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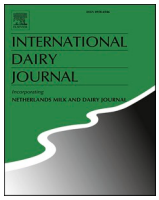

Research paper

Semicarbazide formation during processing and storage of dairy protein ingredients

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A B S T R A C T

Semicarbazide is often used as a marker metabolite for detection of illegal nitrofurazone use in agricultural products, but is not a reliable marker metabolite for analysis of nitrofurazone abuse dairy products because it can also be formed during processing and storage. To further investigate that the semicarbazide found in dairy protein ingredients is formed during processing, rather than from nitrofurazone abuse, a series of studies were conducted. Almost 500 commercial dairy protein ingredients were manufactured and analysed, and semicarbazide was detected in 22.4% of samples. Semicarbazide was detected in <10% of caseinates and >20% of all whey protein concentrate (WPC) samples analysed. Notable differences were observed between different WPC subcategories in terms of the frequency of detection and levels of semicarbazide. Higher semicarbazide levels were observed in WPC with a higher protein content or that were fat-rich. Importantly, semicarbazide was not detected in either the raw materials or intermediate products during dairy protein ingredient production but were only found in final, dried, dairy protein ingredients. This indicates that semicarbazide formation likely occurs during (spray-)drying or during storage after manufacture. Higher semicarbazide levels were found at higher storage temperature and higher water activity of the ingredients. No other nitrofuran metabolites were detected in any of the samples. The investigations presented in this report confirm previous reports that semicarbazide found in dairy ingredients is not present in raw materials but formed during processing and storage.

1. Introduction

Semicarbazide [$H_2NC(=O)NHNH_2$] is a derivative of urea, whereby one of the amino groups in urea [$H_2NC(=O)NH_2$] is replaced with a hydrazine group (-$NHNH_2$). In 1947, a series of now prohibited nitrofuran inhibitory substances was discovered (Stillman & Scott, 1947). These nitrofurans are now prohibited due to carcinogenic and neurotoxic effects. One of these nitrofurans is nitrofurazone, which is a hydrazinecarboxamide 5-nitrofuran. Nitrofurazone is an antibacterial active again both Gram-positive and Gram-negative bacteria and has been used to treat various bacterial infections in animals. Nitrofurazone was synthesised by a condensation reaction between 5-nitrofurfural and semicarbazide (Stillman & Scott, 1947). The reversal of this condensation reaction by hydrolysis to release semicarbazide, and subsequent derivatization with 2-nitrobenzaldehyde, became the basis for a widely used analytical approach to detect illegal use of nitrofurazone in meat (Leitner et al., 2001). Because residues of intact nitrofurazone are so short-lived compared to semicarbazide for nitrofurazone-treated pork meat (Cooper et al., 2005), chicken eggs (Cooper, Le, Kane, & Kennedy, 2008a, 2008b), and fish muscle (Chu et al., 2008) the use of semicarbazide as a marker metabolite became common amongst testing laboratories, including for milk; even though early work had shown intact nitrofurazone residues to remain in milk (Hawkins et al., 1961), with more recent work demonstrating that intact nitrofurazone persists

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in UHT-treated milk stored at ambient temperature for six months (Bendall, Crawford, Evers, Aleksic, & Hutchinson, 2019).

Part of the reason for the method of Leitner et al. (2001), with its reliance on semicarbazide as a marker metabolite, becoming common for all food analysis (including for milk) is that when the European Commission set a Minimum Required Performance Limit (MRPL) of 1 μg/kg for 'nitrofurazone metabolites' (without specifying the identity of the metabolites) in poultry, meat and aquaculture products in 2003 (EU, 2003), test methods that could achieve 1 μg/kg for intact nitrofurazone in milk were unpublished, being the proprietary methods of commercial laboratories. It was not until 2016 when test methodology was published describing analysis of intact nitrofurazone, as well as other nitrofuran inhibitory substances, with an analytical Limit of Detection (LoD) of 0.07 μg/kg in milk (Pearson et al., 2016). This method was further developed into an International Standard test method after multi-laboratory collaborative studies (Bendall, Crawford, Evers, Smythe, et al., 2019, Bendall, Crawford, Evers, Aleksic, & Hutchinson, 2019; ISO|IDF, 2020).

