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(54) **Title:** METHODS FOR SELECTIVE AND SENSITIVE MILK FAT QUANTITATION AND MILK QUALITY CONTROL USING BODIPY-BASED MILK FAT FLUORESCENT SENSORS

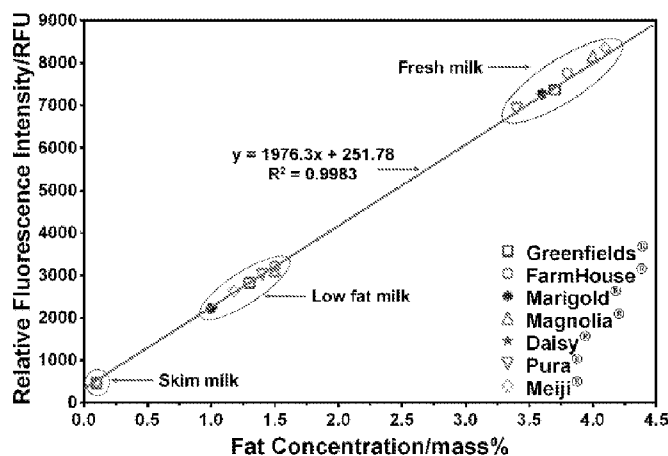


FIG. 4

(57) **Abstract:** Methods for the fluorescence-based detection and quantification of milk fat in milk using a BODIPY-based fluorescent sensor are disclosed. The BODIPY-based fluorescent sensors of the invention are selective for fat molecules being unaffected by the presence of other milk ingredients, such as proteins and carbohydrates. The methods provide a convenient and rapid tool for milk fat detection.

METHODS FOR SELECTIVE AND SENSITIVE MILK FAT QUANTITATION AND MILK QUALITY CONTROL USING BODIPY-BASED MILK FAT FLUORESCENT SENSORS

RELATED APPLICATION(S)

[0001] This application claims the benefit of U.S. Provisional Application No.: 62/011,869 filed June 13, 2014. The entire teachings of the above application are incorporated herein by reference. The entire teachings of the above application(s) are incorporated herein by reference.

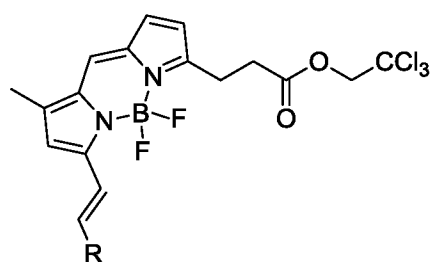
BACKGROUND OF THE INVENTION

[0002] Traditional milk fat detection methods include many procedures, such as destabilization of fat by adding sulfuric acid, separation of the fatty acids by centrifugation and measurement. The complicated handling and use of toxic corrosive chemicals make it unfavorable for untrained people. Instruments utilizing infrared absorbance of specific bond vibrations are available, however their high.

[0003] Currently, there is no easy tool available to measure milk fat level rapidly and quantitatively. Nor is there any fluorescent sensor targeting milk fat. Most methods require sophisticated and expensive instruments and well-trained hands to operate them, thus they are not applicable to resource-limited regions or normal dairy farms. Therefore, a need remains for safer and more cost effective methods for milk fat detection.

SUMMARY OF THE INVENTION

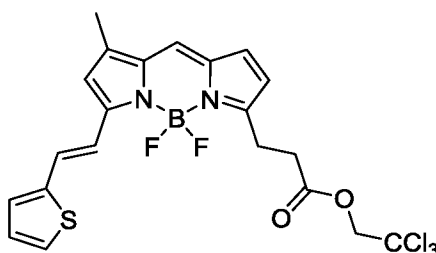
[0004] In a first main aspect, the invention relates to a method for fluorescence-based detection of milk fat in a liquid medium, the method comprising: (a) diluting a sample of milk to a concentration to fully quench background fluorescence signals of the milk, to form a liquid medium; (b) contacting a sample of the liquid medium with a compound of Formula (I), or a pharmaceutically acceptable salt thereof; (c) incubating the sample of step (b) for a period of time sufficient to enable visualization of milk fat by fluorescence if present in the liquid medium; and (d) detecting fluorescence in the incubated sample, wherein a change in fluorescence as compared to fluorescence of the compound of Formula (I) not in the presence of the sample of liquid medium is indicative of the presence of milk fat in the liquid medium. The compound of Formula (I) is represented by the following structure:



(Formula I),

[0005] wherein R is (C₆-C₁₀)aryl, (C₅-C₁₀)heteroaryl, (C₆-C₁₀)aryl(C₂-C₆)alkenyl or 2-4 member polycyclyl, wherein each 2-4 member polycyclyl optionally and independently contains 1-2 ring heteroatoms selected from oxygen, nitrogen and sulfur; and wherein R is optionally and independently substituted with 1-4 substituents independently selected from (C₁-C₆)alkyl, (C₂-C₆)alkenyl, halo(C₁-C₆)alkyl, hydroxy(C₀-C₆)alkyl, (C₁-C₆)alkoxy, halo(C₁-C₆)alkoxy, halogen, amino, nitro, -B(OH)₂, (C₁-C₆)alkyl-S(O)_n, -(C₁-C₆)alkylamino, (C₁-C₆)dialkylamino, (C₃-C₈)cycloalkyl, (C₃-C₈)heterocycloalkyl, (C₆-C₁₀)aryl, and (C₅-C₁₀)heteroaryl, wherein each (C₃-C₈)cycloalkyl, (C₃-C₈)heterocycloalkyl, (C₆-C₁₀)aryl, and (C₅-C₁₀)heteroaryl is optionally and independently substituted with 1-4 substituents independently selected from (C₁-C₆)alkyl, (C₂-C₆)alkenyl, halo(C₁-C₆)alkyl, hydroxy(C₀-C₆)alkyl, (C₁-C₆)alkoxy, halo(C₁-C₆)alkoxy, and halogen; and n is 0, 1, or 2;

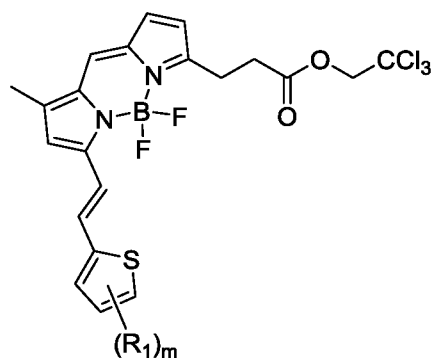
[0006] In one embodiment, R is thiophenyl, optionally substituted with 1-3 substituents independently selected from halogen, halo(C₁-C₆)alkyl, (C₁-C₆)alkoxy, halo(C₁-C₆)alkoxy and hydroxy(C₀-C₆)alkyl. In another embodiment, the compound of Formula (I) is:



[0007] or a pharmaceutically acceptable salt thereof.

