received in revised form
20 November 2018
Accepted 29 November 2018
Available online 31 December 2018

ABSTRACT

An international pilot study involving five laboratories evaluated the performance of a method intended as a joint ISO|IDF international standard for the analysis of fluid milk and powdered dairy products for intact nitrofurazone down to levels of 1 ng g^{-1}. After first establishing that nitrofurazone remains stable to heat treatments in excess of pasteurisation conditions, fluid milk samples that were deliberately spiked with nitrofurazone at low ng g^{-1} levels were measured by the laboratories, despite the milk containing no preservatives and, in some cases, being stored at ambient temperature for several months. Commercial whole milk powder and milk protein concentrate, deliberately spiked with low ng g^{-1} levels of nitrofurazone, were also analysed by the participating laboratories. For both liquid and powdered dairy samples containing nitrofurazone, laboratories could detect and confirm the presence of nitrofurazone. The within-laboratory repeatability and between-laboratory reproducibility estimates were consistent with the Horwitz ratio.

1. Introduction

The international trade of powdered dairy products has experienced problems when border control authorities have tested for compliance against the illegal nitrofurazone treatment of dairy cows. These problems began around 2003 after the test methodology for the banned inhibitory substance changed to rely upon semicarbazide as the marker metabolite. For meat products, using semicarbazide as a marker metabolite makes some sense: (i) because the parent nitrofurazone is unstable in tissues, and any nitrofurazone residues become undetectable within an impractically short time frame (WHO, 1993); (ii) because semicarbazide remains detectable in meat (and eggs) for a period of weeks (Chu, Lopez, Abraham, El Said, & Plakas, 2008; Cooper et al., 2005; Cooper, Le, Kane, & Kennedy, 2008; McCracken, Van Rhijn, & Kennedy, 2005). However, for dairy products that are initially semicarbazide free, semicarbazide has been shown to form in liquids under conditions of temporary high pH, via the Hofmann reaction (Bendall, 2009), and to form in powders during warm storage (Gatermann, Hoenicke, & Mandix, 2004; Pearson, Evans, & Bendall, 2016), most likely via oxidation through hydrazino chemistry (Abernethy, 2015). This makes semicarbazide non-specific as a marker metabolite for milk and powdered dairy products — a point that the Codex Alimentarius Commission has noted in their risk management recommendation for nitrofurazone (Codex, 2018). Specificity is a necessary aspect of a test method's validation (EU, 2002).

As nitrofurazone is known to be carcinogenic (Bartel, Montalto de Mecca, & Castro, 2009; Hiraku et al., 2004; Morris, Price, Lalich, & Stein, 1969), many countries have prohibited its use in agriculture. However, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was unable to establish a maximum residue limit (MRL) for nitrofurazone — in part, because it could not establish an acceptable daily intake from the toxicity data, and, in part, because, at the time of its evaluation, JECFA was unable to

DOI of original article: https://doi.org/10.1016/j.idairyj.2018.11.014.
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https://doi.org/10.1016/j.idairyj.2018.11.013
0958-6946/

identify an appropriate residue in meat (WHO, 1993). Consequently, in 1993, the European Commission decided to place nitrofurazone on a "list of pharmacologically active substances for which no maximum levels can be fixed" (EU, 1993). In effect, this set a "zero limit". In 2003, the EU set Commission Decision 2003/181 with a "minimum required performance limit" (MRPL) for "nitrofuran metabolites" of 1 ng g^{-1} for poultry meat and aquaculture products (EU, 2003).

An MRPL should not be mistaken for an MRL for a regulated agricultural compound; rather, it is defined to be the "minimum content of an analyte in a sample, which at least has to be detected and confirmed" (EU, 2002). This Commission Decision followed shortly after the 2001 publication of methodology that tested meat for semicarbazide (after acid hydrolysis and derivatisation with 2-nitrobenzaldehyde) as the metabolite of nitrofurazone (Leitner, Zöllner, & Lindner, 2001). Although never set by EU regulation, many laboratories, as well as regulatory authorities in other countries, have interpreted there to be a de facto MRL of 1 ng g^{-1} for semicarbazide in all foods. However, the actual regulation is for a "zero" amount of a metabolite that is unspecified, as long as laboratories can reliably measure that metabolite to levels of 1 ng g^{-1} or less. The problems inherent in this de facto interpretation can easily be seen by a reductio ad absurdum argument: ammonium is another non-specific metabolite of nitrofurazone; because all protein-containing foods contain detectable traces of ammonium and almost all foods contain protein, it would therefore follow that all foods are prohibited by EU regulation.

