Abstract:

Title: DETECTION OF MELAMINE

Anti-melamine antibodies, compounds that can be used to generate anti-melamine antibodies, melamine-label conjugates, and methods, devices and kits for detecting melamine in samples suspected of containing melamine.
DETECTION OF MELAMINE

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The invention generally relates to the detection of melamine. More particularly, the invention relates to anti-melamine antibodies, conjugates that can be used to generate anti-melamine antibodies, conjugates useful in melamine immunoassays, and methods and devices for detecting melamine in samples suspected of containing melamine.

Technical Background

[0002] Melamine (2,4,6-triamino-1,3,5-triazine) is an industrial chemical commonly used in the manufacture of plastic materials. Dietary exposure to melamine can cause the formation of kidney stones, and can increase the incidence of kidney inflammation which may lead to infection.

[0003] Melamine is manufactured in large amounts, primarily for use in the synthesis of melamine formaldehyde plastics which are used in a variety of products. Melamine is also used as an adulterant in foodstuffs and milk to make diluted or poor quality material appear to be higher in protein content by elevating the total nitrogen content detected by simple protein tests. Recently, melamine tainted milk has been linked to the illness of hundreds of thousands of people in China. Similarly, melamine tainted pet foods have been recalled in the United States, but not before the illness and death of countless animals. Moreover, because melamine resin is often used in food packaging and tableware, melamine at ppm level (1 part per million) in food and beverages has been reported due to migration from melamine-containing resins. Small amounts of melamine have also been reported in
foodstuffs as a metabolite product of cyromazine, an insecticide used on animals and crops. Melamine targets the urinary system, where it can combine with uric acid to form stones and crystallites. Limits for melamine in powdered infant formula (1 mg/kg or 1 ppm) and in other foods (2.5 mg/kg or 2.5 ppm) would provide a sufficient margin of safety for dietary exposure relative to the World Health Organization tolerable daily intake level of 0.2 mg/kg body weight/day.

Accordingly, attention has turned toward methods for the detection of melamine. Existing methods for melamine determination (e.g., extraction coupled with LC/MS or HPLC; surface-enhanced Raman spectroscopy) are often complex and time consuming. While electrospray ionization methods coupled with mass spectrometry can allow a rapid and direct analysis of samples in complex matrices, they are also limited in that they are equipment-intensive. LC/MS and HPLC methods are also labor-intensive procedures; for example, they typically require sample processing steps and skilled technicians.

Accordingly, the inventors have identified a need in the art for a simple, convenient and cost effective methods and devices for the detection of melamine in samples suspected of containing melamine.

**BRIEF SUMMARY OF THE INVENTION**

In various aspects, the invention is directed to melamine analogs, anti-melamine antibodies, devices, and kits for detecting melamine by immunoassay.

One aspect of the invention is a conjugate having the formula
or a salt thereof, wherein the P moiety is the protein, and m is an integer in the range of 1-1000.

[0008] Another aspect of the invention is an anti-melamine antibody raised against the above-described conjugate.

[0009] Another aspect of the invention is a method of detecting the presence or amount of melamine in a sample. The method includes contacting the sample with an anti-melamine antibody and a melamine analog conjugated to a label, and detecting the presence or amount of the label associated with the antibody, thereby determining the presence or amount of melamine in the sample.

[0010] In another aspect, the method includes contacting the sample with a melamine analog and an anti-melamine antibody conjugated to a label, and, detecting the presence or amount of the label associated with the melamine analog, thereby determining the presence or amount of melamine in the sample.

[0011] In another aspect, the method includes providing a lateral flow assay device comprising a solid phase having non-diffusively bound thereto a melamine analog; providing a sample; contacting the sample with a melamine analog and an anti-melamine antibody conjugated to a label; and detecting the presence or amount of the label associated with the melamine analog, thereby determining the presence or amount of melamine in the sample.
wherein the detectable presence or amount of melamine in the sample is at least about 2.5 parts per million (ppm), about 1 ppm and/or about 0.1 ppm.

[0012] Another aspect of the invention is an anti-melamine antibody exhibiting substantially reduced cross-reactivity to ammeline, ammelide, CDAT, cyanuric acid, 2-chloro-4,6-diaminotriazine, 2-methyl-4,6-diaminotriazine, 6-hydroxy-2,4-diaminopyrimidine, 6-chloro-2,4-diaminopyrimidine, atrazine, desethylatrazine, desisopropylatrazine, 2-hydroxyatrazine, simazine and cyanazine.

[0013] Other aspects of the invention devices and kits for determining the presence or amount of melamine in a sample. The device includes a solid phase having non-diffusively bound thereto an anti-melamine antibody. The kit includes the device and a DAT moiety conjugated to a label.

[0014] Additional features, advantages, and embodiments of the invention may be set forth or apparent from consideration of the following detailed description, drawings, and claims. Moreover, it is to be understood that both the foregoing summary of the invention and the following detailed description are exemplary and intended to provide further explanation without limiting the scope of the invention as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] The accompanying drawings, which are included to provide a further understanding of the invention, are incorporated in and constitute a part of this specification, illustrate embodiments of the invention, and together with the detailed description serve to explain the principles of the invention. No attempt is made to show structural details of the invention in more detail than may be necessary for a fundamental understanding of the invention and various ways in which it may be practiced.
FIGURES 1, 2 and 3 shows the results of an immunoassay for melamine at various concentrations of melamine spiked into commingled milk samples.

FIGURE 4 shows the results of another immunoassay for melamine at various concentrations of melamine spiked into commingled milk samples.

FIGURE 5 shows the results of another immunoassay for melamine at various concentrations of melamine spiked into commingled milk samples.

FIGURE 6 is a graph showing the incorporation of DAT into a DAT-BSA conjugate for a range of CDAT concentrations.

FIGURE 7 is a graph showing the incorporation of DAT into a DAT-ovalbumin conjugate for a range of CDAT concentrations and two different temperatures.

FIGURE 8 is a graph showing the incorporation of DAT into a DAT-ovalbumin conjugate for a range of CDAT concentrations and two different reaction times.

FIGURE 9 is a graph showing the correlation of the ratio of absorbances at 240 nm and 280 nm with DAT/ovalbumin ratio.

FIGURE 10 is a graph showing the results for the use of an OAEM-HRP conjugate as the label in a commercially available melamine immunoassay kit.

FIGURE 11 is a graph showing the results for a competitive melamine immunoassay using anti-DAT-SMCC-HRP as label.

FIGURE 12 is a graph showing minimal interference by CDAT for a competitive melamine immunoassay using anti-DAT-SMCC-HRP as label.
FIGURE 13 is a graph showing the response vs. melamine concentration of an ELISA using anti-DAT-SMCC-HRP and DAT-ovalbumin.

FIGURE 14 is a set of graphs showing the results of immunoassays for melamine spiked at various concentrations into commingled milk samples, for the SNAPSHOT reader.

FIGURES 15, 16 and 17 are graphs showing the results of immunoassays for melamine spiked at 0 and 0.1 ppm into commingled milk samples, hydrated powdered full cream milk, and hydrated powdered skim milk, with varying additions of EDTA.

DETAILED DESCRIPTION OF THE INVENTION

In various aspects, the invention provides methods for detecting melamine in samples such as foodstuffs, milk and environmental samples. In one embodiment, the method includes detecting the presence or amount of melamine in the sample by using an immunoassay format, such as a competitive immunoassay. The assay includes the use of antibodies to melamine, which in certain embodiments can be made using immunogens described herein. In certain embodiments, the assay uses melamine analogs and anti-melamine antibodies are attached to labels and/or solid phases.

Before describing the invention in further detail, a number of terms are defined:

CDAT is 2-chloro-4,6-diamino-l,3,5-triazine.

EDTA is ethylenediaminetetraacetic acid, which can be provided either as the acid or as a salt, e.g., a sodium salt.

The term "analog," as used herein, generally refers to a compound in which one or more individual atoms have been replaced with a different atom(s) or with a different functional group(s). For example, an analog may be a modified form of the analyte which
can compete with the analyte for a receptor, the modification providing a means to join the analyte to another moiety, such as a label or solid support. The analyte analog can bind to an antibody in a manner similar to the analyte. In certain embodiments, the melamine analog includes the 4,6-diamino-1,3,5-triazine-2-yl moiety (DAT):

![Chemical Structure](image)

[0034] The term "antibody," as used herein, generally refers to a glycoprotein produced by B lymphocyte cells in response to exposure to an antigen and binds specifically to that antigen. The term "antibody" is used in its broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

[0035] As used herein, an "anti-melamine," "anti-melamine antibody portion," or "anti-melamine antibody fragment" and/or "anti-melamine antibody variant" and the like include any protein- or peptide-containing molecule that comprises at least a portion of an immunoglobulin molecule, such as, but not limited to, one complementarity determining region (CDR) of a heavy chain or light chain constant region, a framework region, or any portion thereof. An "anti-DAT" antibody is an anti-melamine antibody that was raised against a direct-linked DAT-protein conjugate.

