

## DESCRIPTION

METHOD FOR EVALUATING SULFUR COMPOUND-CONTAINING SUBSTANCE,  
AND METHOD FOR QUANTIFYING VOLATILE LOW MOLECULAR WEIGHT  
SULFUR COMPOUND

### TECHNICAL FIELD

[0001]

The present invention relates to a method for evaluating a sulfur compound-containing substance contained in a sample such as an agricultural crop and a method for quantifying a volatile low-molecular-weight sulfur compound.

### BACKGROUND ART

[0002]

Some agricultural crops rapidly develop oxidative deterioration in their quality and have difficulty in long-term storage. Conventionally, typical methods of evaluating freshness of an agricultural crop have been employed sensory tests in which items such as appearance, touch, and flavor of the agricultural crop are evaluated by evaluators (such as agricultural crop distributors or customers). However, sensory tests have a problem in that they may incorporate subjective views of evaluators, and thus prevent objective evaluation and also fail to produce quantitative evaluation. Hence, an evaluating method which provides objective and quantitative evaluation has been demanded.

[0003]

Examples of methods of quantitatively evaluating freshness of an agricultural crop include methods of measuring change of vitamin C content in the agricultural crop. Agricultural crops lose vitamin C content in accordance with postharvest time passage and storage environment temperature. Thus, freshness of agricultural crops can be evaluated by measuring change of vitamin C content. Examples of methods of measuring vitamin C content

in agricultural crops include a method using ORAC (Oxygen Radical Absorbance Capacity) technique (see, e.g., Non-Patent Literature 1).

#### CITATION LIST

##### NON-PATENT LITERATURE

[0004]

NON-PATENT LITERATURE 1: Cao, G., and Prior, R. (1999) Methods Enzymol. 299:50-62. Measurement of oxygen radical absorbance capacity in biological samples.

#### SUMMARY OF INVENTION

##### TECHNICAL PROBLEM

[0005]

In methods for measuring variation of vitamin C content, the vitamin C content needs to be measured at least once each at harvesting and evaluation of freshness for an agricultural crop. In other words, for measurement of freshness of the agricultural crop by this method, a vitamin C content to be the standard of the freshness needs to be measured at harvesting of the agricultural crop. Accordingly, without grasping the condition of the agricultural crop at harvest, these methods cannot provide evaluation of its freshness, and thus has had a problem in that they cannot provide evaluation of freshness of an agricultural crop at an arbitrary time point.

Moreover, the inventors found that freshness can be evaluated by multivariate analysis on analysis results of a plurality of sulfur compounds and a plurality of sulfur metabolism-related compounds contained in an agricultural crop and the like, although volatile low-molecular-weight sulfur compounds had conventionally been analyzed by gas chromatography, thus creating difficulty in simultaneous quantification with nonvolatile sulfur compound by liquid chromatography mass spectrometry technique (LC-MS/MS technique).

[0006]

The present invention has been made in consideration of the aforementioned circumstances, and has an object to provide a method for evaluating freshness (redox level) or the like of a test substance such as an agricultural crop on the basis of analysis results of a plurality of sulfur compounds and a plurality of sulfur metabolism-related compounds. Additionally, the present invention has another object to provide a method for quantifying a volatile low-molecular-weight sulfur compound by liquid chromatography mass spectrometry technique.

#### SOLUTION TO PROBLEM

[0007]

The issue described above would be solved by performing a method for evaluating a sulfur compound-containing substance for evaluating a plurality of test substances, including the steps of: providing quantitative values of a plurality of sulfur compounds and a plurality of sulfur metabolism-related compounds for each of the test substances; analyzing analysis values of the test substances, including the quantitative values, by multidimensional scaling, and creating a scatter diagram having two or more types of scales selected from redox level, contribution of microbial metabolism, anti-oxidative capacity, or aroma or odor; and assessing the redox level, the contribution of microbial metabolism, the anti-oxidative capacity, or the aroma or odor of the test substances, on the basis of the positions on the scatter diagram, according to the method for evaluating a sulfur compound-containing substance of the present invention.

[0008]

In this context, it is preferred that the scatter diagram be a two-dimensional scatter diagram having two types of scales selected from redox level, contribution of microbial metabolism, anti-oxidative capacity, or aroma or

odor.

[0009]

In this context, it is preferred that the scatter diagram be a three-dimensional scatter diagram having three types of scales selected from redox level, contribution of microbial metabolism, anti-oxidative capacity, or aroma or odor.

[0010]

In this context, it is preferred that the sulfur compounds include glutathione and a derivative thereof, and that the sulfur metabolism-related compounds include an ergothioneine metabolism related compound.

[0011]

In this context, it is preferred that the sulfur compounds include at least 20 types, and that the sulfur metabolism-related compounds include at least 18 types.

[0012]

In this context, it is preferred that the sulfur compounds include sulfite ion, thiosulfate ion, sulfide ion, glutathione, glutathione monosulfide, glutathione disulfide, glutathione trisulfide, glutathione tetrasulfide, oxidized glutathione, oxidized glutathione monosulfide, oxidized glutathione disulfide, oxidized glutathione trisulfide, oxidized glutathione tetrasulfide, cysteine, cysteine monosulfide, methionine, 2-furfurylthiol, benzyl mercaptan, 4-mercapto-4-methyl-pentane-2-one, and 2-mercaptoethyl acetate, and that the sulfur metabolism-related compounds include cystine, cystathionine, S-adenosylmethionine, homocysteine, S-sulfocysteine, O-phosphoserine, O-acetylserine, N-acetylserine, O-succinylhomoserine, adenosine 5'-phosphosulfate, lanthionine, 3'-phosphoadenosine -5'-sulfate, 3'-phosphoadenosine -5'-phosphosulfate, ergothioneine, hercynine, 5-glutamylcysteine, S-hercynyl-cysteine sulfoxide, and 5-glutamyl-S-hercynyl-cysteine sulfoxide.

[0013]

In this context, it is preferred that a method for quantifying a volatile low-molecular-weight sulfur compound be performed, in which the step of providing the quantitative values includes the steps of: pretreating the volatile low-molecular-weight sulfur compound by modification with an alkylating agent; and analyzing the modified volatile low-molecular-weight sulfur compound by a liquid chromatography mass spectrometry technique, and quantifying the volatile low-molecular-weight sulfur compound.

[0014]

In this context, it is preferred that the alkylating agent be one or more types of compounds selected from the group consisting of 5, 5'-dithiobis (2-nitrobenzoic acid), 2, 6-dichlorophenolindophenol, *p*-chloromercuribenzoic acid, iodoacetamide, *N*-ethylmaleimide, and monobromobimane.

[0015]

In this context, it is preferred that the volatile low-molecular-weight sulfur compound be one or more types of compounds selected from the group consisting of 2-furfurylthiol, benzyl mercaptan, 4-mercapto-4-methylpentane-2-one, and 2-mercaptoethyl acetate.