The inadvertent formation of semicarbazide in dairy products during processing, or warm storage, with subsequent detection by analysis, has been interpreted incorrectly by many laboratories, and regulatory authorities outside the EU, to be a breach of EU regulations. However, in reality, they are false violative results. This is not an inconsequential problem for the international trade of dairy products. A survey of 1651 dairy products found that the proportion that contained detectable levels of semicarbazide was 35% for whey protein concentrates (WPCs) and milk protein concentrates (MPCs), 27% for buttermilk powders, 9% for caseinates and 0.5% for whole milk powders (WMPs) and skim milk powders (Stadler, Verzegnassi, Seefelder, & Racault, 2015).

Alternative marker metabolites, other than semicarbazide, have been explored. In early work, 5-nitro-2-furaldehyde was utilised as a generic marker for the total nitrofuran class of antibiotics, rather than a nitrofurazone-specific marker (Ritchie, Clear, & Solly, 1977; Ryan, Lee, Dupont, & Charbonneau, 1975). More recently, modern instrumentation has been used to identify 5-nitro-2-furaldehyde, as its 2,4-dinitrophenylhydrazine derivative, at 1 ng g^{-1} levels in seafood samples. Samples from the wild without exposure to nitrofurazone were found to contain semicarbazide that could have been treated as a false violative result were it not for the absence of 5-nitro-2-furaldehyde; and samples exposed to nitrofurazone, were confirmed as being treated through a conjunctive process in which 5-nitro-2-furaldehyde was present and the side-chain metabolites of the other major nitrofuran antibiotics were absent (Zhang, Guo, Yan, Sun, & Zhang, 2015; Zhang, Li, Yan, Long, & Zhang, 2017).

For dairy production, it has long been established that nitrofurazone will transfer intact from treated cows into their milk (Hawkins, Paar, & Cannon, 1961). And radiolabelling studies with ^{14}C-nitrofurazone have confirmed that both intramammary and intrauterine treatment of dairy cows will transfer intact nitrofurazone into a cow's milk (Smith, Paulson, & Larsen, 1998). Therefore, to create a specific and improved approach from the existing use of non-specific semicarbazide as the marker metabolite of nitrofurazone, the earlier testing approach of analysing for intact nitrofurazone in milk (Noa et al., 2002; Perez et al., 2002; Vilim & MacIntosh, 1979) was revisited. By using modern liquid chromatography–tandem mass spectrometry (LC—MS/MS) instrumentation, a limit of detection of 0.07 ng g^{-1} was achieved for milk, and a similar limit was achieved for powdered dairy products (after reconstitution), comfortably meeting the stringent EU MRPL requirement of 1 ng g^{-1} for both fluid milk and powdered dairy powders on a whole product basis (Pearson et al., 2016).

A joint project was then established by 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. Such an internationally recognised standard would help to bring resolution to the confusion being caused to international dairy trade from the use of semicarbazide by regulatory authorities. The project involved initially conducting an international collaborative pilot study with five laboratories (hereafter referred to as the "Pilot Study"), in accordance with ISO Standard 5725, Part 2 (ISO, 1994), and then conducting a subsequent full international collaborative study (hereafter referred to as the "Collaborative Study") amongst a larger number of laboratories and incorporating any improvements learnt from the Pilot Study. The results from the Pilot Study are described in this present article; and the results from the Collaborative Study are described in the following article (Bendall, Crawford, Evers, Aleksic, & Hutchinson, 2019). The purpose of these two studies was to evaluate the performance of the analytical method, rather than to evaluate the proficiency testing of the laboratories to perform a given analytical method.

The aims of the Pilot Study were fourfold: (i) provide initial precision estimates (repeatability and reproducibility); (ii) determine whether nitrofurazone in fluid milk remains stable to heating, such as might occur during pasteurisation; (iii) experimentally verify whether nitrofurazone in liquid dairy solutions withstands the spray-drying process to remain detectable in dairy powders; (iv) assess whether nitrofurazone remains stable in dairy powders over time frames that are relevant to commercial trade.

2. Materials and methods

2.1. Antibacterial activity of heated nitrofurazone solutions

The cultures used were: *Staphylococcus aureus* NZRM 917 (ATCC 25923; international antibiotic testing reference strain); *Escherichia coli* NZRM 916 (ATCC 25922; international antibiotic testing reference strain); and *Pseudomonas aeruginosa* NZRM 3007 (type strain).

