Journal of Chromatography A, 1373 (2014) 106–113

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Validation of a rapid method of analysis using ultrahigh-performance liquid chromatography - tandem mass spectrometry for nitrogen-rich adulterants in nutritional food ingredients

Jon Draher, Vickie Pound, Todime M. Reddy*

Abbott Laboratories, 3300 Stelzer Road, Columbus, Ohio 43219, United States

ARTICLE INFO

Article history:
Received 24 July 2014
Received in revised form 6 November 2014
Accepted 8 November 2014
Available online 15 November 2014

Keywords:
Economic adulteration
Liquid chromatography–mass spectrometry
Milk
Soy
Stable isotope labeled internal standard
Protein

ABSTRACT

A method for the rapid quantification of 9 potential nitrogen-rich economic adulterants (dicyandiamide, urea, biuret, cyromazine, amidinourea, ammeline, amidinourea, melamine, and cyanuric acid) in five milk and soy derived nutritional ingredients, i.e. whole milk powder, nonfat dry milk, milk protein concentrate, sodium caseinate, and soy protein isolate has been developed and validated for routine use. The samples were diluted tenfold with water followed by treatment with 2% formic acid and acetonitrile to precipitate proteins. Sample extracts were analyzed using hydrophilic interaction chromatography and tandem mass spectrometry (HILIC-MS/MS) under both positive and negative modes. Stable isotope labeled internal standards were used to ensure accurate quantification. In multi-day validation experiments, the average accuracies, relative standard deviations (RSD), and method detection limits (MDL) for all analytes in whole milk powder were 82–101%, 6–13%, and 0.1 mg/kg–7 mg/kg, respectively. The retention times of the analytes in matrix spiked controls were within ±0.06 min of the average retention times of the corresponding analytes in calibration standards. The validated method was proven to be rugged for routine use to quantify the presence of 9 nitrogen-rich compounds in milk and soy derived ingredients and to provide a defense from economically motivated adulteration.

1. Introduction

The economic adulteration of milk and infant formula products in China with melamine and cyanuric acid resulted in the sickening and deaths of infants fed with adulterated milk powder and received wide spread global attention during the melamine scandal in 2007 [1]. The 2007 melamine incident was preceded by pet foods adulteration with melamine and cyanuric acid which resulted in renal failure and deaths of cats and dogs [2]. The intentional adulteration of food ingredients with nitrogen-rich compounds has created lasting ramifications for food safety, regulations, and global commerce [3].

Economic adulteration of nutritional ingredients involves mixing with nitrogen rich compounds for economic gains, which in turn causes an interference with the widely used Kjeldahl protein assay [4,5]. The Kjeldahl method measures total nitrogen as an indirect measure of protein content [6]. The lack of specificity of the Kjeldahl method makes it susceptible to tampering by the addition of nitrogen rich compounds to inflate the apparent protein content.

Today a heightened awareness of the melamine issue, the availability of reliable analytical test methods and increased regulations, collectively decrease the risk of repeat occurrence of melamine–cyanuric acid incidents [7–9]. However motivation of economic adulteration still remains despite the above advances and the potential use of melamine related analogs for adulteration provides opportunity. Hence the continuous development of new analytical screening methodologies which are capable of detecting the presence of externally introduced nitrogen-rich compounds in food ingredients is needed to ensure the safety of ingredients and finished products.

Milk derived ingredients are major components of infant and adult nutritional products. Milk is traded globally mainly in the form of powdered ingredients because of its ease of transport [10]. The breadth and complexity of growing global trade involves supply chains which span various continents and milk derived ingredients being sourced from different regions of the world. Globally sourced ingredients are used to manufacture nutritional products which are in turn sold around the world. Hence rapid determination of residues of nitrogen rich melamine analogs in

* Corresponding author. Tel.: +1 614 6246179; fax: +1 614 7276179.
E-mail address: todime.reddy@abbott.com (T.M. Reddy).

http://dx.doi.org/10.1016/j.chroma.2014.11.019
0021-9673/

powdered ingredients derived from milk is of paramount importance to the nutritional products industry to ensure ingredients safety. Soy derived protein is a major plant derived protein used in the manufacturing of nutritional products and hence warrants protection from economic adulteration.

For a nitrogen rich compound to become a potential economic adulterant, it has to meet a number of criteria which are as follows: odorless, colorless, tasteless, absence of acute toxicity, and ready commercial availability at economically attractive prices. Based on these criteria the Canadian Food Inspection Agency drafted a list of other potential economic adulterants *viz*., dicyandiamide, urea, biuret, triuret, cyromazine, and amidinourea [5]. Other studies have identified two additional potential economic adulterants *viz*., ammeline and amidinourea, in addition to the two well-known compounds, melamine and cyanuric acid [11].

The aforementioned economic adulterants are widely used in agriculture as fertilizers and as industrial chemicals [12]. Urea is a commonly used fertilizer which can contain traces of biuret and triuret. Amidinourea is also used as a fertilizer. Dicyandiamide is used as nitrification inhibitor as well as in the production of melamine. Cyromazine is used as an insect growth regulator and its breakdown product is melamine [5,13]. Melamine and cyanuric acid are widely used industrial chemicals in the production of plastics and disinfectants. Ammeline and ammelide are known metabolites of melamine and are often found as impurities in melamine and cyanuric acid feed stocks [11].

The incident of contamination of milk products with trace levels of dicyandiamide from New Zealand was reported in 2013 [14]. Dicyandiamide is approved for agricultural use in New Zealand where it is primarily sprayed on cattle grazing pastures as a nitrification inhibitor.

