In-house validation method for quantification of formaldehyde in silage and feedingstuffs with the use HPLC-DAD technique

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Abstract

An HPLC-DAD method was developed for the determination of formaldehyde in animal feed and silage. The method is based on the determination of the product of chemical reaction between formaldehyde and 2,4-dinitrophenylhydrazine. A 3 g of feed or silage were extracted with Milli-Q water with phosphoric acid and next formaldehyde was derivative with the use 2,4-dinitrophenylhydrazine in acetonitrile solution. The extract was purified with 0.45 µm syringe filters and separated on Zorbax Eclipse XDB C18 column and detection was carried out at 360 nm. Formaldehyde was eluted with a mobile phase consisting of acetonitrile/water in isocratic elution. This method provided average recoveries of 90.6% to 102.2%, with CVs of 2.6% to 6.4% for feed and from 91.3% to 108.7% with CVs of 1.1% to 4.1% for silage in the ranged of 50 to 1000 mg/kg feeds and silage. The LOD and LOQ for formaldehyde in feed and silage ranged from 1.6 to 2.6 and 2.7 to 5.7 mg/kg, respectively. The methodology was applied for the analysis of feed and silage samples collected from poultry, pigs and cows farms.

Key words: formaldehyde, feed, silage, 2,4-dinitrophenylhydrazine, validation, HPLC-DAD

Introduction

Formaldehyde (FA) (CH₂O, CAS 50-0-0) is a colourless, flammable gas that is commercially available as a 35 – 40% aqueous solution (formalin), as formol (a mixture of formaldehyde, formic acid and methanol in water) or as the precursor hexamethylenetetramine (a complex of formaldehyde with ammonium) (Claeys et al. 2009). FA is commercially produced from methanol and used as a preservative, reducing agent, and a sterilizing agent in food industry (Norliana et al. 2009). Formaldehyde is produced industrially for a large number of applications such as the production of resins that act as adhesive and binders for wood products, pulp, paper, glass wool and rock wool, plastics, paints, industrial chemicals and textile finishing. It is also used in packaging and as a disinfectant and preservative (Claeys et al. 2009). FA is authorised in European Union (EU) as a preservative in cosmetics (Environmental Protection Agency 2010). Formaldehyde is also used in hospitals, research and teaching laboratories as a sterilizing and preserving agent (Luo et al. 2001). Furthermore it is also used in vaccines
as a biocidal agent and to prevent bacterial or fungal contamination. It is a naturally occurring product of normal metabolism in many foods including fruits, vegetable, meats, fish, crustacean and dried mushrooms (Norliana et al. 2009).

Formaldehyde is classified by International Agency for Research on Cancer (IARC) into Group 1, as being carcinogenic for humans. Within the European Union formaldehyde is currently classified as a category 1b carcinogen and category 2 mutagenic. Most studies regarding the toxicity of formaldehyde relate to the inhalation of formaldehyde, which is probably the most important route of exposure (IARC 2006).

Since formaldehyde is water soluble, high reactive with biological macromolecules (formaldehyde induces DNA-protein and protein-protein cross-link), and rapidly metabolised, the effects of exposure are mainly observed in those tissues or organs which come into first contact with formaldehyde, namely the respiratory and gastrointestinal tracts, oral and gastrointestinal mucosa included (erosion, ulceration, inflammation and hyperplasia of stomach were observed in rats) (IARC 2006, Schulte et al. 2006). There is no evidence that formaldehyde is carcinogenic by the oral route. Formaldehyde causes toxicity to the nasal epithelium of rats and mice upon inhalation. Epidemiological data have shown that formaldehyde is cancerogenic in human by the inhalation route (sinonasal and nasopharyngeal cancers) (EFSA 2006).

An oral reference dose (RfD) for formaldehyde of 0.2 mg/kg body weight per day has been set by the US Environmental Protection Agency (1999), based on chronic exposure to free formaldehyde in drinking water studies. A tolerable daily intake (TDI) of 0.15 mg/kg body weight per day has also been set for formaldehyde by the World Health Organization. Symptoms of acute toxicity after ingestion include systemic acidosis and gastrointestinal bleeding (WHO 2002).