However, in 2019, Commission Regulation 2019/1871 (EU, 2019) explicitly stated that semicarbazide was to be the metabolite used for nitrofurazone, removing the previous ambiguity, and also established a Reference Point for Action (RPA) of 0.5 μg/kg in all foods of animal origin, although recognising that for crayfish there was natural occurrence of semicarbazide and that confirmation of illegal nitrofurazone use was needed before enforcing the RPA. Following the 2021 assessment by the European Food Safety Authority (EFSA) that acknowledged the natural formation of semicarbazide in a wider variety of foods during processing, including milk and whey products (EFSA, 2021), Commission Regulation 2023/411 (EU, 2023) modified the 2019 regulation by noting that natural formation of semicarbazide in several different foods, including dairy ingredients, which meant that detection of semicarbazide alone was (temporarily, for one year) insufficient to enforce the RPA and that confirmation of illegal nitrofurazone was required through means of detecting at least one other nitrofuran metabolite. (Although none of the notifications for semicarbazide in the European Rapid Alert System for Food and Feed, RASFF, indicate semicarbazide to have been detected simultaneously with a second nitrofuran metabolite.) For some of those foods, including dairy ingredients, the temporary dispensation was made permanent by Commission Regulation (2024)/2858 (EU, 2024) as a result of those food industries communicating to the European Commission the results of their investigations into the factors leading to formation of semicarbazide during food processing.

The occurrence of semicarbazide in dairy products, via means other than illegal use of nitrofurazone or from migration from sealing gaskets used in the metal lids of jars and bottles (Stadler et al., 2004), has been the subject of several previous studies. An extensive survey of >1600 dairy products and dairy ingredients samples detected semicarbazide in dried dairy protein ingredients, but not in protein-free dairy ingredients (lactose) or in dairy products that were not dried (cheese, yoghurt, butter, fresh milk; Stadler et al., 2015). The proportion of dairy product samples with semicarbazide detection varied greatly: from 35% for whey and milk protein concentrates, to 27% for sweet buttermilk powder, to <10% for caseinates, and only 0.5% in milk powders. In addition to dairy products, Stadler et al. (2015) also reported semicarbazide levels in other animal-based food products with: shellfish powder having a high proportion of samples that tested positive for semicarbazide (33%), and detections for fish powder of 25%, with average measured levels for these seafood products being higher than for dairy products.

To identify contributing factors of semicarbazide formation in dairy protein ingredients, a number of studies have been carried out. Hoenicke et al. (2004) suggested a number of pathways and precursors for semicarbazide formation in foods. Precursor compounds that are naturally present in milk include the amino acids, arginine and histidine, as well as urea; as well as hypochlorite that provides a strong oxidant that can react with these natural precursors, which can be present as a residual from its use as a sanitising agent for dairy processing equipment. Hoenicke et al. (2004) showed that appreciable levels of semicarbazide were formed when arginine and urea were treated with very high quantities of hypochlorite, but not from histidine. Building on that work, Bendall (2009) investigated the potential role of hypochlorite for semicarbazide formation under more realistic processing conditions for dairy ingredients. It was found that hypochlorite, alone, at levels which were higher than likely levels of hypochlorite contamination did not result in semicarbazide formation in milk powders; however, when liquid milk or whey with added hypochlorite was simultaneously subjected to high pH values, >10, semicarbazide formed in both milk and whey via the Hofmann reaction. While pH levels >10 do not commonly occur during dairy processing, they can occur in some specific situations, *e.g.*, during treatment with anion exchange resins for demineralization. High pH conditions alone did not result in semicarbazide formation in milk or whey (Bendall, 2009). It should be noted that these experiments found semicarbazide formation for liquid samples and, as outlined previously, semicarbazide formation is observed primarily in dried dairy protein ingredients. Spray-drying has also been shown to result in the formation semicarbazide in suspensions of red blood cells, as has freeze-drying (Cavlovic et al., 2023).

