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(54) Title: METHOD FOR THE PREPARATION OF A DRY COMPOSITION COMPRISING A STABLE ABTS RADICAL.

(57) Abstract: The invention is in the field of medical and nutritional diagnostics. It provides means and methods for determining the antioxidant status of a sample. The invention provides a method for obtaining a stable radical ABTS in solid form comprising the steps of dissolving ABTS, converting the dissolved ABTS into radical ABTS with a peroxidase in the presence of hydrogen peroxide, freezing the thus obtained radical ABTS solution and freeze drying the frozen solution to obtain radical ABTS in solid form.



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METHOD FOR THE PREPARATION OF A DRY COMPOSITION
COMPRISING A STABLE ABTS RADICAL.

Field of the invention

5 The invention is in the field of diagnostics in particular medical, paramedical, cosmetical or nutritional diagnostics. It provides means and methods for determining the antioxidant status of a sample.

Background of the invention

10 Free radicals and reactive oxygen species (ROS) are highly reactive molecules that are generated by normal cellular processes, environmental stresses, and UV irradiation. ROS react with cellular components, damaging DNA, carbohydrates, proteins, and lipids causing cellular and tissue injury. Excess production of reactive oxygen species can also lead to inflammation, premature aging disorders,
15 and several disease states, including cancer, diabetes, and atherosclerosis. Organisms have developed complex antioxidant systems to protect themselves from oxidative stress, however, excess ROS can overwhelm the systems and cause severe damage.

 Oxidative stress is widely recognized as a factor in many degenerative diseases, as either a cause or effect. The atherosclerotic plaques that
20 obstruct arterial flow and cause cardiovascular disease are laid down by macrophages engorged with oxidized LDL (foam cells). Oxidized bases in DNA are potentially mutagenic and are therefore implicated in the process of carcinogenesis. Diabetes mellitus is associated with oxidative damage to biomolecules, and any inflammatory condition inevitably leads to an increased oxidative burden, because the release of
25 reactive oxygen species by macrophages is part of the body's defense mechanism (1).

 An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a
30 cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols.

 Although oxidation reactions are crucial for life, they can also be
35 damaging; plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as

catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells.

5 As oxidative stress appears to be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. However, it is unknown whether oxidative stress is the cause or the consequence of disease.

10 Antioxidants are widely used as ingredients in dietary supplements and have been investigated for the prevention of diseases such as cancer and coronary heart disease (2-5). In addition to these uses of antioxidants in medicine, these compounds have many industrial uses, such as preservatives in food and cosmetics and preventing the degradation of rubber and gasoline.

15 Antioxidants are found in varying amounts in foods such as vegetables, fruits, grain cereals, eggs, meat, legumes and nuts. Some antioxidants such as lycopene and ascorbic acid can be destroyed by long-term storage or prolonged cooking. Other antioxidant compounds are more stable, such as the polyphenolic antioxidants in foods such as whole-wheat cereals and tea. The effects of cooking and food processing are complex, as these processes can also affect the bioavailability of antioxidants, such as some carotenoids in vegetables. In general,
20 processed foods contain less antioxidants than fresh and uncooked foods.

Besides antioxidants that are derived from food sources, like vitamins, the human body can also synthesize antioxidants. For example, ubiquinol (coenzyme Q) is poorly absorbed from the gut and is made in humans. Another example is glutathione, which is made from amino acids. Any glutathione is broken
25 down in the gut to free cysteine, glycine and glutamic acid before being absorbed. Large amounts of sulfur-containing amino acids such as N-acetylcysteine can increase glutathione. Supplying more of these precursors may be useful as part of the treatment of some diseases, such as acute respiratory distress syndrome, protein-energy malnutrition, or preventing the liver damage produced by paracetamol overdose.

30 Other compounds in the diet can alter the levels of antioxidants indirectly, by enhancing the endogenous production of antioxidants. Some of these compounds, such as isothiocyanates and curcumin, may be chemopreventive agents that either block the transformation of abnormal cells into cancerous cells, or even kill existing cancer cells.

35 The role of antioxidants obtained from the diet in protection against disease is a topic of continuing interest. Several studies revealed beneficial effects of

antioxidants from the diet, for example the effect of n-acetylcysteine on COPD patients (6).