#### ADVANTAGEOUS EFFECTS OF INVENTION

[0016]

The present invention can provide a method for evaluating the freshness (redox level) or the like of a test substance such as an agricultural crop, on the basis of analysis results of a plurality of sulfur compounds and a plurality of sulfur metabolism-related compounds, and a method for quantifying a volatile low-molecular-weight sulfur compound in which a volatile low-molecular-weight sulfur compound is quantified by liquid chromatography mass spectrometry technique.

#### BRIEF DESCRIPTION OF DRAWINGS

[0017]

FIG. 1 depicts a scatter diagram showing results analyzed for 69 types of commercially-available wines as test substances by multidimensional scaling.

FIG. 2 depicts a scatter diagram showing results analyzed for a plurality of sulfur compounds and a plurality of sulfur metabolism-related compounds by multidimensional scaling.

FIG. 3 illustrates results quantified for monobromobimane (mBBBr)-modified glutathione (GS-bimane), mBBBr-modified glutathione monosulfide (GS-S-bimane), mBBBr-modified glutathione disulfide (GS-S<sub>2</sub>-bimane), mBBBr-modified glutathione trisulfide (GS-S<sub>3</sub>-bimane), and mBBBr-modified glutathione tetrasulfide (GS-S<sub>4</sub>-bimane) by liquid chromatography mass spectrometry technique.

FIG. 4 depicts a scatter diagram showing results analyzed for culture liquids after culturing of each of a plurality of microorganisms as test substances by multidimensional scaling.

FIG. 5 depicts a scatter diagram showing results analyzed for commercially-available wines as test substances by multidimensional scaling.

FIG. 6 depicts a scatter diagram showing results analyzed for commercially-available wines as test substances by multidimensional scaling.

FIG. 7 depicts a scatter diagram showing results analyzed for commercially-available beers as test substances by multidimensional scaling.

FIG. 8 depicts a scatter diagram showing results analyzed for commercially-available beers as test substances by multidimensional scaling.

#### DESCRIPTION OF EMBODIMENTS

[0018]

The embodiments of the method for evaluating a sulfur compound-containing substance and the method for quantifying a volatile low-molecular-weight sulfur compound of the

present invention will now be described.

Incidentally, the embodiments are specifically described to provide better understanding of the spirit of the invention, and do not limit the present invention unless otherwise specified.

[0019]

[Method for evaluating a sulfur compound-containing substance]

The method for evaluating a sulfur compound-containing substance of the embodiment is a method for evaluating a sulfur compound-containing substance for evaluating a plurality of test substances, including the steps of: providing quantitative values of a plurality of sulfur compounds and a plurality of sulfur metabolism-related compounds for each of the test substances; analyzing analysis values of the test substances, including the quantitative values, by multidimensional scaling, and creating a scatter diagram having two or more types of scales selected from redox level, contribution of microbial metabolism, anti-oxidative capacity, or aroma or odor; and assessing redox level, contribution of microbial metabolism, anti-oxidative capacity, or aroma or odor of the test substances, on the basis of the positions on the scatter diagram.

In the method for evaluating a sulfur compound-containing substance of the embodiment, the term "test substance" means a substance to be a subject for assessing redox level, contribution of microbial metabolism, or aroma or odor, or the like by the method for evaluating a sulfur compound-containing substance of the embodiment.

[0020]

Test substances in the method for quantifying a volatile low-molecular-weight sulfur compound of the embodiment are not particularly limited, and include, for example, soil, water, foods, beverages, biological samples.

Examples of foods include meat; seafood; eggs; milk; and agricultural crops such as cereals, beans, potatoes,

vegetables, and fruits.

Examples of beverages include brewed products such as beer, wine, and sake; and soft drinks.

Examples of biological samples include blood, saliva, and feces.

[0021]

Furthermore, in the method for evaluating a sulfur compound-containing substance of the embodiment, the steps of pretreating the volatile low-molecular-weight sulfur compound by modification with an alkylating agent (step A); and analyzing the volatile low-molecular-weight sulfur compound modified with the alkylating agent, by a liquid chromatography mass spectrometry technique, and quantifying the volatile low-molecular-weight sulfur compound (step B) can be performed according to the method for quantifying a volatile low-molecular-weight sulfur compound of the embodiment as described later.

[0022]

In the method for evaluating a sulfur compound-containing substance of the embodiment, in addition to an off-flavor compound which is a volatile low-molecular-weight sulfur compound, sulfur compounds, including glutathione and a derivative thereof, and sulfur metabolism-related compounds, including an ergothioneine metabolism related compound, are desirably quantified.

[0023]

Sulfur compounds may include 20 types or more, and sulfur metabolism-related compounds may include 18 types or more.

[0024]

Sulfur compounds are not particularly limited, as long as they can be modified with an alkylating agent and after modification with the alkylating agent, they can be analyzed by liquid chromatography mass spectrometry technique.

The alkylating agent is preferably one or more types of compounds selected from the group consisting of 5,5'-

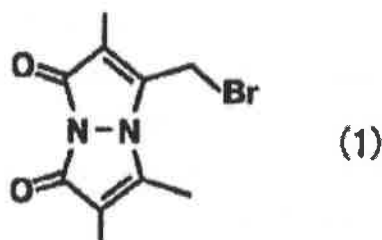
dithiobis(2-nitrobenzoic acid), 2,6-dichlorophenolindophenol, p-chloromercuribenzoic acid, iodoacetamide, N-ethylmaleimide, and monobromobimane (mBBr). These alkylating agents may be used singly with one type or may be used in combination of two or more types.

[0025]

In this context, monobromobimane (mBBr) is represented by the following formula (1). Hereinafter, monobromobimane may be abbreviated as "mBBr".

[0026]

[Formula 1]



[0027]

Sulfur compound preferably include sulfite ion, thiosulfate ion, sulfide ion, glutathione, glutathione monosulfide, glutathione disulfide, glutathione trisulfide, glutathione tetrasulfide, oxidized glutathione, oxidized glutathione monosulfide, oxidized glutathione disulfide, oxidized glutathione trisulfide, oxidized glutathione tetrasulfide, cysteine, cysteine monosulfide, methionine, 2-furfurylthiol, benzyl mercaptan, 4-mercapto-4-methylpentane-2-one, and 2-mercaptoethyl acetate. The quantitative values of these sulfur compounds are to be factors for assessing redox level of a test substance.

