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(54) **EXTRACTION AND QUANTIFICATION OF VITAMINS A & D IN FLUID SAMPLES**

EXTRAKTION UND QUANTIFIZIERUNG VON VITAMIN A UND VITAMIN D IN FLÜSSIGKEITSPROBEN

EXTRACTION ET QUANTIFICATION DES VITAMINES A & D DANS DES ECHANTILLONS DE FLUIDES

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Description

FIELD OF THE INVENTION

[0001] The present invention relates to methods for extracting and quantifying fat-soluble vitamins from fluid samples. In particular, it relates to antibodies, methods for their use, and kits therefore, to quantify vitamins A and D in dairy products and in other fluids.

BACKGROUND OF THE INVENTION

[0002] Milk which is' marketed in Canada and the United States must be fortified with vitamin A and D3 (1,2). Regulatory agencies have set standards specifying the minimum amount of vitamins A and D3 to be added to milk products. Fortified fluid milk products add value to the agricultural and dairy industry in that consumers seek products with essential vitamins and nutrients. Milk processors typically assert general claims of vitamin fortification levels because current methodology is too costly and time consuming to implement testing on a batch basis.

[0003] Vitamins A and D3 are potentially toxic to humans at higher concentrations. Since the margin between the nutritionally desirable intake of vitamins and harmful excess is small (3,4,5) it is important that errors in fortification levels be detectable in the shortest possible time. Currently available methodology for these analyses is laborious, tedious, and expensive, and adds to the high cost of production to dairy processors. Currently available methods for detecting vitamin A and D3 metabolites include binding assays, receptor proteins, high performance liquid chromatography (HPLC), and gas chromatography-mass spectrometry (GC-MS) (6,7,8,9). Such analyses in any laboratory are time consuming, require skill and expertise on the part of analysts, and require large capital investments for equipment.

[0004] Vitamins A and D3 are fat soluble and they are bound by fat molecules in a dairy product such as milk. Accordingly, they must be extracted in lengthy extraction steps. Since vitamins A and D are both labile to heat, light, and oxidation, laborious extraction results in loss of the vitamin in the preparative steps, and often requires 3 to 5 days for the completion of the analyses.

[0005] Vitamin D plays an active role in the homeostatic mechanism that controls the concentration of calcium ion in plasma. Vitamin D is transported to various sites in the body where it is activated.

[0006] The activated forms of the vitamin act on the target tissues, thereby causing an increase in calcium content. The activation of vitamin D is regulated in a negative feedback system by plasma calcium. The most biologically active form of vitamin D is 1,25-dihydroxycholecalcifrerol or calcitriol, which is formed by two successive hydroxylations of vitamin D. That is, calcitriol is formed by the sequential hydroxylation of vitamin D at C-25 in the liver and at C-1 in the kidney. Various other analogs can be produced by hydroxylation at C-24 and C-26. The above mentioned biologically active forms of hydroxylated vitamin D are synthesized in the body only and are not found in dairy or agri-food products. Vitamin D3, or cholecalciferol, is the form used as an additive and is also produced in the body when the skin, which contains the provitamin 7-dehydrocholesterol, is exposed to sunlight.

[0007] Currently, the principal assay for Vitamin D3 in dairy samples which has been developed is a two step high pressure liquid chromatography (HPLC) assay, whereby the extracted vitamin in about 200 ml of the hexane is lyophilized to about 2 ml and the fraction corresponding to vitamin D is isolated by HPLC and collected manually. This collected fraction of vitamin D is again lyophilized, and dissolved in about 0.5 ml of methanol and loaded again on reverse phase HPLC. This assay employs an expensive and costly laboratory set up and requires trained and skilled personnel to handle advanced instrumentation like HPLC (8,9,10).

[0008] The principal assays for vitamin A include laborious extractions and the use of HPLC, or a direct extraction with the detection of vitamin A by spectrofluorometer. The latter process has limitations due to the interference from other compounds that has fluorescence at the same wavelength. Moreover, because vitamins A and D3 are sensitive to UV-light, they might lose some of their activity due to extensive extractions, purifications and storage conditions.