An alternative, the molecular epidemiologic approach, has several advantages, notably the small number of subjects needed and the short time scale. Its success depends, however, on the use of reliable validated biomarkers, which are rare. A notable example involves chromosomal aberrations, which were shown in prospective studies to be good indicators of future risk of cancer (7, 8). DNA damage is clearly the ultimate cause of cancer, because DNA base changes can be mutagenic, but, except for some special cases (9), DNA damage is probably better regarded as a marker of exposure to genotoxic agents than as an indicator of the likelihood that cancer will occur in an individual.

The sum of endogenous and food-derived antioxidants represents the total antioxidant activity of the extracellular fluid.

Cooperation of different antioxidants provides greater protection against oxidative stress. Thus, the overall antioxidant capacity gives more relevant biological information compared to that obtained by the measurement of individual components, as it considers the cumulative effect of all antioxidants present in plasma and body fluids.

Measurement of antioxidants is not a straightforward process, as this is a diverse group of compounds with different reactivities to different reactive oxygen species. Several antioxidant capacity assays have been developed and are used for testing. Each test has specific characteristics and limitations. The debate about a standard measurement is still ongoing. Previous research revealed that, from the specific oxidative stress biomarkers, the TEAC is the most sensitive biomarker (10).

There are several other assays described and on the market that measure anti-oxidant status. The Trolox Equivalent Antioxidant Assay measures the total antioxidant capacity of plasma, serum, urine, saliva or other solutions/fluids. The assay relies on the ability of antioxidants to reduce the ABTS radical (2,2'-azino-di-[3-ethylbenzothiazoline-sulphonate] radical). The capacity of the antioxidants in the sample to reduce the ABTS radical is compared with that of Trolox, a water soluble tocopherol analogue, and is quantified as Trolox equivalents (11, 12). This assay however can only be performed in the laboratory, using specialized equipment.

There is still a need in the art for reliable easy-to-use assays that measure the antioxidant status of a sample. The present invention addresses that need.

Summary of the invention

The invention provides a method for obtaining a stable radical ABTS in solid form comprising the steps of dissolving ABTS, converting the dissolved ABTS into radical ABTS with a peroxidase in the presence of hydrogen peroxide, freezing the thus obtained radical ABTS solution and freeze drying the frozen solution to obtain radical ABTS in solid form

The invention also relates to a stable radical ABTS in solid form obtainable by that method.

The invention also relates to a method for obtaining a solid support wherein a stable radical ABTS in solid form is dissolved in an organic solvent and applied to the solid support.

The invention also relates to a solid support thus obtainable.

The invention also relates to the use of such a solid support for determining the antioxidant status of a sample.

Detailed description of the invention

We found that ABTS (2,2-azino-bis-3 ethylbenzothiazoline-6-sulfonic acid) may be used in a simple and reliable ready-for-use test in order to determine the antioxidant status of a sample. For that purpose, ABTS is dissolved in water or a buffered aqueous solution at a pH between 7 and 8. ABTS is then converted into radical ABTS, frozen and freeze dried.

Such a method has been described by Durmaz (12). It is described therein that a radical ABTS powder was prepared in 2.45 mM potassium persulfate. The thus prepared ABTS radical appeared not to be stable upon storage for as short a period of 10 days; it lost at least 20% of its activity within 10 days after production (figure 3 at page 1661 of Durmaz, supra).

This confirmed the prejudice in the art that radical ABTS is not stable. Contrary to this prejudice, we were able to obtain a stable ABTS radical powder when we prepared radical ABTS in the presence of a peroxidase and hydrogen peroxide before freeze drying the radical ABTS solution. In that way we were able to generate a radical ABTS that was stable for at least 40 days. In a representative experiment, the dried powder did not lose more than 10% of its activity in a period of 40 days.

The thus obtained stable radical ABTS powder may be used to determine the antioxidant status of a sample. For instance, by immobilizing the ABTS radical on a solid support. This may be accomplished by contacting the ABTS radical solution with a solid support such as a boron silica strip. The solid support comprising

the radical ABTS solution is then frozen and freeze dried in order to obtain a test strip.

When the radical ABTS radical powder is contacted with a sample containing antioxidants, the color of the powder changes from green to colourless.

5 The assay was found to be sensitive in the micromolar range, i.e. it provided reliable results in a broad range, such as between 1 micromolar and 1 millimolar of ascorbic acid in solution. The test strips may be designed in such a way that they cover a broad range of anti-oxidant capacity (TAC), such as between 5 µg/mL and 50 µg/mL.