For some time in the European Union there has been a debate on the extension of the scope of registration of formaldehyde as a feed additive to compound feed belonging to the category of “technological additives” and the functional group “preservatives”. The use of formaldehyde as a feed additive was limited to silage in order to stop fermentation processes and skimmed milk for piglets (Xu et al. 2011). In skimmed milk, formaldehyde could be used to feed piglets up to 6 months at the maximum authorized dose of 600 mg/kg, for silage has not set maximum dose for formaldehyde. The proposal to authorize formaldehyde as a feed additive for all animal species has been widely discussed by EU member states. This was the result of the described side effects on humans as well as the dose of formaldehyde proposed by the applicants in feed, which would be from a minimum of 200 to a maximum of 1000 mg/kg of complete feed. Scientific opinions issued by the European Food Safety Authority (EFSA) in 2014 indicated that doses of 470 mg formaldehyde/kg feed are safe for chicken broilers, laying hens and Japanese quails, and 630 mg formaldehyde/kg feed is safe for piglets but no safe level can be determined for all species and categories of animals, including all species of poultry and pigs. In addition, the concentration of formaldehyde, which would be safe for the reproduction of target species, can not be determined on the basis of available studies. When formaldehyde is administered as a feed additive, similar side effects are seen in animals as in people exposed to this substance. In a study conducted in poultry, it was found that the administration of formaldehyde to broilers did not affect feed intake, but focal necrosis and blood effusions in the gut were observed. Necrotic and ulcerative abomasum changes were also observed in calves fed with formaldehyde-containing milk. Moreover, the use of formaldehyde in cattle feed can move the release of formaldehyde with milk, which can be a risk to human health. In animals under chronic exposure, inflammatory and reactive lesions in the nasal cavity were found. The desire to market formaldehyde as a feed additive stems from its high biocidal efficacy compared to Salmonella. On 7th February 2018, Commission Implementing Regulation (EU) 2018/183 issued refusal to authorize the use of formaldehyde as a feed additive belonging to the functional groups “preservatives” and “hygiene improving substances”.

The official method for the determination of formaldehyde in foodstuffs is based on a colorimetric reaction where sample distillates are mixed with sulphuric acid yielding a purple colour if formaldehyde is present. The intensity of the colour is proportional to formaldehyde concentration and can be measured by ultraviolet (UV) spectrophotometer (AOAC 1931). Titrations and acetyloholine have also been used to detect and quantify relatively the presence of formaldehyde in food (European Pharmacopoeia 6.0). For the detection and determination of formaldehyde in food, water and air spectrofluorimetry are also used (Yilmaz et al. 1992, EFSA 2006, Weng et al. 2009, European Pharmacopoeia 6.0), isotope dilution mass spectrometry (MS) (Sakuragawa et al. 1999), liquid chromatography-mass spectrometry (LC-MS) (Schulte et al. 2006, You et al. 2009), gas chromatography (GC) (Kim and Kim 2005), GC-MS (Bianchi et al. 2007) and high performance liquid chromatography (HPLC) (Zegota 1999, Heyden et al. 2002, Oliva-Teles et al. 2002, Iqbal and Novalin 2009). In recent years HPLC is broadly applied to the separation of formaldehyde from possible interferents.
and improvement of the detection limit (Jones et al. 1999, Chen et al. 2008). Formaldehyde can react with 2,4-dinitrophenylhydrazine (2,4-DNPH) to form the corresponding hydrazone before HPLC analysis (Liu et al. 2005, Chen et al. 2008). In this work, for the first time liquid chromatography with diode array detector after precolumn derivatization with 2,4-dinitrophenylhydrazine are used for detection and quantification of formaldehyde in feed and silage. This paper reports the development of a selective and sensitive method for analysing formaldehyde from feed and silage using a liquid-liquid extraction, 2,4-dinitrophenylhydrazine derivatization and high performance liquid chromatography with diode array detection (HPLC-DAD) analysis.