In a 4 × 4 × 3 experimental trial, in triplicate, vials of sterilised trypticase soy broth (TSB) solution (4 mL) were spiked with nitrofurazone to a concentration of 12.5 μg mL^{-1}. They were then heated at four various times (0, 10, 20 and 30 min) and four various temperatures (70, 80, 90 and 100 °C). The temperature was monitored by a digital thermometer probe; typically, 10 min was required before the set temperature was reached, when the timing would start. After cooling, the vials were inoculated with three various bacteria (*S. aureus*, *E. coli* and *P. aeruginosa*) (10 μL of a 1 × 10^5 cfu mL^{-1} solution in 0.1% sterile peptone diluent, 250 cfu mL^{-1}) followed by incubation at 30 °C overnight. The vials were then visually assessed for turbidity (Lichstein & Soule, 1944).

2.2. Samples and sample preparation

Three sample types were used: fluid milk, WMP and MPC with a minimum 85% protein content (MPC85, hereafter simply referred to as "MPC"). The WMP and MPC were commercially manufactured by Fonterra Co-operative Group Ltd (Auckland, New Zealand), and the fluid milk was prepared from the WMP by reconstituting one part of powder with seven parts of water.

For each fluid milk sample, a 400 mL aliquot was put into a glass Schott bottle, the lid was sealed and the bottle and its contents were heated in an autoclave at 121 °C for 20 min. After cooling to ambient temperature, each bottle was opened under aseptic handling conditions, a solution of nitrofurazone in ethanol was added via a gastight micro syringe and then the lid was re-sealed. The final levels of nitrofurazone in the samples were 0, 1, 5, 10 and 20 ng g^{-1}.

For the WMP and MPC samples, a "stock spike concentrate" of each sample type was first prepared by reconstituting each powder (500 g) in water (1.5 L for WMP and 2.5 L for MPC) and adding nitrofurazone (500 μL of a 1 mg mL^{-1} solution in ethanol, 0.5 mg, 1 mg kg^{-1} powder basis) via a gastight micro syringe. The reconstituted WMP 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 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 re-dried powders with a high nitrofurazone spiking level 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 (40 g) with final nitrofurazone concentrations of 2, 10 and 20 ng g^{-1} on a powder basis. The powder samples were then packed into foil sachets and heat sealed. To avoid potential problems of non-homogeneity arising from the stock spike concentrate and the non-spiked powder possibly not having been thoroughly mixed, laboratories were requested to analyse the entire content of each 40 g sample sachet.

Each laboratory was provided with a pack containing blind duplicate samples of each product at each spiking level, viz., 10 samples of spiked liquid milk, six samples of spiked WMP, six samples of spiked MPC and non-spiked matrix blanks of WMP and MPC, making a total of 22 spiked samples and two non-spiked powder blanks. The matrix blank of WMP also served as a matrix blank for fluid milk after reconstitution with water. All samples were labelled with a code containing the prefix "LM" for liquid milk, "WMP" or "MPC" and a randomly generated three-digit code for the suffix.

For homogeneity and stability testing, a further 13 samples each of liquid milk (400 g), WMP (40 g) and MPC (40 g) were prepared at a single spiking level of 20 ng g^{-1}. To check the sample homogeneity, 10 samples of each type were analysed soon after their preparation — seven of which were in a single analytical run and three of which were analysed in duplicate with only the first measurement being used. For stability testing, the three samples of each type that were analysed in duplicate served as a baseline, and were compared against the results from the analysis of a further three samples of each type that were also analysed in duplicate after 3 months of accelerated aging at 30 °C.

2.3. Method of analysis

Instructions were sent to the participating laboratories, asking them to analyse the samples by closely following the draft international standard ISO/CD 22186 | IDF 245, to note any difficulties and to offer suggested improvements. The 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.

2.3.1. Principle

2.3.1.1. Proposed method. The method followed that described by Pearson et al. (2016). In brief, using Carrez reagents, proteins are first precipitated from the samples and the protein-free samples are then extracted using solid phase extraction cartridges. Sample extracts are then analysed by HPLC with MS/MS detection. Quantification is achieved using the matrix-matched calibration approach in comparison with an isotopically labelled internal standard that is spiked prior to extraction.