[0036] The term "antibody fragment," as used herein, refers to a portion of a full length antibody, generally the antigen binding or variable domain thereof. Specifically, for
example, antibody fragments may include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies from antibody fragments.

[0037] The term "antigen," as used herein, generally refers to a substance that is capable, under appropriate conditions, of reacting with an antibody specific for the antigen.

[0038] The term "sample," as used herein, generally refers to a sample of material for testing. The range of materials that can be tested varies greatly, and can be any material that can be prepared for use in an immunoassay. Samples can be obtained from a variety of sources, such as agricultural products (e.g., milk, cream, meat products, fruits and vegetables); processed foodstuffs (e.g., pet food, powdered milk/cream, foods containing milk products) and environmental samples (e.g., soil). Milk samples can be raw, or processed, for example, milk containing various amounts of fat, e.g., skim milk, 1% fat, 2% fat or higher. The sample can be provided in a number of different forms depending on the source and the particular immunological method used (e.g., the material as received, or an extract, concentrate, or pulverized version thereof). Accordingly, in certain embodiments, assays are performed on powdered milk or powdered cream samples (e.g., suspended in an aqueous solvent).

[0039] The term "immunoassay," as used herein, generally refers to a test that employs antibody and antigen complexes to generate a measurable response. An "antibody:antigen complex" may be used interchangeably with the term "immuno-complex." Immunoassays, in general, include noncompetitive immunoassays, competitive immunoassays, homogeneous immunoassays, and heterogeneous immunoassays. In "competitive immunoassays," unlabeled analyte (or antigen) in the test sample is measured by its ability to compete with labeled antigen in the immunoassay. The unlabeled antigen blocks the ability of the labeled
antigen to bind because the binding site on the antibody is already occupied. Alternatively, the label could be on the antibody, wherein the free antigen in the sample (if present) competes for binding with the labeled antibody. In "competitive immunoassays," the amount of antigen present in the test sample is inversely related to the amount of signal generated from the label. Conversely, in "noncompetitive immunoassays," also known as "sandwich" immunoassays, the analyte is bound between two highly specific antibody reagents to form a complex and the amount of antigen is directly proportional to the amount of signal associated with the complex. Immunoassays that require separation of bound antibody:antigen complexes are generally referred to as "heterogeneous immunoassays," and immunoassays that do not require separation of antibody:antigen complexes are generally referred to as "homogeneous immunoassays." One of skill in the art would readily understand the various immunoassay formats.

[0040] The term "immune complexes," as used herein, generally refers to the complexes formed by the binding of antigen and antibody molecules, with or without complement fixation. When one of either the antibody or antigen is labeled, the label is associated with the immune complex as a result of the binding between the antigen and antibody. Therefore, when the antibody is labeled, the label becomes associated with the antigen as a result of the binding. Similarly, when the antigen is labeled (e.g., an analyte analog having a label), the label becomes associated with the antibody as a result of the binding between the antigen and the antibody.

[0041] The term "label," as used herein, refers to a detectable compound, composition, or solid support, which can be conjugated directly or indirectly (e.g., via covalent or non-covalent means, alone or encapsulated) to an antibody, melamine analog, or antigen of the invention. The label may be detectable by itself (e.g., radioisotope labels, chemiluminescent
dye, electrochemical labels, metal chelates, latex particles, or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable (e.g., enzymes such as horseradish peroxidase, alkaline phosphatase, and the like). The label employed in the current invention could be, but is not limited to: alkaline phosphatase; glucose-6-phosphate dehydrogenase (G6PDH); horseradish peroxidase (HRP); chemiluminescers such as isoluminol, fluorescers such as fluorescein and rhodamine compounds; ribozymes; and dyes. The label may also involve a specific binding molecule which itself may not be detectable (e.g., biotin, avidin, streptavidin, digoxigenin, maltose, oligohistidine, 2, 4-dinitrobenzene, phenylarsenate, ssDNA, dsDNA, and the like). The label may be bound to another molecule or solid support and that is chosen for specific characteristics that allow detection of the labeled molecule. The utilization of a label produces a signal that may be detected by means such as detection of electromagnetic radiation or direct visualization, and that can optionally be measured.

[0042] The term "solid support," as used herein, refers to a non-aqueous matrix to which the antibody or melamine analog of the present invention can adhere. Example of solid support include supports formed partially or entirely of glass (e.g., controlled pore glass), synthetic and natural polymers, polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohols and silicones, magnetic particles, latex particles, chromatographic strips, microtiter polystyrene plates, or any other substances that will allow bound antigens and/or antibodies to be washed or separated from unbound materials. In certain embodiments, depending on the application, the solid support can be the well of an assay plate or can be a purification column (e.g., an affinity chromatography column).
"Receptor" refers to any compound or composition capable of recognizing a particular spatial and polar organization of a molecule, e.g., epitopic or determinant site. Illustrative receptors include antibodies, Fab fragments, and the like.

"Substantial binding" or "substantially bind" refers to an amount of specific binding or recognizing between molecules in an assay mixture under particular assay conditions. In its broadest aspect, substantial binding relates to the difference between a first molecule's incapability of binding or recognizing a second molecule, and the first molecule's capability of binding or recognizing a third molecule, such that the difference is sufficient to allow a meaningful assay to be conducted distinguishing specific binding under a particular set of assay conditions, which includes the relative concentrations of the molecules, and the time and temperature of an incubation. In another aspect, one molecule is substantially incapable of binding or recognizing another molecule in a cross-reactivity sense where the first molecule exhibits a reactivity for a second molecule that is less than 25%, less than 10%, less than 5% or less than 1% of the reactivity exhibited toward a third molecule under a particular set of assay conditions. Specific binding can be tested using a number of widely known methods, e.g., an immunohistochemical assay, an enzyme-linked immunosorbent assay (ELISA), a radioimmunoassay (RIA), or a western blot assay.

The term "salt," as used herein, means a salt formed between an acid and a basic functional group of a compound. Illustrative salts include, but are not limited, to sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, and pamoate (i.e., 1,1'-methylene-bis-(2-hydroxy-3-
naphthoate) salts. The term "salt" also refers to a salt formed between a compound having an acidic functional group, such as a carboxylic acid functional group, and an inorganic or organic base. Suitable bases include, but are not limited to, hydroxides of alkali metals such as sodium, potassium, and lithium; hydroxides of alkaline earth metal such as calcium and magnesium; hydroxides of other metals, such as aluminum and zinc; ammonia, and organic amines, such as unsubstituted or hydroxy-substituted mono-, di-, or trialkylamines; dicyclohexylamine; tributyl amine; pyridine; N-methyl, N-ethylamine; diethylamine; triethylamine; mono-, bis-, or tris-(2-hydroxy-lower alkyl amines), such as mono-, bis-, or tris-(2-hydroxyethyl)amine, 2-hydroxy-tert-butylamine, or tris-(hydroxymethyl)methylamine, N,N-di-lower alkyl-N-(hydroxy lower alkyl)-amines, such as N,N-dimethyl-N-(2-hydroxyethyl)amine, or tri-(2-hydroxyethyl)amine; N-methyl-D-glucamine; and amino acids such as arginine, lysine, and the like.

[0046] Turning now to the various aspects of the invention, in one aspect, the invention relates to melamine-protein conjugates and methods for their production and use. One embodiment of the invention is a melamine-protein conjugate comprising a DAT moiety directly bound to a protein. For example, certain melamine-protein conjugates according to the present invention have the following Formula I:

```
\[
\text{\begin{array}{c}
\text{H}_2\text{N} \\
\text{N} \\
\text{N} \\
\text{H}_2\text{N} \\
\end{array}} \quad \text{P}
\]
```

Formula I
or a salt thereof, in which the P moiety is the protein, and m is an integer in the range of 1-1000. DAT moieties are covalently bound to the protein, chiefly to the peptide's lysine residues (which include a side chain having a reactive amino group). Accordingly, when the DAT moiety is covalently bound to (e.g., to the lysine residues of) the protein, a linker is not present or necessary. DAT can also bind to the free amino group of the N-terminus amino acid, and may also bind in relatively smaller amounts to other amino acids (e.g., serine, cysteine). Any suitable protein can be used, for example, bovine serum albumin, keyhole limpet hemocyanin, lactalbumin, ovalbumin, thyroglobulin, immunoglobulins and label proteins (e.g., enzymes such as HRP).

[0047] In general, m is limited by the number of available CDAT-reactive groups (e.g., amines) present in the protein and the number of equivalents of DAT reacted with the protein. For example m can be in the range of 1 to about 50, 1 to about 25, or even 1 to about 15. In certain embodiments, m is at least 2, at least 3, or even at least 5. As the person of skill in the art will appreciate, desirable values of m will depend on the application. Generally, higher values of m are desired when the DAT-protein conjugate is to be used as an immunogen than when it is bound to a solid phase in an immunoassay. For example, for use as an immunogen with BSA as the protein, m greater than about 5 (e.g., greater than about 8) can be sufficient for rapid and specific production of anti-melamine antibodies. For use as a solid phase with ovalbumin as the protein, m can be, for example, greater than about 2 (e.g., in the range of about 3-5). For use as a solid phase with thyroglobulin as the protein, m can be, for example, in the range of about 5-15.