[0008] In another aspect, the invention relates to a method for fluorescence-based detection of milk fat in a liquid medium, the method comprising: (a) diluting a sample of milk to a concentration to fully quench background fluorescence signals of the milk, to form a liquid medium; (b) contacting a sample of the liquid medium with a compound of Formula (II), or a pharmaceutically acceptable salt thereof; (c) incubating the sample of step (b) for a period of time sufficient to enable visualization of milk fat by fluorescence if present in the liquid medium; and (d) detecting fluorescence in the incubated sample, wherein a change in fluorescence as

compared to fluorescence of the compound of Formula (II) not in the presence of the sample of liquid medium is indicative of the presence of milk fat in the liquid medium. The compound of Formula (II) is represented by the following structure:



[0009] wherein: each R_1 is independently (C₁-C₆)alkyl, (C₂-C₆)alkenyl, halo(C₁-C₆)alkyl, hydroxy(C₀-C₆)alkyl, (C₁-C₆)alkoxy, halo(C₁-C₆)alkoxy, halogen, amino, nitro, -B(OH)₂, (C₁-C₆)alkyl-S(O)_n, -(C₁-C₆)alkylamino, (C₁-C₆)dialkylamino, (C₃-C₈)cycloalkyl, (C₃-C₈)heterocycloalkyl, (C₆-C₁₀)aryl, or (C₅-C₁₀)heteroaryl, wherein each (C₃-C₈)cycloalkyl, (C₃-C₈)heterocycloalkyl, (C₆-C₁₀)aryl, and (C₅-C₁₀)heteroaryl is optionally and independently substituted with 1-4 substituents independently selected from (C₁-C₆)alkyl, (C₂-C₆)alkenyl, halo(C₁-C₆)alkyl, hydroxy(C₀-C₆)alkyl, (C₁-C₆)alkoxy, halo(C₁-C₆)alkoxy, and halogen; n is 0, 1, or 2; and m is 0, 1, or 2;

[0010] In one embodiment, detecting the incubated sample by fluorescence comprises analysis by fluorescence reader, fluorescence meter or fluorescence spectroscopy. In another embodiment, detecting the incubated sample by fluorescence comprises qualitative visual analysis of the sample.

[0011] The change in fluorescence in the method of the invention can be a change in fluorescence intensity or a change in the color of the fluorescence. In one embodiment, the change in fluorescence intensity is an increase in fluorescence intensity. In another embodiment, the change in the color of the fluorescence is detectable under visible light or a wavelength portion thereof or ultraviolet light.

[0012] In one embodiment, the change in the color of the fluorescence to a yellow-colored fluorescence at a wavelength between about 530 nm and about 700 nm is indicative of the presence of whole milk in the liquid medium. In another embodiment, the change in the color of the fluorescence to a pinkish orange-colored fluorescence at a wavelength between about 530 nm

and about 700 nm is indicative of the presence of low fat milk in the liquid medium. In yet another embodiment, the change in the color of the fluorescence to a pale pink-colored fluorescence at a wavelength between about 530 nm and about 700 nm is indicative of the presence of skim milk in the liquid medium.

[0013] In second aspect, the method of the invention further comprises quantification of the amount of fat present in the liquid medium by measuring the change in fluorescence as compared to fluorescence of the compound of Formula (I) not in the presence of the sample of liquid medium sample.

[0014] In another aspect, the method of the invention further comprises quantification of the amount of fat present in the liquid medium by measuring the change in fluorescence as compared to fluorescence of the compound of Formula (II) not in the presence of the sample of liquid medium sample.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] FIG. 1 shows the structure and chemical/physical information of a milk fat sensor, Milk Orange (MO).

[0016] FIG. 2 illustrates a fluorescence image-based hyper-throughput screening approach. FIG. 2(a) is a schematic of the setup of the black box imaging system used in the hyper-throughput screening. FIG. 2(b) shows a schematic of a 96-well plate that is used for the hyper-throughput screening and fluorescent images acquired before and after addition of milk. FIG. 2(c) shows fluorescence spectrum analysis of a hyper-throughput screening library containing various BODIPY-based fluorescent ligands and milk.

[0017] FIG. 3(a) shows fluorescence spectrum analysis of MO-fat interaction at various at a concentration of fat (0.1% to 3.7%) and 10 μ M MO. FIG. 3(b) is a linear graph of fluorescence intensity and increasing fat concentration.

[0018] FIG. 4 is a graph of illustrating the change in fluorescence intensity as a result of a change in fat concentration. The linear line is derived from one type of milk (Greenfields). All other data sets are plotted on the graph based on their respective fat concentration and fluorescence intensity.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

[0019] All definitions of substituents set forth below are further applicable to the use of the term in conjunction with another substituent.

[0020] “Alkyl” as used alone or as part of a larger moiety as in “arylalkyl” or “aryloxyalkyl” means a saturated aliphatic branched or straight-chain monovalent hydrocarbon radicals, typically C₁-C₁₆, preferably C₁-C₁₂. For example, “(C₁-C₆) alkyl” means a radical having from 1- 6 carbon atoms in a linear or branched arrangement. “(C₁-C₆)alkyl” includes methyl, ethyl, propyl, butyl, *tert*-butyl, pentyl and hexyl.

[0021] “Alkylene” means a saturated aliphatic straight-chain divalent hydrocarbon radical and is represented by $-\text{[CH}_2\text{]}_z-$, wherein *z* is a positive integer, preferably from one to eight, more preferably from one to four. Thus, “(C₁-C₆)alkylene” means a divalent saturated aliphatic radical having from 1- 6 carbon atoms in a linear arrangement. “(C₁-C₆)alkylene” includes methylene, ethylene, propylene, butylene, pentylene and hexylene.

[0022] An “alkenylene group” is an alkylene in which at least a pair of adjacent methylenes are replaced with $-\text{CH}=\text{CH}-$.

[0023] “Heterocyclyl” means a saturated or partially unsaturated (3-7 membered) monocyclic heterocyclic ring containing one nitrogen atom and optionally 1 additional heteroatom independently selected from N, O or S. When one heteroatom is S, it can be optionally mono- or di-oxygenated (*i.e.*, $-\text{S}(\text{O})-$ or $-\text{S}(\text{O})_2-$). Examples of monocyclic heterocycle include, but not limited to, azetidine, pyrrolidine, piperidine, piperazine, hexahydropyrimidine, tetrahydrofuran, tetrahydropyran, morpholine, thiomorpholine, thiomorpholine 1,1-dioxide, tetrahydro-2H-1,2-thiazine, tetrahydro-2H-1,2-thiazine 1,1-dioxide, isothiazolidine, or isothiazolidine 1,1-dioxide.

[0024] “Heterocycloalkyl” means a cyclic 4- to 12-membered saturated aliphatic ring containing 1, 2, 3, 4 or 5 heteroatoms independently selected from N, O or S. When one heteroatom is S, it can be optionally mono- or di-oxygenated (*i.e.* $-\text{S}(\text{O})-$ or $-\text{S}(\text{O})_2-$). When one heteroatom is N, it can be optionally substituted with alkyl, cycloalkyl, alkylene-cycloalkyl, heterocycloalkyl, alkylene-heterocycloalkyl, aryl, alkylene-aryl, heteroaryl, alkylene-heteroaryl, each of which can be optionally substituted with one or more halogen, =O, hydroxy, alkoxy, haloalkyl, alkyl, etc.