[0009] The standard assays, which employ one or two step (HPLC) are illustrated, for example, by references (6-9) and (10). Other assays are described, for example, in U.S. Patent No. 5,232,836 to Bouillon et al. which describes an assay method for metabolites of vitamine D using tracers consisting of a conjugation of the derivative and a labelling component. Granado et al. (2001, J. Food Comp. Anal. 14(5):479-489) describes a saponification method to extract carotenoids in vegetables. Bouillon et al. (1984, Clin. Chem. 30(11):1731-1736) describes binding assays for determination of 25-hydroxyvitamin D_3 in serum. Tanner et al. (1988, J. Assoc. Off. Anal. Chem. 71(3);607-610) describes methods using liquid chromotography for the determination of vitamins A and D in milk. Sharpless et al. (1999, J. Assoc. Off. Anal. Chem. 82(2):288-296) describes methods using extraction, saponification and liquid chromatography to quantify vitamins A and E in a standard reference material.

[0010] Antibodies have been used in analytical methods; for example, Zhou et al. (1991, J. Immunol. Methods 138(2):211-223) describes a method to produce a hybridoma cell line which secretes a monoclonal antibody against retinoic acid, a derivative of vitamin A. Both Jean et al. (1999, J. Immunol. Methods 223(2):155-163) and Kobayashi et al. (1997, J. Steroid Biochem. Mol. Biol. 63 (1-3):127-137) describe methods to produce and characterize antibodies against vitamin D and a vitamin D_3 metabolite, respectively.

[0011] Therefore, there is a need in the art for methods and kits for quantifying vitamins A and D in a fluid sample,

and dairy products in particular.

SUMMARY OF THE INVENTION

[0012] This invention discloses methods for quantifying the level of vitamins A and D3 in a fluid dairy product. The methods disclosed herein comprise the extraction of a fluid sample with an extraction solvent to isolate fat-soluble vitamins A and D3 from the rest of the fluid mixture. The vitamins are then quantified with assays which include using antibodies to vitamins A and D3. Either monoclonal or polyclonal antibodies may be used.

[0013] The method would allow dairy processors to test for Vitamins A and D3 internally and obtain a result within few hours, so that the percentage of recommended daily amounts of vitamin D contained in the product tested can be indicated directly on the package of milk or other dairy product containing the product. In one aspect, the invention comprises a method of quantifying vitamin A or D3, or vitamin A and D3, in a fluid dairy sample, comprising the steps of:

(a) extracting the fluid dairy sample with an alcoholic solution of potassium hydroxide, potassium chloride, sodium hydroxide, sodium bicarbonate or sodium ascorbate, and an extraction solvent comprising a mixture of a non-polar organic solvent and a polar organic solvent, to isolate vitamin A or D3 or vitamins A and D3 from the fluid dairy sample into an organic fraction; and

(b) determining the amount of vitamin D3 or vitamin A, or both vitamin D3 and vitamin A, in the organic fraction, wherein the vitamin is quantified with an antibody which specifically binds to the vitamin in the organic fraction; wherein the non-polar organic solvent comprises an aliphatic hydrocarbon having from 4 to 10 carbon atoms, or a mixture thereof, and the polar organic solvent is a chloroalkane or ethyl acetate.

[0014] In one embodiment, the amount of vitamin D3 and/or vitamin A is determined using a monoclonal antibody to vitamin D3 and/or vitamin A respectively. The antibody may be used in a competitive ELISA or a sandwich ELISA to quantify the vitamin of interest.

[0015] Isolated cell lines that synthesize the monoclonal antibody to vitamin A and vitamin D3 are described herein. In other embodiments, direct measurement assays may be utilized.

[0016] In yet another aspect, this invention is a kit comprising a monoclonal antibody, said kit being used to detect vitamin D3 or vitamin A, or both vitamin D3 and vitamin A in a sample, which may include any fluid such as those described herein. The kit may include means for quantifying the vitamin which may include assay reagents, glassware and plasticware.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0017] The present invention provides a method of quantifying fat-soluble vitamins, provitamins and their metabolites in a fluid. In one embodiment, the method is applied to vitamins A and D3 present in a dairy product such as milk. Generally, the method comprises the steps of providing an antibody that specifically binds to the fat-soluble vitamin, which is preferably a monoclonal antibody, extracting the vitamin from the fluid using an extraction solvent, and assaying the vitamin extracted from the fluid.

[0018] In this application, the sample fluid is a fluid dairy product such as milk, but may also include, without limitation, other dairy products.