[0028]

Sulfur metabolism-related compounds are not particularly limited, as long as they can be modified with the alkylating agent described above and after modification with the alkylating agent, they can be analyzed by liquid chromatography mass spectrometry technique. Nonetheless,

they preferably include cystine, cystathionine, S-adenosylmethionine, homocysteine, S-sulfocysteine, O-phosphoserine, O-acetylserine, N-acetylserine, O-succinylhomoserine, adenosine 5'-phosphosulfate, lanthionine, 3'-phosphoadenosine -5'-sulfate, 3'-phosphoadenosine -5'-phosphosulfate, ergothioneine, hercynine, 5-glutamylcysteine, S-hercynyl-cysteine sulfoxide, and 5-glutamyl-S-hercynyl-cysteine sulfoxide. The quantitative values of these sulfur metabolism-related compounds are to be factors for assessing contribution of microbial metabolism of a test substance.

[0029]

In the method for evaluating a sulfur compound-containing substance of the embodiment, quantitative values of a plurality of sulfur compounds and a plurality of sulfur metabolism-related compounds are provided beforehand for each of test substances.

Examples of the methods for quantifying beforehand sulfur compounds and sulfur metabolism-related compounds contained in a test substance include liquid chromatography mass spectrometry technique (LC-MS/MS), gas chromatography mass spectrometry technique (Gas Chromatography-Mass Spectrometry, GC-MS), gas chromatography mass spectrometry technique (Gas Chromatography-tandem Mass Spectrometry, GC-MS/MS), capillary electrophoresis-mass spectrometry technique (Capillary Electrophoresis-Mass Spectrometry, CE-MS). Among these, due to ease for connection with quantitative values of the off-flavor compound described later, liquid chromatography mass spectrometry technique is preferably used. Additionally, quantitative values derived from a method other than liquid chromatography mass spectrometry technique can be connected with quantitative values derived from liquid chromatography mass spectrometry technique.

[0030]

In the method for evaluating a sulfur compound-

containing substance of the embodiment, analysis values of test substances, including quantitative values of a plurality of sulfur compounds and a plurality of sulfur metabolism-related compounds, provided beforehand for each of the test substances are analyzed by multidimensional scaling to create a two-dimensional or three-dimensional scatter diagram. Then, on the basis of the positions on the scatter diagram, redox level, contribution of microbial metabolism, anti-oxidative capacity, or aroma or odor of the test substances are assessed.

In this context, scales of the scatter diagram (parameters for each axis) can be selected from redox level, contribution of microbial metabolism, anti-oxidative capacity (action of microorganisms), or aroma or odor, but are not limited to these scales.

[0031]

In the method for evaluating a sulfur compound-containing substance of the embodiment, a two-dimensional or three-dimensional scatter diagram is created by analysis with multidimensional scaling. Examples of the scatter diagrams thus obtained include the two-dimensional scatter diagrams shown in FIG. 1 and FIG. 2.

In the method for evaluating a sulfur compound-containing substance of the embodiment, analysis by multidimensional scaling is performed using multidimensional scaling (Vegan), which is one of packages in the free software for statistical analysis "R".

[0032]

FIG. 1 depicts a scatter diagram showing results analyzed for 69 types of commercially-available wines as test substances by multidimensional scaling. FIG. 2 depicts a scatter diagram showing results analyzed for the aforementioned sulfur compounds and sulfur metabolism-related compounds by multidimensional scaling. Incidentally, the items indicated as "labeled" in FIG. 2 represent the sulfur compounds or sulfur metabolism-related compounds

having modification with an alkylating agent as described above.

[0033]

By comparing FIG. 1 to FIG. 2, the redox states of the test substances in the scatter diagram of FIG. 1 can be evaluated. More precisely, it is known whether each of the sulfur compounds and sulfur metabolism-related compounds shown in FIG. 2 is in an oxidized state or in a reduction state: the redox states of the test substances can therefore be evaluated by comparing the positions of these sulfur compounds and sulfur metabolism-related compounds on the scatter diagram to the positions of the test substance on the scatter diagram. In FIG. 2, for example, since the compounds in a reduction state (such as sulfite ion and glutathione) are located in the right-side area based on 0 on the x-axis, the right-side area is defined to represent being in a reduction state. Meanwhile, in FIG. 2, for example, since oxidized compounds (such as oxidized glutathione) are located in the left-side area based on 0 on the x-axis, the left-side area is defined to represent being in an oxidized state. Moreover, in FIG. 2, for example, since ergothioneine is located in the upper area based on 0 on the y-axis, the upper area is defined to represent having high contribution of microbial metabolism. Ergothioneine is one of sulfur-containing amino acids, and known to be biosynthesized solely in some microorganisms. Therefore, a test substance containing more ergothioneine represents one having higher contribution of microbial metabolism.

[0034]

Additionally, though not shown in FIG. 1 and FIG. 2, for example, compounds located the upper area based on 0 on the z-axis perpendicular to the x-axis and y-axis (the front side of the paper plane) may be defined to be in a state with strong odor, while compounds located in the bottom area based on 0 on the z-axis perpendicular to the x-axis and y-axis (the back side of the paper plane) may be defined to be

in a state with weak odor. For example, if 2-furfurylthiol is located in the upper area based on 0 on the z-axis, the upper area is defined to represent having strong coffee-like flavor.

In this way, on the basis of a position on a scatter diagram, redox level, contribution of microbial metabolism, anti-oxidative capacity, or aroma or odor of a test substance can be assessed.

[0035]

In the method for evaluating a sulfur compound-containing substance of the embodiment, results assessed for redox states and aroma of test substances may be shown in a two-dimensional scatter diagram. Moreover, in the method for evaluating a sulfur compound-containing substance of the embodiment, results assessed for contribution of microbial metabolism and aroma of test substances may also be shown in a two-dimensional scatter diagram. Furthermore, in the method for evaluating a sulfur compound-containing substance of the embodiment, results assessed for redox states, anti-oxidative capacity, or odor or aroma of test substances may be shown in a three-dimensional scatter diagram.

[0036]

Meanwhile, in the method for evaluating a sulfur compound-containing substance of the embodiment, analysis values of test substances, including quantitative values of a sulfur compound with a thiol group and polysulfide of a sulfur compound quantified by liquid chromatography mass spectrometry technique, are analyzed by multidimensional scaling, and then redox states of the test substances can also be assessed from the analysis results on the basis of types and quantitative values of a sulfur compound with a thiol group and polysulfide of a sulfur compound.

[0037]

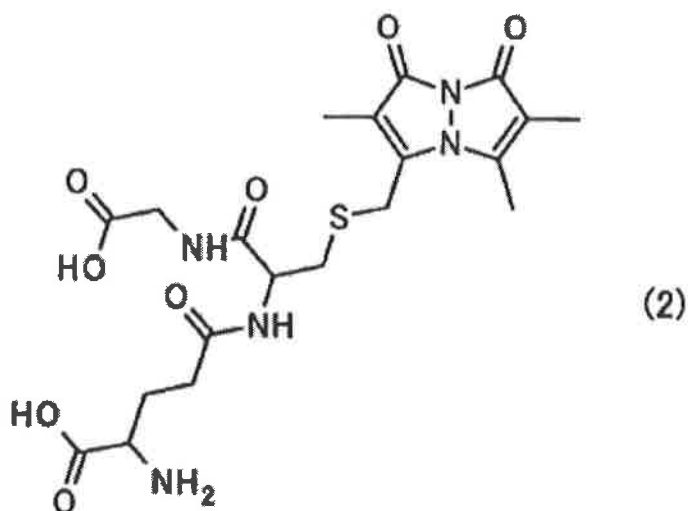
For example, as quantified by liquid chromatography mass spectrometry technique, mBBR-modified glutathione, glutathione monosulfide, glutathione disulfide, glutathione

trisulfide, and glutathione tetrasulfide form grades in this order as shown in FIG. 3.