[0048] In one embodiment, a melamine analog such as DAT is conjugated to a carrier protein to form a "hapten-carrier" immunogen that can be used to stimulate an immune response to melamine. Exemplary immunogenic carrier proteins include, but are not limited
of bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), lactalbumin and ovalbumin. In certain embodiments, DAT is directly conjugated to the protein, without the intermediary of a linker. When the carrier protein (i.e., the P moiety) is BSA, the hapten-carrier immunogen is "DAT-BSA." When the carrier protein is KLH, the hapten-carrier immunogen is "DAT-KLH." When the carrier protein is lactalbumin, the hapten-carrier immunogen is "DAT-lactalbumin." When the carrier protein is ovalbumin, the hapten-carrier immunogen is "DAT-ovalbumin." Protocols for conjugating haptens to immunogenic proteins are known in the art (see, e.g., Antibodies: A Laboratory Manual, E. Harlow and D. Lane, eds., Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, 1988) pp. 78-87).

[0049] In various embodiments, the P moiety is BSA and m is greater than about 3. In various other embodiments when the P moiety is BSA, m is in a range of about 3 to about 80. In particular examples, m is 10, 20, 30, 40, 50, 60 or 70. DAT can generally be incorporated in a DAT-BSA conjugate in an amount corresponding to up to about one third or about one half of the total number of lysines. In other embodiments, the P moiety is KLH and m is greater than about 5. In various particular embodiments when the P moiety is KLH, m is the range of 5-1000, and, for example, m is 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000. In other embodiments, P is lactalbumin and m is greater than about 5. In various particular embodiments when the P moiety is lactalbumin and m is the range of 5-100; for example, m is 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100. DAT can generally be incorporated in a DAT-lactalbumin conjugate in an amount corresponding to up to about three quarters or even nearly all of the total number of lysines. In other embodiments, P is ovalbumin and m is greater than about 3. In various particular embodiments when the P moiety is ovalbumin and m is the range of 3-100; for example, m is 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100. In certain embodiments, P is ovalbumin and m is in the range of 3-5. DAT can generally be
incorporated in a DAT-ovalbumin conjugate in an amount corresponding to up to about one third or about one half of the total number of lysines.

[0050] In another embodiment, the P moiety is a label protein. For example, a melamine-label protein conjugate can be suitable as a detectable conjugate for use in receptor binding assays, such as immunoassays for melamine. For example, in certain embodiments, the P moiety is horseradish peroxidase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, avidin or streptavidin. A detectable melamine-label protein conjugate may be used in various homogenous, sandwich, competitive, or non-competitive assay formats, to generate a signal that is related to the presence or amount of an melamine in a test sample.

[0051] The melamine-protein conjugate of structural formula (I) can be prepared using 2-chloro-4,6-diamino-1,3,5-triazine (CDAT) as shown the following illustrative synthetic Scheme 1:

Scheme 1:

[0052] In the reaction of Scheme 1, CDAT is reacted directly with the protein (i.e., in the absence of a linker compound). Unlike in Formula I, the -NH$_2$ moieties of the protein (e.g., of the lysine residues) are explicitly shown in Scheme 1. Surprisingly, the inventors have determined that, contrary to reports in the literature, see e.g., Abuknesha, J. Immunol. Meth. vol. 306, pp 211-217 (2005), CDAT will react directly with lysine residues on the protein to form a direct DAT-protein conjugate. The inventors have determined that reaction of CDAT
with protein and octanesulfonic acid in 25% DMSO/75% carbonate buffer pH 8-11 at
moderate temperatures (e.g., 56 °C) and for moderate times (e.g., 12 h) can lead to a high
degree of reaction without significant gelation or denaturation. For certain heat-stable
proteins (such as ovalbumin and lactalbumin), the use of DMSO as a cosolvent is not strictly
necessary; for example, the reaction can be performed in borate buffer for 24 h at 56 °C. The
reaction of CDAT with protein can be performed under other conditions; for example, at
room temperature CDAT will react with BSA to form DAT-BSA conjugates, albeit with
relatively lower levels of DAT incorporation. Accordingly, one aspect of the invention is a
method for making a DAT-protein conjugate, by reacting CDAT with the protein at a pH in
the range of about 8 to about 11, for at least about 4 hours. The reaction can, for example, be
performed with or without an organic (e.g., DMSO) cosolvent, and can in certain
embodiments be performed at temperatures in the range of about 40 °C to about 75 °C, or
even in the range of about 50 °C to about 60 °C. Examples of synthetic procedures for the
synthesis of direct DAT-protein conjugates are provided below in Example 1.

[0053] The resulting conjugate can be purified using methods known to those skilled in
the art including, but not limited to column chromatography, for example, using gel-filtration
column chromatography with Sephadex™ brand cross-linked dextran gel filtration media (for
example, Sephadex™ G-25M) as the solid support (commercially available from Sigma-
Aldrich).

[0054] The DAT/protein ratio can be determined using pH difference spectroscopy. UV
spectra can be acquired at pH 2.5 and pH 9.0; the net (pH 2.5 - pH 9.0) spectrum has a peak
around 240 nm that can be used to quantify the amount of melamine (absorptivity 9200
L/mol-cm). For a given protein, these results can be correlated with the ratio of absorbances
at 240 nm and 280 nm in 10 mM phosphoric acid at pH 2.4. With such a calibration, a single pH scan can be used to determine DAT/protein ratio.

[0055] The hapten-carrier immunogens described above can be used to generate antibodies that substantially bind melamine. For example, a DAT-protein conjugate (e.g., DAT-BSA, DAT-ovalbumin) can be used as an immunogen to generate antibodies that substantially bind melamine. While the prevailing immunochemical model for producing antibodies against small molecules maintains that a spacer is required for separating the immunochemical response of the hapten from that of the carrier, the inventors have surprising found that the direct-linked melamine-protein conjugates produce antibodies that are more specific for melamine than antibodies raised again melamine with conjugates with typical spacers. In certain embodiments of the invention, the mole ratio of DAT to carrier protein (e.g., BSA or ovalbumin) is greater than about 5 (e.g., greater than about 8). For example, in certain embodiments the mole ratio of DAT to carrier protein can be in the range of about 5 to about 100, for example, in the range of about 5 to about 50, about 5 to about 20, or about 5 to about 15.

[0056] Accordingly, another embodiment of the invention is an anti-melamine antibody raised in an animal exposed to a melamine-protein conjugate as described above. The anti-melamine antibody can be, for example, a polyclonal antibody. Suitable animals include, for example, rabbits, goats, sheep, chicken, rats, and mice. Methods for producing antibodies according to the present invention include administering one or more melamine-protein conjugates to an animal using a suitable immunization protocol, and separating an appropriate antibody from a body fluid(s) of the animal, as described, for example, in Example 4, below. Alternatively, the melamine-protein conjugates of the invention may be used in phage display methods to select phage displaying on their surface an appropriate
antibody, followed by separation of nucleic acid sequences encoding at least a variable
domain region of an appropriate antibody. Phage display methods are well known to those of
ordinary skill in the art. (See, e.g., Antibody Phage Display; Methods in Molecular Biology,
Vol. 178, O'Brien, Philippa M.; Aitken, Robert (Eds.) 2002). In addition, monoclonal
antibodies to melamine can be prepared by methods generally known in the art. In certain
embodiments, fluids from the animal are collected after 6 weeks, or even after 25 weeks of
exposure to the melamine-protein conjugate.

[0057] Anti-melamine antibodies can be linked to a label to provide detectable anti-
melamine antibodies for use in receptor binding assays, such as immunoassays for melamine.
The anti-melamine antibodies can be linked to a label using methods well known to those
skilled in the art. E.g., Immunochemical Protocols; Methods in Molecular Biology, Vol. 295,
edited by R. Burns (2005). The detectable anti-melamine antibodies may be used in various
homogenous, sandwich, competitive, or non-competitive assay formats, to generate a signal
that is related to the presence or amount of an melamine in a test sample.

[0058] In certain embodiments, the antibodies disclosed herein exhibit substantially
reduced cross-reactivity to various other compounds, such as CDAT, cyanuric acid, 2-
methyl-4,6-diaminotriazine, 6-chloro-2,4-diaminopyrimidine, triazine herbicides, their
metabolites, and melamine metabolites. For example, in certain embodiments, the antibodies
exhibit substantially reduced cross-reactivity to ammeline, ammelide, CDAT, cyanuric acid,
2-chloro-4,6-diaminotriazine, 2-methyl-4,6-diaminotriazine, 6-hydroxy-2,4-
diaminopyrimidine; 6-chloro-2,4-diaminopyrimidine, atrazine, desethylatrazine,
desisopropylatrazine, 2-hydroxyatrazine, simazine and cyanazine. For example, an antibody
can have a reactivity with these compounds that is less than its reactivity to melamine, for
example, less than 50% of its reactivity to melamine, or even less than 10% of its reactivity to
melamine. This property is of particular importance because many commercially available kits cross react with triazine herbicides, analogs and metabolites (e.g. CDAT, an atrazine metabolite).