Materials and Methods

Chemicals and reagents

Formaldehyde solution in water (37% v/v) was obtained from Sigma Aldrich (St. Louis, USA), 2,4-dinitrophenylhydrazine was from Spectrum Chemical (New Brunswick, USA). HPLC-grade acetonitrile was from Baker (Deventer, The Netherlands) and phosphoric acid 85% was purchased from POCH (Gliwice, Poland). Water was deionised (>18 MΩ cm⁻¹) by a Milli-Q water purification system (MO, USA).

Instrumentation

For sample preparation, vortex mixer (Select Bio- Products, N.J, USA), magnetic stirrer (IKA, Germany), ultrasonic bath (Bandelin, Germany) and laboratory centrifuge (Sigma, Germany) were used. The chromatographic system consisted of HP 1100 series HPLC from Agilent Technologies (USA) equipped with automatic injector, degasser system, quaternary pump with four solvent channels, column thermostat, and diode array detector.

Chromatography

The separation of the formaldehyde was performed on an Agilent Eclipse XDB C18 (150 × 4.6 mm, 5 μm) column (Agilent Technologies, MO, USA) protected by a RP18 guard column (4.0 × 3.0 mm, 5 μm) from Phenomenex (USA), using a mobile phase consisting of HPLC-grade acetonitrile and water (70:30 v/v) mixture prepared in one glass bottle. The flow rate was 0.45 mL/min and the column thermostat was set at 30°C. The injection volume was 5 μL. The UV detection was monitored at 360 nm.

Validation procedure

The proposed HPLC-DAD method was in-house validated. Linearity and working range were calculated by preparing five series of matrix-matched calibration curves (for silage and feed). The matrix-matched calibration curve was prepared by spiking the samples (at the beginning of sample preparation) with different volumes of the standard solution to obtain an appropriate final concentration of formaldehyde (50, 200, 600, 1000 and 1500 mg/kg). The curve was prepared with every batch of samples and the concentrations of the analytes were calculated. Correlation coefficient values in this concentration range were >0.99 for formaldehyde in feed and silage.

Limit of detection (LOD) and limit of quantification (LOQ) values were the concentrations in a matrix-matched samples which gave a signal-to-noise ratio higher than three and LOQ concentration which gave a signal-to-noise ratio higher to ten of blank feed and silage samples spiked with formaldehyde. In the accuracy and precision study, feed and silage samples were spiked with formaldehyde at three different levels (50, 200, and 1000 mg/kg).

In the selectivity/specificity study, 20 blank feed
and 20 blank silage samples were analysed. For the repeatability study, three series of feed and silage samples were analysed under the identical conditions (six samples for each spiking level). Standard deviations (SD) and coefficients of variation (CV, %) were calculated for each level. The within-laboratory reproducibility was obtained by the analysis of two additional series (at three levels of concentration) in reproducibility conditions (another technician, on two different days) of feed and silage and overall SD and CV were calculated. The overall mean concentrations obtained in the reproducibility study were used to calculate accuracy (%). The recoveries were evaluated by comparing the measured concentrations with the fortified concentrations of the samples.

The uncertainty (U) was calculated as the ratio of coverage factor (k = 2) and standard deviation (SD) of within-laboratory reproducibility, and expressed in percent.

\[ U = k \times \text{SD within-laboratory reproducibility} \]

**Sample collection**

Feed and silage samples were provided to the laboratory by inspectors from pig and poultry farms and feed producers and were collected from all over Poland. The samples were taken as a part of the national feed monitoring programme. During 2018–2019, a total of 97 samples of feed and silage were analysed.

**Results**

The developed procedure was designed to elaborate a qualitative and quantitative method of determination of formaldehyde in feed and silage. In this work liquid chromatographic conditions were optimised to improve separation, sensitivity and selectivity of the formaldehyde. A two different mobile phases were investigated including methanol and water and acetonitrile and water. For the separation of formaldehyde the most popular are C18 chromatographic columns such as Hypersil ODS C18 (Rivero and Topiwala 2004), XDB C18, Luna C18 (Wahed et al. 2016), Supelcosil C18 (Claeys et al. 2009), Zorbax StableBond SB-C18 (Luo et al. 2001) or Zorbax Bonus-RP-C18 (Weng et al. 2009). In our work two different C18 chromatographic columns (Zorbax Eclipse XDB and Thermo BDS C18) were tested. The best results were achieved using acetonitrile and water 70:30 v/v, using an isocratic elution and Zorbax Eclipse XDB C18 chromatographic column. Under selected conditions, FA displayed high UV absorption at 360 nm, while no interference of the matrix was observed (Figs. 1, 3).