[0025] A heterocycloalkyl moiety can be monocyclic, fused bicyclic, bridged bicyclic, spiro bicyclic, or polycyclic. For example, monocyclic (C₃-C₈) heterocycloalkyl means a 3- to 8 membered saturated aliphatic ring containing 1, 2, 3, 4, or 5 heteroatoms independently selected from N, O or S arranged in a monocyclic ring. Examples of monocyclic heterocycloalkyls include, but are not limited to, azetidine, pyrrolidine, piperidine, piperazine, azepane, hexahydropyrimidine, tetrahydrofuran, tetrahydropyran, morpholine, thiomorpholine, thiomorpholine 1,1-dioxide, tetrahydro-2H-1,2-thiazine, tetrahydro-2H-1,2-thiazine 1,1-dioxide, isothiazolidine, isothiazolidine 1,1-dioxide.

[0026] “Cycloalkyl” means saturated aliphatic cyclic hydrocarbon ring. Thus, “C₃-C₈ cycloalkyl” means (3-8 membered) saturated aliphatic cyclic hydrocarbon ring. C₃-C₈ cycloalkyl includes, but is not limited to cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl. Preferably, cycloalkyl is C₃-C₆ cycloalkyl.

[0027] The term “alkoxy” means -O-alkyl; “arylalkoxy” means an alkoxy group substituted at any carbon by an aryl group; “hydroxyalkyl” means alkyl substituted with hydroxy; “arylalkyl” means alkyl substituted with an aryl group; “alkoxyalkyl” mean alkyl substituted with an alkoxy group; “alkylamine” means amine substituted with an alkyl group; “cycloalkylalkyl” means alkyl substituted with cycloalkyl; “dialkylamine” means amine substituted with two alkyl groups; “alkylcarbonyl” means -C(O)-A*, wherein A* is alkyl; “alkoxycarbonyl” means -C(O)-OA*, wherein A* is alkyl; and where alkyl is as defined above. Alkoxy is preferably O(C₁-C₆)alkyl and includes methoxy, ethoxy, propoxy, butoxy, pentoxy and hexoxy.

[0028] “Cycloalkoxy” means an cycloalkyl-O- group wherein the cycloalkyl is as defined above. Exemplary (C₃-C₇)cycloalkyloxy groups include cyclopropoxy, cyclobutoxy, cyclopentoxy, cyclohexoxy and cycloheptoxy.

[0029] “Hetero” refers to the replacement of at least one carbon atom member in a ring system with at least one heteroatom selected from N, S, and O. A hetero ring system may have 1 or 2 carbon atom members replaced by a heteroatom.

[0030] “Halogen” and “halo” are interchangeably used herein and each refers to fluorine, chlorine, bromine, or iodine.

[0031] “Cyano” means -C≡N.

[0032] “Nitro” means -NO₂.

[0033] As used herein, an amino group may be a primary ($-\text{NH}_2$), secondary ($-\text{NHR}_x$), or tertiary ($-\text{NR}_x\text{R}_y$), wherein R_x and R_y may be any alkyl, aryl, heterocyclyl, cycloalkyl or alkenylene, each optionally and independently substituted with one or more substituents described above. The R_x and R_y substituents may be taken together to form a “ring”, wherein the “ring”, as used herein, is cyclic amino groups such as piperidine and pyrrolidine, and may include heteroatoms such as in morpholine.

[0034] The terms “haloalkyl”, “halocycloalkyl” and “haloalkoxy” mean alkyl, cycloalkyl, or alkoxy, as the case may be, substituted with one or more halogen atoms. The term “halogen” or “halo” means F, Cl, Br or I. Preferably the halogen in a haloalkyl or haloalkoxy is F.

[0035] The term “acyl group” means $-\text{C}(\text{O})\text{B}^*$, wherein B^* is an optionally substituted alkyl group or aryl group (*e.g.*, optionally substituted phenyl).

[0036] The term “(C₆-C₁₀)aryl” used alone or as part of a larger moiety as in “arylalkyl”, “arylalkenyl”, “arylalkoxy”, “aryloxy”, or “aryloxyalkyl”, means carbocyclic aromatic rings. The term “carbocyclic aromatic group” may be used interchangeably with the terms “aryl”, “aryl ring”, “carbocyclic aromatic ring”, “aryl group” and “carbocyclic aromatic group”. An aryl group typically has 6-10 ring atoms. A “substituted aryl group” is substituted at any one or more substitutable ring atom. The term “C₆-C₁₆ aryl” as used herein means a monocyclic, bicyclic or tricyclic carbocyclic ring system containing from 6 to 16 carbon atoms and includes phenyl (Ph), naphthyl, anthracenyl, 1,2-dihydronaphthyl, 1,2,3,4-tetrahydronaphthyl, fluorenyl, indanyl, indenyl and the like. The (C₆-C₁₀)aryl(C₁-C₆)alkyl group connects to the rest of the molecule through the (C₁-C₆)alkyl portion of the (C₆-C₁₀)aryl(C₁-C₆)alkyl group.

[0037] The term benzyl (Bn) refers to $-\text{CH}_2\text{Ph}$.

[0038] The term “heteroaryl”, “heteroaromatic”, “heteroaryl ring”, “heteroaryl group” and “heteroaromatic group”, used alone or as part of a larger moiety as in “heteroarylalkyl” or “heteroarylalkoxy”, refers to aromatic ring groups having five to fourteen total ring atoms selected from carbon and at least one (typically 1 - 4, more typically 1 or 2) heteroatoms (*e.g.*, oxygen, nitrogen or sulfur). They include monocyclic rings and polycyclic rings in which a monocyclic heteroaromatic ring is fused to one or more other carbocyclic aromatic or heteroaromatic rings. The term “5-14 membered heteroaryl” as used herein means a monocyclic, bicyclic or tricyclic ring system containing one or two aromatic rings and from 5 to 14 total atoms of which, unless otherwise specified, one, two, three, four or five are heteroatoms independently selected from N, NH, N(C₁₋₆alkyl), O and S. (C₃-C₁₀)heteroaryl includes furyl,

thiophenyl, pyridinyl, pyrrolyl, imidazolyl, and in preferred embodiments of the invention, heteroaryl is (C₃-C₁₀)heteroaryl.

[0039] The term “2-4 member polycyclyl” is a cyclic compound with 2-4 hydrocarbon loop or ring structures (*e.g.*, benzene rings). The term generally includes all polycyclic aromatic compounds, including the polycyclic aromatic hydrocarbons, the heterocyclic aromatic compounds containing sulfur, nitrogen, oxygen, or another non-carbon atoms, and substituted derivatives of these.

[0040] The term “alkenyl” means a straight or branched hydrocarbon radical having a specified number of carbon atoms and includes at least one double bond. An alkenyl group generally has between 2 and 6 carbon atoms. The (C₆-C₁₀)aryl(C₂-C₆)alkenyl group connects to the remainder of the molecule through the (C₂-C₆)alkenyl portion of (C₆-C₁₀)aryl(C₂-C₆)alkenyl.

[0041] Pharmaceutically acceptable salts of the compounds of the present invention are also included. For example, an acid salt of a compound of the present invention containing an amine or other basic group can be obtained by reacting the compound with a suitable organic or inorganic acid, resulting in pharmaceutically acceptable anionic salt forms. Examples of anionic salts include the acetate, benzenesulfonate, benzoate, bicarbonate, bitartrate, bromide, calcium edetate, camsylate, carbonate, chloride, citrate, dihydrochloride, edetate, edisylate, estolate, esylate, fumarate, glyceptate, gluconate, glutamate, glycolylarsanilate, hexylresorcinate, hydrobromide, hydrochloride, hydroxynaphthoate, iodide, isethionate, lactate, lactobionate, malate, maleate, mandelate, mesylate, methylsulfate, mucate, napsylate, nitrate, pamoate, pantothenate, phosphate/diphosphate, polygalacturonate, salicylate, stearate, subacetate, succinate, sulfate, tannate, tartrate, teoclate, tosylate, and triethiodide salts.