The following formula (2) represents mBBBr-modified glutathione (GS-bimane), the following formula (3) represents mBBBr-modified glutathione monosulfide (GS-S-bimane), the following formula (4) represents mBBBr-modified glutathione disulfide (GS-S<sub>2</sub>-bimane), the following formula (5) represents mBBBr-modified glutathione trisulfide (GS-S<sub>3</sub>-bimane), and the following formula (6) represents mBBBr-modified glutathione tetrasulfide (GS-S<sub>4</sub>-bimane).

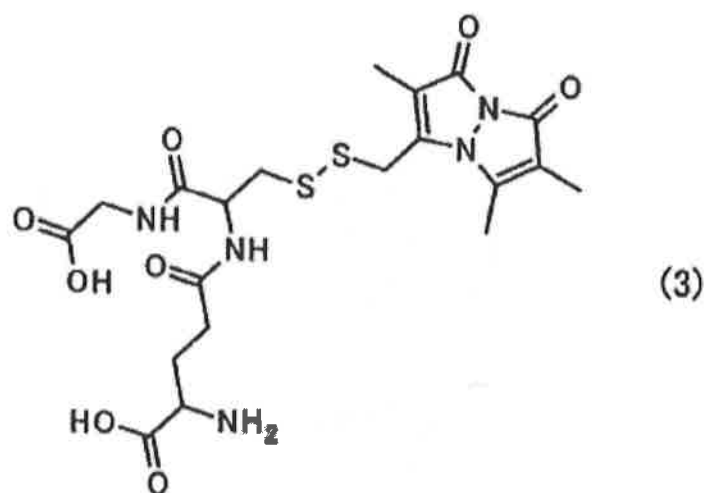
[0038]

[Formula 2]



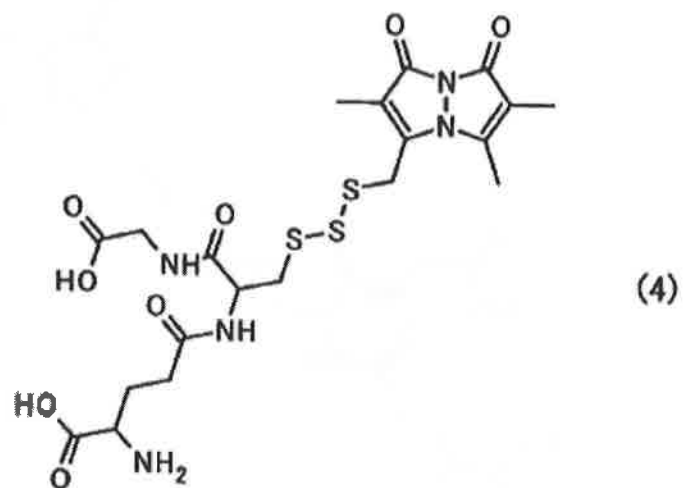
[0039]

[Formula 3]



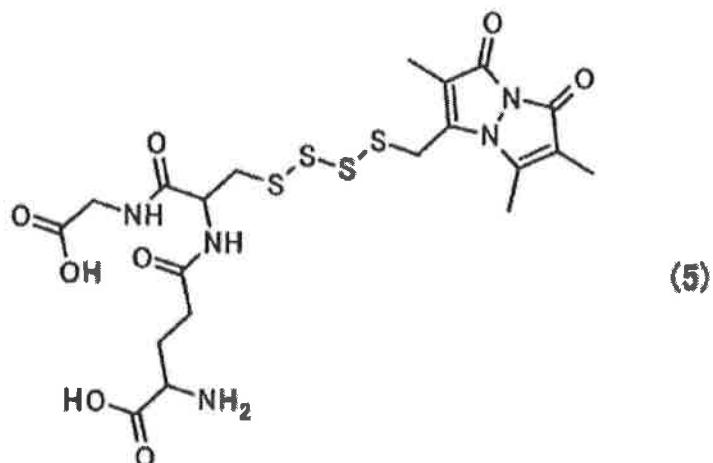
[0040]

[Formula 4]



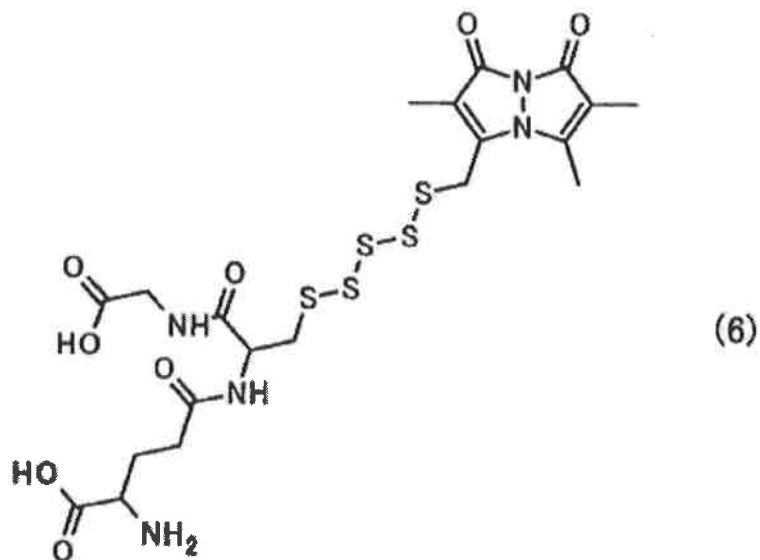
[0041]

[Formula 5]



[0042]

[Formula 6]



[0043]

GS-bimane, GS-S-bimane, GS-S<sub>2</sub>-bimane, GS-S<sub>3</sub>-bimane, and GS-S<sub>4</sub>-bimane also form grades in this order. These grades indicate redox states of test substances, and if GS-bimane shows a high quantitative value, such test substance is to be judged as having low oxidation level. On the other hand, if GS-S<sub>4</sub>-bimane shows a high quantitative value, such test substance is to be judged as having high oxidation level.

[0044]

Since food or the like increases in oxidation level with the passage of time, freshness of a test substance such as food can be evaluated by assessing a redox state, i.e., oxidation level, of the test substance.

[0045]

Moreover, since brewed products such as beer, wine, and sake increase in umami with progress in fermentation, umami of a test substance can be evaluated by assessing contribution of microbial metabolism.