In the presented work different extraction methods for FA in feed and silage were tested. In a first approach method of extraction was tested described by Wahed et al. (2016). Formaldehyde was extracted with the use of acetonitrile and samples were put in an ultrasonic bath for 30 min. A second option was the use of a mixture of phosphoric acid in water and samples were placed in heated magnetic stirrer. In this work effect of extraction time was examined for a range from...
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10 to 40 min. The HCHO recovery increased from 65.0% to 100.0% with the increasing of the extraction time from 10 to 20 min. The recovery did not significant change between 20 to 40 min. So the extraction time of 20 min was chosen in the study.

Due to the lack of certified reference material for analyte under investigation, the accuracy and precision assay of the method in terms of repeatability (CV %, intra-day precision), reproducibility (CV % inter-day precision) and uncertainty were determined using blank feed and silage samples fortified with know amounts of the analyte. Feed and silage were fortified with concentrations of 50 to 1500 mg/kg of FA. Correlation coefficient (R) values in this concentration range were 0.999 for FA in feed and silage. The accuracy of the method and repeatability were evaluated by analyzing formaldehyde spiked feeds at levels of 50, 200, and 1000 mg/kg feed (six replicates for each level). The results (Table 1) show that the assay recovery for feed and silage were in the range of 90.6% to 102.2% and 88.9% to 108.7%, respectively and CVs were less than 9.0% for both matrices. The reproducibility was...
established by analyzing another sets of samples spiked at the same concentration levels as for the repeatability. Specificity is the ability of a method to distinguish between the analyte and other substances that may be present in a sample. In the evaluation of the specificity, blank feed and silage was analyzed by the established protocol. The results obtained with blank samples were compared with fortified samples. No interfering peaks were observed.

The limits of detection and quantification for the formaldehyde in feed and silage were from 1.6 to 2.7 and 2.6 to 5.2 mg/kg, respectively. The expanded uncertainty, obtained by multiplying the combined uncertainty by the coverage factor of $k=2$, was found to be 17.4% for feed and 18.4% for silage. Parameters obtained during the validation of the method are presented in Table 1. The method was shown to be appropriate for the identification and quantification of formaldehyde with acceptable accuracy and precision.

### Discussion

This work was aimed to develop a reversed-phase HPLC method for the detection of FA from feed and silage. To ensure the safety of food and feed, efficient methods are required for the simultaneous monitoring of presence of prohibited substances in feed and silage. In the literature there are not described methods of detection of formaldehyde in the feed and silage although formaldehyde was authorized for use in silage and skim milk for food producing animals as a preservative.

Chromatographic techniques such as LC or GC are used for the detection and determination of formaldehyde in various matrices such as: water, air, fish tissue, shrimp, animal tissue, blood and beer, fruit and vegetables (Luo et al. 2001, Rivero and Topiwala 2004, Weng et al. 2009, Afkhami and Begheri 2012, Wahed et al. 2016, Borges de Freitas Rezende et al. 2017). For chromatographic detection, the researchers used gas chromatography with mass spectrometry (Bianchiet et al. 2007), mass spectrometry with isotope dilution.
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(Sakuragawa et al. 1999) and liquid chromatography-mass spectrometry (Rivero and Topiwala 2004), but the most often use liquid chromatography with an ultraviolet detector (Zegota 1999, Claeyts et al. 2009, Wahed et al. 2016, Borges de Freitas Rezende et al. 2017). The most popular for formaldehyde separation are C18 chromatographic columns, such as Hypersil ODS C18 (Rivero and Topiwala 2004), XDB C18, Luna C18 (Wahed et al. 2016), Supelcosil C18 (Claeyts et al. 2009), Zorbax StableBond SB-C18 (Luo et al. 2004) or Zorbax Bonus-RP-C18 (Weng et al. 2009). As a mobile phases for the analysis of formaldehyde from various matrices, researchers typically use water or acetic acid in water in combination with methanol or acetonitrile. Weng et al. (2009) used acetonitrile and 0.5% acetic acid in water (60:40, v/v) and isocratic elution to separate the formaldehyde from beer samples. Wahed et al. (2016) developed a method for analyzing FA in mango, milk and fish. They used acetonitrile for extraction and 2,4-DNPH for derivatization, next, samples were incubated at 40°C for 60 min. They used HPLC with a diode array detector. The wavelength was set to 355 nm and the separation was achieved using an isocratic elution with water/methanol mixture (35:65, v/v). In our work, the experimental results showed that a mixture of acetonitrile and water (70:30, v/v), and isocratic elution with the use Zorbax Eclipse C18 chromatographic column gave the best results.