[0042] Salts of the compounds of the present invention containing a carboxylic acid or other acidic functional group can be prepared by reacting with a suitable base. Such a pharmaceutically acceptable salt may be made with a base which affords a pharmaceutically acceptable cation, which includes alkali metal salts (especially sodium and potassium), alkaline earth metal salts (especially calcium and magnesium), aluminum salts and ammonium salts, as well as salts made from physiologically acceptable organic bases such as trimethylamine, triethylamine, morpholine, pyridine, piperidine, picoline, dicyclohexylamine, N,N'-dibenzylethylenediamine, 2-hydroxyethylamine, bis-(2-hydroxyethyl)amine, tri-(2-hydroxyethyl)amine, procaine, dibenzylpiperidine, dehydroabietylamine, N,N'-

bisdehydroabietylamine, glucamine, N-methylglucamine, collidine, quinine, quinoline, and basic amino acids such as lysine and arginine.

[0043] As used herein, “spectroscopy” encompasses any method by which matter reacts with radiated energy. This includes, but is in no way limited to, microscopy, fluorescence microscopy, UV/Vis spectrometry, and flow cytometry.

[0044] A “liquid medium” as used herein, is a mixture of milk and a solvent. The types of milk that can be used to generate a liquid medium include, but are not limited to, non-fat milk containing between about 0% to about 1.0% fat, low fat milk containing between about 1% to about 3.0% fat, and whole milk containing greater than about 3.0% fat. The solvents that can be used in the liquid medium include, but are not limited to, DMSO, water, methanol, and ethanol, or a combination thereof. The liquid medium may be a homogenous mixture such as a solution or a heterogeneous mixture and can have a concentration of milk between about 1% to about 99%. In one embodiment, the liquid mixture is a mixture of milk and a solvent wherein the milk is diluted to 50 times the original concentration. In another embodiment, the liquid mixture is a mixture of milk and a solvent wherein the milk is diluted to 100 times the original concentration. In yet another embodiment, the liquid mixture is a mixture of milk and a solvent wherein the milk is diluted to 150 times the original concentration.

[0045] A “change in fluorescence” as used herein, can be used to indicate a change in the fluorescence intensity of a sample after exposure to an analyte, as compared to a baseline exposure. For example, a fluorophore, such as a BODIPY-based fluorophore having the structure of Formula (I), exhibits a change in fluorescence intensity after exposure to an analyte such as milk fat. In some embodiments of the invention, the change in fluorescence intensity is an increase in fluorescence intensity. Alternately, a change in fluorescence can be a change in the color of the fluorescence. A change in the color of the fluorescence can be a change in the color hue of the fluorescence (*e.g.* a yellow hue versus a pinkish-orange hue), or can be a change in the tint or saturation of the fluorescence (*e.g.* a light or pale pink versus a dark pink).

[0046] As used herein, “incubating” a sample means mixing a sample. Alternately, incubating means mixing and heating a sample. “Mixing” can comprise mixing by diffusion, or alternately by agitation of a sample.

[0047] In some embodiments of the invention, “detecting, determining, or analyzing a sample by fluorescence” means detecting, determining, or analyzing utilizing a fluorescence reader, fluorescence spectroscopy, fluorescence meter or another method that can quantify

fluorescence. In alternate embodiments of the invention, “analyzing a sample by fluorescence” or “detecting a sample by fluorescence” means a visual analysis carried out by the human eye. In some embodiments of the invention, fluorescence analysis by visual analysis is carried out under visible light. In other embodiments of the invention, fluorescence analysis by visual analysis is carried out under certain wavelengths of light, *e.g.* about 365 nm (ultra-violet light), 530 nm (green laser light).

[0048] Herein we describe the discovery of the first fluorescent sensor for milk fat and dubbed it as Milk Orange (MO). (FIG. 1) It exhibits magnificent, yet selective turn-on feature towards fat molecules even in complicated milk matrix. Its fluorescence response is not affected by the amount of proteins or carbohydrates. Furthermore, it provides a convenient and rapid tool to measure fat amount, simply by mixing with diluted milk samples. We believe this discovery could revolutionize the milk fat detection process and provide end-point consumer-friendly products for easy milk fat level testing.

[0049] A description of example embodiments of the invention follows.

[0050] Image-based screening method was used to conduct a hyper-throughput sensor development process. Firstly, we setup a black box with formalized signal detection system. The box consists of a sample chamber and a signal concentration chamber on the top. The signal concentration chamber could efficiently filter off noises from outside the box and from the light source itself. The light source emits 365 nm ultra-violet (UV) signals, which is able to excite versatile fluorescent scaffolds. To fully expand the chances of hit discovery, the auto-fluorescence signals of milk itself were carefully measured and the milk sample was serially diluted until the background was fully quenched. Removal of any background signals ensures that any change of fluorescence signals observed will be derived from the dyes. With the help of our hyper-throughput imaging system, thousands of fluorescent dyes were rapidly screened in the format of 96-well plates.

CHEMICAL STRUCTURE AND OPTICAL PROPERTIES OF MILK ORANGE

[0051] By analyzing the pictures taken before and after the addition of milk, we were able to identify any dramatic signal change. We then proceeded to a more systematic confirmation step and measured the dose-dependent spectra change of milk fat towards the selected fluorescent dyes. Different milk fat concentrations were prepared from a combination of skim milk, low fat milk and whole milk, which were chosen from the same brand with identical amounts of ingredients other than milk fat. Screening of more than 5,000 fluorescent dyes, allowed

identification of various BODIPY-based sensors, which exhibited strong responses towards milk fat and directly visible emission colour differences. One dye identified was Milk Orange (MO) (FIG. 1) which was further analyzed.

APPLICATION OF MILK ORANGE INTO VERSATILE MILK FAT DETECTOR

[0052] MO displays high quantum yield of 0.99 in DMSO and has great potential for visualized milk fat detection. By carefully analyzing the milk-MO response pictures, it was observed that skim milk (0.1% fat) displays a very dim signal, which is almost invisible through naked eyes. However, an orange colour from MO appears as an intrinsic orange fluorescence upon increasing the amount of milk fat in the sample from 0.5% to 2.0%. Further increasing the milk fat will lead to higher density of fat emulsion particles, which in turn increases the scattering of milk auto-fluorescence, providing a sample that is green in colour. The mixture of milk auto-fluorescence and the MO fluorescence providing clear yellow colour, indicates a milk fat range of 2.3% to 3.7%. Furthermore, the fluorescence emission signals exhibit highly linear response towards milk fat, with the Pearson Coefficient of Determination R^2 of more than 0.99. This indicates that MO can be directly used to quantitatively measure milk fat amount in milk samples. Thus based on the milk fat-dependent fluorescence colour change and linear response curve, it was determined that MO is able to achieve both qualitative and quantitative differentiation of skim milk, low fat milk and whole milk.