2.3.1.2. Modified method using the QuEChERS protocol. One laboratory modified the method using the QuEChERS protocol of EN 15662:2018 (CEN, 2018) with some alterations. The liquid milk sample (10 g) or dairy powder sample (2.5 g of powder reconstituted in 10 mL of water) was added to a 50 mL polypropylene tube, into which was added an isotopically labelled internal standard of $^{13}C^{15}N_2$-nitrofurazone (Witega, Berlin, Germany) at 2.5 ng g^{-1} equivalent-in-sample concentration. This mixture was extracted with acetonitrile (10 mL) with mechanical shaking for 10 min at 300 rev min^{-1} using a horizontal shaker and with a ceramic homogeniser added to the tube. Liquid–liquid partitioning was then performed using a QuECheERS salt mixture containing magnesium sulphate (4.0 g) and sodium chloride (1.0 g). After immediate hand shaking for a few seconds, the contents were mechanically shaken for 10 min at 300 rev min^{-1} using a horizontal shaker. The contents were centrifuged at 4000 *g* for 5 min at room temperature. Supernatant clean-up was performed by transferring the supernatant (6 mL) into a 15 mL polypropylene tube that already contained a mixture of $MgSO_4$ (1200 mg), C-18-sorbent (400 mg) and PSA-sorbent (400 mg). The contents were shaken on a horizontal shaker for 10 min at 300 rev min^{-1} and centrifuged at 4000×*g* for 5 min at room temperature. An aliquot of the supernatant (1 mL for liquid milk and 4 mL for reconstituted powder) was evaporated under a stream of nitrogen to dryness at 35 °C, and was reconstituted in methanol (250 μL), vortexed and sonicated for 1 min. The solution was then filtered through a 0.45 μm nylon syringe filter (Millex, Merck KGaA, Darmstadt, Germany) into an HPLC vial before LC–MS/MS analysis in scheduled multiple reaction monitoring mode using negative electrospray ionisation. Positive identification of nitrofurazone in the sample was conducted according to the confirmation criteria defined in European Commission Decision 2002/657/EC (EU, 2002).

2.4. Statistical design and analysis

The statistical design and the analysis for the estimation of precision parameters were based upon ISO Standard 5725, Part 2 (ISO, 1994).

3. Results and discussion

3.1. Heat sensitivity of nitrofurazone in solution

A significant challenge for the project was to find a means of transporting fluid milk samples to laboratories in multiple locations around the world, including potentially extensive delays at border control, without having the milk spoil. Whilst nitrofurazone is an inhibitory substance against bacteria above a minimum inhibitory concentration (MIC), at the trace levels to which it was spiked, the inhibitory effect of nitrofurazone would have been overwhelmed by rampant bacterial growth. Freezing samples of fluid milk can work for international shipments, but was considered to be impractical for multiple laboratories and multiple border controls. Noa et al. (2002) have shown that, with the addition of preservatives, nitrofurazone remains stable in refrigerated milk for 7 days; however, that would still have required temperature-controlled transportation, whilst adding an extra variable, and without a sufficiently long timeframe. Therefore, sterilising the fluid milk samples by high heat treatment was explored.

As a rapid means for detecting the presence/absence of therapeutic levels of nitrofurazone (μg g^{-1}), a microbiological approach of scoring growth was used. Nitrofurazone has MIC values for *S. aureus* and *E. coli* of 8 μg mL^{-1} and for *P. aeruginosa* of >128 μg mL^{-1} (Johnson, Berggren, & Conway, 1993). After an initial sense check of these literature values in both TSB and sterile reconstituted skim milk (results not shown), vials of TSB were spiked with nitrofurazone, heated for various times/temperatures, cooled, inoculated with either *S. aureus*, *E. coli* or *P. aeruginosa*, and then incubated overnight at 30 °C. As expected, for the unheated control samples, growth of both *S. aureus* and *E. coli* was inhibited by 12.5 μg mL^{-1} of nitrofurazone but growth of *P. aeruginosa* was not inhibited. For the samples heated to 70 °C, nitrofurazone remained inhibitory for 30 min of heating. For the samples heated to 80 °C, nitrofurazone remained inhibitory for ≤10 min of heating for *S. aureus* and for ≤20 min for *E. coli*. For the samples heated to 90 °C, nitrofurazone inhibition was destroyed within 10 min of heating (Table 1).