[0046]

According to the method for evaluating a sulfur compound-containing substance of the embodiment, analysis values of test substances, including quantitative values of a plurality of sulfur compounds and a plurality of sulfur metabolism-related compounds, provided beforehand for each of the test substances are analyzed by multidimensional scaling to create a two-dimensional or three-dimensional scatter diagram, thereby allowing to assess redox level, contribution of microbial metabolism, anti-oxidative capacity, or aroma or odor of the test substances.

[0047]

[Method for quantifying a volatile low-molecular-weight sulfur compound]

Like carbon cycle, nitrogen cycle, or the like, sulfur also has a cycle. In term of sulfur metabolism, every organism has their own strengths and weaknesses clearly. In particular, only limited microorganisms excels in "assimilation", which converts an inorganic sulfur compound to an organic sulfur compound. Most microorganisms and plants (agricultural crops) has poor assimilation of sulfur compounds, and animals including human have no capacity in assimilation of sulfur compounds. In other words, comprehensive analysis of sulfur compounds provides estimation for living environment, growth environment, or the like of human to agricultural crops: what they have ingested and what environment they grew up in.

Based on the findings as described above, the inventors came to complete the present invention.

[0048]

The method for quantifying a volatile low-molecular-weight sulfur compound of the embodiment includes the steps of pretreating the volatile low-molecular-weight sulfur compound by modification with an alkylating agent (hereinafter referred to as step A); and analyzing the modified volatile low-molecular-weight sulfur compound by a liquid chromatography mass spectrometry technique, and quantifying the volatile low-molecular-weight sulfur compound (hereinafter referred to as step B).

[0049]

In the method for quantifying a volatile low-molecular-weight sulfur compound of the embodiment, a volatile low-molecular-weight sulfur compound contained in the following test substances is quantified.

[0050]

The test substances in the method for quantifying a volatile low-molecular-weight sulfur compound of the embodiment are not particularly limited, and include, for example, soil, water, foods, beverages, biological samples.

Examples of foods include meat; seafood; eggs; milk; and agricultural crops such as cereals, beans, potatoes, vegetables, and fruits.

Examples of beverages include brewed products such as beer, wine, and sake; and soft drinks.

Examples of biological samples include blood, saliva, and feces.

[0051]

The volatile low-molecular-weight sulfur compound in the method for quantifying a volatile low-molecular-weight sulfur compound of the embodiment is a liquid that has a low molecular weight and a characteristic likely to be evaporated.

Additionally, the volatile low-molecular-weight sulfur compound in the method for quantifying a volatile low-

molecular-weight sulfur compound of the embodiment has a weight-average molecular weight (Mw) of 50 or more and 300 or less.

[0052]

The volatile low-molecular-weight sulfur compounds in the method for quantifying a volatile low-molecular-weight sulfur compound of the embodiment include a compound that causes off-flavor, such as unusual odor, altered odor, or deteriorate odor, which is secondarily produced following deterioration of food quality due to chemical change of a food component itself, contamination of an exogenous substance, or the like (hereinafter referred to as "off-flavor compound").

[0053]

Such off-flavor compound (volatile low-molecular-weight sulfur compound) is one or more types of compounds selected from the group consisting of 2-furfurylthiol, benzyl mercaptan, 4-mercapto-4-methylpentane-2-one, and 2-mercaptoethyl acetate. In other words, two or more types of off-flavor compounds may be contained in the sample described above.

[0054]

In the method for quantifying a volatile low-molecular-weight sulfur compound of the embodiment, the volatile low-molecular-weight sulfur compound described above is pretreated by modification with an alkylating agent in step A.

Pretreatment in step A is a treatment in which a volatile low-molecular-weight sulfur compound is modified with an alkylating agent so as to allow for analysis by liquid chromatography mass spectrometry technique (Liquid Chromatography-tandem Mass Spectrometry, LC-MS/MS) in the later step B.

[0055]

Liquid chromatography mass spectrometry apparatuses for performing liquid chromatography mass spectrometry

technique have a component separation (LC) part, a first mass spectrometry (MS) part, and a second mass spectrometry (MS) part. Liquid chromatography mass spectrometry technique is an analysis method including: separating a test substance into individual components in accordance with their affinity in the component separation (LC) part; further dissociating and fragmenting only a component with a specific mass in the first mass spectrometry part; and detecting (quantifying) a specific ion in the second mass spectrometry part.

[0056]

The volatile low-molecular-weight sulfur compound as described above has a too low molecular weight and thus has difficulty in quantifying as it is by liquid chromatography mass spectrometry technique. Hence, in the method for quantifying a volatile low-molecular-weight sulfur compound of the embodiment, in step A, a volatile low-molecular-weight sulfur compound and an alkylating agent are reacted to modify the volatile low-molecular-weight sulfur compound with the alkylating agent to produce a volatile low-molecular-weight sulfur compound having a quantifiable molecular weight and modification with the alkylating agent.

[0057]

In step A, at first, a volatile low-molecular-weight sulfur compound contained in a test substance is extracted as follows.

For example, an extraction solution is added to the test substance, followed by thorough mixing of the test substance and the extraction solution, and then the mixture is centrifuged to recover the supernatant. In this way, the volatile low-molecular-weight sulfur compound is extracted.

[0058]

The amount of the test substance to be collected is preferably 0.001  $\mu\text{g}$  or more and 10  $\mu\text{g}$  or less, and more preferably 0.01  $\mu\text{g}$  or more and 0.1  $\mu\text{g}$  or less.

[0059]

As the extraction solution, a solution containing D-camphor-10-sulfonic acid sodium salt diluted with ultra-pure water at a final concentration of 0.1  $\mu$ M or more and 100  $\mu$ M or less and methanol at a final concentration of 99% (w/w) is used.

The amount of the extraction solution is preferably 50  $\mu$ L or more and 1000  $\mu$ L or less, and more preferably 100  $\mu$ L or more and 500  $\mu$ L or less.

[0060]

In centrifugation, rotation speed is preferably 10000 rpm or more and 15000 rpm or less, and more preferably 13000 rpm or more and 15000 rpm or less.

In centrifugation, temperature is preferably 1°C or more and 10°C or less, and more preferably 4°C or more and 5°C or less.

Centrifugation is preferably performed for 1 minute or more and 10 minutes or less, and more preferably performed for 3 minutes or more and 5 minutes or less, at the rotation speed and temperature as described above.

[0061]

Then, Tris-hydrochloride buffer is added to the recovered supernatant, followed by further addition of an alkylating agent, and then the mixture of them is stirred to modify the volatile low-molecular-weight sulfur compound with the alkylating agent.

[0062]

The alkylating agent is preferably one or more types of compounds selected from the group consisting of 5, 5'-dithiobis (2-nitrobenzoic acid), 2, 6-dichlorophenolindophenol, *p*-chloromercuribenzoic acid, iodoacetamide, *N*-ethylmaleimide, and monobromobimane. These alkylating agents may be used singly with one type or may be used in combination of two or more types.