Abernethy (2015) investigated the role of oxaziridine chemistry to form a pool of hydrazine compounds in foods that can go on to form semicarbazide from urea without the need for high pH conditions (Fig. 1). Because urea is both a natural and abundant component of milk, this is a plausible route for semicarbazide formation when high pH conditions do not exist. The addition of urea to suspensions of red blood cells has been shown to increase semicarbazide formation on spray-drying and freeze-drying (Cavlovic et al., 2023). The pathway for oxaziridine chemistry requires an oxidant (*e.g.*, hydrogen peroxide, peracetic acid, atmospheric oxygen, or hypochlorite) and a Schiff base to generate hydrazino compounds via an oxaziridine intermediate, followed by reaction with a urea compound (Abernathy, 2015). This chemistry also raises the possibility that any carbamates in food contact materials could react with hydrazine to form semicarbazide, which might be relevant for the sanitisation of food contact surfaces. Furthermore, Abernethy (2015) also noted that the detection of 2-nitrobenzaldehyde semicarbazone could be entirely artefactual because acid conditions required for analysis using the Leitner et al. (2001) method are identical to the conditions needed to generate semicarbazide from a pool of hydrazine compounds in foods and the urea that is abundant in many foods. From the above, it is clear that there are different mechanisms for semicarbazide formation during processing of dairy products and further mechanisms may yet be discovered, particularly around drying processes.

As well as there being formation pathways, semicarbazide levels in foods can also decline. In the case of egg powders from chickens fed nitrofurazone, the spray drying process was found to cause 72% and 17% of the semicarbazide that was initially present in the albumin and yolk components, respectively, being destroyed, resulting in an overall loss of 51% for the whole egg. This came as a surprise to the authors who noted that the cooking process of pork only resulted in a 15% loss of semicarbazide (Cooper et al., 2008a, 2008b).

In the case of dairy protein ingredients, Pearson et al. (2016) carried out a two-year storage trial of milk protein concentrate (MPC) at various temperatures and oxygen levels. It was found that even though the MPC85 contained no detectable semicarbazide immediately after manufacture, semicarbazide did form during storage with faster initial rates of formation at higher storage temperatures, reaching a peak before subsequently declining with further storage time. Given that semicarbazide contains a reactive hydrazide functional group that is susceptible to further oxidation, it should be expected that semicarbazide is not a stable end-product (Pearson et al., 2016).

This study describes the investigations which were presented to the European Commission about parameters and factors in the

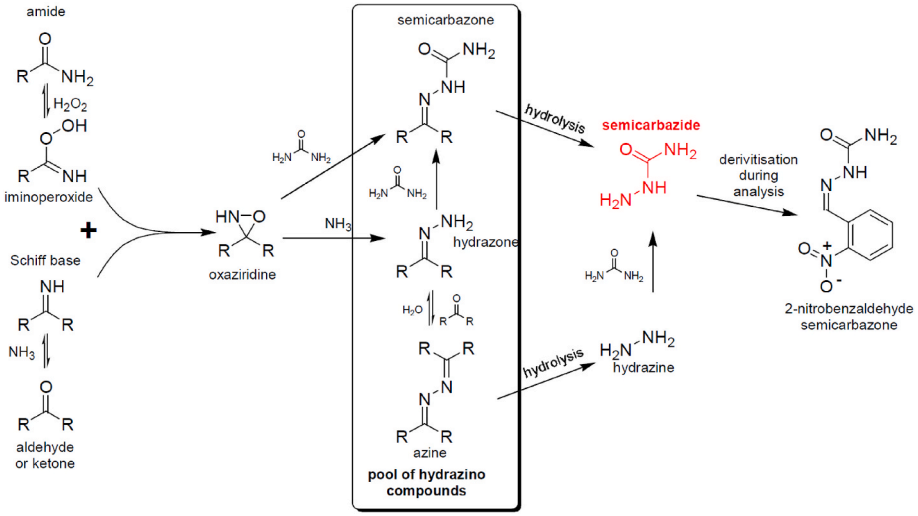

Fig. 1. Scheme for semicarbazide formation from a pool of hydrazine compounds in foods formed by oxaziridine chemistry. Adapted from Abernethy (2015).

commercially-used processing steps that result in semicarbazide formation in dairy protein ingredients. For this purpose, a large number (almost 500) of commercial dairy protein ingredients were analysed. In addition, conditions contributing to process-induced semicarbazide formation were investigating by in-process samples collected during processing, as well as finished product samples collected during storage under different conditions of temperature and water activity (A_w).

2. Materials and methods

2.1. Samples

In the current investigations, commercially produced dairy protein ingredients were manufactured by several different production sites located across European Union countries. An overview of the product classes included and analysed in the investigations is shown in Table 1. Additionally, some samples were modified in the laboratory to explore effects from different A_w. Storage trials were conducted under controlled conditions at 20, 22 or 40 °C.

