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(54) Title: PLANT EXTRACTION PROCESS

(57) Abstract: A method for producing a plant extract comprises incubating a plant material with an enzyme composition comprising a lipolytic enzyme.

PLANT EXTRACTION PROCESS

FIELD OF THE INVENTION

The present invention relates to an improved method for extraction of plant compounds.

BACKGROUND OF THE INVENTION

A number of plant compounds, e.g. polyphenols, have desired properties, e.g. as antioxidants, colouring or flavouring agent. Plant polyphenols may be added to beverages, dairy products, jams, jellies, soups, fruit porridge, preserves and confectionary products. Furthermore, a high intake of plant polyphenols is believed to reduce the risk of coronary heart diseases, to lower cholesterol, as well as having an inflammatory effect. Thus improved processes for extracting such polyphenols and other plant compounds are highly desirable.

SUMMARY OF THE INVENTION

The present inventors have now discovered that the extraction of plant compounds, e.g. polyphenols from plant material, can be facilitated by contacting the plant material with a lipolytic enzyme, preferably a cutinase.

Accordingly the invention provides in a first aspect a process for producing a plant extract comprising incubating a plant material with an enzyme composition comprising a lipolytic enzyme.

Furthermore the invention provides in a second aspect a process comprising incubating a plant material with an enzyme composition comprising a lipolytic enzyme, and separating an aqueous liquid from said plant material.

Furthermore the invention provides in a third aspect a process for extraction of a compound from a plant material comprising incubating said plant material with an enzyme composition comprising a lipolytic enzyme, and separating an aqueous liquid from said plant material, said liquid comprising said compound.

DETAILED DESCRIPTION OF THE INVENTION

By treating the plant material with a lipolytic enzyme, preferably a cutinase, more of the plant solids can be solubilized and brought into suspension in an aqueous liquid.

The aqueous liquid separated from the enzyme treated plant material may comprise one or more compounds. The compound may be any compound present in a plant material. The compound may be a desired compound having any desired property, e.g. as antioxidative, colouring or flavouring. The compound may be selected from the group consisting of 5-(hydroxymethyl)-2 furaldehyde, benzoeic acid, cinnamic acid; phenols, preferably benzoic acid derivatives or cinnamic acid derivatives, more preferably glycosyl benzoates, glycosyl cinnamates, salicylic acid, isovanillic acid, gallic acid, coumaric acid, ferulic acid, caffeic acid, sinapic acid, sinapine; polyphenols, preferably stilbene derivatives

or flavonoides, more preferably stilbeneols flavonols anthocyanins isoflavones flavons, most preferably stilbenol glycosides, flavanol glycosides, anthocyanin glycosides, isoflavon glycosides, flavon glycosides, stilbene, phloridzin, resveratrol, kaempferol, quercetin, isorhamnetin, myrecetin, pelagonidin, cyanidin, peonidin, delphinidin, apigenin or luteolin; terpenes, preferably carotenoides, more preferably phytoene neurosporene lycopene zeacarotene carotene; pyrrols preferably tetrapyrrol more preferably tetrapyrrol glycosides, chlorophyll a/b, chlorophyllide a/b, pheophytin a/b, pheophorbide a/b or pyropheophytin a.

The plant material may be any plant material, i.e. vegetable material, comprising a desired compound. Preferred plant material may be derived from berries, pome, citrus fruit, drupes, vegetables, and seed. The plant material preferably comprises material from one or more plant selected from the list comprising grapes (red and green), cranberry, black currant, red currants, lingonberry, elderberry, blueberry, bilberry, gooseberry, crowberry, raspberry, strawberry, blackberry, kiwi, lemon, orange, lime, cherry, plum, peach, mango, apples, pears, carrots, black carrots, lettuce, cabbage, red cabbage, cauliflower, broccoli, leak, celery, onion, garlic, ginseng, pepper fruit, chilli, tomato, tea, spinach, ginko biloba, pepper (black or white), coffe berries, coffee beans, rape seed, canola.