[0053] To investigate the visual differentiation capacity of MO and to fully explore the applicability of MO, we systematically examined its performance in various milk samples using various brands of milk. The high quantum yield and significant differentiation capacity of MO allows us to visually identify milk samples with high, low or skim milk fat amount. The milk used in our studies came from batches of different brands. From each brand, we obtained at least two types of milk, ensuring consistent milk processing procedures and largely identical ingredients. By comparing fluorescence images of milk with different fat amount within the same brand, it was observed that whole milk displays a yellow colour, low fat milk displays a pinkish orange colour, and skim milk displays a pale pink fluorescence. This phenomenon was consistent for all the five brands of milk we acquired, indicating that MO shows remarkable reproducibility. Due to the various milk sources, procession procedures and delivery time of different brands of milk, there can be significant differences in fat conformation or variations of other ingredients. Yet MO is able to tell the approximate milk fat amount with their distinct

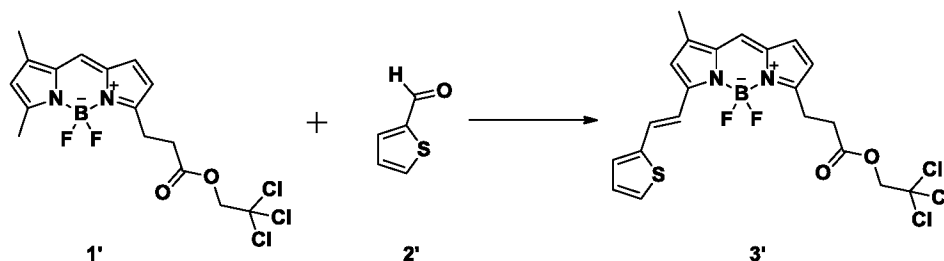
colour changes. Thus, MO can be used and developed into a rapid and versatile milk fat detector.

[0054] To corroborate the observed fluorescence-based milk fat differentiation, we proceeded to estimate the specific milk fat amount based on fluorescence intensity and the standard linear line acquired. Through the information listed in the ingredient table we were able to determine the exact concentrations of milk fat found in each sample. The milk fat concentrations that were measured with the fluorimeter corresponded remarkably with the listed milk fat data. The results clearly indicate that MO is capable of not only qualitatively estimating the amount of milk fat, but also qualitatively calculating the fat concentration in various milk samples. The ingredient tables showed significant variations among proteins, sugars and minerals in the different milk brands, yet despite these discrepancies MO provided a rapid qualitative estimation and quantitative measurement of milk fat.

EXPERIMENTAL

[0055] All reactions were performed in oven-dried glassware under a positive pressure of nitrogen. Unless otherwise noted, starting materials and solvents were purchased from Aldrich and Acros organics and used without further purification. Analytical TLC was carried out on Merck 60 F254 silica gel plate (0.25 mm layer thickness) and visualization was done with UV light. Column chromatography was performed on Merck 60 silica gel (230-400 mesh). Mass of all the compounds was determined by LC-MS of Agilent Technologies with an electrospray ionization source. All fluorescence assays were performed with a SpectraMax M2 plate reader.

EXAMPLE 1: Synthesis of Milk Orange



Scheme 1: synthetic scheme of Milk orange through aldehyde condensation.

[0056] BODIPY precursor **1'** (25 mg, 60 μ mol) and thiophene-2-carbaldehyde **2'** (27 mg/21 μ L, 240 μ mol, 4 equiv.) were dissolved in 2.5 mL acetonitrile, with 4 equiv. of pyrrolidine (29 μ L) and 4 equiv. of acetic acid (20 μ L). The mixture was shaken at 95 $^{\circ}$ C for 5 minutes, followed by immediate cooling down to 0 $^{\circ}$ C. The resulting crude mixture was concentrated

under vacuum and purified by flash column chromatography on silica gel (dichloromethane-methanol: 99.8:0.2) to afford **3'** as a dark purple solid (12.5 mg, 24 μ mol, 40% yield, 99.9% purity). ^1H NMR (500 MHz, CDCl_3) δ 7.47 (s, 1H), 7.46 (s, 1H), 7.43 (s, 1H), 7.38 – 7.32 (m, 2H), 7.06 (s, 1H), 6.88 (d, J = 3.9 Hz, 1H), 6.69 (s, 1H), 6.31 (d, J = 3.9 Hz, 1H), 4.78 (s, 2H), 3.39 (t, J = 7.5 Hz, 2H), 2.96 (t, J = 7.5 Hz, 2H), 2.30 (s, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ 171.03, 157.27, 155.73, 143.03, 139.38, 136.80, 133.75, 132.51, 127.24, 126.75, 126.38, 125.52, 122.12, 118.72, 116.66, 116.56, 94.92, 74.10, 31.92, 22.68, 11.38. HRMS ($\text{C}_{21}\text{H}_{18}\text{BCl}_3\text{F}_2\text{N}_2\text{O}_2\text{S}$): calc. $[\text{M} + \text{Na}]^+$: 539.0120, found $[\text{M} + \text{Na}]^+$: 539.0112; calc. $[\text{M} - \text{F}]^+$: 497.0236, found $[\text{M} - \text{F}]^+$: 497.0230.

EXAMPLE 2: High-Throughput Screening of Fluorescent Dyes for Milk Fat Detection

[0057] In high-throughput screening approach, black Greiner 96-well plates were pre-filled with the fluorescence dyes at 1 nmol quantity and were dissolved in 1 μL DMSO to make 1 mM solutions. In each well, 98 μL DI water was added, mixed well and the plate image was taken one time as dye background. After taking image, 1 μL of milk sample was added into the well, mixed well and the plate image was taken again. Both images were imported into Adobe Photoshop program and compared side-by-side to grant discovery of potential hit compounds. In other non-plate tests such as cuvettes, 1 mM sensor solution was first prepared and diluted with DI water, followed by addition of milk (1% (v/v)). The samples were then inserted into SpectroMax M2 plate reader or measured by a constructed fat detector.

EXAMPLE 3: Milk fat fluorescence determination

[0058] 98 μL DI water was added, mixed well and an image was taken one time as dye background. After taking an image, 1 μL of milk of several brands of milk including Greenfields, Pura, Meiji, Farmhouse, Marigold, Magnolia, and Daisy milk was added into 99 μL of dye-water mixture, mixed well by pipetting, and an image of each sample was taken.

[0059] The official milk nutrients (fat, protein, carbohydrates) were obtained directly from labeling on milk packages. Through milk fat and fluorescence measurements of Greenfields milk, a linear line graph of milk fat relative to fluorescence intensity ($y = 1976.3x + 251.78$) was generated. The fluorescence data obtained for the other samples of known fat concentrations were then plotted on the linear line graph. (FIG. 4).

[0060] The results clearly indicate that MO is capable of not only qualitatively estimating the milk fat amount, but also qualitatively calculating the fat concentration in various milk samples.

The ingredient tables for each brand showed significant variations among proteins, sugars and minerals contained within the different milk brands, yet MO could largely overlook these discrepancies and provide us with a rapid qualitative estimation and quantitative measurement of milk fat.

Table 1. Comparison of fat concentrations acquired from fluorescence measurement and ingredient tables listed on milk package.