[0059] Melamine associated with various labels is also useful in immunoassays. The melamine conjugated to the label can be, for example, a melamine-label protein species as described above (e.g., Formula 1 and Scheme 1). Other melamine-label conjugates can be used. For example, the melamine-label conjugate can include a linker between a melamine moiety and the label. In certain embodiments of the invention, the linker reacts with a carboxylate residue (e.g., glutamic acid) of the protein. Scheme 2 illustrates the synthesis of two linked melamine-label protein conjugates, which are described in further detail in Example 4. These melamine-label conjugates comprise $N^2$-(2-(2-aminoethoxy)ethyl)-1,3,5-triazine-2,4,6-triamine (OAEM).
Other aspects of the invention are directed to immunological methods, devices and kits for detecting the presence of an amount of melamine in a sample. The method may include controls, calibrators or standards comprising one or more melamine analogs. In particular, the method may be accomplished using immunoassay techniques well known to those of skill in the art, including, but not limited to, using microplates and lateral flow devices.

The solid phase assay format is a commonly used binding assay technique. There are a number of assay devices and procedures wherein the presence of an analyte is indicated by the analyte's binding to a conjugate and/or an immobilized complementary binding member. In one particular aspect, the immobilized binding member (e.g., anti-melamine
antibody) is bound, or becomes bound during the assay, to a solid phase such as a reaction well, dipstick, test strip, flow-through pad, paper, fiber matrix or other suitable solid phase material. The binding reaction between melamine in the sample and immobilized antibody can be determined by adding to the sample an analog of melamine conjugated to a label. The melamine in the sample and the labeled melamine analog are allowed to bind the immobilized antibody. Following this reaction, unbound reactants are removed from the solid phase. The amount of the label that becomes associated with the antibody through binding of the antibody to the analog is measured. The amount of the label associated with the antibody is inversely proportional to the amount of melamine in the sample.

[0062] Immobilization of one or more antibodies to melamine onto a device or solid support is performed so that the antibodies will not be washed away by the sample, diluent and/or wash procedures. One or more antibodies can be attached to a surface by physical adsorption (i.e., without the use of chemical linkers) or by chemical binding (i.e., with the use of chemical linkers). Chemical binding can generate stronger attachment of antibodies on a surface and provide defined orientation and conformation of the surface-bound molecules.

[0063] In another aspect, anti-melamine antibodies raised in a particular species are bound to a solid support by interaction with an anti-species antibody that is bound to the support. In one particular aspect, anti-melamine antibodies are raised in rabbits, and the support has bound thereto anti-rabbit antibody that recognizes the anti-melamine antibody raised in rabbits. In this aspect, the antibody may be in the form of anti-serum obtained from the species. The anti-melamine antibodies can either be applied to the solid phase having the anti-species antibody prior to adding the sample to the solid phase, or the anti-melamine antibodies can be mixed with the sample prior to adding the sample to the solid phase. In either case, the anti-melamine antibodies become bound to the solid phase through binding to
the anti-species antibody on the solid phase. Or, in another embodiment, the antibodies can be coated on a microparticle that becomes bound to the solid phase as the result of entrapment.

[0064] In another aspect, the invention includes one or more labeled anti-melamine antibodies (e.g., anti-DAT antibodies) that can be mixed with a test sample prior to application of the mixture to a solid support. In this case, a melamine analog can be attached to the solid support so that the analog will not be washed away by the sample, diluent and/or wash procedures. The anti-melamine-label conjugate can be, for example, anti-DAT-SMCC-HRP (i.e., anti-DAT antibody linked to HRP through an SMCC linker). Labeled antibodies in the sample bind to melamine in the sample and are, therefore, not available for binding with the melamine analog on the solid support. After application of the mixture to the solid support, and an appropriate incubation, the mixture is washed from the solid support. Antibodies that have not bound to sample melamine will become bound to the melamine analog on the solid support. The presence or amount of melamine in the sample is inversely proportional to the amount of anti-melamine antibody that has become bound to the melamine analog on the solid phase. The signal associated with the label on the antibody can be measured by the appropriate method.

[0065] The melamine analog can be, for example, DAT-ovalbumin-latex microparticles, which can be deposited on a porous carrier matrix such as porous high MW polyethylene and adhered thereto by passive absorption. One example of a porous carrier matrix is a high molecular weight porous polyethylene strip coated with surfactant to make it wettable. Of course, other materials, such as nitrocellulose, can also be used by the person of skill in the art.
In other embodiments, The DAT-ovalbumin can be mixed with polystyrene particles in a buffer system to allow binding. The DAT-ovalbumin bound particles can then be centrifuged, washed, mixed with a buffer and blocked with BSA. Finally the particles can be resuspended in a buffer containing sucrose. Alternatively, CDAT can be reacted with NH2-terminated polystyrene particles using methods similar to those described below.

In another aspect of the disclosure, a DAT-protein conjugate is suitable for use in a solid phase in an immunoassay. For example, in certain embodiments, a DAT-protein conjugate has a DAT/protein ratio of about 1 to about 15; of about 2 to about 6; or even about 3 to about 5. The protein can be, for example, ovalbumin. In other embodiments, the protein is BSA, lactalbumin or thyroglobulin. For example, in one example, the ratio of DAT to protein is in the range of about 3 to about 5, or about 3.4 to about 4.2 (e.g., when the protein is ovalbumin). In another example, the ratio of DAT to protein is in the range of about 5 to about 15 (e.g., when the protein is thyroglobulin). The inventors have determined that such DAT/protein conjugates can exhibit increased sensitivity in immunoassays.

Detection of the antibody:antigen complexes may be achieved through a variety of techniques well known in the art, such as, for example, turbidimetry, enzymatic labeling, radiolabeling, luminescence, or fluorescence. Immunoassay methodologies are known by those of ordinary skill in the art and are appreciated to include, but not limited to, radioimmunoassay (RIA), enzyme immunoassays (EIA), fluorescence polarization immunoassays (FPIA), microparticle enzyme immunoassays (MEIA), and chemiluminescent magnetic immunoassays (CMIA). In RIA, an antibody or antigen is labeled with radioactivity and used in a competitive or noncompetitive format. In EIA, an antibody or antigen is labeled with an enzyme that converts a substrate to a product with a resulting signal that is measured, such as a change in color. In FPIA, an antigen is labeled with fluorescent
label and competes with unlabeled antigen from the specimen. The amount of analyte measured is inversely proportional to the amount of signal measured. In MEIA, a solid phase microparticle is coated with antibodies against an antigen of interest and is used to capture the analyte. The antibody for detection is labeled with an enzyme as in the EIA method. The concentration of analyte measured is proportional to the amount of signal measured. In CMIA, a chemiluminescent label is conjugated to the antibody or antigen, and produces light when combined with its substrate. CMIA can be configured in a competitive or noncompetitive format, and yields results that are inversely or directly proportional to the amount of analyte present, respectively.

[0069] The use of reagent-impregnated test strips in specific binding assays is also well-known. In such procedures, a test sample is applied to one portion of the test strip and is allowed to migrate or wick through the strip material. Thus, the analyte to be detected or measured passes through or along the material, possibly with the aid of an eluting solvent which can be the test sample itself or a separately added solution. The analyte migrates into a capture or detection zone on the test strip, wherein a complementary binding member to the analyte is immobilized. The extent to which the analyte becomes bound in the detection zone can be determined with the aid of the conjugate which can also be incorporated in the test strip or which can be applied separately. In one embodiment, an anti-melamine antibody is immobilized on a solid support at a distinct location. Following addition of the sample, detection of melamine-antibody complexes on the solid support can be by any means known in the art. For example, U.S. Patent No. 5,726,010, which is incorporated herein by reference in its entirety, describes an example of a lateral flow device, the SNAP® immunoassay device (IDEXX Laboratories), useful in the present invention. Using the SNAP® immunoassay, the experiment can be performed by mixing 20 µL of a mixture of anti-melamine:HRP (or melamine analogue:HRP), Goat-anti chicken:HRP and 450 µL of a
sample (e.g., milk). The mixture is incubated at 45° C for 2 minutes then added to the SNAP® device. When the sample reaches the activation circle, the device is activated to release the onboard reagents. At the end of 8 min, the device is read both visually and in a SNAPshot® reader (IDEXX Laboratories). A control to sample ratio less than 1.06 indicates a negative result.

In certain embodiments, it can be helpful to add EDTA (i.e., ethylenediaminetetraacetic acid or a salt thereof) to the sample. For example, EDTA can be added at concentrations in the range of about 0.1 to about 25 mM, or in the range of about 0.5 to about 15 mM. For example, in certain embodiments, and as described in more detail below, EDTA is added at concentrations in the range of about 1.1 to about 7.7 mM. As described below with respect to FIGURES 15-17, the use of EDTA can improve sensitivity, and assist in making possible assays operating even below 0.1 ppm melamine.