When extracting a desired compound belonging to the carotenoides (e.g., phytoene, neurosporene, lycopene, zeacarotene or carotene), which compounds are pH stable, the pH during the extraction step a) is not critical. However, most of the other desired compounds are pH unstable. Examples of pH unstable compounds are cinnamoyl derivatives, flavonoids and flavonoid glycosides. Thus preferably the pH during the incubation in step a) is preferably no more than pH 7.0, no more than pH 6.5, or even no more than pH 6.0, such as no more than pH 5.5. Preferably the pH is at least 3.0, at least 3.5, or even at least 4.0.

The plant material is preferably macerated the prior to or during step a) to reduce the particle size and increase extraction. Preferred is also pulp or pomace from fruit processing, e.g. grape pomace, such as a by-product from wine making, comprising the seeds and skin of the grape and the compound may be polyphenols, such as flavonoids. The plant material may be the residues from a juice process, i.e. the pulp, e.g. black currant pulp or apple pulp. Also preferred as a plant material is any fraction of a plant comprising skin or seed of fruits *or* vegetables as these parts are especially rich in e.g. polyphenols and/or carotenoides.

The plant material may prior to or simultaneous with the incubation during step a) be contacted with a second enzyme, e.g. a pectinase, and/or a cellulase.

The process of the present invention may be integrated in a process for production of vegetable products such as fruit juice, jams, jellies, soups, fruit porridge, preserves, thereby increasing the content of the desired compound in the vegetable products. Thus the plant material may be incubated with additional enzymes, such as e.g. pectinases, as well as with a lipolytic enzyme, preferably a cutinase, according to the present invention. The process of the present invention may be integrated in a wine making process thereby increasing the content of polyphenols in the grape juice and in the wine following fermentation.

In an embodiment all, or substantially all the plant material is solubilized and brought into suspension in the aqueous liquid.

Following extraction the desired compound may be added to or used in beverages, dairy products, infant foods, juice, jams, jellies, soups, fruit porridge preserves and/or confectionary products.

Enzymes

Lipolytic enzyme

A lipolytic enzyme is an enzyme which is capable of hydrolyzing carboxylic ester bonds to release a carboxylic acid or carboxylate (EC 3.1.1), e.g. a lipase, a phospholipase, a cutinase.

The lipolytic enzymes may be prokaryotic, particularly bacterial, or eukaryotic, e.g. from fungal or animal sources. Lipolytic enzymes may be derived, e.g. from the following genera or species: *Thermomyces, T. lanuginosus (also known as Humicola lanuginosa), Humicola, H. insolens, Fusarium, F. oxysporum, F. solani, F. heterosporum, Aspergillus, A. tubigensis, A. niger, A. oryzae, Rhizomucor, Candida, C. antarctica, C. rugosa, Penicillium, P. camembertii, Rhizopus, Rhizopus oryzae, Absidia. Dictyostelium, Mucor, Neurospora, Rhizopus, R. arrhizus, R. japonicus, Sclerotinia, Trichophyton, Whetzelinia, Bacillus, Citrobacter, Enterobacter, Edwardsiella, Erwinia, Escherichia, E. coli, Klebsiella, Proteus, Providencia, Salmonella, Serratia, Shigella, Streptomyces, Yersinia, Pseudomonas, P. cepacia, Verticillium, Septoria* and *Gliocladium.*

Some particular examples of lipolytic enzymes are listed as follows:

Phospholipase from bee or snake venom or from mammal pancreas, e.g. porcine pancreas.

Phospholipase from *Aspergillus oryzae* (EP 575133, JP-A 10-155493), *Hyphozyma* (U.S. Pat. No. 6127137).

Lipase from *Thernomyces lanuginosus* (also called *Humicola lanuginosa*) (EP 305216, U.S. Pat. No. 5869438), *A. tubigensis* (WO 9845453), *Fusarium solani* (U.S. Pat. No. 5990069).

Lipase/phospholipase from *Fusarium oxysporum* (WO 98/26057).

Lipolytic enzyme from *F. culmorum* (U.S. Pat. No. 5830736) or as described in WO 02/00852 (PCT/DK 01/00448) or DK PA 2001 00304.

A variant derived from one of the above enzymes by substituting, deleting or inserting one or more amino acids, e.g. as described in WO 2000/32758, particularly Examples 5 4, 5, 6 and 13, such as variants of lipase from *Thermomyces lanuginosus* (also called *Humicola lanuginosa*).