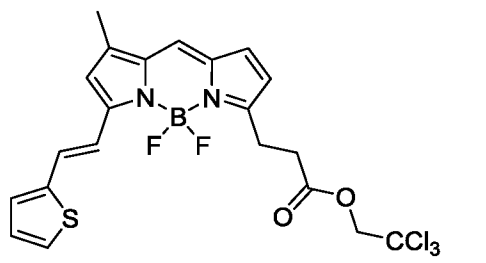
Brands of milk		Official report (g/100ml)		Fluorescence test (g/100ml)	
		Protein	Carbohydrate	Fat	Fat
Pura	Skim	4.0	5.4	0.1	0.11 ± 0.01
	Low fat	3.6	5.0	1.4	1.42± 0.01
	Fresh	3.2	4.9	3.4	3.42± 0.02
Meiji	Skim	3.2	6.9	0.1	0.10± 0.01
	Low fat	3.2	7.0	1.2	1.18± 0.01
	Fresh	3.3	~	4.1	4.09± 0.02
Farmhouse	Low fat	4.0	5.5	1.5	1.52± 0.01
	Fresh	3.2	4.8	3.8	3.80± 0.02
Marigold	Low fat	5.0	5.3	1.0	1.00± 0.01
	Fresh	3.5	5.0	3.6	3.59± 0.01
Magnolia	Low fat	3.7	5.0	1.5	1.52± 0.00
	Fresh	4.0	5.0	4.0	3.98± 0.01
Daisy	Low fat	3.5	5.2	1.0	1.00± 0.01

[0061] While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

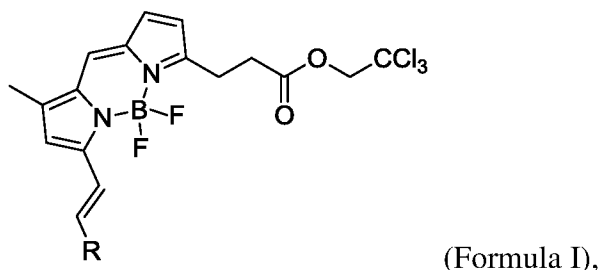
What is claimed is:

1. A compound of the following structural formula:



or a pharmaceutically acceptable salt thereof.

2. A method for fluorescence-based detection of milk fat in a liquid medium, the method comprising:
- diluting a sample of milk to a concentration to fully quench background fluorescence signals of the milk, to form a liquid medium;
 - contacting a sample of the liquid medium with a compound of Formula (I), or a pharmaceutically acceptable salt thereof:



wherein:

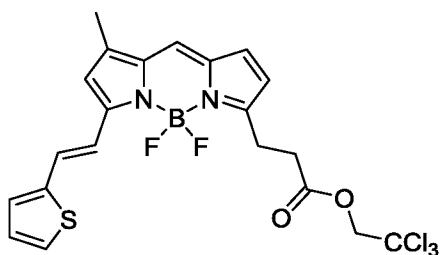
R is (C₆-C₁₀)aryl, (C₅-C₁₀)heteroaryl, (C₆-C₁₀)aryl(C₂-C₆)alkenyl or 2-4 member polycyclyl, wherein each 2-4 member polycyclyl optionally and independently contains 1-2 ring heteroatoms selected from oxygen, nitrogen and sulfur; and wherein R is optionally and independently substituted with 1-4 substituents independently selected from (C₁-C₆)alkyl, (C₂-C₆)alkenyl, halo(C₁-C₆)alkyl, hydroxy(C₀-C₆)alkyl, (C₁-C₆)alkoxy, halo(C₁-C₆)alkoxy, halogen, amino, nitro, -B(OH)₂, (C₁-C₆)alkyl-S(O)_n, -(C₁-C₆)alkylamino, (C₁-C₆)dialkylamino, (C₃-C₈)cycloalkyl, (C₃-C₈)heterocycloalkyl, (C₆-C₁₀)aryl, and (C₅-C₁₀)heteroaryl, wherein each (C₃-C₈)cycloalkyl, (C₃-C₈)heterocycloalkyl, (C₆-C₁₀)aryl, and (C₅-C₁₀)heteroaryl is optionally and independently

substituted with 1-4 substituents independently selected from (C₁-C₆)alkyl, (C₂-C₆)alkenyl, halo(C₁-C₆)alkyl, hydroxy(C₀-C₆)alkyl, (C₁-C₆)alkoxy, halo(C₁-C₆)alkoxy, and halogen; and

n is 0, 1, or 2;

- (c) incubating the sample of step (b) for a period of time sufficient to enable visualization of milk fat by fluorescence if present in the liquid medium; and
- (d) detecting fluorescence in the incubated sample, wherein a change in fluorescence as compared to fluorescence of the compound of Formula (I) not in the presence of the sample of liquid medium is indicative of the presence of milk fat in the liquid medium.

3. The method of Claim 2, wherein R is thiophenyl, optionally substituted with 1-3 substituents independently selected from halogen, halo(C₁-C₆)alkyl, (C₁-C₆)alkoxy, halo(C₁-C₆)alkoxy and hydroxy(C₀-C₆)alkyl.
4. The method of Claim 2 or 3, wherein the compound of Formula (I) is:

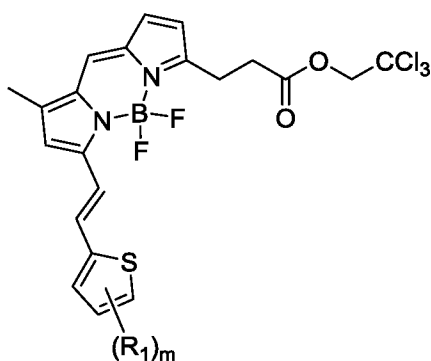


or a pharmaceutically acceptable salt thereof.

5. The method of any one of Claims 2-4, wherein detecting the incubated sample by fluorescence comprises analysis by fluorescence reader, fluorescence meter or fluorescence spectroscopy.
6. The method of any one of Claims 2-5, wherein detecting the incubated sample by fluorescence comprises qualitative visual analysis of the sample.

7. The method of any one of Claims 2-6, wherein the change in fluorescence is a change in fluorescence intensity or a change in the color of the fluorescence.
8. The method of Claim 7, wherein the change in fluorescence intensity is an increase in fluorescence intensity.
9. The method of Claim 7, wherein the change in the color of the fluorescence is detectable under visible light or a wavelength portion thereof or ultraviolet light.
10. The method of Claim 9, wherein the change in the color of the fluorescence to a yellow-colored fluorescence at a wavelength between about 530 nm and about 700 nm is indicative of the presence of whole milk in the liquid medium.
11. The method of Claim 9, wherein the change in the color of the fluorescence to a pinkish orange-colored fluorescence at a wavelength between about 530 nm and about 700 nm is indicative of the presence of low fat milk in the liquid medium.
12. The method of Claim 9, wherein the change in the color of the fluorescence to a pale pink-colored fluorescence at a wavelength between about 530 nm and about 700 nm is indicative of the presence of skim milk in the liquid medium.
13. The method of any one of Claims 2-12, further comprising quantification of the amount of fat present in the liquid medium by measuring the change in fluorescence as compared to fluorescence of the compound of Formula (I) not in the presence of the sample of liquid medium sample.