Other detection technologies employ magnetic particles or microbeads, for example, superparamagnetic iron oxide impregnated polymer beads. These beads are associated with, for example, a specific binding partner for the analyte. The beads bind with the target analytes in the sample being tested and are then typically isolated or separated out of solution magnetically. Once isolation has occurred, other testing may be conducted, including observing particular images or labels, whether directly optically or by means of a camera.

In one specific embodiment, the immunoassay methodologies are competitive immunoassays for detection of melamine. The competitive immunoassay may be carried out in the following illustrative manner. A sample to be analyzed for melamine is contacted with a melamine analog conjugated to a solid support and with an anti-melamine antibody conjugated to a detectable label. Any melamine present in the sample competes with the
melamine analog conjugated to the solid support for binding with the anti-melamine antibody conjugated to the detectable label. The amount of the label associated with the melamine analog conjugated to the solid support can be determined after unbound antibodies and antibodies bound to the melamine from the sample. In an alternative embodiment, the competitive immunoassay is carried out in the following illustrative manner. A sample to be analyzed for melamine, is contacted with an melamine analog linked to a detectable label and with an anti-melamine antibody conjugated to a solid support. Any melamine present in the sample competes with the melamine analog linked to the detectable label for binding with the anti-melamine antibodies conjugated the solid support. In either case, the signal obtained is inversely related to the amount of melamine present in the sample.

[0073] FIGURES 1, 2 and 3 show the results of a lateral flow immunoassay for melamine in commingled bovine milk spiked with melamine. A melamine analog was immobilized on a solid phase and an anti-melamine antibody-HRP conjugate (anti-DAT-SMCC-HRP) was mixed with the sample. After the sample was applied to the solid phase, the solid phase was washed and the amount of signal from the HRP was determined after the addition of the substrate appropriate for the label. The amount of the label on the solid phase is inversely proportional to the amount of melamine in the sample.

[0074] FIGURE 1 shows the relative signals associated with the background, and the control and test areas on a solid phase. The signal from the sample was compared to the signal from a negative control signal associated with anti-chicken IgG antibody spotted on the solid phase. As shown in FIGURE 2, the control/sample (C/S) ratio shows that for samples that had no melamine, the C/S ratio was 0.72 and 0.69 on day 1 and day 2, respectively. The C/S ratio increased in for samples containing 1.5, 2.0 and 2.5 ppm melamine, reflecting a decrease in signal from the sample as compared to the control. Ten
samples were run at each concentration on day 1. On day 2, 15 samples were run for 0 and 2.5 ppm, and 10 samples were run for 1.5 and 2.0 ppm. The 1.06 ratio C/S threshold indicates a color change that is visibly different to an ordinary observer. FIGURE 3 is based on the same data as shown in FIGURE 1, and shows that 100% of samples containing 2.0 or 2.5 ppm provided a positive signal (i.e., C/S > 1.06). FIGURE 3 also shows that the method did not provide any false positive results for samples that did not contain melamine.

[0075] FIGURE 4 shows the results for another lateral flow immunoassay for the detection of melamine in commingled bovine milk samples. In this immunoassay, an anti-DAT antibody (raised against DAT-BSA as described herein) was bound to the solid phase on a test strip. Melamine conjugated to horseradish peroxidase through a PEG linker was mixed with the sample before disposing it onto the test strip. Increasing levels of melamine in the milk sample showed increased C/S ratios.

[0076] FIGURE 5 shows the results of another lateral flow immunoassay for detecting melamine in commingled bovine milk samples spiked with melamine. In this experiment, DAT-ovalbumin was bound to the solid phase. Anti-DAT-SMCC-HRP conjugate (prepared as described herein) was mixed with the sample prior to adding the sample to a test strip. C/S ratios were calculated as described for the experiment depicted in FIGURE 1. Increasing levels of melamine in the milk samples showed increased C/S ratios.

[0077] In one aspect the device of the invention is directed to the use of a method that provides a qualitative determination of the presence of melamine in a sample. For example, a device can provide a positive result when the amount of melamine in a sample is above a predetermined cut-off amount. In various embodiments, the cut off is commercially and physiologically significant. In one example, the device provides a positive result for a sample containing greater than about 2.5 ppm melamine, a level that the United States Food and Drug
Administration and the World Health Organization has suggested to be generally tolerable.

In one aspect, the device provides a positive result above a cut-off value, but does not provide a false positive signal for samples containing less than the cut-off value. In addition, the assays disclosed herein can be engineered to detect melamine levels below 2.5 ppm if desired. For example, in another embodiment, the device provides a positive result for a sample containing greater than about 1 ppm melamine, for a sample containing greater than about 0.5 ppm melamine, or even for a sample containing greater than about 0.1 ppm melamine.

[0078] The invention further provides diagnostic kits containing reagents for use in detecting melamine. Typically, such a kit contains at least one melamine analog and/or at least one anti-melamine antibody. Kits typically also includes directions or instructions describing how to perform the above-described diagnostic assays, and/or how to interpret the results thereby obtained. In some kits, anti-melamine antibodies are linked to an immobilized solid support and/or the melamine analog is immobilized on a solid support. The kits can also include an anti-melamine antibody linked to an appropriate label, or a melamine analog linked to an appropriate label. For example, in one embodiment, a kit comprises an anti-melamine antibody (e.g., an anti-DAT antibody), and a DAT moiety conjugated to a label (either directly or through a linker). In another embodiment, a kit comprises an anti-melamine antibody (e.g., an anti-DAT antibody) conjugated to a label, and a DAT moiety bound to a solid phase. The DAT moiety conjugated to the solid phase can be linked directly to a protein (e.g., DAT-ovalbumin), or in other embodiments linked to a protein or to the solid phase through the intermediary of a linker.

[0079] In one particular example, such a kit would include a device complete with specific binding reagents (e.g., a non-immobilized labeled specific binding reagent and an
immobilized analyte capture reagent) and wash reagent, as well as detector reagent and positive and negative control reagents, if desired or appropriate. In addition, other additives can be included, such as stabilizers, buffers, and the like. The relative amounts of the various reagents can be varied, to provide for concentrations in solution of the reagents that substantially optimize the sensitivity of the assay. Particularly, the reagents can be provided as dry powders, usually lyophilized, which on dissolution will provide for a reagent solution having the appropriate concentrations for combining with a sample. For example, the anti-DAT conjugates described herein can be provided as lyophilized powders, with good assay results.

[0080] The device may also include a liquid reagent that transports unbound material (e.g., unreacted fluid sample and unbound specific binding reagents) away from the reaction zone (solid phase). A liquid reagent can be a wash reagent and serve only to remove unbound material from the reaction zone, or it can include a detector reagent and serve to both remove unbound material and facilitate analyte detection. For example, in the case of a specific binding reagent conjugated to an enzyme, the detector reagent includes a substrate that produces a detectable signal upon reaction with the enzyme-antibody conjugate at the reactive zone. In the case of a labeled specific binding reagent conjugated to a radioactive, fluorescent, or light-absorbing molecule, the detector reagent acts merely as a wash solution facilitating detection of complex formation at the reactive zone by washing away unbound labeled reagent.

[0081] Two or more liquid reagents can be present in a device, for example, a device can comprise a liquid reagent that acts as a wash reagent and a liquid reagent that acts as a detector reagent and facilitates analyte detection.
A liquid reagent can further include a limited quantity of an "inhibitor", i.e., a substance that blocks the development of the detectable end product. A limited quantity is an amount of inhibitor sufficient to block end product development until most or all excess, unbound material is transported away from the second region, at which time detectable end product is produced.

Without further elaboration, it is believed that one skilled in the art using the preceding description can utilize the invention to the fullest extent. Other features and advantages of the invention will be apparent from the following Examples. The following are provided for exemplification purposes only and are not intended to limit the scope of the invention described in broad terms above. All references cited in this disclosure are incorporated herein by reference.

**Examples**

**Example 1: Conjugation of DAT to Proteins**

DAT-BSA conjugates were synthesized by reacting concentrations of 13 mM CDAT, 38 mg/mL BSA, 6.3 mM octanesulfonic acid in 0.4 M sodium carbonate buffer, pH 8-11, with 25% added DMSO for 12 hours at 56 °C. The DAT/BSA ratio was measured using the methods described below in Example 2. Results are summarized in Table 1, below. For these examples, the molar ratio of DAT to BSA ranged from 4.5 to 9.7. The molar ratio was determined by measuring absorbance of the conjugate at 237 nm (A\textsubscript{237}) and 280 nm (A\textsubscript{280}). The absorbance at 237 nm arising from the BSA part of the DAT-BSA conjugate was estimated using the formula BSA A\textsubscript{237} = 2.54-(A\textsubscript{280}). Subtracting the BSA A\textsubscript{237} from A\textsubscript{237} yielded DAT A\textsubscript{237}, the absorbance at 237 nm of the DAT portion of the DAT/BSA conjugate. The extinction coefficient at 237 nm of the DAT portion is estimated to be 14250 M\textsuperscript{-1}cm\textsuperscript{-1}. 