[0019] In one embodiment, the assay methods of the present invention utilize antibodies to vitamin D3 and vitamin A, which are preferably monoclonal antibodies. Accordingly, there is disclosed the generation and purification of a monoclonal antibody against vitamin D3 or other forms of vitamin D. The monoclonal antibody which specifically binds to vitamin D3 does not exhibit significant or substantial binding activity to other variants or biologically active forms of vitamin D. As used herein, vitamin D, also known as calciferol, includes all available forms of vitamin D including vitamin D3 also known as cholecalciferol. As well, there is disclosed the generation and purification of a monoclonal antibody against vitamin A. As used herein, vitamin A, also known as retinol palmitate, includes all active forms of vitamin A and provitamin A carotenoids. The monoclonal antibody is of course distinct from the commercially available polyclonal antibody of vitamin A.

[0020] Methods for preparing and isolating polyclonal and monoclonal antibodies are well known in the art. See, for example, Current Protocols in Immunology, Cooligan, et al. (eds.), National Institutes of Health, John Wiley and Sons, Inc., 1995; Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N. Y., 1989; and Hurrell, J. G. R., Ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, Fla., 1982. As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from a variety of warm-blooded animals, such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats with Vitamin A or D.

[0021] There is disclosed monoclonal antibodies which specifically bind to vitamin A, as well as monoclonal antibodies which specifically bind to vitamin D3. These monoclonal antibodies are not known in the prior art. In general terms, commercially available pure vitamin A or vitamin D3 may be conjugated to keyhole limpet hemocyanin and used to immunize mice. Spleen cells may be recovered from the immunized mice and fused with known cell lines. Those hybridomas which produce the desired monoclonal antibody may be detected by ELISA. These procedures are well known and include standard

techniques available to one skilled in the art.

[0022] As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as F(ab').sub.2 and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included.

[0023] Antibodies are determined to be specifically binding if: 1) they exhibit a threshold level of binding activity, and/or 2) they do not significantly cross-react with related molecules.

[0024] Antibodies described herein specifically bind if they bind to either Vitamin A or Vitamin D3 with a binding affinity (K_a) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most preferably 10^9 M^{-1} or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art (Scatchard, G., Ann. NY Acad. Sci. 51: 660-672,1949).

[0025] The methods of the present invention utilize an extraction solvent which comprises organic solvents with high selectivity in extracting polar compounds from fat molecules.

[0026] The extraction solvent comprises a polar organic solvent and a non- polar organic solvent as described in combination with an alcoholic solution of potassium hydroxide, potassium chloride, sodium hydroxide, sodium bicarbonate or sodium ascorbate, to extract fat-soluble compounds from fluids, such as dairy products. As used herein, an "organic solvent" includes, without limitation, liquid aliphatic hydrocarbons preferably containing 4 to 10 carbon atoms, or halogenated hydrocarbons containing 1 to 4 carbon atoms. The potassium hydroxide, potassium chloride, sodium hydroxide, sodium bicarbonate or sodium ascorbate is preferably dissolved in an alcoholic solution, such as a 65% ethanol solution.

[0027] It is preferred that the fluid sample have an alkaline pH for the extraction. The use of a potassium or sodium hydroxide of course produces suitable alkaline conditions.

[0028] Solvent polarity has been defined and measured in several different ways, one of the most common being the dielectric constant (ε). Aliphatic hydrocarbon solvents typically have dielectric constant values less than about 2.0. As used herein, "non-polar" shall refer to any solvent having a dielectric constant less than 3.0, and preferably less than about 2.0. Halogenated hydrocarbon solvents typically have dielectric constants greater than about 8.0. As used herein, "polar" shall refer to any organic solvent having a dielectric constant greater than 3.0, and preferably greater than about 4.0, and more preferably greater than about 8.0.

[0029] In one embodiment, the extraction solvent comprises a mixture of hexane and petroleum ether as the non-polar solvent and methylene chloride (dichloromethane) as the polar solvent. Preferably, equal amounts of hexane and petroleum ether are mixed with a small amount of methylene chloride. In one embodiment, the solvent comprises hexane, petroleum ether and methylene chloride in a 49:49:2 ratio (49:1 non-polar to polar). Suitable non-polar solvents may include butane, pentane, hexane, heptane and octane, including mixtures thereof such as petroleum ether, benzene and acetonitrile, amongst others. Suitable polar organic solvents are chloroalkanes such as methylene chloride and ethyl acetate. It is preferred that the potassium chloride, potassium hydroxide, or sodium hydroxide, sodium bicarbonate, and sodium ascorbate raise the pH of the fluid sample to an alkaline pH.