Although it was encouraging to learn that nitrofurazone in solution could withstand heat treatments well in excess of pasteurisation conditions (either 63 °C for 30 min or 72 °C for 15 s), to reliably prevent the growth of bacterial spores, far greater heat treatments are required. Therefore, a decision was made to first sterilise the fluid milk by autoclaving filled sample containers at 121 °C for 3 min, being the standard conditions for 12-*D* reduction [the time taken, at a given temperature, to achieve a 12 × 10-fold (or decimal) decrease in the number of viable organisms] of *Clostridium botulinum* spores used in the canning of low acid foods (Pflug, 1987), and to subsequently add nitrofurazone as a solution in ethanol under aseptic handling conditions.

3.2. Statistical analysis of laboratory data

3.2.1. Evaluation of the validity of the data

All five participating laboratories were able to detect the ng g^{-1} quantities of spiked nitrofurazone in all three sample types. The raw data are given (Supplementary material, Table S1) and, as an illustrative example, the results from laboratory A are shown in Fig. 1.

In general, individual laboratories gave results that were linear with the spiked nitrofurazone concentration. There was no systematic bias. Some laboratories produced results that were higher than the actual spiking level and other laboratories gave lower results. The method was not prescriptive about which ion transition should be monitored, as the optimal ion transition can vary between different instruments. Hence, that decision is best left to the operator. Two of the laboratories provided two sets of results from the transitions *m/z* 197 → 124 and *m/z* 197 → 154. When precision estimates were calculated utilising the full data set, including the *m/z* 197 → 124 transition data but excluding the *m/z* 197 → 154 transition data, or vice versa, there was negligible difference in the relative repeatability and reproducibility between the two approaches (data not shown). Because the data for the two transitions were not derived from true replicate analyses, but merely from alternative instrument readings, it would not be appropriate to retain both sets of data for the calculation of precision estimates. As the laboratories had indicated a preference for the data for the *m/z* 197 → 124 transition because of its greater instrumental sensitivity, the data for the *m/z* 197 → 154 transition were removed from the overall evaluation.

Cochran's and Grubbs' outliers were identified and were removed from the evaluation. Cochran's and Grubbs' stragglers were identified but were retained for the calculation of precision estimates.

Laboratory B provided data for the fluid milk samples but not for the powdered samples.

For laboratory D, the data for the non-spiked fluid milk samples yielded false positive results and were rejected. The data for fluid milk spiked at 20 ng g^{-1} were Cochran's outliers and were therefore removed from the analysis.

For laboratory E, the data for all spiked fluid milk samples were approximately 2–3 times the average of the other laboratories and approximately twice the known spiked levels, but its results for the powdered dairy products were similar to those of the other laboratories. Consultation with laboratory E indicated that the fluid milk samples had been analysed separately from the powdered products and that its internal standard solution had possibly been prepared incorrectly. Therefore, laboratory E's data for fluid milk samples were rejected for technical reasons.

3.2.2. Precision of analytical method

For each pair of blind duplicate samples, the mean nitrofurazone concentrations and other statistical parameters were calculated (Table 2). As expected, between-laboratory reproducibility, R, was greater than within-laboratory repeatability, r.

Fig. 2 shows the average measured values obtained for nitrofurazone, with each spiking level and sample type plotted against either the estimated standard deviation of repeatability, s_r (Fig. 2A), or the estimated standard deviation of reproducibility, s_R (Fig. 2B).

The data in Fig. 2 demonstrates that both s_r and s_R increase with the concentration. When a linear fit is applied to the data, the relationship can be described, irrespective of the matrices, by the equations:

$$s_r = 0.054 \times \text{nitrofurazone concentration}$$

$$s_R = 0.35 \times \text{nitrofurazone concentration}$$

This means that, for the range 1–20 ng g^{-1} of nitrofurazone, it is possible to express repeatability as: “on 95% of occasions, duplicate analyses are expected to agree within 15% of their average”. And reproducibility can be expressed as: “on 95% of occasions, replicate analyses of the same sample are expected to agree within 97% of their average”.

Although not formally included in ISO 5725 (ISO, 1994), the Horwitz ratio can be a helpful tool to assess whether the inter-laboratory reproducibility is reasonable. If there has been unreported averaging, the relative standard deviation of reproducibility (RSD_R) will be lower than expected; if there has been inhomogeneity of the samples, the RSD_R will be higher than

Table 1
Growth of bacteria inoculated in heat-treated nitrofurazone solutions subjected to various heat treatments.[a]

Temperature (°C)/Time (min)	Bacteria		
	P. aeruginosa	*S. aureus*	*E. coli*
TSB Control	+++	+++	+++
70/0	+++	−	−
70/10	+++	−	−
70/20	+++	−	−
70/30	+++	−	−
80/0	+++	−	−
80/10	+++	−	−
80/20	+++	+	−
80/30	+++	+++	+++
90/0	+++	−	−
90/10	+++	+++	+++
90/20	+++	+++	+++
90/30	+++	+++	+++

[a] Nitrofurazone concentration was 12.5 μg mL^{-1}; growth is expressed as: −, no growth; +, poor growth; ++, good growth; +++, heavy growth.