30
using ε-caproyl-melamine as a calibrator. This extinction was used to calculate the DAT concentration, which in turn was used to calculate the molar ratio.

Table 1

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp °C</th>
<th>A237 (cm⁻¹)</th>
<th>A280 (cm⁻¹)</th>
<th>BSA A237 (cm⁻¹)</th>
<th>DAT A237 (cm⁻¹)</th>
<th>Protein (mg/mL)</th>
<th>Protein (μM)</th>
<th>DAT (μM)</th>
<th>mol DAT/mol BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>56</td>
<td>0.978</td>
<td>0.219</td>
<td>0.556</td>
<td>0.422</td>
<td>0.335</td>
<td>5</td>
<td>29.6</td>
<td>5.9</td>
</tr>
<tr>
<td>9</td>
<td>56</td>
<td>1.475</td>
<td>0.269</td>
<td>0.683</td>
<td>0.792</td>
<td>0.411</td>
<td>6.1</td>
<td>55.6</td>
<td>9.1</td>
</tr>
<tr>
<td>10</td>
<td>56</td>
<td>1.158</td>
<td>0.231</td>
<td>0.587</td>
<td>0.571</td>
<td>0.353</td>
<td>5.3</td>
<td>40.1</td>
<td>7.6</td>
</tr>
<tr>
<td>11</td>
<td>56</td>
<td>1.12</td>
<td>0.218</td>
<td>0.526</td>
<td>0.566</td>
<td>0.275</td>
<td>4.1</td>
<td>39.7</td>
<td>9.7</td>
</tr>
<tr>
<td>11</td>
<td>25</td>
<td>0.914</td>
<td>0.228</td>
<td>0.579</td>
<td>0.335</td>
<td>0.349</td>
<td>5.2</td>
<td>23.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

DAT-BSA conjugates were also synthesized in a DMSO-free preparation, by reacting concentrations of 2-20 mM CDAT, 45 mg/mL BSA, 10 mM octanesulfonic acid in 0.3 M borate buffer, at pH 9.5, 24-48 hours at 56 °C. FIGURE 6 is a graph of the mole ratio of DAT/BSA in the product conjugate vs. the concentration of CDAT used in the preparation.

DAT-ovalbumin and DAT-lactalbumin conjugates were synthesized by reacting 1 mM protein with 2-20 mM CDAT at pH 9.5 in 0.3 M borate buffer for 24 hours at 56 °C. As shown in FIGURE 7, the ratio of DAT/ovalbumin is relatively linear with CDAT concentration. FIGURE 7 is a graph showing the incorporation of DAT into a DAT-ovalbumin conjugate for a range of CDAT concentrations and two different temperatures. FIGURE 8 is a graph showing the incorporation of DAT into a DAT-ovalbumin conjugate for a range of CDAT concentrations and two different reaction times. As shown in FIGURES 7 and 8, incorporation of DAT is not critically dependent on temperature or time.

DAT-ovalbumin with an average DAT/protein ratio of 3.65 was produced by incubating 200 mL of 46 mg/mL ovalbumin in 0.3 M borate buffer, pH 9.5, with 134.5 mg CDAT at 56 °C for 24 hours, followed by removal of a small amount of aggregated protein.
by centrifugation for 5 minutes at 10000 x g or vacuum filtration using a 0.8 µm filter fitted with a glass fiber pad. The filtrate is then dialyzed for 6-24 hours against 2 changes of at least 50 volumes each of 10 mM phosphate/150 mM sodium chloride buffer, pH 8.0.

**Example 2: Assay to determine coupling efficiency**

[0088] UV pH difference spectroscopy at pH 2.5 and pH 9.0, and wavelengths of 240 nm and 280 nm was used to quantify the amount of DAT coupled to ovalbumin. ε-Aminocaproylmelamine (synthesized by reaction of 10 mM CDAT, 20 mM ε-Aminocaproic acid and 20 mM triethylamine at 85 °C for 4 hours) was used to create a standard curve; the extinction coefficient of ε-aminocaproylmelamine in the difference spectrum was determined to be 9200 M⁻¹cm⁻¹ at 240 nm. The ratio of absorbances at 240 nm and 280 nm in 10 mM phosphoric acid correlates with DAT/ovalbumin ratio, as shown in FIGURE 9.

[0089] Accordingly, once a standard curve is created for a given DAT/protein combination using UV pH difference spectroscopy, the ratio of absorbances can be used to determine the DAT/protein ratio of a sample.

**Example 3: Synthesis of melamine-label conjugates**

[0090] One equivalent of CDAT was reacted with one equivalent of di(2-aminoethyl)ether in the presence of one equivalent of triethylamine at 85 °C for 5 h in aqueous solution to formN 2-(2-(2-aminoethoxy)ethyl)-1,3,5-triazine-2,4,6-triamine ("OAEM").

[0091] 1.14 mM HRP was reacted with 50 mM OAEM and 6, 12, and 24 mM 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in MES buffer for one hour at room
temperature to yield melamine-label conjugates having average incorporation of melamine of 0.7, 0.9, and 1.2 mol OAEM/mol HRP, respectively.

[0092] 0.41 mM succinyl-HRP was reacted with 50 mM OAEM and 7.5, 15, and 30 nM EEDQ in MES buffer for one hour at room temperature to yield melamine-label conjugates having average incorporation of melamine of 2.5, 3.2 and 4.2 mol OAEM/mol HRP, respectively.

[0093] A variety of OAEM-HRP conjugates having a variety of OAEM/HRP ratios were used as the label in a commercially available melamine immunoassay kit from Beacon Analytical Systems (Portland, Maine). Results are shown in FIGURE 10. Notably, the OAEM-HRP conjugates produced comparable or much higher signal for 0.5 ppm melamine than that obtained using the label from the kit at comparable concentrations (1 µg/mL).

Example 4: Production of anti-melamine antibodies

[0094] Antibodies were produced using standard immunization protocol at Strategic Diagnostics Inc. (Newark, Delaware). Rabbits were immunized by subcutaneous administration of 0.2 mg DAT-BSA having an average DAT:BSA mole ratio of 8.5 in Freund's complete adjuvant. Animals were boosted biweekly with 0.2 mg DAT-BSA in incomplete Freund's adjuvant, resulting in maximal titer after the second boost. Blood was collected at week six, then periodically thereafter. Continued antibody production using monthly boosts produced similar titers for at least several months afterward. The assays described with reference to FIGS. 1-3 were performed with blood collected at 6 weeks after immunization. The assays described with reference to FIGS. 14-17 were performed with blood collected at 25 weeks after immunization.
Antibodies were collected and purified as follows: Anti-DAT rabbit antiserum was diluted with an equal volume of 0.1 M phosphate buffered saline ("PBS"), pH 7.2, then centrifuged at 8000 x g for 20 min at 4 °C, filtered through a 0.45 µm filter, and applied to a Protein G column equilibrated with PBS, pH 7.2. After washing with PBS, antibody was eluted with 0.1 M sodium citrate, 25% ethylene glycol, pH 2.5, pooled and neutralized to pH 7.2 with 0.2 M sodium carbonate, pH 9.4.

The anti-DAT antibody was shown to be an IgG by size exclusion chromatography, (MW=150 kDa for rabbit IgG) and immunoreactivity against goat anti-rabbit IgG.

**Example 5: Competitive immunoassay for detecting melamine**

Anti-DAT-SMCC-HRP was prepared using rabbit polyclonal anti-DAT antibodies (made as described above using DAT-BSA). The conjugate was prepared by reducing a 5 mg/mL solution of antibody with 1/100 volume of 1M dithiothreitol ("DTT"), gel-filtering to remove excess DTT, then adding to HRP modified with 0.5 - 1.5 mol/mol of SMCC. 1.5 mg of SMCC-HRP was added per mg of antibody.

DAT-ovalbumin having a DAT/ovalbumin ratio of 4.7 was prepared as described above.

Competitive assays were performed for melamine as well as a variety of possible interferents. Competitive ELISA of aqueous samples of melamine, CDAT and other potential interferents were performed according to the procedure of Magnotti et al., Clinical Chemistry 1989;35:1371-1375, using 0.3 µg/mL anti-DAT-SMCC-HRP for the conjugate, coating with 0.1 µg/mL DAT-ovalbumin instead of albumin, and using a commercial TMB reagent ("TMBHK," Moss, Inc.) for color development. Samples were diluted from 300 ppm stocks.
made up in either water, 5 mM HCl, or 5 mM NaOH, then diluted 10 - 1,000 fold into ELISA sample diluent. Data for the melamine assay is presented in FIGURE 11, and data for CDAT as an interferent is shown in FIGURE 12.

[00100] In addition to the small (but significant) interference from CDAT, slight interference was observed at 30 ppm levels from the congeners 2-methyl-4,6-diaminotriazine and 6-chloro-2,4-diaminopyrimidine. There was no interference up to at least 30 ppm from the triazine herbicides (atrazine, simazine and cyanazine) or their metabolites (desethylatrazine and desisopropylatrazine), or from the melamine metabolites ammeline and ammelide. A barely detectable level of interference was observed for 30 ppm cyanuric acid, which will also interfered with the Abraxis kit (LDD 0.4 ppm). Table 2 compares the responses of the ELISA of this example to those of a commercially available kit (Abraxis Melamine ELISA kit; data presented is derived from the package insert).