14. A method for fluorescence-based detection of milk fat in a liquid medium, the method comprising:
- diluting a sample of milk to a concentration to fully quench background fluorescence signals of the milk, to form a liquid medium;
 - contacting a sample of the liquid medium with a compound of Formula (II), or a pharmaceutically acceptable salt thereof:



(Formula II),

wherein:

each R_1 is independently (C_1-C_6) alkyl, (C_2-C_6) alkenyl, halo (C_1-C_6) alkyl, hydroxy (C_0-C_6) alkyl, (C_1-C_6) alkoxy, halo (C_1-C_6) alkoxy, halogen, amino, nitro, $-B(OH)_2$, (C_1-C_6) alkyl-S(O) $_n$, $-(C_1-C_6)$ alkylamino, (C_1-C_6) dialkylamino, (C_3-C_8) cycloalkyl, (C_3-C_8) heterocycloalkyl, (C_6-C_{10}) aryl, or (C_5-C_{10}) heteroaryl, wherein each (C_3-C_8) cycloalkyl, (C_3-C_8) heterocycloalkyl, (C_6-C_{10}) aryl, and (C_5-C_{10}) heteroaryl is optionally and independently substituted with 1-4 substituents independently selected from (C_1-C_6) alkyl, (C_2-C_6) alkenyl, halo (C_1-C_6) alkyl, hydroxy (C_0-C_6) alkyl, (C_1-C_6) alkoxy, halo (C_1-C_6) alkoxy, and halogen;

n is 0, 1, or 2; and

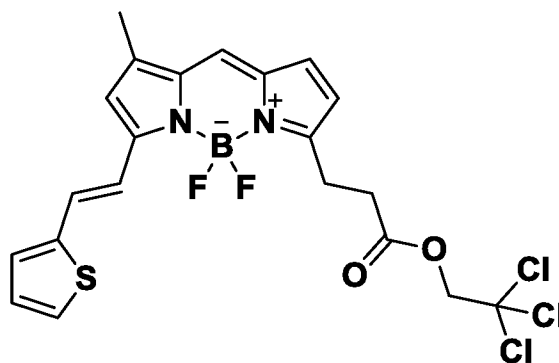
m is 0, 1, or 2;

- incubating the sample of step (b) for a period of time sufficient to enable visualization of milk fat by fluorescence if present in the liquid medium; and
- detecting fluorescence in the incubated sample, wherein a change in fluorescence as compared to fluorescence of the compound of Formula (II) not in the presence of the sample of liquid medium is indicative of the presence of milk fat in the liquid medium.

15. The method of Claim 14, wherein detecting the incubated sample by fluorescence comprises analysis by fluorescence reader, fluorescence meter or fluorescence spectroscopy.
16. The method of Claim 14 or 15, wherein detecting the incubated sample by fluorescence comprises qualitative visual analysis of the sample.
17. The method of any one of Claims 14-16, wherein the change in fluorescence is a change in fluorescence intensity or a change in the color of the fluorescence.
18. The method of Claim 17, wherein the change in fluorescence intensity is an increase in fluorescence intensity.
19. The method of Claim 17, wherein the change in the color of the fluorescence is detectable under visible light or a wavelength portion thereof or ultraviolet light.
20. The method of Claim 19, wherein the change in the color of the fluorescence to a yellow-colored fluorescence at a wavelength between about 530 nm and about 700 nm is indicative of the presence of whole milk in the liquid medium.
21. The method of Claim 19, wherein the change in the color of the fluorescence to a pinkish orange-colored fluorescence at a wavelength between about 530 nm and about 700 nm is indicative of the presence of low fat milk in the liquid medium.
22. The method of Claim 19, wherein the change in the color of the fluorescence to a pale pink-colored fluorescence at a wavelength between about 530 nm and about 700 nm is indicative of the presence of skim milk in the liquid medium.

23. The method of any one of Claims 14-22, further comprising quantification of the amount of fat present in the liquid medium by measuring the change in fluorescence as compared to fluorescence of the compound of Formula (II) not in the presence of the sample of liquid medium sample.

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**Chemical Formula: C₂₁H₁₈BCl₃F₂N₂O₂S****Molecular Weight: 517.61****DMSO, Φ = 0.11** **$\lambda_{\text{abs max}}$ = 565 nm** **$\lambda_{\text{em max}}$ = 580 nm****FIG. 1**