2.2. Analysis

Products were analysed for the presence of semicarbazide (SEM) and the nitrofuran metabolites (3-amino-2-oxazolidinone, AOZ, from furazolidone; 5-morpholino-3-amino-2-oxazolidinone, AMOZ, from furaltadone; 1-aminohydantoin, AHD, from nitrofurantoin; and 3,5-dinitrosalicylic acid hydrazide, DNSAH, from nifursol) by liquid chromatography–tandem mass spectroscopy (LC–MS/MS) in DIN EN ISO/IEC 17025:2018 accredited laboratories. The analysis is based on the work of Leitner et al. (2001) and is described in further detail by Čavlović et al. (2023). The basic principle of the method is based on combined acid hydrolysis and derivatization with the derivatization reagent 2-Nitrobenzaldehyde, followed by neutralization and extraction with ethyl acetate, evaporation and measurement of the redissolved analytes by LC-MS/MS. A complete description is provided by Čavlović et al. (2023). The limit of quantification (LOQ) for SEM, AOZ, AMOZ, AHD and DNSAH was 0.5 µg/kg.

Table 1
Categories of milk protein ingredients investigated in this research.

Ingredients categories	Subcategories included
Whey protein concentrates	Whey protein concentrate (WPC) Fat-rich whey protein concentrate (fWPC) Whey protein isolate (WPI) Whey protein hydrolysates (hWPC)
Caseinates	Sodium caseinate (NaCas) Calcium caseinate (CaCas) Magnesium caseinate (MgCas) Potassium caseinate (KCas) Casein hydrolysates (hCas)
Milk powder	Skim milk powder (SMP)
Milk protein concentrates	Milk protein concentrate (MPC) Micellar casein isolate (MCI)

3. Results

3.1. Nitrofuran metabolites in milk protein ingredients

In monitoring programs from a number of dairy production sites based in the European Union over the period 2020–23, almost 500 dairy protein ingredients in the categories outlined in Table 1 were tested for the presence of the nitrofuran metabolites SEM, AOZ, AMOZ, and AHD. Some of those samples were additionally tested for DNSAH. As outlined in Table 2, semicarbazide was detected in 22.4% of the dairy protein ingredients analysed but AOZ, AMOZ, AHD and DNSAH were never detected in any sample. By the criteria of Commission Regulation (2023)/411, this suggests that the semicarbazide in dairy ingredients was not from illegal nitrofurazone but is instead a process-induced contaminant. This is further supported by data shown in Table 3 where, for a number of fat-rich whey protein concentrate samples, intact nitrofurazone was monitored for as a confirmatory test in addition to nitrofuran metabolites. Whilst semicarbazide was detected in these samples, neither intact nitrofurazone nor any other nitrofuran

Table 2
Nitrofuran metabolite detection frequency in milk protein ingredients (n = 486).

Nitrofuran metabolite[a]	Samples analysed	Samples negative	Samples positive	Detection frequency
SEM	486	377	109	22.4%
AOZ	486	486	0	0%
AMOZ	486	486	0	0%
AHD	486	486	0	0%
DNSAH	89	89	0	0%

[a] SEM = semicarbazide, AOZ = 3-amino-2-oxazolidinone; AMOZ = 3-amino-5-methylmorpholino-2-oxazolidinone; AHD = 1-aminohydantoin; DNSAH = 3,5-dinitrosalicyclic acid hydrazide.

Table 3
Occurrence and levels (expressed in μg/kg product) of nitrofuran metabolites and intact nitrofurazone in fat-rich whey protein concentrate (n = 3).

Component[a]	Samples tested	Samples negative n	Samples positive n		
				average	Range
SEM	3	0	3	2.6	0.7–5.9
AOZ	3	3	0	–	–
AMOZ	3	3	0	–	–
AHD	3	3	0	–	–
NFZ	3	3	0	–	–

[a] SEM = semicarbazide, AOZ = 3-amino-2-oxazolidinone; AMOZ = 3-amino-5-methylmorpholino-2-oxazolidinone; AHD = 1-aminohydantoin; NFZ = nitrofurazone.

metabolites were detected, which is consistent with the results in Table 2.