[0063]

5, 5'-dithiobis (2-nitrobenzoic acid) reacts with a thiol group, and thus is preferably used for quantification

(detection) of a thiol group-containing compound.

2, 6-dichlorophenolindophenol reacts with a thiol group, and thus is preferably used for quantification (detection) of a thiol group-containing compound.

*p*-chloromercuribenzoic acid reacts with a thiol group, and thus is preferably used for quantification (detection) of a thiol group-containing compound.

Iodoacetamide reacts with a thiol group, and thus is preferably used for quantification (detection) of a thiol group-containing compound.

*N*-ethylmaleimide reacts with a thiol group, and thus is preferably used for quantification (detection) of a thiol group-containing compound.

Monobromobimane (Monobromobiman, 3-(bromomethyl)-2,5,6-trimethylpyrazolo[1,2-*a*]pyrazole-1,7-dione) reacts with a sulfide ion ( $S^{2-}$ ) and a thiol group, and thus is preferably used for quantification (detection) of a sulfur compound with a thiol group and polysulfide of a sulfur compound.

[0064]

The amount of the supernatant solution is preferably 50  $\mu$ L or more and 100  $\mu$ L or less, and more preferably 60  $\mu$ L or more and 90  $\mu$ L or less.

[0065]

The concentration of Tris-hydrochloride buffer is preferably 1 M or more and 2 M or less, and more preferably 1.5 M or more and 2 M or less.

The pH of Tris-hydrochloride buffer is preferably 8 or more and 9 or less, and more preferably 8.7 or more and 8.9 or less.

[0066]

The added amount of the alkylating agent is preferably 1  $\mu$ L or more and 20  $\mu$ L or less, and more preferably 5  $\mu$ L or more and 10  $\mu$ L or less.

[0067]

In step A, the volatile low-molecular-weight sulfur

compound modified with the alkylating agent is further treated as follows so as to be quantifiable by liquid chromatography mass spectrometry technique in step B.

[0068]

The mixture containing the volatile low-molecular-weight sulfur compound modified with the alkylating agent is centrifuged to recover a supernatant.

[0069]

In centrifugation, rotation speed is preferably 10000 rpm or more and 15000 rpm or less, and more preferably 13000 rpm or more and 15000 rpm or less.

In centrifugation, temperature is preferably 1°C or more and 10°C or less, and more preferably 4°C or more and 5°C or less.

Centrifugation is preferably performed for 1 minute or more and 10 minutes or less, and more preferably performed for 3 minutes or more and 5 minutes or less, at the rotation speed and temperature as described above.

[0070]

Then, the post-centrifugal supernatant is recovered, subjected to drying treatment in a centrifugal evaporator, and dried to solid.

Then, ultra-pure water is added to the supernatant dried to solid and resuspended to prepare a suspension.

[0071]

The amount of the supernatant is preferably 50  $\mu$ L or more and 100  $\mu$ L or less, and more preferably 60  $\mu$ L or more and 90  $\mu$ L or less.

[0072]

The drying treatment by a centrifugal evaporator is performed preferably for 2 hours or more and 48 hours or less, and more preferably for 12 hours or more and 16 hours or less.

[0073]

The amount of ultra-pure water to be added to the supernatant dried to solid is preferably 10  $\mu$ L or more and

100  $\mu\text{L}$  or less, and more preferably 30  $\mu\text{L}$  or more and 60  $\mu\text{L}$  or less.

[0074]

Then, the suspension is centrifuged to recover a supernatant.

[0075]

In centrifugation, rotation speed is preferably 10000 rpm or more and 15000 rpm or less, and more preferably 13000 rpm or more and 15000 rpm or less.

In centrifugation, temperature is preferably 1°C or more and 10°C or less, and more preferably 4°C or more and 5°C or less.

Centrifugation is preferably performed for 1 minute or more and 10 minutes or less, and more preferably performed for 3 minutes or more and 5 minutes or less, at the rotation speed and temperature as described above.

[0076]

In the method for quantifying a volatile low-molecular-weight sulfur compound of the embodiment, in step B, the modified volatile low-molecular-weight sulfur compound is analyzed by liquid chromatography mass spectrometry technique, and the volatile low-molecular-weight sulfur compound is quantified. In other words, in step B, a portion of the supernatant obtained by centrifugation of the suspension is analyzed by liquid chromatography mass spectrometry technique, and the volatile low-molecular-weight sulfur compound contained in the supernatant is quantified.

[0077]

In step B, a portion of the supernatant obtained by centrifugation of the suspension is transferred into a sample cup.

The amount of the supernatant to be transferred to the sample cup is preferably 30  $\mu\text{L}$  or more and 100  $\mu\text{L}$  or less, and more preferably 40  $\mu\text{L}$  or more and 60  $\mu\text{L}$  or less.

[0078]

Additionally, the amount of the supernatant used for quantification by liquid chromatography mass spectrometry technique is preferably 1  $\mu$ L or more and 10  $\mu$ L or less, and more preferably 3  $\mu$ L or more and 5  $\mu$ L or less.

[0079]

According to the method for quantifying a volatile low-molecular-weight sulfur compound of the embodiment, composition of an extract extracted from a test substance can be clarified by quantifying a volatile low-molecular-weight sulfur compound by liquid chromatography mass spectrometry. In this way, the degree of progression of damage of the test substance can be evaluated. For example, among volatile low-molecular-weight sulfur compounds, if 2-furfurylthiol is detected in large amounts, such test substance can be deemed to be in a state containing coffee-like flavor. If benzyl mercaptan is detected in large amounts, such test substance can be deemed to be in a state containing roast-like flavor. If 4-mercapto-4-methylpentane-2-one is detected in large amounts, such test substance can be deemed to be in a state containing wine-like flavor. If 2-mercaptoethyl acetate is detected in large amounts, such test substance can be deemed to be in a state containing rubber-like flavor.

[Example]

[0080]

The present invention will now be more specifically described below with reference to the Experimental examples, but the present invention is not limited to the following Experimental examples.

[0081]

[Experimental example 1]

Sixty-nine kinds of commercially-available wines were prepared as test substances.

Volatile low-molecular-weight sulfur compounds contained in these wines were modified with an alkylating

agent, and then analyzed by liquid chromatography mass spectrometry technique to quantify the volatile low-molecular-weight sulfur compounds contained in the wines.

An aliquot of 0.5 mL of wine was taken, followed by addition of 0.5 mL of an extraction solution (5  $\mu$ L of D-camphor-10-sulfonic acid sodium salt diluted with ultra-pure water at a final concentration of 500  $\mu$ M, and 495  $\mu$ L of methanol at a final concentration of 99% (w/w)). After thorough mixing of the wine and the extraction solution, the mixture was centrifuged under the condition of 15000 rpm, 4°C, and 3 minutes to recover a supernatant.