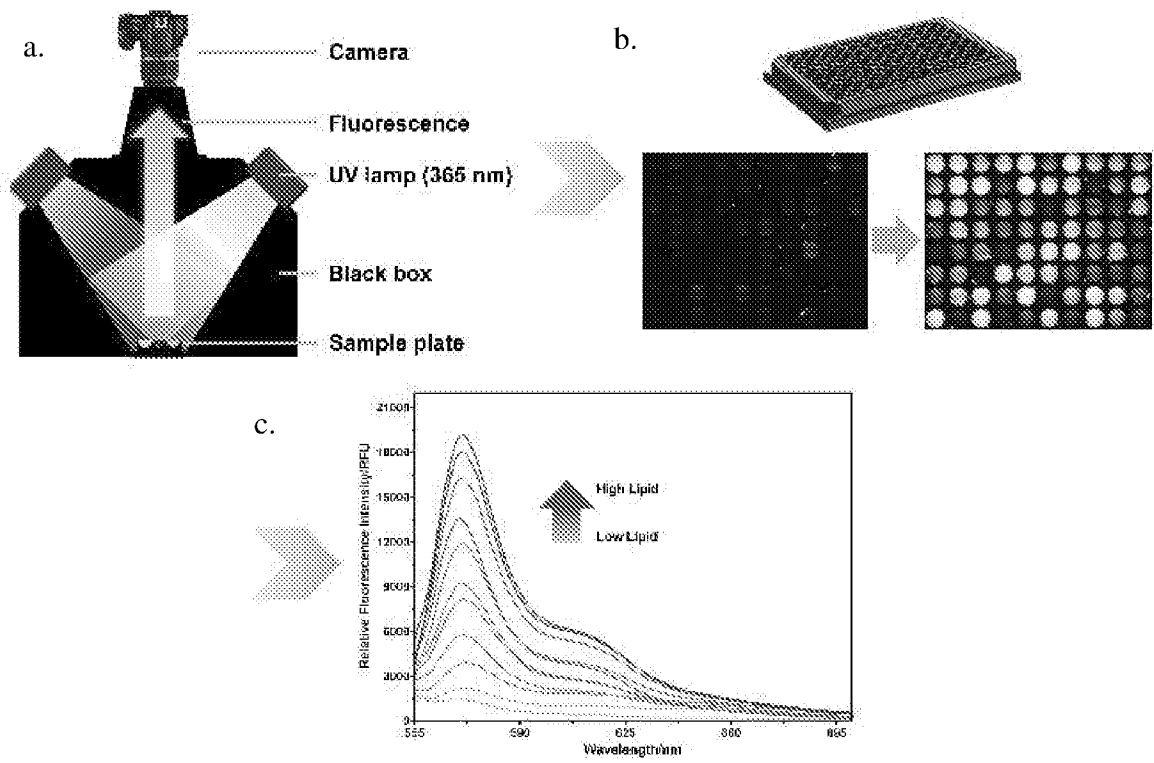


FIG. 2

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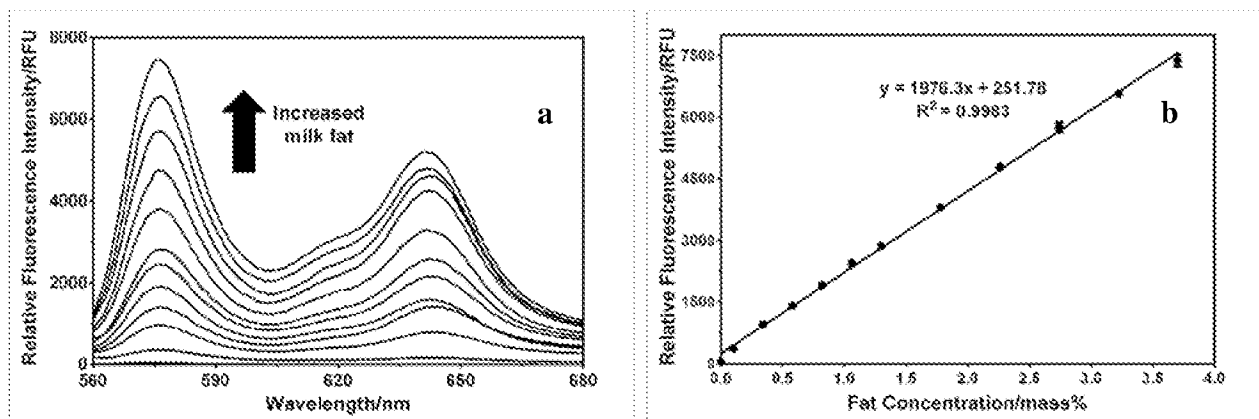


FIG. 3

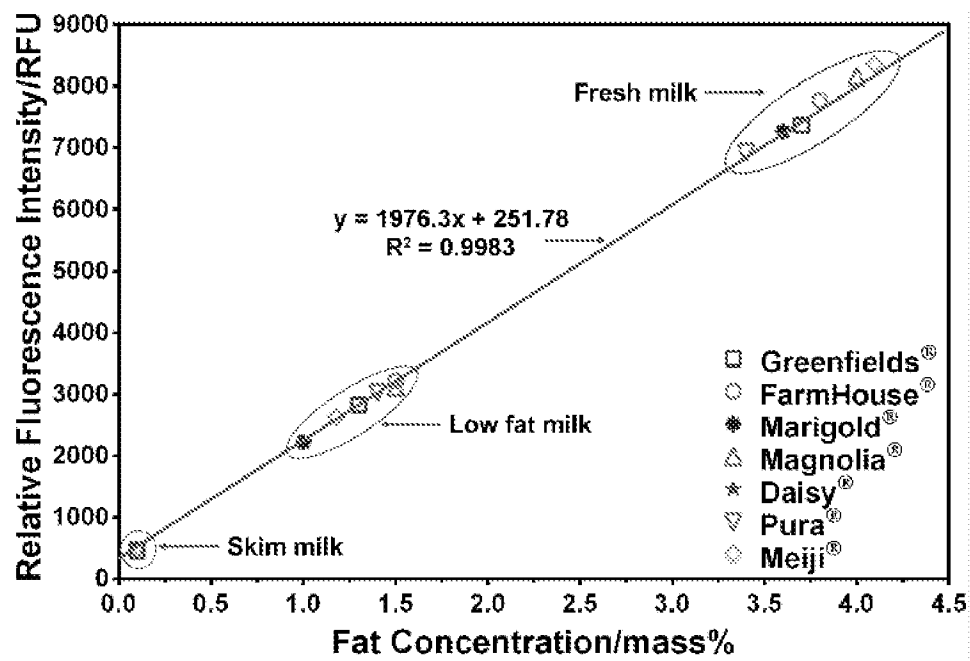


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2015/050146

A. CLASSIFICATION OF SUBJECT MATTER

C07F 5/02 (2006.01) G01N 33/06 (2006.01) G01N 21/64 (2006.01)

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)

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 practicable, search terms used)

EPOQUE (epodoc, WPIAP, Medline); Keywords: milk fat, milkfat, butter fat, butterfat, dairy fat, dairyfat, triglyceride, fatty acid, dairy, milk, cream, BODIPY, fluorescence, fluorophore, fluorochrome, dye, tracer, tag, reporter, label, sensor, detect, determine, analyse, analyze, visualise, visualize, quantify & like terms. STN (Registry; HCAPlus): Structure based on Formula (I) of claim 2. STN (Registry; HCAPlus): BODIPY ring identifier search: 1409.195.3/RID; Keywords: BODIPY, fluorescence, fluorophore, fluorochrome, milkfat, milk fat, butterfat, butter fat, dairyfat, dairy fat, triglyceride, fatty acid, dairy, milk, cream & like terms. STN (Biosis, Embase, Agricola, FROSTI, FSTA): Keywords: BODIPY, fluorescence, fluorophore, fluorochrome, milkfat, milk fat, butterfat, butter fat, dairyfat, dairy fat, triglyceride, fatty acid, dairy, milk, cream & like terms. PatentScope; Espacenet; Google Patents; IP Australia internal databases: INTESS, PAMS: Applicant: National University of Singapore; Inventors: CHANG, XU, BAI; Keywords: BODIPY, milk, fat, fluorescent.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"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 August 2015		Date of mailing of the international search report 28 August 2015	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au		Authorised officer Ross Heisey AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262833185	

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2015/050146
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHAI, D., et al., "Synthesis of a novel BODIPY library and its application in the discovery of a fructose sensor", ACS Combinatorial Science, 2012, vol. 14(2), pages 81-84, and Supporting Information pages 1-21 Whole document, in particular Abstract; Scheme 1 at page 81; Supporting Information Table S1 at page 6 (aldehyde L423) and Table S2 at page 9 (Compound BDD423)	1
A	SU 1698715 A1 (UKR AGRIC ACAD) 15 December 1991, Derwent WPI Online Abstract Accession No. 1992-329882, Class D13 J04 Whole document, in particular col. 2 and Fig. 1; and DWPI Abstract	2-23
A	SU 365636 A1 (SCIENTIFIC INSTRUMENTATIO) 08 January 1973, Derwent WPI Online Abstract Accession No. 1973-59287U, Class D13 E23 Whole document, in particular col. 2 and Fig. 1; and DWPI Abstract	2-23
A	SCOLOZZI, C., et al., "A method for identification and characterization of ewe's milk fat globules", Milchwissenschaft, 2003, vol. 58, pages 490-493 Whole document, in particular Abstract; Results, RH col., page 491; Fig. 2	2-23
A	NTAKATSANE, M.P., et al., "Rapid detection of milk fat adulteration with vegetable oil by fluorescence spectroscopy", Journal of Dairy Science, 2013, vol. 96(4), pages 2130-2136 Whole document, in particular Abstract; Fig. 3	2-23
P,X	XU, W., et al., "Milk quality control: instant and quantitative milk fat determination with a BODIPY sensor-based fluorescence detector", Chemical Communications, 2014, vol. 50, pages 10398-10401 (E.Pub 08 July 2014) Whole document, in particular Figs. 1, 3	1-23
Form PCT/ISA/210 (fifth sheet) (July 2009)		

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/SG2015/050146	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
SU 1698715 A1	15 December 1991	SU 1698715 A1	15 Dec 1991
SU 365636 A1	08 January 1973	SU 365636 A1	08 Jan 1973
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			