[0030] The volume ratio of non-polar to polar in the extraction solvent may be greater than 2:1, and is preferably greater than 5:1, and is most preferably greater than about 10:1.

[0031] The extraction solvent may be used in ratio of 1:1 or lower with the fluid. In one embodiment, 20 ml of a milk sample may be extracted with 15 ml of the preferred extraction solvent described herein. Optionally, an antioxidant such as pyrogallol may be added to the fluid to prevent oxidation of the vitamins A and D3 during the extraction procedure.

[0032] The mixture of organic solvents in combination with the alcoholic solution as described has the ability to extract fat-soluble compounds that can be directly detected by immunoassay without any further treatment. The extracted vitamins can be quantified directly by well-known enzyme-immunoassays, such as by a competitive ELISA, a sandwich ELISA or by radioimmunoassay, which are standard quantitative assays well known in the art (Persoon T. Immunochemical assays in the clinical laboratory. Clinical Laboratory Science; 5(1):31-40, 1992).

[0033] The extracted vitamins and antibodies can be used for detection and quantification of vitamins A and D3 by direct measurement. The Fourier Transform-Infrared Spectroscopy (FT-IR) method is well known and may be applied through incorporation to biochip technology in the present invention. FT-IR is a nondestructive technique that enables the identification of the unique chemical bonds (a "fingerprint") of a given organic substance, as each chemical bond in a molecule absorbs different frequencies at a different wave number. A molecular fingerprint of the protein/vitamin complex in solution may be obtained, in terms of infrared spectra. This spectrum is a unique identity of the protein/vitamin complex at a given concentration in solution. Other direct measurement techniques are well-known in the art.

[0034] The methods and antibodies disclosed herein can also be used to prepare a kit that can be used to determine the levels of vitamins A and D3 in a fluid dairy sample according to claim 11. In one embodiment, the kit comprises an extraction solvent for extraction of the vitamins from the fluid dairy sample, a sample of the monoclonal antibody, and the reagents required for the assay, which may be an ELISA assay. The kit may additionally

comprise hardware, required to perform the analysis, such as tubes, assay plates or other glassware or plasticware.

[0035] While the invention has been described in conjunction with the disclosed embodiments, it will be understood that the invention is not intended to be limited to these embodiments. As will be apparent to those skilled in the art, various modifications, adaptations and variations of the foregoing specific disclosure can be made without departing from the scope of the invention claimed herein.

[0036] The following examples are intended only to illustrate and describe the invention rather than limit the claims that follow.

EXAMPLES

Materials and Methods

[0037] All chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.) unless otherwise noted. Vitamin D3 (Cholecalciferol) was from Merck. Hexane, petroleum ether, methylene chloride, potassium hydroxide were from Caledon Laboratories. Bovine serum albumin was from Gibco BRL, and TMB substrate was from KPL Laboratories.

Generation and Identification of Monoclonal Antibodies

1. Conjugation of Retinol Palmitate to Keyhole limpet hemocyanin (KLH)

[0038] 4.1 mg of vitamin A (retinol palmitate) was mixed with KLH and stirred overnight at room temperature. After dialysis, glutaraldehyde was added to the mixture to a final concentration of 1%. The resulting mixture was stirred for 6 hrs. The conjugated mixture was dialysed in phosphate buffered saline (pH 7.4) for 4 hr. Conjugated KLH-vitamin A was filter sterilized and stored in sterile vials at -20°C.

2. Conjugation of Vitamin D3 to Keyhole Limpet Hemocyanin (KLH)

[0039] 5.4 mg of vitamin D3 was mixed with 10 mg of KLH and stirred overnight at room temperature. After dialysis, glutaraldehyde was added to the mixture to a final concentration of 1%. The resulting mixture was stirred for 6 hrs. The conjugated mixture was dialysed in phosphate buffered saline (pH 7.4) for 4 hr. Conjugated KLH-vitamin D3 was filter sterilized and stored in sterile vials at -20°C.