Of the dairy protein ingredient categories analysed, semicarbazide was not detected for samples in the milk powders category but was detected in the milk protein concentrate, caseinate, and whey protein concentrate categories. Semicarbazide occurrence, both in terms of frequency of observation and levels found, was highest for the whey protein concentrate category (Table 4). Although it is tempting to attribute these differences between ingredient classes to the different (dominant) protein species between the ingredients (*i.e.*, whey proteins in whey protein concentrates; caseins in milk powders, caseinates, and milk protein concentrates) it is important to realize that these ingredients also have very different production processes and that there are components other than proteins also present. Furthermore, as will be discussed below, even for products with similar protein composition, very different levels of semicarbazide were found, *e.g.*, within the category of whey protein concentrates (Table 5).

Within the category of whey protein concentrates, some distinctions between subcategories of products can be distinguished. In general, products with a higher protein content showed a higher semicarbazide content in a manner that is non-linear with protein content (Table 5). Furthermore, for WPC products of comparable protein content, higher levels of semicarbazide were typically found for the fat-rich WPC products than for products with a lower fat content (Table 5). Furthermore, in the subcategory of WPC products with >85% protein, higher levels of semicarbazide were found for products prepared using ion-exchange chromatography than for those prepared with membrane filtration (data not shown). These differences indicate, as outlined above, that it is the processing conditions that are likely to have a dominant role on the formation of semicarbazide in dairy protein ingredients.

3.2. Influence of processing steps on semicarbazide formation

Dairy protein ingredient manufacture can involve various unit operations including heat treatment, membrane filtration, and drying. Three different WPC products with a protein content in the range 70–85% were monitored on multiple occasions at various stages during manufacture. In all cases, no semicarbazide was detected in any raw materials or intermediate products, and semicarbazide was only detected in final products after spray-drying (Table 6). This indicates the strong impact of the spray-drying step on process-induced semicarbazide formation in dairy ingredients and also suggests that conditions encountered during drying can accelerate semicarbazide formation reactions. One of these conditions is the elevated temperatures encountered during drying, whereby, albeit for very brief times, the dairy material can be exposed to temperatures >80 °C. In addition, the drying process results in a decrease in the A_w of the material. The liquid dairy material entering the spray-drier will have an initial A_w close to 1.0, whereas the final dairy protein ingredient will typically have an A_w of <0.2. Whilst for processes involving biochemical reactions, such as microbial growth and enzymatic reactions, reaction rates are often higher at higher A_w, for chemical reactions in foods the highest reaction rates are typically observed at intermediate A_w levels. For instance, for non-enzymatic browning reactions, reaction rates are typically highest at A_w 0.5–0.7 (Labuza & Dugan, 1971). Whilst this is not the A_w of either the raw material or final product, it is an A_w range through which the product transitions during drying and this could impact semicarbazide formation. Another important result, shown in Table 6, are the very high levels of semicarbazide in dairy products which were spray-dried at pH 10.

Table 4
Occurrence of semicarbazide in different categories of milk protein ingredients.

Ingredient category	Samples tested	Samples negative	Samples positive	semicarbazide (μg/kg product)	
				average	Range
Caseinate	41	38	3	3.3	1.0–4.6
Milk protein concentrate	3	1	2	1.2	1.1–1.3
Whey protein concentrate	251	147	104	2.2	0.6–13

Table 5
Occurrence of semicarbazide in different categories of whey protein concentrate (WPC) ingredients.

WPC category	Samples tested	Samples negative	Samples positive	semicarbazide (μg/kg product)	
				average	Range
WPC <70% protein	75	75	0	–	–
WPC 70–85% protein	107	51	56	1.3	1.0–8.1
WPC >85% protein	19	4	15	4.0	1.0–13
Fat-rich WPC	25	8	17	4.7	0.6–13
WPC hydrolysate	25	9	16	2.7	1.2–7.1

3.3. Influence of storage on semicarbazide formation

As outlined in section 3.2, the combination of time, temperature, and A_w contribute to semicarbazide formation. Whilst high temperature conditions may only be in combination with transitory time periods during processing, during storage the lower temperatures are combined

Table 6
Occurrence of semicarbazide in raw materials, intermediate products, and final spray-dried products for three different whey protein concentrate products.