The dicyandiamide incident was yet another reminder of the need to remain continuously vigilant regarding the potential for the adulteration/contamination of milk and plant derived ingredients with nitrogen-rich compounds because of their wide spread availability and use.

The concentrations of economic adulterants in milk are typically expected to be in parts per million (mg/kg) range. For instance, to achieve a gain of 1–10% nitrogen (0.035–0.350% apparent increased protein in milk), an addition of melamine in the range of 82–820 mg/kg is required. However the method detection limits required to detect incidences of economic adulteration need to be in the low parts per billions (ppb) range. This is necessary because milk may be pooled from various suppliers and hence the original adulteration levels of economic adulterants may under further dilution. It is also possible to achieve economic adulteration using a combination of adulterants which are added individually at low enough concentrations to avoid detection.

The present effort is aimed at developing a high throughput, sensitive, rugged, and simple to use HILIC chromatography based MS/MS based method for the quantification of nine economic adulterants in milk derived ingredients. Several LC/MS/MS based methods for the analysis of melamine and related compounds have been published in the literature for food matrices [5,15,22]. Many of the published methods involve protein precipitation or solid-phase extraction based sample preparation prior to analysis. The present effort is aimed at developing a simple dilute and analyze sample preparation procedure followed by sensitive and specific detection at levels that can detect instances of economic adulteration.

Out of the ten listed economic adulterants, nine economic adulterants (ammeline, ammelide, cyromazine, amidinourea, dicyandiamide, urea, biuret, melamine, and cyanuric acid) were incorporated in the present effort (Fig. 1). Despite extensive searches, a commercial source of triuret could not be located and hence the compound was not included the present work. The aim of the study was to fully validate the method for the above listed nine compounds using four milk derived matrices (whole milk powder, nonfat dry milk, milk protein concentrate, and sodium caseinate) and one plant protein matrix (soy protein isolate) (Fig. 2).

2. Experimental

2.1. Reference materials

Melamine (MEL), cyanuric acid (CYA), amidinourea sulfate (AMU), biuret (BU), dicyandiamide (DCD), and urea were purchased from Sigma–Aldrich, St. Louis, MO, USA. Ammeline (AMN) and ammelide (AMD) were purchased from TCI America Portland, OR, USA. Cyromazine (CYR) was purchased from Fluka Sigma–Aldrich, St. Louis, MO, USA. $^{13}C_3$,$^{15}N_3$-melamine, $^{13}C_3$,$^{15}N_3$-cyanuric Acid, $^{13}C_3$-ammelide, and $^{13}C_3$-ammeline were purchased from Cambridge Isotope Laboratories, Andover, MA, USA. $^{15}N_3$-biuret, $^{15}N_4$-dicyandiamide, and ^{13}C, $^{15}N_2$-urea were purchased from Sigma–Aldrich, St. Louis, MO, USA. Cyromazine-d_4 was purchased from CDN Isotopes, Pointe-Claire, Quebec, Canada.

2.2. Samples

Three unique lots of milk protein concentrate, nonfat dry milk, whole milk powder, soy protein isolate, and sodium caseinate were used for validation testing.

2.3. Reagents

Formic acid (ACS Reagent Grade >96%), and diethylamine (puriss) were purchased from Sigma Aldrich, St. Louis, MO, USA. Acetonitrile (LCMS Grade) was purchased from Fisher Scientific Pittsburgh, PA, USA. Ammonium acetate was purchased from Fluka Sigma–Aldrich, St. Louis, MO, USA. Laboratory water (18.2 MΩ cm) was filtered using a Millipore Milli-Q water filtration unit with and EDSPAK001 filter attached.

2.4. Preparation of stock and spiking solutions

Individual stock standard solutions of approximately 100 μg/mL melamine, cyanuric acid, dicyandiamide, cyromazine, and amidinourea, 1000 μg/mL of biuret and approximately 2000 μg/mL urea were prepared by weighing the appropriate amount of each reference standard (corrected for composition and purity) into separate 100 mL volumetric flasks and bringing to volume with deioinzed laboratory water. Individual stock solutions of approximately 100 μg/mL ammelide and ammeline were prepared by weighing the appropriate amount of each reference standard into separate 100 mL volumetric flasks and bringing to volume with deionized water: diethylamine (80:20, v/v).

Individual QC (quality control) stock solutions were also prepared at the same concentrations and using the same procedure described above. All individual stock standard and individual QC stock solutions were stored in amber vials at laboratory room temperature conditions. A mixed intermediate QC spiking solution with concentrations of approximately 0.8 μg/mL melamine and cyromazine, 4.0 μg/mL cyanuric acid, 2.0 μg/mL amidinourea, 2.5 μg/mL ammeline and ammelide, 3.0 μg/mL dicyandiamide, 60 μg/mL biuret, and 80 μg/mL urea was prepared by adding the appropriate amount of each individual QC stock solution to a 10 mL volumetric flask and diluting to volume with 2% formic acid in acetonitrile (v/v).

A mixed internal standard working solution with concentrations of approximately 25 μg/mL $^{13}C_3$,$^{15}N_3$-melamine, cyromazine-d_4, $^{15}N_4$-dicyanodiamide, and $^{13}C_3$,$^{15}N_3$-cyanuric acid was prepared by adding the appropriate amount of each internal standard stock solution to an autosampler vial. A second mixed internal standard