Preferred for the present invention are lipolytic enzymes having activity at acid pH, e.g. at pH 7 and below, such as from pH 2 to pH 7, e.g. from pH 3 to pH 5.

Cutinase

Preferably the lipolytic enzyme is a cutinase. For the present purposes a cutinase is an enzyme classified as EC 3.1.1.74. While the cutinase to be used for the present invention may be of any origin including mammalian, plant or animal origin it is preferred that the cutinase is of microbial origin. In particular the cutinase may be one derivable from a filamentous fungus or a yeast. Preferred for the present invention are cutinases having activity at acid pH, e.g. at pH 7 and below, such as from pH 2 to pH 7, e.g. from pH 3 to pH 5.

Preferred is a cutinase derived from *Humicola insolens*, e.g. such as a cutinase encoded by the DNA sequence shown in SEQ ID NO:1 or SEQ ID NO:2, or any sequence being at least 50 %, preferably at least 60 %, more preferably at least 70 %, most preferably at least 80 %, or even at least 90 %, such as at least 95 % identical to the sequence shown in SEQ ID NO:1 or SEQ ID NO:2, or to a fragment thereof, said fragment having cutinase activity. Especially preferred are the variants shown in WO9613580 in table A.

Also preferred is a cutinase derived from *Fusarium solani* f.sp. *pisi* (*Nectria haematococca*), e.g. a cutinase according to WO 94/14964.

Polypeptide identity

The term "polypeptide identity" is understood as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The degree of identity may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. The following settings for amino acid sequence comparison are used: GAP creation penalty of 3.0 and GAP extension penalty of 0.1. The relevant part of the amino acid sequence for the homology determination is the mature polypeptide, i.e. without the signal peptide.

MATERIALS AND METHODS

Lipolytic enzyme and cutinase activity

The lipolytic enzyme and/or cutinase activity may be determined as lipolytic activity using tributyrine as substrate. The method is based on the rate at which the enzyme hydrolyses tributyrin to form butyric acid. The butyric acid is titrated with hydroxide and the consumption of the latter is recorded as a function of time.

One Lipase Unit (LU) is defined as the amount of enzyme which, under standard conditions (i.e. at 30.0°C; pH 7.0; with Gum Arabic as emulsifier and tributyrine as substrate) liberates 1 micromol titrable butyric acid per minute.

A folder AF 95/5 describing this analytical method in more detail is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

Enzymes

A cutinase composition comprising the polypeptide shown in SEQ ID NO:2 derived from *Humicola insolens.*

A cutinase composition derived from *Septoria* sp.

A cutinase composition derived from *Verticillium* sp.

A cutinase composition derived from *Gliocladium.*sp.

A pectinase, - a composition produced from a strain of Aspergillus aculeatus comprising polygalacturonase activity 26.000 PG/ml (Pectinex® Ultra SP-L).

Example 1

Extraction of polyphenols and anthocyanins from black currant pomace using cutinases from *Humicola insolens.*

5.0 gram black currant pomace was mixed with 25.0 mL 100 mM acetate buffer pH 5.8 in 50 mL centrifuge vials and 1.00 mL enzyme solution (15000 LU/ml) or blank was added prior to incubation in a waterbath at 40 °C for 120 min. The enzyme used was a cutinase encoded by the DNA sequence shown herein as SEQ ID NO:1. After incubation the samples were centrifuged at 3500 rpm for 15 min. at 4 °C and the supernatant was decanted. Sugar content (°Brix), turbidity (NTU), and pH were measured directly. 1 mL supernatant was diluted 25 times with 0.1 M acetic acid and left overnight at 4 °C prior to UV-Visual spectrofotometry at 430 and 520 nm for estimation of the color and anthocyanin content. Brown index is the ratio between the 520 og 430 nm valuues and indicates the ratioo between anthocyanins and total phenols. All samples were run in duplicates. The results are shown in Table 1. Color results are given as relative to the blank treatments

Table 1: Extraction of black currant pomace

Treatment	Sugar		Turbidity		Absorbance %				Brown index	
	°Brix		NTU		430 nm		520 nm		520/430	
Blank	1.35	±0.07	10.5	±0.6	100.0	±0.6	100.0	±1.0	1.44	±0.02
Cutinase	1.40	±0.00	27.8	±3.3	116.9	±0.8	111.6	±0.8	1.41	±0.08

The cutinase treatment increased the extraction yield of anthocyanins (O.D. 520 nm) by 12 % and total polyphenols (O.D.430 nm) by 17 % compared to blank.