Ingredient	Product type	semicarbazide (μg/kg product)	
		average	range
WPC 70–85% protein (n = 2)	Raw material	–	–
	Intermediate product	–	–
	Spray-dried product	2.9	2.3–3.5
WPC 70–85% protein (n = 5)	Raw material	–	–
	Spray-dried Dried product	1.2	0.9–1.6
WPC 70–85% protein (n = 3)	Raw material	–	–
	Intermediate product adjusted to pH 10	–	–
	Spray-dried product	79	51–120

with long time periods that can similarly lead to semicarbazide formation. The impact of storage was previously outlined by Pearson et al. (2016) for milk protein concentrates, and there are unpublished cases where semicarbazide is not detectable immediately after production of commercially-manufactured milk protein ingredients but may only become notable during (prolonged) storage of the ingredients. To evaluate the influence of storage on semicarbazide formation in milk protein ingredients, two critical parameters were investigated, *i.e.*, storage temperature and A_w. Results for a number of WPC products stored at different conditions are shown in Table 7 and clearly indicated that next to storage temperature, which was already reported by Pearson et al. (2016), A_w also had a very strong role in the development of semicarbazide in products. In all cases, increasing storage temperature accelerated storage-induced formation of semicarbazide, as did the increase of A_w from (<)0.1 to 0.3. In most cases, the effect of A_w was notably larger than that of temperature (Table 7). Table 7 also indicates the impact of product pH, where in comparable products at pH 3.3 and 6.7, semicarbazide formation was notably less in the acidic product, even when A_w was raised to 0.3.

Table 7
Levels of semicarbazide, expressed in μg/kg product, in different whey protein concentrate stored for various times at different temperature and for powder of different water activity. All powders were spray-dried, unless specifically indicated otherwise.

Ingredient	Storage condition	Products A_w	semicarbazide (μg/kg product)
WPC >85% protein	35 days at 20 °C	<0.1	1.4
	90 days at 20 °C	<0.1	3.7
	35 days at 20 °C + 55 d at 40 °C	<0.1	5.3
	35 days at 20 °C	0.3	8.1
	90 days at 20 °C	0.3	14
	35 days at 20 °C + 55 d at 40 °C	0.3	15
Fat-rich WPC	35 days at 20 °C	<0.1	4.6
	90 days at 20 °C	<0.1	8.6
	35 days at 20 °C + 55 d at 40 °C	<0.1	19
	35 days at 20 °C	0.3	20
	90 days at 20 °C	0.3	33
	35 days at 20 °C + 55 d at 40 °C	0.3	45
Fat-rich WPC (freeze-dried)	14 days at 20 °C	<0.1	<0.5
	56 days at 20 °C	<0.1	1.0
	56 days at 40 °C	<0.1	2.3
	14 days at 20 °C	0.3	18
	56 days at 20 °C	0.3	37
	56 days at 40 °C	0.3	46
WPC >85% protein pH 3.3	14 d at −20 °C	0.1	0.8
	14 d at 22 °C	0.1	0.8
	14 d at 22 °C	0.3	1.0
WPC >85% protein pH 6.7	14 d at −20 °C	0.1	4.0
	14 d at 22 °C	0.1	4.4
	14 d at 22 °C	0.3	15
WPC 70–85% pH 7.4	14 d at −20 °C	0.1	3.8
	14 d at 22 °C	0.1	4.4
	14 d at 22 °C	0.3	22

4. Discussion

The results from the investigations presented in this report highlight a number of important aspects. First and foremost, it is apparent that whilst semicarbazide was detected in some of the dairy ingredients investigated, other nitrofuran metabolites, *i.e.*, AOZ, AMOZ, AHD, and DNSAH were not detected (Table 1), and nor was intact nitrofurazone detected (Table 2). This agrees with expectations that semicarbazide in these dairy protein ingredients is not related to the use of nitrofurans in primary production, but rather that semicarbazide is formed during processing. This is further supported by the fact that whilst semicarbazide is detected in the dried ingredients, it is not detected in either raw materials or intermediate dairy materials prior to drying (Table 6). Overall, the findings from these investigations strongly support previous suggestions that semicarbazide is either formed during processing of the dairy ingredients, or during subsequent storage of those ingredients.