3. Immunization and Hybridoma Production for vitamin A

[0040] For general procedures related to the generation of monoclonal antibodies, refer to Harlow and Lane, Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory, 1988. BALB/c mice were immunized, via intraperitoneal injection, with 50 μg/mouse of the vitamin A- KLH conjugate emulsified in complete Freund's adjuvant. After two weeks, a second injection of the antigen was given in Freund's incomplete adjuvant, followed by another dose of the antigen in sterile PBS (pH- 7.4), after three days. An intrasplenic injection of the antigen was given 10 days following the third dose of antigen. Three days after the intrasplenic injection, spleen cells from the mice were isolated and fused with the Sp2/0-Ag 14 cell line as described by Shulman et al. in Nature 276: 269 (1978) using the method of Galfre et al., disclosed in Nature 266: 550 (1977). Cells were cultured in 96-well tissue culture plates in RPMI media supplemented with 20% fetal bovine serum. After 24 hrs, selection medium HAT (hypoxanthine, aminopterin and thymidine) was added as described by J. W. Littlefield in Science 145: 709 (1964).

4. ELISA with Monoclonal Antibody to Vitamin A

[0041] Monoclonal antibodies that bind to vitamin A were detected from culture supernatants by using ELISA. Microtitre plates (Falcon) were coated with retinol palmitate (20ug/ml in PBS, pH 7.4) and incubated at 4°C overnight. Plates were washed with PBS (pH 7.4) and blocked with 3% bovine serum albumin for 3 hrs. Plates were washed again with PBS, and air-dried. Wells exhibiting hybridoma growth were marked and the supernatant (100 ul) from each well was transferred to the vitamin A coated plate and incubated overnight at 4°C. Commercially available polyclonal antibody for vitamin A, and antisera collected as blood from tail vein of the mice after the third intraperitoneal injection of vitamin A (positive control representing the high titre antibody against vitamin A), were run concurrently as controls. Pre-bleed sera (collected as blood from tail vein of the mice before the vitamin A injection) were also run concurrently as negative control. Bound monoclonal antibody was detected with enzyme-labeled antibody (1:6,000 dilution, Sigma) using TMB as substrate.

[0042] Cells from positive wells were transferred to 24-well plates followed by 6-well plates and then into T25 tissue culture flasks. The cell lines producing high titre of antibody are SM-1.4A and SM-4.12A.

5. Immunization and Hybridoma Production for Vitamin D3

[0043] For general procedures related to the generation of monoclonal antibodies, refer to Harlow and Lane, Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory, 1988. BALB/c mice were immunized, via intraperitoneal injection, with 50 μg/mouse of the vitamin D- KLH conjugate emulsified in complete Freund's adjuvant. After two weeks, a second injection of the antigen was given in Freund's incomplete adjuvant, followed by another dose of the antigen in sterile PBS (pH- 7.2), after

three days. An intrasplenic injection of the antigen was given 10 days following the third dose of antigen. Three days after the intrasplenic injection, spleen cells from the mice were isolated and fused with the Sp2/0-Ag 14 cell line as described by Shulman et al. in Nature 276: 269 (1978) using the method of Galfre *et al.,* disclosed in Nature 266: 550 (1977). Cells were cultured in 96-well tissue culture plates in RPMI media supplemented with 20% fetal bovine serum. After 24 hrs, selection medium HAT (hypoxanthine, aminopterin and thymidine) was added as described by J. W. Littlefield in Science 145: 709 (1964).

6. ELISA with Monoclonal Antibody to Vitamin D3

[0044] Monoclonal antibodies that bind to vitamin D3 were detected from culture supernatants by using ELISA. Microtitre plates (Falcon) were coated with vitamin D3 (20ug/ml in PBS, pH 7.4) and incubated at 4°C overnight. Plates were washed with PBS (pH 7.4) and blocked with 3% bovine serum albumin for 3 hrs. Plates were washed again with PBS, and air-dried. Wells exhibiting hybridoma growth were marked and the supernatant (100 ul) from each well was transferred to the vitamin D3 coated plate and incubated overnight at 4°C. Commercially available monoclonal antibody for 1-25 dihydroxy vitamin D3 and anti-sera collected as blood from tail vein of the mice after the third intraperitoneal injection of vitamin D3 (positive control representing a high titre antibody against vitamin D3), were run concurrently as positive controls. Pre-bleed sera (collected as blood from tail vein of the mice before the vitamin D3 injection) were also run concurrently as negative control. Bound monoclonal antibody was detected with enzyme-labeled antibody (1:6,000 dilution, Sigma) using TMB as substrate.