Then, 10  $\mu$ L of 2 M Tris-hydrochloride buffer (pH 8.8) was added to 100  $\mu$ L of the recovered supernatant solution, followed by further addition of 10  $\mu$ L of 20 mM monobromobimane, subjected to 10 minutes of stirring operation, and centrifuged under the condition of 15000 rpm, 4°C, and 3 minutes to recover a supernatant.

Then, 87  $\mu$ L of the recovered supernatant was subjected to about 2 hours of drying treatment in a centrifugal evaporator, and dried to solid.

Then, 60  $\mu$ L of ultra-pure water was added to the supernatant dried to solid, resuspended, and then centrifuged under the condition of 15000 rpm, 4°C, and 3 minutes. An aliquot of 50  $\mu$ L of the supernatant thus obtained was transferred to a sample cup, and 5  $\mu$ L of the aliquot was used to perform liquid chromatography mass spectrometry to quantify volatile low-molecular-weight sulfur compounds.

In liquid chromatography mass spectrometry, a liquid chromatography mass spectrometry apparatus (model name: LC<S-8030, manufactured by Shimadzu Corporation) was used. As a column, ACQUITY® UPLC CSH C18, 1.7  $\mu$ m manufactured by Waters Corporation was used.

As a mobile phase, acetonitrile containing 0.1% formic acid was used.

Measurement time was 20 minutes.

[0082]

Quantitative values (analysis values), derived by liquid chromatography mass spectrometry, of sulfur compounds and sulfur metabolism-related compounds for each of the test substances, and quantitative values, provided beforehand, of the sulfur compounds and sulfur metabolism-related compounds were analyzed by multidimensional scaling of the free software for statistical analysis "R" (Vegan), and then a two-dimensional scatter diagram as shown in FIG. 1 was created.

Meanwhile, in this Experimental example, quantitative values of sulfite ion, thiosulfate ion, sulfide ion, glutathione, glutathione monosulfide, glutathione disulfide, glutathione trisulfide, glutathione tetrasulfide, oxidized glutathione, oxidized glutathione monosulfide, oxidized glutathione disulfide, oxidized glutathione trisulfide, oxidized glutathione tetrasulfide, cysteine, cysteine monosulfide, methionine, 2-furfurylthiol, benzyl mercaptan, 4-mercapto-4-methyl-pentane-2-one, and 2-mercaptoethyl acetate, as sulfur compounds, had been provided (analyzed) beforehand for each of the test substances, and registered in a database.

Additionally, in this Experimental example, quantitative values of cystine, cystathionine, S-adenosylmethionine, homocysteine, S-sulfocysteine, O-phosphoserine, O-acetylserine, N-acetylserine, O-succinylhomoserine, adenosine 5'-phosphosulfate, lanthionine, 3'-phosphoadenosine -5'-sulfate, 3'-phosphoadenosine -5'-phosphosulfate, ergothioneine, hercynine, 5-glutamylcysteine, S-hercynyl-cysteine sulfoxide, and 5-glutamyl-S-hercynyl-cysteine sulfoxide, as sulfur metabolism-related compounds, had been provided (analyzed) beforehand for each of the test substances, and registered in a database.

[0083]

In FIG. 1, large dots represent wines without addition

of antioxidant, and small dots represent wines with addition of antioxidant. Results in FIG. 1 revealed that the wines without addition of antioxidant show high oxidation level and thus are likely to be oxidized. In contrast, the wines with addition of antioxidant show high reduction level and thus are unlikely to be oxidized. Furthermore, both types of wines without addition of antioxidant and with addition of antioxidant include those with high and low contribution of microbial metabolism. Thus, it was found that in order to select a wine to be defined as more delicious one, it is only necessary to select a wine with low oxidation level (high reduction level) and high contribution of microbial metabolism in FIG. 1. In FIG. 1, wines present in the right area based on 0 on the x-axis and the upper area based on 0 on the y-axis can be evaluated as more delicious wines.

[0084]

[Experimental example 2]

Culture liquids after culturing of each of a plurality of microorganisms were prepared as test substances.

Volatile low-molecular-weight sulfur compounds contained in these culture liquids were modified with an alkylating agent, and then analyzed by liquid chromatography mass spectrometry technique to quantify the volatile low-molecular-weight sulfur compounds contained in the culture liquids in a manner similar to Experimental example 1.

[0085]

Quantitative values (analysis values), derived by liquid chromatography mass spectrometry, of sulfur compounds and sulfur metabolism-related compounds for each of the test substances, and quantitative values, provided beforehand, of the sulfur compounds and sulfur metabolism-related compounds were analyzed by multidimensional scaling of the free software for statistical analysis "R" (Vegan), and then a three-dimensional scatter diagram as shown in FIG. 4 was created. In particular, three scales of redox level (freshness), anti-oxidative capacity, and odor were selected

as the x-axis, y-axis, and z-axis, thereby creating the three-dimensional scatter diagram.

[0086]

Meanwhile, in this Experimental example, quantitative values of sulfite ion, thiosulfate ion, sulfide ion, glutathione, glutathione monosulfide, glutathione disulfide, glutathione trisulfide, glutathione tetrasulfide, oxidized glutathione, oxidized glutathione monosulfide, oxidized glutathione disulfide, oxidized glutathione trisulfide, oxidized glutathione tetrasulfide, cysteine, cysteine monosulfide, methionine, 2-furfurylthiol, benzyl mercaptan, 4-mercapto-4-methyl-pentane-2-one, and 2-mercaptoethyl acetate, as sulfur compounds, had been provided (analyzed) beforehand for each of the test substances, and registered in a database.

Additionally, in this Experimental example, quantitative values of cystine, cystathionine, S-adenosylmethionine, homocysteine, S-sulfocysteine, O-phosphoserine, O-acetylserine, N-acetylserine, O-succinylhomoserine, adenosine 5'-phosphosulfate, lanthionine, 3'-phosphoadenosine -5'-sulfate, 3'-phosphoadenosine -5'-phosphosulfate, ergothioneine, hercynine, 5-glutamylcysteine, S-hercynyl-cysteine sulfoxide, and 5-glutamyl-S-hercynyl-cysteine sulfoxide, as sulfur metabolism-related compounds, had been provided (analyzed) beforehand for each of the test substances, and registered in a database.

[0087]

In FIG. 4, A1-A3 represent culture liquids of bacillus; B1-B8 represent culture liquids of *E. coli*; C1-C5 represent culture liquids of actinomycetes; and D represents a culture liquid of lactic acid bacteria. Results in FIG. 4 revealed that in multidimensional scaling, sulfur compounds look promising and determine axes of the first major component and the second major component. Accordingly, it was found that sulfur compounds are to be new indexes and evaluation

systems indicating action, freshness, or the like of microbial flora in intestinal flora (intestinal bacterial flora), soil flora, or an agricultural crop or food.