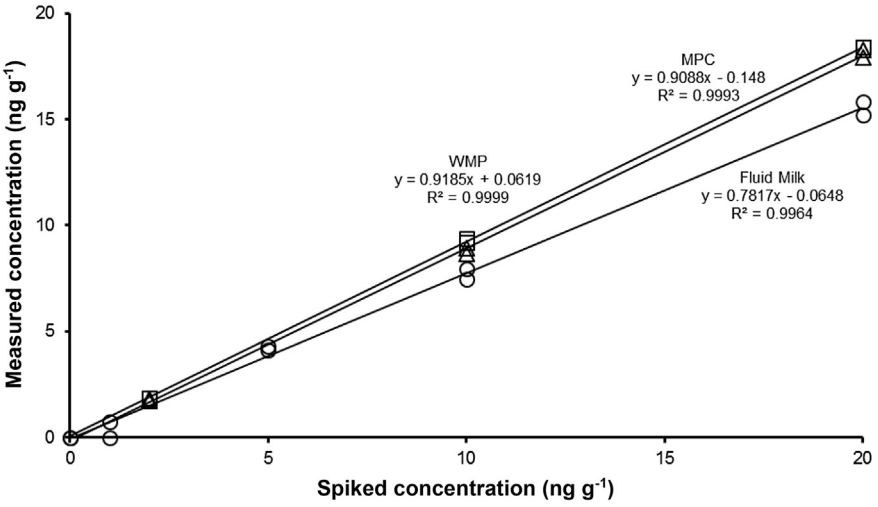

Fig. 1. Measurements of nitrofurazone in spiked samples of fluid milk (○), WMP (□) and MPC (△) from laboratory A as an illustrative example.

Table 2
Statistical results from Pilot study of nitrofurazone determination in fluid milk, WMP and MPC.

Parameter	Sample type									
	Fluid milk				WMP			MPC		
Spiking level (ng g^{-1})	1	5	10	20	2	10	20	2	10	20
N (number of laboratories with valid data)	3	4	4	3	4	4	4	4	4	4
n (number of replicates)	2	2	2	2	2	2	2	2	2	2
M (average)	0.82	4.46	8.16	16.45	2.68	11.78	23.87	2.00	10.67	20.36
s_r (within-laboratory repeatability, SD)	0.32	0.10	0.59	0.74	0.32	0.83	1.24	0.28	0.15	1.72
r (within-laboratory repeatability, 95%)	0.89	0.28	1.65	2.06	0.89	2.31	3.48	0.78	0.41	4.82
RSD_r (within-laboratory repeatability, relative SD) (%)	39	2	7	4	12	7	5	14	1	8
s_R (between-laboratory reproducibility, SD)	0.32	1.00	1.87	5.49	0.96	3.70	7.69	0.71	3.64	7.65
R (between-laboratory reproducibility, 95%)	0.89	2.79	5.23	15.38	2.70	10.36	21.53	1.98	10.19	21.41
RSD_R (between-laboratory reproducibility, relative SD) (%)	39	22	23	33	36	31	32	36	34	38

expected. The Horwitz ratio is calculated by the equation, $RSD_R = 2\ C^{-0.15}$, where C is the concentration ratio (i.e., 1 for 1000 mg g^{-1} and 0.001 for 1 mg g^{-1}) (Horwitz & Albert, 2006). For an analyte at concentrations of 1, 2, 5, 10 and 20 ng g^{-1}, the expected RSD_R values would be 45, 40, 35, 32 and 29% respectively. The measured values for RSD_R shown in Table 2 were found to be consistent with those expected from the Horwitz ratio.

3.3. Homogeneity

When evaluating the performance of an analytical method, it is important that the sample sets issued to participating laboratories are homogeneous. Ten samples of each product type, deliberately spiked with 20 ng g^{-1} of nitrofurazone, were analysed and the standard deviation between the ten results for each matrix was compared with the repeatability standard deviation for the matrix to see if there was any significant between-sample variation, which would have indicated inhomogeneity. *F*-tests were calculated and used to obtain *p*-values for these measurements (Supplementary material, Table S2). Because none of the *p*-values were <0.05, the null hypothesis that the samples are homogeneous could not be rejected.