It is worth noting that when monitoring for nitrofurazone abuse during dairy farming, testing of semicarbazide as a marker metabolite is no longer necessary because validated confirmatory test methodology for intact nitrofurazone is now available for this purpose (Bendall, Crawford, Evers, Smythe, et al., 2019, Bendall, Crawford, Evers, Aleksic, & Hutchinson, 2019; ISO|IDF, 2020). Unlike the case for meat products, where nitrofurazone is readily metabolized in the liver and other organs of the animal, and may not be detectable by the time that sampling occurs, the aforementioned work indicated that nitrofurazone in milk and dairy products remains stable for at least six months to allow for detection of illegal use (Bendall et al., 2019a, 2019b). Hence, the detection of intact nitrofurazone rather than the metabolite, semicarbazide, in milk is regarded as being the appropriate confirmatory test. In data shown in Tables 3 and it was indeed shown that in fat-rich WPC samples which did contain semicarbazide, no intact nitrofurazone was detected.

Semicarbazide was detected in several of the dairy ingredient categories investigated (Table 4). However, the frequency of detection and the levels of semicarbazide that were detected varied between the different ingredients. In general, a higher frequency of detection and the highest levels of semicarbazide were found in WPC ingredients compared to caseinate ingredients (Table 4). Average levels of semicarbazide detected in WPC ingredients were also higher in WPC ingredients than in caseinates or MPC ingredients (Table 4). This is in line with previous findings by Stadler et al. (2015), who reported the highest frequency of detection for semicarbazide in the (combined) category of WPC and MPC (35%; n = 118), whereas for caseinates, the frequency of detection was notably lower (9.3%; n = 204). For milk powder (skim milk and whole milk), semicarbazide was detected in only 0.5% of samples (n = 924), but for another product in the milk powder category, buttermilk powder, Stadler et al. (2015) detected semicarbazide in 27% of samples analysed (n = 75).

Interestingly, Stadler et al. (2015) did not detect semicarbazide in fresh milk (n = 104), cheese (n = 35), butter (n = 4), or yoghurt (n = 29; including yoghurt and yoghurt powder), in line with results presented above that semicarbazide is only found in dried dairy products and not in dairy products that have not been dried. This points to the importance of the drying step in semicarbazide formation in milk protein ingredients. The fact that semicarbazide formation was also not detected in yoghurt powder (Stadler et al., 2015) may be attributable to the low pH of this material (pH < 5), which is in line with data shown in Table 7 where acidified products have notably lower semicarbazide formation, whereas those dried at elevated pH have notably higher semicarbazide formation. Likewise, products dried at increased pH (pH 10; Table 6) showed even higher levels of semicarbazide. As outlined previously, this effect of pH may be related to the protonation state of the amino acid arginine, which has been implicated as important in processed-induced semicarbazide formation (Hoenicke et al., 2004). With increasing pH, the proportion of arginine residues in the neutral (free base) state, which is believed to be the reactive state, increases. High pH may also a

contributing factor to the higher levels of semicarbazide found in whey protein ingredients in which ion exchange treatment had been used compared to whey samples where no ion-exchange treatment was used. Bendall (2009) previously showed that after treatment with anion exchange resins strong increases in pH can occur. In the presence of hypochlorite, this could even lead to some semicarbazide formation prior to drying (Bendall, 2009) but data in Table 6 suggest that the high pH alone, without presence of hypochlorite, could also lead to semicarbazide formation during drying.

As outlined earlier, while it may be tempting to attribute this difference in process-induced semicarbazide formation between WPC and caseinates (Table 4) to the different dominant protein source in the ingredients, *i.e.*, whey protein *vs*. casein, this may an oversimplification. The amino acid arginine, considered to be important in processed-induced semicarbazide formation (Hoenicke et al., 2004), is present both in caseins and whey proteins at comparable levels. Furthermore, within the category of WPC, there are also notable differences of levels of process-induced formation of semicarbazide (Table 5). It is, therefore, more likely that differences in process-induced semicarbazide formation between different categories and subcategories of milk protein ingredients may also be attributable to differences in processing.