<table>
<thead>
<tr>
<th>%inhibition</th>
<th>Ex. 5 ppm</th>
<th>Abraxis kit - derived from package insert</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEL</td>
<td>CDAT</td>
</tr>
<tr>
<td>50</td>
<td>0.4</td>
<td>12</td>
</tr>
<tr>
<td>90</td>
<td>3.4</td>
<td>79</td>
</tr>
<tr>
<td>LDD</td>
<td>0.1</td>
<td>5</td>
</tr>
</tbody>
</table>

[00101] A graph of response vs. melamine concentration for a similarly-performed set of ELISA experiments is presented in FIGURE 13, which demonstrates a limit of detection on the order of 3 ppb.

**Example 6: Lateral flow immunoassays for detecting melamine**

[00102] The lateral flow immunoassay of FIGURES 1, 2 and 3 was performed as follows: The solid phase of the SNAP® assay device includes both sample and control spots. The
sample spot has bound thereto melamine-PEG4 particles, which were made by conjugating DAT-PEG4-COOH with Amino-link Particles. The DAT-PEG4-COOH was synthesized by dissolving PEG4-COOH (0.1 g) in 0.1 M sodium carbonate (pH 11.46, 10 mL), then adding CDAT (0.065 g) and heating the resulting white suspension in an oil bath at 70 °C. The reaction was monitored by Thin Layer Chromatography (TLC). The reaction mixture was transferred to a 100 mL round bottom flask and concentrated by rotary evaporation to yield a white solid residue, which was dissolved in methanol (30 mL) filtered through filter paper, and concentrated to roughly 10 mL volume by rotary evaporation. TLC of the crude methanolic solution showed two spots (via UV visualization). The crude material in methanol was purified by column chromatography (silica gel, 1.25" x 6" column, eluting with 1:2 ethyl acetate methanol (~200 mL), then methanol). Solvent was removed to give DAT-PEG4-COOH as a clear to white solid. DAT-PEG4-COOH was conjugated to Amino-link Particles by putting a suspension of the Amino-link particles (0.5 µm, 8 mL, 5% solid) into a 50 mL conical tube, centrifuging (13 g, 10 min), then removing the supernatant. DAT-PEG4-COOH (0.1 g) was weighed into a 50 mL conical tube. DMSO (4 mL) was first added to the tube to dissolve the solid, and then PBS (16 mL) was added to give a clear solution, followed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) (0.1 M, 0.38 g) and N-hydroxysuccinimide (NHS) (0.1 M, 0.23 g). The resulting mixture was added to the conical tube containing the Amino-link Particles. The particles were resuspended by probe sonication. The reaction mixture was rotated at room temperature for 12 hour then the particles were spun down by centrifugation (13 g, 10 min), and supernatant was removed. The particles were resuspended in CAPS buffer (0.05 M CAPS, 2.5% Sucrose, 0.05% Tween, pH 10.5). These particles were spotted in the sample spot of the solid phase of a SNAP® immunoassay device. Particles for the control spot were made by passively coating chicken IgG (0.2 mg/ml) on 0.5 µM polystyrene particles. The particles and chicken IgG were mixed
in 10 mM sodium phosphate buffer (pH 7.2). The mixture was rotated overnight at 4 °C. The following day the particles were centrifuged and resuspended in 10 mM sodium phosphate buffer containing 2% BSA for 1 h at room temperature. The particles were then centrifuged in 10 mM sodium phosphate buffer containing 2.5% sucrose. Finally, the particles were brought to 1% solids again and spotted on the solid phase. Goat anti chicken-HRP conjugate was made using periodate chemistry as is common in the art.

[00103] Lateral flow immunoassays were performed by mixing 20 µL of a mixture of anti-DAT-SMCC-HRP and goat-anti chicken-HRP conjugate with 450 µL of a sample (commingled milk spiked with melamine). The mixture was incubated at 45 °C for 2 minutes then added to the SNAP® device. When the sample reached the activation circle, the device was activated to release the onboard reagents, including 400 µL of wash reagent (aqueous solution of barbital buffer (60 mM), sodium chloride (58 mM), fish gelatin (0.1%), PROCLIN 150 preservative (0.05%), Dow Corning 193 fluid (0.05%)), followed by 600 µL of tetramethylbenzylidine as the color development substrate. At the end of 8 min, the device was read both visually and in a SNAPSHOT reader (IDEXX Laboratories). A control to sample ratio less than 1.06 indicated a negative result. Data for these experiments is provided in FIGURES 1, 2 and 3.

[00104] The lateral flow immunoassay of FIGURE 4 was performed as follows: Anti-DAT antibody (0.5 mg/mL) was passively coated onto polystyrene particles as described above for the coating of chicken IgG. Chicken IgG-coated polystyrene particles for the control spot were prepared as described above. A DAT-PEG-HRP conjugate was prepared by mixing DAT-PEG4-COOH (5.0 mg), EDAC (3.0 mg), NHS (3.0 mg) and DMSO (0.1 mL) in a 1.5mL Eppendorf tube. The tube was briefly sonicated (< 1 min) until all solids were dissolved, then shook at room temperature for 15 min. Then 0.9 mL PBS was added to
give a clear solution. This solution was then added to another 1.5 mL Eppendorf tube containing HRP (40 mg). Upon addition, the clear solution became dark brown. The tube was then wrapped in aluminum foil, placed on a rotator and the reaction mixture was allowed to react overnight (12 hours) at room temp. The reaction mixture was purified using a zepak desalting column to provide DAT-PEG-HRP conjugate.

[00105] Lateral flow immunoassays were performed as described above with respect to FIGURES 1, 2 and 3, but using the DAT-PEG-HRP conjugate in place of the anti-DAT-SMCC-HRP. Data for these experiments is provided in FIGURE 4.

[00106] The lateral flow immunoassay of FIGURE 5 was performed as described above with respect to FIGURES 1, 2 and 3 but using DAT-ovalbumin in the solid phase: DAT-ovalbumin (0.5 mg/mL) was passively coated onto polystyrene particles as described above for the coating of chicken IgG. Lateral flow immunoassays were performed as described above with respect to FIGURES 1, 2 and 3. Data for these experiments is provided in FIGURE 5.

[00107] The lateral flow immunoassays of FIGURE 14 were performed as described above with respect to FIGURES 1, 2 and 3, with two differences. First, only 300 µL (instead of 400 µL) of wash reagent was used. Second, the blood used for antibody collection was collected at 25 weeks after immunization (instead of 6 weeks after immunization), as noted above in Example 4.

[00108] FIGURE 14 shows the control/sample ratio for melamine concentrations of 0 - 2.5 ppm in commingled raw milk samples. The average control/sample ratio for 0.1 ppm, as well as all points measured for 0.1 ppm, are well above 1.06, the threshold for a positive assay result. Accordingly, this assay is capable of measuring melamine at 0.1 ppm.
The lateral flow immunoassays of FIGURES 15, 16 and 17 were performed as described above with respect to FIGURE 14, with the following differences. In the assay of FIGURE 15, the samples were raw milk, both with and without 0.1 ppm melamine, and both with and without 1.1 mM added EDTA (as ethylenediaminetetraacetic acid, disodium salt). In the assay of FIGURE 16, the samples were hydrated full cream milk powder. 11.2 g full cream milk powder was mixed in 100 mL of 75 °C deionized water spiked with 0 or 0.1 ppm melamine, for at least 5 minutes, ensuring that the powder was homogenously distributed in the solution. The solution was allowed to mix at room temperature for a minute, then cooled on ice. Samples of 450 µL of cooled milk were spiked with EDTA (at 3.9, 5.9 or 7.7 mM), then assayed as above. In the assay of FIGURE 17, the samples were hydrated skim milk powder. 8.3 g skim milk powder was mixed in 100 mL of 40 °C deionized water spiked with 0 or 0.1 ppm melamine for at least 1 minute, ensuring that the powder was homogenously distributed in the solution. The solution was allowed to mix at room temperature for 5 minutes. Samples of 450 µL of cooled milk were spiked with 0 or 7.7 mM EDTA, then assayed as above.

FIGURES 15 and 17 show the results for these immunoassays. In each, the assays performed with EDTA exhibited higher sensitivity than did the corresponding assays performed without EDTA. In FIGURE 16, assays performed with increased concentrations of EDTA exhibit higher sensitivities. The water solubilized full cream milk sample would not flow on the SNAP® device without EDTA). However, all assays—both those with EDTA and those without—exhibited sensitivity at 0.1 ppm melamine.

The examples given above are merely illustrative and are not meant to be an exhaustive list of all possible embodiments, applications or modifications of the invention. Thus, various modifications and variations of the described methods and systems of the
invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology, immunology, chemistry, biochemistry or in the relevant fields are intended to be within the scope of the appended claims.