3.4. Stability

Six samples of each product type, deliberately spiked with 20 ng g^{-1} of nitrofurazone, were analysed in duplicate. Three of the samples were analysed soon after their preparation and the remaining three samples were analysed after accelerated storage at 30 °C for 3 months. For the WMP and MPC samples, the average nitrofurazone concentrations were, for all practical purposes, unchanged after this storage. However, for the fluid milk samples, after accelerated storage, the nitrofurazone levels were only 5% of the level measured initially.

The findings from this accelerated storage experiment for fluid milk contrasted with the results from the Pilot Study, in which the samples were unavoidably kept stored at ambient temperature for prolonged periods because of: delays in shipping; delays with international border control; delays with the scheduling of laboratory time. Analysis of the samples was not completed until 3, 6, 8, 17 and 25 weeks after their preparation for laboratories A–E respectively. The fact that low ng g^{-1} levels of spiked nitrofurazone remained detectable in samples of fluid milk, without preservatives, at ambient temperature for several months, and without excessive inter-laboratory variation, is quite astonishing. The results from the accelerated storage experiment at 30 °C are therefore considered to have less relevance than the more realistic, albeit unintentional, storage results obtained from the main part of this Pilot Study. Further work on the stability of nitrofurazone in spiked dairy product samples is described in the following Collaborative Study (Bendall et al., 2019).

3.5. Practicality of the analytical method

The participating laboratories were encouraged to offer suggested improvements on the method. Most of the laboratories

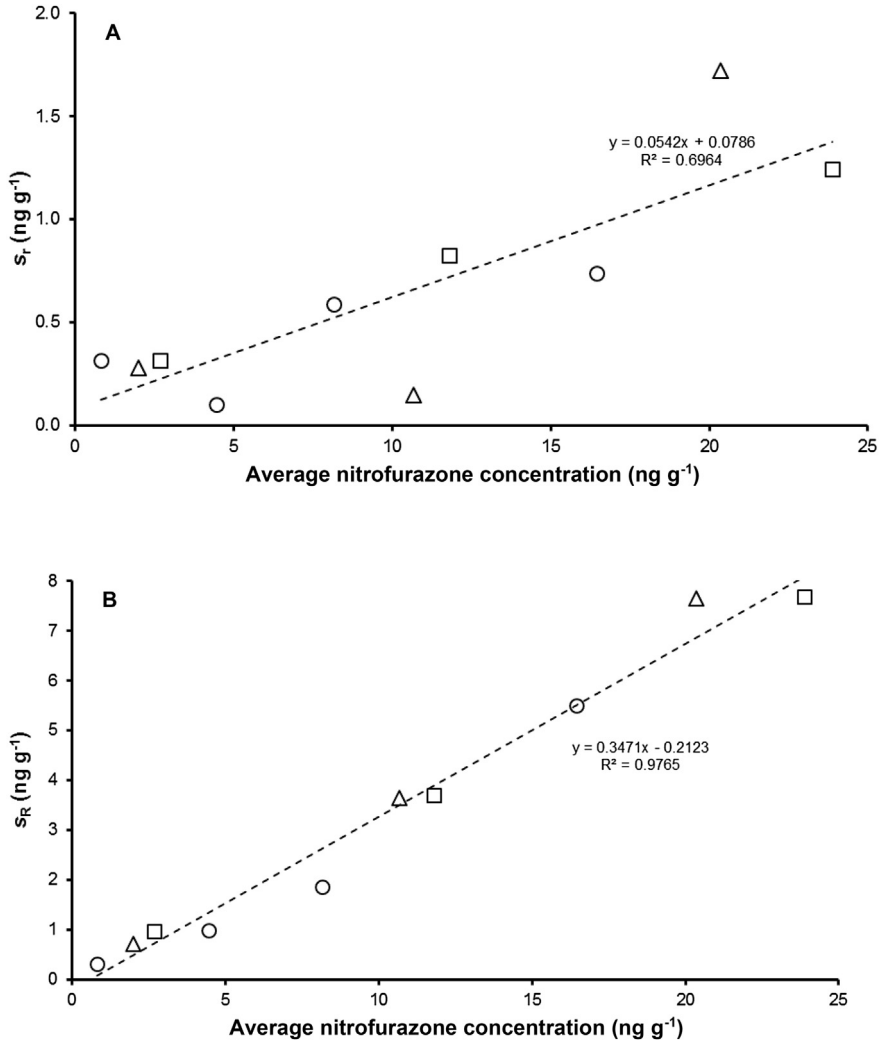

Fig. 2. Standard deviation of (A) repeatability, s_r, and (B) reproducibility, s_R, versus average measured nitrofurazone concentration for fluid milk (○), WMP (□) and MPC (△).