[0045] Cells from positive wells were transferred to 24-well plates, followed by 6-well plates and then into T25 tissue culture flasks. The cell lines producing high titre of antibody are SM 4.9A, SM 4.9B and SM 1.1E.

Extraction of Vitamins A and D3 from Milk

[0046] 20 ml of well-mixed fluid milk, at room temperature, was placed into amber colored bottle, followed by addition of 2 ml of 10% pyrogallol antioxidant, and mixed for 5 min. Then slowly from the sides of the bottle, cold alcoholic potassium hydroxide solution was added to achieve a ratio of potassium, ethanol and water of 1:2: 0.3. Samples were placed in the incubator, and extracted with 15 ml of a mixture of organic solvents. The solvent mixture comprised hexane: petroleum ether: methylene chloride, in a ratio of 49:49:2. The sample was then either centrifuged or placed on the table for 10 min, to separate the the phases. The supernatant may be used directly for immunoassay for the quantification of vitamins A & D3.

Quantification of Vitamin A and D3 in Dairy Samples using ELISA

[0047] Conventional assays utilizing monoclonal or polyclonal antibodies for vitamin D (including its metabolites and analogs) and vitamin A (including precursors or provitamins, metabolites and analogs) are well known in the art. Such assays include competitive binding assays and enzyme-linked immunoassays which are well known in the art. For example, methods to assay for 1,25-dihydroxyvitamin D are described (11) in Chen et al., J. Nutr. Biochem. 1:320-327 (1990), and in U.S. Patent Nos. 4,297,289, 4,816,417,4,585,741 and 5,232,836.

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Claims

1. A method of quantifying vitamin A or D3, or vitamins A and D3, in a fluid dairy sample, comprising the steps of:

   (a) extracting the fluid dairy sample with an alcoholic solution of potassium hydroxide, potassium chloride, sodium hydroxide, sodium bicarbonate or sodium ascorbate,
   and
   an extraction solvent comprising a mixture of a non-polar organic solvent and a polar organic solvent, to isolate vitamin A or D3, or vitamins A and D3 from the fluid dairy sample into an organic fraction; and
   (b) determining the amount of vitamin D3 or vitamin A, or both vitamin D3 and vitamin A, in the organic fraction, wherein the vitamin is quantified with an antibody which specifically binds to the vitamin in the organic fraction;
   wherein the non-polar organic solvent comprises an aliphatic hydrocarbon having from 4 to 10 carbon atoms, or a mixture thereof, and the polar organic solvent is a chloroalkane or ethyl acetate.

2. The method of claim 1 wherein the antibody is a monoclonal antibody.

3. The method of claim 1 wherein the antibody is a polyclonal antibody.

4. The method of claim 1 wherein the alcoholic solution comprises greater than 50% and less than 75% ethanol in water (v:v).

5. The method of claim 1 wherein the non-polar organic solvent comprises a mixture of hexane and petroleum ether and the polar organic solvent comprises methylene chloride.

6. The method of claim 1 wherein the vitamin D3 or vitamin A, or vitamin D3 and vitamin
   A, is detected by an immunosorbent assay.

7. The method of claim 1 wherein the vitamin is quantified by direct measurement.

8. The method of claim 1 wherein the fluid dairy sample is extracted with an alcoholic solution of potassium hydroxide.

9. The method of claim 1, wherein the non-polar organic solvent is selected from butane, pentane, hexane, heptane, octane, or a mixture thereof.

10. The method of claim 1, wherein the chloroalkane is methylene chloride.

11. A kit for assaying for vitamin D3 or vitamin A, or vitamin D3 and vitamin A, in a fluid dairy sample comprising:

    (a) an alcoholic solution of potassium hydroxide, potassium chloride, sodium hydroxide, sodium bicarbonate or sodium ascorbate, and an extraction solvent comprising a mixture of a non-polar organic solvent and a polar organic solvent, to produce an organic fraction, wherein the non-polar organic solvent comprises an aliphatic hydrocarbon having from 4 to 10 carbon atoms, or a mixture thereof, and the polar organic solvent is a chloroalkane or ethyl acetate,
    (b) a monoclonal antibody which specifically binds to vitamin D3 or vitamin A,
    and
    (c) means for performing a quantitative assay with the antibody.