[0012] It is understood that the invention is not limited to the particular methodology, protocols, and reagents, etc., described herein, as these may vary as the skilled artisan will recognize. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only, and is not intended to limit the scope of the invention. It also is to be noted that, as used herein and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a linker" is a reference to one or more linkers and equivalents thereof known to those skilled in the art.

[0013] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which the invention pertains. The embodiments of the invention and the various features and advantageous details thereof are explained more fully with reference to the non-limiting embodiments and/or illustrated in the accompanying drawings and detailed in the following description. It should be noted that the features illustrated in the drawings are not necessarily drawn to scale, and features of one embodiment may be employed with other embodiments as the skilled artisan would recognize, even if not explicitly stated herein.
Any numerical values recited herein include all values from the lower value to the upper value in increments of one unit provided that there is a separation of at least two units between any lower value and any higher value. As an example, if it is stated that the concentration of a component or value of a process variable such as, for example, size, angle size, pressure, time and the like, is, for example, from 1 to 90, specifically from 20 to 80, more specifically from 30 to 70, it is intended that values such as 15 to 85, 22 to 68, 43 to 51, 30 to 32, etc. are expressly enumerated in this specification. For values which are less than one, one unit is considered to be 0.0001, 0.001, 0.01 or 0.1 as appropriate. These are only examples of what is specifically intended and all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application in a similar manner.

Particular methods, devices, and materials are described, although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention. The disclosures of all references and publications cited above are expressly incorporated by reference in their entireties to the same extent as if each were incorporated by reference individually.

U.S. Provisional Patent Application serial no. 61/158,258 is hereby incorporated herein by reference in its entirety.
WHAT IS CLAIMED IS:

1. A conjugate having the formula

```
    H2N
   /   \
  N---N
   \   /
    H2N
```

or a salt thereof, wherein the P moiety is the protein, m is an integer in the range of 1-1000.

2. The conjugate according to claim 1 wherein the protein is a carrier protein, and the melamine-protein conjugate is a hapten-carrier immunogen.

3. The conjugate according to claim 2, wherein the protein is BSA, KLH, lactalbumin or ovalbumin.

4. The conjugate according to any of claims 1-3, wherein the DAT/protein ratio is greater than about 5.

5. The conjugate according to claim 1 wherein the protein is a label.

6. The conjugate according to claim 5, wherein the protein is horseradish peroxidase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, avidin or streptavidin.

7. The conjugate according to any of claims 5-6, wherein the DAT/protein ratio is in the range of 1-15.

8. An anti-melamine antibody raised against the conjugate of any of claims 1-4.
9. An anti-melamine antibody according to claim 8, exhibiting substantially reduced cross-reactivity to ammeline, ammelide, CDAT, cyanuric acid, 2-chloro-4,6-diaminotriazine, 2-methyl-4,6-diaminotriazine, 6-hydroxy-2,4-diaminopyrimidine; 6-chloro-2,4-diaminopyrimidine, atrazine, desethylatrazine, desisopropylatrazine, 2-hydroxyatrazine, simazine and cyanazine.

10. A method for detecting the presence or amount of melamine in a sample, the method comprising:
   
   contacting the sample with the anti-melamine antibody of claim 8 or claim 9, and a melamine analog conjugated to a label, and
   
   detecting the presence or amount of the label associated with the antibody, thereby determining the presence or amount of melamine in the sample.

11. The method of claim 10, wherein the melamine analog comprises an OAEM moiety.

12. A method for detecting the presence or amount of melamine in a sample, the method comprising:
   
   contacting the sample with the anti-melamine antibody of claim 8 or claim 9 conjugated to a label, and a melamine analog;
   
   detecting the presence or amount of the label associated with the melamine analog, thereby determining the presence or amount of melamine in the sample.

13. The method of claim 12, wherein the melamine analog comprises DAT-ovalbumin, DAT-thyroglobulin or DAT-BSA.

14. The method of claim 12 or claim 13, wherein the sample is also contacted with EDTA.
15. A device for determining the presence or amount of melamine in a sample, the device comprising a solid phase having non-diffusively bound thereto the antibody of claim 8 or claim 9.

16. The device of claim 15 wherein the device is a lateral flow device and the solid phase is a porous carrier matrix.

17. A kit for determining the presence or amount of melamine in a sample, the kit comprising DAT moiety conjugated to a label and the device of claim 15.

18. A kit for determining the presence or amount of melamine in a sample comprising the antibody of claim 8 or claim 9 conjugated to label and a device comprising a solid phase having non-diffusively bound thereto a melamine analog.

19. The kit of claim 18 wherein the device is a lateral flow device and the solid phase is a porous carrier matrix.

20. The kit of claim 18, wherein the melamine analog comprises DAT-BSA, DAT-ovalbumin or DAT-thyroglobulin.

21. A method for detecting the presence or amount of melamine in a sample, the method comprising:

   providing a lateral flow assay device comprising a solid phase having non-diffusively bound thereto a melamine analog;

   providing a sample;

   contacting the sample with a melamine analog and an anti-melamine antibody conjugated to a label; and

   detecting the presence or amount of the label associated with the melamine analog, thereby determining the presence or amount of melamine in the
sample, wherein the detectable presence or amount of melamine in the sample is at least about 2.5 parts per million.

22. The method according to claim 21, wherein the detectable presence or amount of melamine in the sample is at least about 1 ppm.

23. The method according to claim 21, wherein the detectable presence or amount of melamine in the sample is at least about 0.1 ppm.

24. An anti-melamine antibody exhibiting substantially reduced cross-reactivity to ammeline, ammelide, CDAT, cyanuric acid, 2-chloro-4,6-diaminotriazine, 2-methyl-4,6-diaminotriazine, 6-hydroxy-2,4-diaminopyrimidine, 6-chloro-2,4-diaminopyrimidine, atrazine, desethylatrazine, desisopropylatrazine, 2-hydroxyatrazine, simazine and cyanazine.
FIGURE 1

FIGURE 2
FIGURE 3

FIGURE 4
values provided are average C/S ratio values

FIGURE 5

CDAT RXN with BSA at 56°C

\[ y = 3.8728x^{0.8338} \]

\[ R^2 = 0.9937 \]

FIGURE 6
**FIGURE 7**

24 hr vs. 48 hr at 56°C

**FIGURE 8**

CDAT-Ovalbumin RXN, 51°C vs. 56°C
FIGURE 15
FIGURE 16

FIGURE 17
A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/53 G01N33/531 C07K16/00

According to International Patent Classification (IPC) or to both national classification and IPC:

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched:

Electronic data base consulted during the international search (name of data base and, where practical, search terms used):

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
</table>

Further documents are listed in the continuation of Box C

See patent family annex

* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another document, or to put the invention in a different context, or to be an earlier disclosure
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search

28 May 2010

Date of mailing of the international search report

28/06/2010

Name and mailing address of the ISA:

European Patent Office, P B 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel (+31-70) 340-2040, Fax (+31-70) 340-3016

Authorized officer

Gunster, Marco
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

Form PCT/ISA/210 (continuation of second sheet) (April 2005)
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>x,P</td>
<td>DATABASE WPI Week 201020 Thomson Scientific, London, GB; AN 2010-C31571 XP002584797 &amp; CN 101 643 454 A (BEIJING WDW BIOTECH CO LTD) 10 February 2010 (2010-02-10) abstract</td>
<td>1-24</td>
</tr>
<tr>
<td>x,P</td>
<td>DATABASE WPI Week 201022 Thomson Scientific, London, GB; AN 2010-C31572 XP002584798 &amp; CN 101 643 453 A (BEIJING WDW BIOTECH CO LTD) 10 February 2010 (2010-02-10) abstract</td>
<td>1-24</td>
</tr>
<tr>
<td>Category</td>
<td>Citation of document, with indication, where appropriate, of the relevant passages</td>
<td>Relevant to claim</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Patent document cited in search report</td>
<td>Publication date</td>
<td>Patent family member(s)</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>-----------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>CN 101183105 A</td>
<td>21-05-2008</td>
<td>NONE</td>
</tr>
<tr>
<td>CN 101206223 A</td>
<td>25-06-2008</td>
<td>NONE</td>
</tr>
<tr>
<td>CN 101363850 A</td>
<td>11-02-2009</td>
<td>NONE</td>
</tr>
<tr>
<td>CN 101402683 A</td>
<td>08-04-2009</td>
<td>NONE</td>
</tr>
<tr>
<td>CN 101464354 A</td>
<td>10-02-2010</td>
<td>NONE</td>
</tr>
<tr>
<td>CN 101643453 A</td>
<td>10-02-2010</td>
<td>NONE</td>
</tr>
<tr>
<td>CN 101597267 A</td>
<td>09-12-2009</td>
<td>NONE</td>
</tr>
<tr>
<td>CN 101566627 A</td>
<td>28-10-2009</td>
<td>NONE</td>
</tr>
<tr>
<td>CN 101477124 A</td>
<td>08-07-2009</td>
<td>NONE</td>
</tr>
</tbody>
</table>