10 The test strip can be used in nutritional and clinical research for quick testing of the antioxidant capacity of many samples. In a representative test, the sample is contacted with the test strip and the degree of decolorization is quantified. In a preferred embodiment, the test strip is dipped into the sample fluid, and the degree of decolorization is quantified.

15 The test strip may also conveniently be used for testing the anti-oxidant status of products for topical applications, or ingredients thereof. Cosmetic products such as deodorants, lotions, ointments, creams, gels, hairprays or parfums may conveniently and quickly be tested for their anti-oxidant status thereby avoiding animal testing.

20 General practitioners and analysts in clinical chemistry departments can use this test strip for a quick determination of the antioxidant status of patients. The sample is therefore placed on the test strip and the degree of decolorization is then quantified. Preferred samples for measuring the antioxidant status are blood, plasma, saliva, urine, vaginal fluid, tear, semen, stool, sweat or serum samples.

25 In food production, the test strip can be used to test the quality of ingredients and products. It can also be used to test the shelf life of the products. The sample is placed on the test strip and the degree of decolorization is quantified. Otherwise, the test strip is dipped into the sample fluid, and the degree of decolorization is quantified.

30 Consumers can use the test strip at home to measure the quality of their antioxidant containing products. Potentially, it can also be used to assess their own health status. The sample is placed on the test strip and the degree of decolorization is quantified. Otherwise, the test strip is dipped into the sample fluid, and the degree of decolorization is quantified.

35 A particular useful method for obtaining a stable radical ABTS in solid form comprises the steps of dissolving ABTS, converting the dissolved ABTS into radical ABTS with a peroxidase in the presence of hydrogen peroxide, freezing the thus

obtained radical ABTS solution and freeze drying the frozen solution to obtain radical ABTS in solid form. The concentration of ABTS is not critical and can easily be determined by a skilled person, depending on the desired application.

5 The ABTS may advantageously be dissolved in water or in a buffered solution depending on the intended application. A skilled person may empirically determine the most desired solvent for his application. In the below examples, the ABTS is dissolved in a buffered aqueous solution at a pH between 7 and 8 or in water.

Advantageously, a polymer is added to the radical ABTS solution before freeze drying if the powder is to be applied on a strip. The addition of a polymer ensures that the radical ABTS may be applied to the strip more homogeneously. PVPVA was found to be a particularly useful polymer.

The invention also relates to a stable radical ABTS in solid form obtainable by the method according to claims 1 - 4. Such a stable radical ABTS in solid form was not attainable by any of the prior art methods.

15 The stable radical ABTS may advantageously be applied to a test strip in order to obtain a solid support comprising an immobilized radical ABTS.

It is particularly advantageous to apply the solid radical ABTS to a strip while dissolved in an organic solvent, such as ethyl acetate or dichloro methane. The invention therefore also relates to a method for obtaining a solid support wherein a stable radical ABTS in solid form is dissolved in an organic solvent and applied to the solid support.

Particular useful test strips were found to be made of borosilicate filter paper. The appropriate pore size of that paper may vary and may be determined by the skilled person using routine experimentation. Appropriate pore sizes were found to be within the range of 0,5 micrometer and 3 micrometer, however, other pore sizes may be equally well suited, depending on the desired characteristics of the eventual device.

It was also found that the ABTS powder lost some activity during drying which could not be avoided. However, it was also found that the loss of activity could be greatly reduced if not diminished by allowing the ABTS powder to dry slowly, such as for instance at 22 degrees Celsius for several hours. When the powder was dried under vacuum at 95 degrees Celsius, the powder lost up to half of its activity. In any case, after drying, the powder was stable for at least 40 days, irrespective of the method of drying. Stable in this context is to be interpreted as that the powder or dispersion retained more than 80% of its activity after 10 days of production, such as more than 80% after 20 days or even 40 days. Preferably, the dried powder retained

more than 90% of its activity on day 40 after drying in comparison to the activity immediately after drying.