When considering the potential impact of enzymatic reactions, via their metabolites, that could occur during this holding period, several dairy-relevant enzymes are known to produce hydrogen peroxide. As an example, one enzyme to consider is xanthine oxidoreductase (XOR). XOR is able to produce hydrogen peroxide (H_2O_2) from aldehydes and some other substrates and H_2O_2 can play a role in process-induced semicarbazide formation (Abernethy, 2015) (Fig. 1). Hence, potential involvement of XOR in semicarbazide formation is worthy of further investigation. XOR is part of the milk fat globule membrane (MFGM) and for whey protein concentrate ingredients prepared with membrane filtration, comparatively high levels were observed for the subcategory of fat-rich WPC products (Mestawet et al., 2024; Gadesgaard et al., 2025). In these products, levels of MFGM material, and therefore also XOR, are notably higher than in other subcategories of WPC products. The link between MFGM material and potentially XOR activity may also be applicable to the results of Stadler et al. (2015), who showed a high frequency of observation for semicarbazide in sweet buttermilk powder, whereas for whole milk powder and skim milk powder, semicarbazide was detected in only 0.5% of >900 samples analysed. One difference between buttermilk powder and skim milk powder is the notably higher levels of MFGM material in buttermilk powder, due to the release during the churning process of cream.

As was clear from previous work by Pearson et al. (2016) on milk protein concentrates, semicarbazide not only forms during drying of products but can also form during storage of ingredients. For samples stored at 4 °C, semicarbazide was detected only after 12 months of storage, whereas at higher storage temperatures, semicarbazide was detected at earlier time points (Pearson et al., 2016). The influence of storage temperature on semicarbazide formation was confirmed in a series of investigations performed on both spray-dried as well as freeze-dried WPC products (Table 7). These investigations further highlighted the importance of A_w of the powder during storage. With increasing A_w in the range 0.1–0.3, strong increases in the level of semicarbazide formed during storage were observed, indicating a critical role of A_w. Another condensation-induced reaction pathway that also occurs with milk protein ingredients, non-enzymatic browning, has the reaction rate also increasing with increasing A_w in this range and has a maximum in the range 0.5–0.7 (Labuza & Dugan, 1971). Such effects have been related previously to a combination between increased concentrations of reactive species (compared to materials at higher A_w) and sufficient mobility of the reactive species (compared to samples at lower A_w). Interesting to note is that honey, in which semicarbazide has also been found (Crews, 2014), has a typical A_w in the 0.5–0.6 range. Finally, pH also appears to have a significant effect on SEM formation during storage at high A_w, i.e., higher pH leads to higher SEM formation.

5. Conclusions

Semicarbazide can be present in dairy protein ingredients due to either formation during processing or formation during product storage. Semicarbazide has been used by laboratories as a marker metabolite for the presence of nitrofurazone in food products, particularly for meat. However, the absence of semicarbazide in raw dairy materials and intermediate dairy materials during the production of dairy protein ingredients indicates that semicarbazide in the dried dairy protein ingredients does not derive from the illegal agricultural use of nitrofurazone. In particular, the spray-drying step of the production process of dairy protein ingredients, wherein the dairy material is temporarily exposed to a combination of high temperature and intermediate A_w, can accelerate semicarbazide formation from precursors present in those materials. Semicarbazide formation can occur by different mechanisms and reactions involving the amino side chain of the amino acid arginine may be important. Another notable contributing factor to semicarbazide formation in dairy protein ingredients is the exposure to high pH values, even if such exposure is transitory. Semicarbazide formation can also occur during storage of powdered dairy protein ingredients, and such reactions occur more rapidly and extensively at elevated temperature and in powders with an elevated A_w. Further studies identifying the exact mechanisms for semicarbazide formation in dried dairy ingredients are required and should enable limiting semicarbazide formation during production and storage of dairy ingredients via process control. Such experiment should identify key effects of pH, and time, temperature and water activity conditions during thermal processing, concentration, drying and storage in conjunction with detailed raw materials analysis. Qualitative and quantitative insights derived therefrom can form the basis for product and process optimization to minimize semicarbazide levels.

CRediT authorship contribution statement

Thom Huppertz: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Geertje van de Rijdt:** Writing – review & editing, Data curation. **Peter B. Skou:** Writing – review & editing, Data curation. **Ove N. Jensen:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Anna Kousholt:** Writing – review & editing, Data curation. **Mirka Marescot:** Writing – review & editing, Formal analysis, Data curation. **Diane Durand-Reville:** Writing – review & editing. **Nicolas Marechal:** Writing – review & editing, Formal analysis, Data curation. **Kinga Adamaszwili:** Writing – review & editing, Project administration. **Christian B. Kastrup:** Writing – review & editing, Project administration. **Justin G. Bendall:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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