Example 2

Extraction of polyphenols and anthocyanins from apple peel using cutinase from *Humicola insolens.*

5 gram apple peel (Danish Ingrid Marie apples) was weighed into 50 mL centrifuge vials. 40 mL 0.1 M acetate buffer pH 5.5 containing the selected enzymes were added followed by incubation in waterbath at 40 °C. After 120 min. the samples were filtered using 1.2 μm vacuum filter. pH were adjusted to 1.0 with 1 M hydrochloric acid and volume were adjusted to 50 mL. After storage overnight at 5 °C the extraction yield was measured by spectrophotometry using absorbance at 360 nm as estimate for the total polyphenol yield and absorbance at 520 nm as estimate for anthocyanin yield.

The following enzyme combinations were used (mL/kg apple peel: Blank containing no enzyme; Pectinase (6.5 PG(3.5)/g); Cutinase (6.5 LU/g); Pectinase (6.5 PG(3.5)/g) + Cutinase (6.5 LU/g). The results are shown in Table 2. Results are given as relative to the blank treatments

Table 2: Extraction of black currant pomace, yield relative to blank

	Total Polyphenol	Anthocyanins
Blank	100	100
Pectinase	89	67
Cutinase	108	128
Cutinase + Pectinase	119	116

The cutinase increased the extraction yield of polyphenols by 8 % and anthocyanins by 28 % relative to the blank sample. By addition of pectinase and cutinase the polyphenol yield increased 19 % relative to the blank treatment.

Example 3

Extraction of polyphenols and anthocyanins from apple peel using cutinase from *Septoria sp.*

10 gram apple peel (Danish grown Ingrid Marie apples) was suspended in 80 mL 0.1 M acetate buffer pH 5.5 containing the selected enzymes, followed by incubation in waterbath at 40 °C for 10 min followed by vacuum filtration through a 1.2 microM filter. pH were adjusted to 1.0 with 1 M hydrochloric acid. After storage overnight at 5 °C the extraction yield was measured by spectrophotometry using absorbance at 360 nm as estimate for the total polyphenol yield and absorbance at 520 nm as estimate for anthocyanin yield.

The following enzyme combinations were used: Blank - containing no enzyme; Pectinase (250 mL/MT); *Septoria* cutinase (8000 LU/kg apple peel); Pectinase (0.25 mL/kg

apple peel) + Septoria cutinase (8000 LU/kg apple peel). The results are shown in Table 3. Results are given as relative to the blank treatments.

Table 3: Extraction of apple peel, yield relative to blank.

	Total Polyphenol	Anthocyanins
Blank	100	100
Pectinex Ultra SP-L	105	101
Septoria cutinase	108	121
Septoria cutinase + Pectinase	116	115

The pectinase alone does not change the concentration of the phenolics in the extract. The cutinase increases the extraction yield of polyphenols by 8 % and anthocyanins by 21 % relative to the blank sample. Using cutinase in combination with pectinase the polyphenol yield is further increased.

Example 4

Extraction of polyphenols and anthocyanins from apple peel using cutinases from *Verticillium sp.*, resp. *Gliocladium sp.*

Apple peel (Danish Ingrid Marie apples) was extracted as in the previous example except that 2.5 gram apple peel was suspended in 20 mL 0.1 M acetate buffer pH 5.5.

The following enzyme combinations were used: Blank - containing no enzyme; Pectinase (250 mL/MT); *Verticillium* cutinase (3200 LU/kg apple peel); Pectinase (0.25 mL/kg apple peel) + *Verticillium* cutinase (3200 LU/kg apple peel); *Gliocladium* cutinase (1600 LU/kg apple peel); Pectinase (0.25 mL/kg apple peel) + *Gliocladium* cutinase (1600 LU/kg apple peel). The results are shown in Table 4. Results are given as relative to the blank treatments.

Table 4: Extraction of apple peel, yield relative to blank.