For some applications it may be advantageous to have a homogenous suspension of radical ABTS powder. This is for instance the case when the powder is coated onto a solid support for quantitative measurements. We found that a very homogenous suspension could be obtained with the procedures as described herein. In particular, we found that the powder consists of microscopic particles having an average size of less than 10 micrometer, or even less than 1 micrometer, when the procedure according to example 3 was followed. The powder could be dispersed in a suspension and the dispersions thus obtained were found to be stable for at least 4 months. Dispersions with smaller particle size were also found more suitable for application on borosilicate test strips since they showed an improved adhesion to the strips. Homogenous suspensions with smaller particle sizes were also found to be particularly suited for application on other solid supports, such as for instance ELISA plates. In that way a homogeneously coated ELISA test plate may be obtained allowing for reproducible and quantitative testing of the oxidative status of a sample in an ELISA procedure.

It was also found that when stabilized ABTS radical was adsorbed onto a solid support, this may advantageously be done in a range of 0.055 to 0.125 ug powder per square millimeter of solid support. Other ranges may be applied equally well, depending on the particular purpose of the test strip.

Examples

Example 1: preparation of ABTS radical powder

ABTS (2,2-azino-bis-3 ethylbenzothiazoline-6-sulfonic acid) was dissolved in a 145 mM sodiumphosphate buffer, pH 7.4 at room temperature at a concentration of 10 mM. Four units of horseradish peroxidase were added to the solution and hydrogen peroxide was subsequently added to reach a concentration of 70 nM (ABTS radical solution). It was observed that the solution obtained a dark green color within 10 minutes.

The solution was then kept at -80 degrees Celsius overnight and then freeze dried to obtain ABTS radical powder.

Example 2: alternative preparation of ABTS radical powder

ABTS (2,2-azino-bis-3 ethylbenzothiazoline-6-sulfonic acid) was dissolved in water to a concentration of 500 micromolar. Ten microliters of horse radish

peroxidase (1mg/ml) were added to the solution followed by hydrogen peroxide to a final concentration of 200 micromolar of hydrogen peroxide. It was observed that the solution obtained a dark green color within 10 minutes.

5 The solution was then quickly frozen in liquid nitrogen and freeze dried to obtain ABTS radical powder.

Example 3: Preparation of strips containing ABTS radical powder

For the preparation of strips, the radical powder was dissolved in a polymer solution. The procedure was as follows. ABTS (2,2-azino-bis-3
10 ethylbenzothiazoline-6-sulfonic acid) was dissolved in water to a concentration of 500 micromolar. Ten microliters of horse radish peroxidase (1mg/ml) was added to the solution followed by H_2O_2 to a final concentration of 200 micromolar of H_2O_2 . It was observed that the solution obtained a dark green color within 10 minutes. Polyvinylpyrrolidone vinyl acetate (PVPVA) was added to a final concentration of 0.1
15 milligram PVPVA per milliliter of solution.

The solution was then quickly frozen in liquid nitrogen and then freeze dried to obtain ABTS radical powder.

The powder was then suspended into dichloromethane to a concentration of 1 mg powder per gram of solvent. The suspension was sonicated and
20 dripped onto borosilicate filters (Type A/E or A/D). After air drying the filters were glued onto a base support material to form a solid support comprising the immobilized radical ABTS.

Example 4: Alternative preparation of ABTS antioxidant strips.

25 The dark green ABTS radical solution was absorbed on Boron silica strips. The strips were then placed in sealed tubes and placed at -80 degrees Celsius overnight. The strips were freeze dried while in the tubes for several days. The strips were then stored in a water free environment.

30 Example 5. Use of ABTS antioxidant strips to determine the antioxidant status of a sample.

The ABTS powder and ABTS strips were tested for their reactivity with several concentrations of ascorbic acid in a buffer, ranging from 1 microMolar to 1 milliMolar. Therefore, the test strips were contacted with the different concentrations of
35 ascorbic acid for 1 minute. Decolorization was observed after 10 minutes. The results are displayed in the table below.

It was found that the decolorization of the ABTS radical powder on the test strip was proportional to the concentration of ascorbic acid.