[0088]

[Experimental example 3]

Commercially-available wines were prepared as test substances. Volatile low-molecular-weight sulfur compounds contained in these wines were modified with an alkylating agent, and then analyzed by liquid chromatography mass spectrometry technique to quantify the volatile low-molecular-weight sulfur compounds contained in the wines in a manner similar to Experimental example 1.

[0089]

Results in FIG. 5 revealed that in the horizontal (x-axis) direction, the measured wines with lower oxidation level (with more sulfite, thiosulfate, and sulfide ions) are positioned in order from the right side correspondingly, while that in the perpendicular (y-axis) direction, those with larger amounts of ergothioneine produced by microorganisms (action of microorganisms) are positioned in order from the upper side correspondingly.

Furthermore, results in FIG. 6 revealed that upon including glucose into the data of FIG. 5 and performing multidimensional scaling, completely different results then came out, in which positions in the perpendicular (y-axis) direction was defined by sweetness.

[0090]

[Experimental example 4]

Twenty kinds of commercially-available beers were prepared as test substances. Using 9 kinds of beers, 7 kinds of low-malt beers, 4 kinds of non-alcohol beers, multivariate analysis was performed only with compounds which are to form polysulfide. In particular, volatile low-molecular-weight sulfur compounds were modified with an alkylating agent, and then analyzed by liquid chromatography mass spectrometry technique to quantify the volatile low-molecular-weight

sulfur compounds contained in the beers in a manner similar to Experimental example 1.

[0091]

Results in FIG. 7 revealed that in the horizontal (x-axis) direction, the measured beers with lower oxidation level (with more sulfite, thiosulfate, and sulfide ions) are positioned in order from the right side correspondingly, while that in the perpendicular (y-axis) direction, those with larger amounts of ergothioneine produced by microorganisms (action of microorganisms) are positioned in order from the upper side correspondingly. As shown in FIG. 7, it was found that a scatter diagram is divided into four areas: beers are located within the area surrounded by a solid line at the upper left; low-malt beers are located within the area surrounded by a dash-dot-dash line at the lower left; non-alcohol beers are located within the area surrounded by a dotted line at the right (the upper right and lower right). This results revealed that different types of beverages, i.e., beers, low-malt beers, and non-alcohol beers, can be separately identified using the evaluating method.

[0092]

Moreover, results of 9 kinds of beers in FIG. 8 revealed that in the perpendicular (y-axis) direction, high-priced beers are depicted to be positioned at the upper region relative to low-priced beers. In other words, it can be seen that beers having high contribution of microbial metabolism are high-quality beers, and that the 17th beer in FIG. 8 has higher contribution of microbial metabolism among low-priced beers and thus is a high cost performance beer. Beer developers can use the evaluation technique to obtain an objective index for producing a high-quality beer.

## CLAIMS

1. A method for evaluating a sulfur compound-containing substance for evaluating a plurality of test substances, comprising performing the steps of:

providing quantitative values of a plurality of sulfur compounds and a plurality of sulfur metabolism-related compounds for each of the test substances;

analyzing analysis values of the test substances, including the quantitative values, by multidimensional scaling, and creating a scatter diagram having two or more types of scales selected from redox level, contribution of microbial metabolism, anti-oxidative capacity, or aroma or odor of the test substances; and

assessing redox level, contribution of microbial metabolism, anti-oxidative capacity, or aroma or odor of the test substances, on the basis of the positions on the scatter diagram.

2. The method for evaluating a sulfur compound-containing substance according to claim 1, wherein the scatter diagram is a two-dimensional scatter diagram having two types of scales selected from redox level, contribution of microbial metabolism, anti-oxidative capacity, or aroma or odor.

3. The method for evaluating a sulfur compound-containing substance according to claim 1, wherein the scatter diagram is a three-dimensional scatter diagram having three types of scales selected from redox level, contribution of microbial metabolism, anti-oxidative capacity, or aroma or odor.

4. The method for evaluating a sulfur compound-containing substance according to any one of claims 1 to 3, wherein the sulfur compounds include glutathione and a derivative thereof, and wherein the sulfur metabolism-related compounds include an ergothioneine metabolism related compound.

5. The method for evaluating a sulfur compound-containing substance according to any one of claims 1 to 4, wherein the sulfur compounds include at least 20 types, and wherein the sulfur metabolism-related compounds include at least 18 types.

6. The method for evaluating a sulfur compound-containing substance according to any one of claims 1 to 5,

wherein the sulfur compounds include sulfite ion, thiosulfate ion, sulfide ion, glutathione, glutathione monosulfide, glutathione disulfide, glutathione trisulfide, glutathione tetrasulfide, oxidized glutathione, oxidized glutathione monosulfide, oxidized glutathione disulfide, oxidized glutathione trisulfide, oxidized glutathione tetrasulfide, cysteine, cysteine monosulfide, methionine, 2-furfurylthiol, benzyl mercaptan, 4-mercapto-4-methylpentane-2-one, and 2-mercaptoethyl acetate, and

wherein the sulfur metabolism-related compounds include cystine, cystathionine, *S*-adenosylmethionine, homocysteine, *S*-sulfohomocysteine, *O*-phosphoserine, *O*-acetylserine, *N*-acetylserine, *O*-succinylhomoserine, adenosine 5'-phosphosulfate, lanthionine, 3'-phosphoadenosine 5'-sulfate, 3'-phosphoadenosine 5'-phosphosulfate, ergothioneine, hercynine, 5-glutamylcysteine, *S*-hercynyl-cysteine sulfoxide, and 5-glutamyl-*S*-hercynyl-cysteine sulfoxide.

7. The method for evaluating a sulfur compound-containing substance according to any one of claims 1 to 6, comprising performing a method for quantifying a volatile low-molecular-weight sulfur compound, wherein the step of providing the quantitative values comprises:

pretreating the volatile low-molecular-weight sulfur compound by modification with an alkylating agent, and

analyzing the modified volatile low-molecular-weight

sulfur compound by liquid chromatography mass spectrometry technique, and quantifying the volatile low-molecular-weight sulfur compound.

8. The method for evaluating a sulfur compound-containing substance according to claims 7, wherein the alkylating agent is one or more types of compounds selected from the group consisting of 5, 5'-dithiobis (2-nitrobenzoic acid), 2, 6-dichlorophenolindophenol, *p*-chloromercuribenzoic acid, iodoacetamide, *N*-ethylmaleimide, and monobromobimane.

9. The method for evaluating a sulfur compound-containing substance according to claim 7 or 8, wherein the volatile low-molecular-weight sulfur compound is one or more types of compounds selected from the group consisting of 2-furfurylthiol, benzyl mercaptan, 4-mercapto-4-methylpentane-2-one, and 2-mercaptoethyl acetate.