Sample preparation is a critical step in determining the active ingredient in feed and silage. To develop an appropriate method for formaldehyde, it is important to isolate the analyte and to minimize co-extracted interfering compounds. There are many analytical methods available in the literature for the extraction of this compound from biological matrices, e.g. in milk, fish, shrimp, fruit, vegetable, water or air samples (Rivero and Topiwala 2004, Wahed et al. 2006, Afkhani and Begheri 2012, Borges de Freitas Rezende et al. 2017). Researchers use various solutions to extract formaldehyde, such as methanol, acetonitrile, acidified methanol, or acidified acetonitrile. Xu et al. (2011) used acetonitrile and microwave extraction for the extraction of formaldehyde from beer, cola, apple, orange and peach juices. Borgas de Freitas Rezende et al. used 1M sulfuric acid for analyzing formaldehyde in milk sample (Borges de Freitas Rezende et al. 2017). Wahed et al. used acetonitrile and ultrasonic extraction for extraction of FA from fruit and fish samples (Wahed et al. 2016). On the other hand, Bianchi et al. used solid phase micro-extraction and in situ derivatization with pentafluorobenzyl-hydroxyamine hydrochloride for extraction of formaldehyde from fish samples (Bianchi et al. 2007).

For the determination of formaldehyde 2,4-DNPH derivative reagent before the analysis is often used. 2,4-DNPH reacting with formaldehyde to form the corresponding hydrazone (Chen et al. 2008). The 2,4-DNPH technique has been used in food analysis as well as to determine the aldehyde content in polluted air. We chose 2,4-DNPH for the derivatization of formaldehyde because its high selectivity in combination with HPLC indicates that it could be a promising method for determining low concentrations of formaldehyde in feed and silage. In addition, heating of the samples was used during the formaldehyde derivatization step with DNPH. In the developed method, the temperature of 80°C was assumed as the optimal temperature for the derivatization of formaldehyde to hydrazone and the derivatization time was set at 30 minutes. The use of phosphoric acid in water and 2,4-DNPH in acetonitrile allows to obtain high recoveries of FA without an additional purification step. The combination of the extraction method and the use of a magnetic stirrer heated at 80°C allows obtaining clear chromatographic images indicating the effectiveness of the procedure. The greatest advantage of the proposed method is a simple and robust procedure that saves time and is economical for the analysis of feed and silage samples. Figures 1, 2, 3, 4 show chromatograms of blank and spiked feed and silage samples at the concentration 200 mg/kg.

In 2018 and 2019, a total of 97 samples were analyzed (9 samples of alfalfa, corn and grass silage, wet beet root pulp and 88 samples of feed for broiler chickens, laying hens, chickens for fattening, turkeys, geese, pigs and dairy cows). Our study showed that only 1 feed sample for dairy cows was contaminated with formaldehyde. The formaldehyde concentration in this sample was 59.8 mg/kg. Our research showed that feed and silage produced in Poland did not contain formaldehyde. Good production practice and good hygiene practice allow for a low level of contamination of feed and silage with Salmonella, therefore formaldehyde is not used as a preservative in feed and silage in Poland.

The developed procedure is simple, fast and inexpensive, and the obtained validation results indicate that it can be used for the quantification of formaldehyde in feed and silage for control purposes. The presented HPLC-DAD method is efficient, precise and suitable for routine analyzes. The presented results confirmed the suitability of the method for testing formaldehyde in samples of feed and silage.
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