Concentration ascorbic acid [micro Molar]	Color of ABTS test strip
0	Bright green
1	Bright green
10	Bright green
50	Light green
100	very faint green
1000	Colorless

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CLAIMS

1. Method for obtaining a stable radical 2,2-azino-bis-3 ethylbenzothiazoline-6-sulfonic acid (ABTS) in solid form comprising the steps of dissolving ABTS,
5 converting the dissolved ABTS into radical ABTS with a peroxidase in the presence of peroxide, freezing the thus obtained radical ABTS solution and freeze drying the frozen solution to obtain radical ABTS in solid form.
2. Method according to claim 1 wherein the ABTS is dissolved in a buffered aqueous solution at a pH between 7 and 8.
- 10 3. Method according to claim 1 wherein the ABTS is dissolved in water.
4. Method according to any of claims 1 - 3 wherein a polymer is added to the radical ABTS solution before freeze drying.
5. Method according to claims 1 - 4 wherein the peroxide is hydrogen peroxide.
6. Method according to claim 5 wherein the polymer is polyvinylpyrrolidone vinyl
15 acetate (PVPVA).
7. A stable radical ABTS in solid form obtainable by the method according to claims 1 - 6.
8. A solid support comprising an immobilized radical ABTS according to claim 7.
9. A solid support according to claim 8 which is a test strip or an ELISA plate.
- 20 10. Method for obtaining a solid support according to claims 8 or 9 wherein a stable radical ABTS in solid form according to claim 7 is dissolved in an organic solvent and applied to the solid support.
11. Method according to claim 10 wherein the organic solvent is dichloromethane.
12. Use of a solid support according to claims 8 or 9 for determining the antioxidant
25 status of a sample.
13. Use according to claim 12 wherein the sample is obtained from a food or feed or a food or feed ingredient.

14. Use according to claim 12 wherein the sample is derived from a human.
15. Use according to claim 12 wherein the sample is a blood, plasma, saliva, urine, vaginal fluid, tear, semen, stool, sweat or serum sample.
16. Use according to claim 12 wherein the sample is derived from a product intended
5 for topical use, such as a cosmetic product.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/054208

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/52
ADD.

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

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)

EPO-Internal, EMBASE, INSPEC, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GÖKHAN DURMAZ: "Freeze-dried ABTS+ method: A ready-to-use radical powder to assess antioxidant capacity of vegetable oils", FOOD CHEMISTRY, vol. 133, no. 4, 18 February 2012 (2012-02-18), pages 1658-1663, XP055036370, ISSN: 0308-8146, DOI: 10.1016/j.foodchem.2012.02.064 Note: Available online 18 February 2012 Point 2.2; abstract page 1658, paragraph 1 page 1660, right-hand column, paragraph 1 page 1663, paragraph 2 ----- -/--	1-16



Further documents are listed in the continuation of Box C.



See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/054208

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KADNIKOVA EKATERINA N ET AL: "Oxidation of ABTS by hydrogen peroxide catalyzed by horseradish peroxidase encapsulated into sol-gel glass. Effects of glass matrix on reactivity", JOURNAL OF MOLECULAR CATALYSIS B ENZYMATIC, vol. 18, no. 1-3, 13 September 2002 (2002-09-13), pages 39-48, XP002695482, ISSN: 1381-1177 abstract	1-6
Y	----- VILLANO D ET AL: "The antioxidant activity of wines determined by the ABTS(+) method: influence of sample dilution and time.", TALANTA 8 OCT 2004, vol. 64, no. 2, 8 October 2004 (2004-10-08), pages 501-509, XP002695483, ISSN: 1873-3573 page 502, right-hand column, last paragraph - page 503, left-hand column, paragraph 1	1-6
A	----- ROBERTA RE ET AL: "Antioxidant activity applying an improved ABTS radical cation decolorization assay", FREE RADICAL BIOLOGY & MEDICINE, vol. 26, no. 9-10, 1 May 1999 (1999-05-01), pages 1231-1237, XP055036352, ISSN: 0891-5849, DOI: 10.1016/S0891-5849(98)00315-3 abstract page 1231, right-hand column, last paragraph - page 1232, right-hand column, last paragraph	1-6
A	----- IRIS CATIANA ZAMPINI ET AL: "Autographic Assay for the Rapid Detection of Antioxidant Capacity of Liquid and Semi-solid Pharmaceutical Formulations Using ABTS-+ Immobilized by Gel Entrapment", AAPS PHARMSCITECH, vol. 11, no. 3, 1 September 2010 (2010-09-01), pages 1159-1163, XP055036362, ISSN: 1530-9932, DOI: 10.1208/s12249-010-9484-y abstract p.1160, right-hand column, "Staining procedures"	1-6