12. The kit of claim 11 wherein the means for performing a quantitative assay comprises reagents and hardware for a competitive ELISA or a sandwich ELISA.

Patentansprüche

1. Verfahren zum Quantifizieren von Vitamin A oder D3 oder der Vitamine A und D3 in einer fluiden Milchprobe mit den Schritten:

   (a) Extrahieren der fluiden Milchprobe mit einer alkoholischen Lösung von Kaliumhydroxid, Kaliumchlorid, Natriumhydroxid, Natriumbicarbonat oder Natriumascorbat und
   einem Extraktionslösungsmittel mit einer Mischung aus einem nicht-polaren organischen Lösungsmittel und einem polaren organischen Lösungsmittel, um die Vitamine A oder D3 oder die Vitamine A und D3 aus der fluiden Milchprobe in eine organische Fraktion zu isolieren; und
   (b) Bestimmen der Menge von Vitamin D3 oder Vitamin A oder sowohl der Vitamine D3 und A in der organischen Fraktion, wobei das Vitamin

mit einem Antikörper quantifiziert wird, welcher spezifisch an das Vitamin in der organischen Fraktion bindet;
wobei das nicht-polare organische Lösungsmittel einen aliphatischen Kohlenwasserstoff mit 4 bis 10 Kohlenstoffatomen oder eine Mischung davon enthält, und das polare organische Lösungsmittel ein Chloralkan oder Ethylacetat ist.

2. Verfahren nach Anspruch 1,
wobei der Antikörper ein monoklonaler Antikörper ist.

3. Verfahren nach Anspruch 1,
wobei der Antikörper ein polyklonaler Antikörper ist.

4. Verfahren nach Anspruch 1,
wobei die alkoholische Lösung mehr als 50% und weniger als 75% Ethanol in Wasser (v:v) enthält.

5. Verfahren nach Anspruch 1,
wobei das nicht-polare organische Lösungsmittel eine Mischung von Hexan und Petroleumether enthält und das nicht-polare organische Lösungsmittel Methylenchlorid enthält.

6. Verfahren nach Anspruch 1,
wobei das Vitamin D3 oder Vitamin A oder die Vitamine D3 und A durch einen Immunoadsorptionsassay nachgewiesen werden.

7. Verfahren nach Anspruch 1,
wobei das Vitamin durch eine direkte Messung quantifiziert wird.

8. Verfahren nach Anspruch 1,
wobei die fluide Milchprobe mit einer alkoholischen Lösung von Kaliumhydroxid extrahiert wird.

9. Verfahren nach Anspruch 1,
wobei das nicht-polare organische Lösungsmittel ausgewählt ist aus Butan, Pentan, Hexan, Heptan, Oktan oder einer Mischung davon.

10. Verfahren nach Anspruch 1,
wobei das Chloralkan Methylenchlorid ist.

11. Bausatz zum Nachweis von Vitamin D3 oder Vitamin A oder der Vitamine D3 und A in einer fluiden Milchprobe mit:

(a) einer alkoholischen Lösung von Kaliumhydroxid, Kaliumchlorid, Natriumbicarbonat oder Natriumascorbat und einem Extraktionslösungsmittel mit einer Mischung aus einem nicht-polaren organischen Lösungsmittel und einem polaren organischen Lösungsmittel, um eine organische Fraktion zu erzeugen, wobei das nicht-polare organische Lösungsmittel einen aliphatischen Kohlenwasserstoff mit 4 bis 10 Kohlenstoffatomen oder eine Mischung davon enthält und das polare organische Lösungsmittel ein Chloralkan oder Ethylacetat ist,
(b) einem monoklonalen Antikörper, der spezifisch an Vitamin D3 oder Vitamin A bindet und
(c) einem Mittel zum Durchführen eines quantitativen Assays mit dem Antikörper.

12. Bausatz nach Anspruch 11,
wobei das Mittel zum Durchführen eines quantitativen Assays Reagenzien und Hardware für einen kompetitiven ELISA oder einen Sandwich-ELISA enthält.