	Total Polyphenol	Anthocyanins
Blank	100	100
Pectinase	108	92
Verticillium cutinase	124	98
Gliocladium cutinase	130	106
Verticillium cutinase + Pectinase	163	132
Gliocladium cutinase + Pectinase	140	124

The pectinase alone does not increase extraction of the phenolics. Both cutinases increase the extraction yield of polyphenols by > 20 % but does not influence the yield of anthocyanins. Applying cutinase in combination with pectinase increases both polyphenol and anthocyanin yields.

CLAIMS

1. A process for producing a plant extract comprising incubating a plant material with an enzyme composition comprising a lipolytic enzyme.

2. A process comprising
   a. incubating a plant material with an enzyme composition comprising a lipolytic enzyme, and
   b. separating an aqueous liquid from said plant material.

3. A process for extraction of a compound from a plant material comprising
   a. incubating said plant material with an enzyme composition comprising a lipolytic enzyme, preferably a cutinase, and
   b. separating an aqueous liquid from said plant material, said liquid comprising said desired compound.

4. The process of any of claims 1 to 3 wherein the pH during the incubation during step a) is from pH 3.0 to 7.0.

5. The process of any of claims 1 to 4 wherein the compound is a colour compound, an antioxidant, an aroma compound, and/or a lipid.

6. The process of any of claims 1 to 5 wherein the compound is selected from the group consisting of tri-, di-, and monoglycerides, fatty acids, anthocyanins, tannins, pro-anthocyanidins, stilbenoides, resveratrol, cinnamic acid derivatives, benzoic acid derivatives, chlorophyll, flavanoids, gallic acid, flavan-3ols, flavonols, phloridzin, cinnemates, and hydroxymethyl furfural.

7. The process of any of claims 1 to 6 wherein the plant material is a berry: such as grapes (red and green), cranberry, black currant, lingonberry, red currants, elderberry, blueberry, bilberry, gooseberry, crowberry, raspberry, strawberry, blackberry, kiwi, a citrus fruit: such as lemon, orange, lime, a drupe: such as cherry, plum, peach, mango, a pome: such as apples, pears, a vegetables: such as carrots, black carrots, lettuce, cabbage, red cabbage, cauliflower, broccoli, leak, celery, onion, garlic, ginseng, pepper fruit, chilli, tomato, tea, spinach, ginko biloba, a seed: such as pepper (e.g. black or white), coffee berries, coffee beans, rape seed, canola.

8. The process of any of claims 1 to 7 wherein the lipolytic enzyme is a cutinase.

9. The process of any of claims 1 to 8 wherein the cutinase is derived from a species within *Humicola*, *Verticillium* or *Gliocladium*.

10. The process of any of claims 1 to 9 wherein the cutinase is derived from *Humicola insolens*.

11. The process of any of claims 1 to 10 wherein the cutinase comprises an amino acid sequence having at least 50% identity to the sequence shown as SEQ ID NO:1 or SEQ ID NO:2, or a fragment thereof having cutinase activity.

12. The process of any of claims 1 to 11 comprising macerating the plant material prior to or during step a).

13. The process of any of claims 1 or 12 wherein the plant material is or comprises pomace and/or waste material from fruit processing.

14. The process of any of claims 1 to 13 wherein the plant material comprises the peel of a fruit or vegetable.

15. The process of any of claims 1 to 14 wherein the plant material prior to or simultaneous with the incubation during step a) is contacted with a pectinase.

16. The process of any of claims 1 to 15 wherein the plant material comprises grapes and the aqueous liquid is fermented with a yeast to produce wine.

Sequence 10811-WO.ST25.txt
SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2006/000081

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23L1/30

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)
A23L

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 200382 Derwent Publications Ltd., London, GB; Class B03, AN 2003-878079 XP002380901 & CN 1 394 869 A (ZHANG W) 5 February 2003 (2003-02-05) abstract	1-3,5,7, 12
X	PATENT ABSTRACTS OF JAPAN vol. 1996, no. 02, 29 February 1996 (1996-02-29) & JP 07 274977 A (SHIKOKU SOGO KENKYUSHO:KK), 24 October 1995 (1995-10-24)	1,7
A	abstract	2,3

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Saunders, T

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2

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