noted that the large sample portions and solvent volumes required by the method made it physically laborious to perform the shaking, filtration and centrifugation steps. Filtration of the deproteinated samples was time consuming, which meant that only 15 samples could be analysed in a typical 8-h workday. This was considered to be a serious limitation of the method, particularly amongst contract laboratories that aim for high-throughput analyses.

One laboratory developed a modification of the proposed method using the QuEChERS protocol (Section 2.3.1.2.). The recoveries for samples of fluid milk, WMP and whey protein concentrate 87 (WPC) spiked with nitrofurazone at levels of 1, 2, 5, 10 and 20 ng g^{-1} ranged from 90 to 109% (Supplementary material, Table S3). The results obtained by the modified method for liquid milk using the Pilot Study samples were slightly higher than those obtained with the proposed method (results not shown), but the difference was smaller than the reproducibility.

The major advantages of the modified method included it being faster (throughput 30 samples per day versus 8 samples per day), cheaper (less chemical consumables), more practical in terms of sample size (10 g versus 320 g for fluid milk and 2.5 g versus 40 g for dairy powder) and more environmentally friendly (10 mL versus 125 mL of solvent), while having the same performance as the proposed method (i.e. capable of detecting the presence of nitrofurazone at the 1 ng g^{-1} level). As a consequence of these findings, the modified method was selected to be subjected to the full international Collaborative Study to determine its precision estimates. The results of that study are described in a following article (Bendall et al., 2019).

4. Conclusions

The performance of an analytical method to test for intact nitrofurazone in fluid milk and powdered dairy products, using a draft ISO|IDF standard operating procedure based on the study of Pearson et al. (2016), was evaluated against several criteria by five laboratories in a Pilot Study. Both the within-laboratory repeatability and the between-laboratory reproducibility were acceptable for dairy product samples containing deliberately spiked nitrofurazone at low ng g^{-1} levels.

For fluid milk with a 1 ng g^{-1} nitrofurazone content, such a presence can be “detected and confirmed” using this method, thus meeting the needs of the EU MRPL (EU, 2002), even though the inter-laboratory variation will mean that different laboratories will generate a range of values. As one of the “substances for which no permitted limit has been established” (EU, 2003), any detection of nitrofurazone is a compliance breach; therefore, it matters not whether replicate analyses of the same sample will range within 68% of their average values on 95% of occasions.

For WMP and MPC samples, the lowest spiking levels were 2 ng g^{-1}, which the participating laboratories measured comfortably. This provided confidence that measurement at the 1 ng g^{-1} level was achievable for the subsequent Collaborative Study (Bendall et al., 2019).

The proposed method suffered from various practical drawbacks. A modified method using the QuEChERS protocol significantly improved the method and was chosen to be internationally studied to obtain estimates of precision parameters to finalise the draft ISO|IDF standard (ISO|IDF, 2019). These results are reported in the subsequent study (Bendall et al., 2019).

An important consideration, beyond estimating precision parameters for the method, has been the need to dispel concerns about the suitability of using intact nitrofurazone as the analyte with dairy products. Although the older literature demonstrated that nitrofurazone remains stable in pasteurised fluid milk, hitherto it was unknown whether that also applied to low ng g^{-1} levels for heat-treated fluid milks and for spray-dried dairy powders. This study has demonstrated 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 in fluid milk and spray-dried dairy powders for up to 25 weeks. This is relevant to the commercial trade of dairy products across international borders. Further work would be required to verify whether nitrofurazone remains stable for longer periods of time, such as those associated with the entire shelf life of powdered dairy products.

Acknowledgements

The authors wish to thank the following collaborators: E. Daeseleire, P. De Neve, D. Van Elst and A. Twarogowska, ILVO, Melle, Belgium; C. Felici and M. Meschiari, Neotron, Madena, Italy; B. D. Gill and J. E. Wood, Fonterra Co-operative Group Ltd., Waitoa, New Zealand; C. Evans, L. Fleury and M. Thoma, AsureQuality, Wellington, New Zealand; K. Kraehenbuehl, Nestlé Research Centre, Lausanne, Switzerland.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2018.11.013.

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