Revendications

1. Procédé destiné à quantifier la vitamine A ou D3, ou les vitamines A et D3, dans un échantillon de produit laitier liquide, comprenant les étapes consistant à :

(a)extraire l'échantillon de produit laitier liquide avec une solution alcoolique d'hydroxyde de potassium, de chlorure de potassium, d'hydroxyde de sodium, de bicarbonate de sodium ou d'ascorbate de sodium,
et
un solvant d'extraction comprenant un mélange d'un solvant organique non polaire et d'un solvant organique polaire, afin d'isoler la vitamine A ou D3, ou les vitamines A et D3 de l'échantillon de produit laitier liquide en une fraction organique ; et
(b)déterminer la quantité de vitamine D3 ou de vitamine A, ou à la fois de vitamine D3 et vitamine A, dans la fraction organique, où la vitamine est quantifiée avec un anticorps qui se lie spécifiquement à la vitamine dans la fraction organique ;
où le solvant organique non polaire comprend un hydrocarbure aliphatique possédant 4 à 10 atomes de carbone, ou un mélange de celui-ci, et le solvant organique polaire est un chloroalcane ou l'acétate d'éthyle.

2. Procédé selon la revendication 1, dans lequel l'anticorps est un anticorps monoclonal.

3. Procédé selon la revendication 1, dans lequel l'anticorps est un anticorps polyclonal.

4. Procédé selon la revendication 1, dans lequel la solution alcoolique comprend plus de 50 % et moins de 75 % d'éthanol dans de l'eau (v:v).

5. Procédé selon la revendication 1, dans lequel le sol-

vant organique non polaire comprend un mélange d'hexane et d'éther de pétrole et le solvant organique polaire comprend du chlorure de méthylène.

6. Procédé selon la revendication 1, dans lequel la vitamine D3 ou la vitamine A, ou la vitamine D3 et la vitamine A, est/sont détectée(s) par un dosage d' immunoabsorption.

7. Procédé selon la revendication 1, dans lequel la vitamine est quantifiée par une mesure directe.

8. Procédé selon la revendication 1, dans lequel l'échantillon de produit laitier liquide est extrait avec une solution alcoolique d'hydroxyde de potassium.

9. Procédé selon la revendication 1, dans lequel le solvant organique non polaire est sélectionné parmi le butane, le pentane, l'hexane, l'heptane, l'octane, ou un mélange de ceux-ci.

10. Procédé selon la revendication 1, dans lequel le chloroalcane est le chlorure de méthylène.

11. Kit pour doser la vitamine D3 ou la vitamine A, ou la vitamine D3 et la vitamine A, dans un échantillon de produit laitier liquide, comprenant :

(a) une solution alcoolique d'hydroxyde de potassium, de chlorure de potassium, d'hydroxyde de sodium, de bicarbonate de sodium ou d'ascorbate de sodium, et un solvant d'extraction comprenant un mélange d'un solvant organique non polaire et d'un solvant organique polaire, afin de produire une fraction organique, où le solvant organique non polaire comprend un hydrocarbure aliphatique possédant 4 à 10 atomes de carbone, ou un mélange de celui-ci, et le solvant organique polaire est un chloroalcane ou l'acétate d'éthyle,
(b) un anticorps monoclonal qui se lie spécifiquement à la vitamine D3 ou à la vitamine A, et
(c) un moyen pour réaliser un dosage quantitatif avec l'anticorps.

12. Kit selon la revendication 11, dans lequel le moyen pour réaliser un dosage quantitatif comprend des réactifs et du matériel pour un ELISA par compétition ou un ELISA en sandwich.

REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	流体样品中维生素a和d的提取和定量		
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摘要(译)

本发明公开了维生素A（视黄醇棕榈酸酯）和D3（胆钙化醇）的单克隆抗体;本发明公开了一种使用单克隆抗体和本文公开的单克隆抗体的方法，用于定量流体如乳制品和血液中的这些维生素，以及原料或加工的农业食品和饮料产品。该方法包括使样品与极性和非极性有机溶剂的混合物与无机盐接触以将脂肪分子除去成有机部分，并通过涉及单克隆抗体的免疫测定法测定有机部分。非极性和极性有机溶剂的混合物的存在增加了维生素与脂肪分子的分离，并且使得测试样品能够通过免疫测定来定量，而无需任何进一步处理。