METHODS OF HYDROLYSIS

FIELD OF THE INVENTION

[0001] The present invention relates generally to the field of biotechnology. In particular, the present invention relates to the use of recombinant enzymes and methods of hydrolysis.

BACKGROUND OF THE INVENTION

[0002] Oryzanol is a mixture of ferulic acid esters of plant sterols and ferulic acid esters of triterpene alcohols. Alkaline hydrolysis method is the conventional method used for the commercial hydrolysis of oryzanol. Alkaline hydrolysis indiscriminately hydrolyses both ferulic esters of plant sterols and triterpene alcohols, resulting in, for example, a reduction in product yield and/or quality. Pancreatin and cholesterol esterases (CEs) from bovine and porcine sources have been reportedly used in oryzanol hydrolysis but were shown to often result in low yield and/or quality of ferulic acids.

[0003] Thus, there is an unmet need for improved methods of hydrolysis.

SUMMARY

[0004] In one aspect, the present disclosure refers to a method of hydrolysing oryzanol to obtain ferulic acid, wherein the method comprises: i. hydrolysing an oryzanol comprising material in a first hydrolysis step; and ii. exposing product of i. to a second hydrolysis step; wherein the first hydrolysis step is enzymatic, and the second hydrolysis step is chemical.

[0005] In another aspect, the present disclosure refers to a method of extracting ferulic acid, wherein the method comprises: i. incubating oryzanol with a cholesterol esterase in a first hydrolysis solution; ii. extracting ferulic acid and phytosterols from the first hydrolysis solution in a first extraction step; iii. subjecting the first hydrolysis solution after extraction of ferulic acid and phytosterols to chemical hydrolysis to obtain a second hydrolysis solution; iv. extracting ferulic acid and triterpene alcohol from the second hydrolysis solution in a second extraction step; wherein the cholesterol esterase preferentially hydrolyses phytosterol esters.

[0006] In yet another example, the present disclosure refers to a method of extracting ferulic acid from oryzanol-comprising material, comprising treating the material according to the methods disclosed herein to extract ferulic acid.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0008] **Fig. 1** shows a schematic diagram summarizing the “two-step” process of oryzanol hydrolysis.

[0009] **Fig. 2** shows a HPLC chromatogram illustrating the hydrolysis profile of oryzanol. Fig. 2 shows that ferulic acid esters of phytosterols (in this example, campesteryl ferulae and sitosteryl ferulate) were

preferentially hydrolysed by enzymes disclosed herein to yield ferulic acids and phytosterols. The oryzanol hydrolysis profile showed that more than 90% of phytosterol esters in oryzanol was hydrolysed during the enzymatic hydrolysis step, leaving triterpene esters as primary component in the oryzanol which can be further hydrolysed during, for example, a subsequent chemical hydrolysis step.

[0010] **Fig. 3** shows an exemplary vector map, in this example of pFAi. The sequence of this vector map is provided in Table 3.

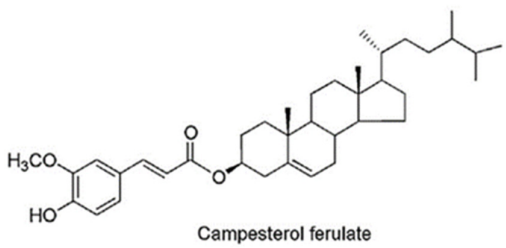
[0011] **Fig. 4**shows an image of an SDS PAGE gel. Bands indicate the presence of His-tagged cholesterol esterases. L refers to the ladder used, showing the protein size in kilo dalton (kDa). EE – *Erinaceus europaeus*; MPF – *Mustela putorius furo*; NS – *Neomonachus schauinslandi*.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

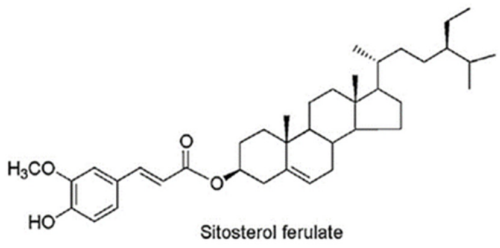
[0012] Ferulic acid (FA) is widely used in the cosmetic industry due to its anti-aging function. It has also been employed in other sectors, such as, but not limited to, an antioxidant in food and an anti-diabetic in medical applications. Ferulic acid is often derived from oryzanol, a compound often obtained from, for example, seeds of the Poaceae family, and is a well-known source of esters of ferulic acid with phytosterols or triterpene alcohols.

[0013] Oryzanol (also referred to as γ-oryzanol) has been shown to contain a multitude of compounds, more specifically, a mixture of ferulic acid esters of phytosterols and triterpenoids (also referred to as triterpene alcohols). The compounds comprised in oryzanol include, but are not limited to, ferulic acid esters of triterpenoids, such as, but not limited to, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, cycloartenol ferulate, 24-methylenecycloartanol ferulate, cycloblanol ferulate, cycloartanol ferulate, cyclobranyl ferulate, and cyclosadyl ferulate,and campesteryl ferulate, as well as 4-desmethylsterols (also referred to as phytosterols, such as but not limited to, campesterol, and β-sitosterol, and stanols such as campestanol), and ferulic acids thereof, such as campersterol ferulate, sitosterol ferulate, sitosteryl ferulate, sitostanyl ferulate, campesteryl ferulate, campestanyl ferulate, stigmasteryl ferulate, Δ^7-stigmateryl ferulate, and Δ^7-campestenyl ferulate.

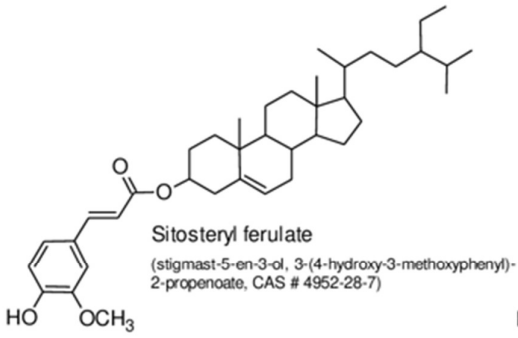
[0014] Examples of ferulic acid esters of phytosterols that can be found in oryzanol are, but are not limited to:



Campesterol ferulate

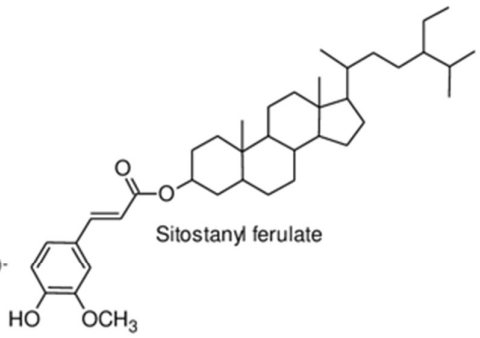


Sitosterol ferulate

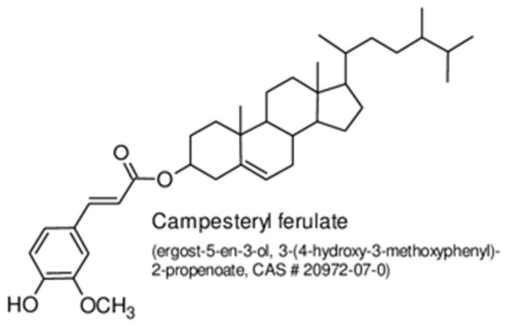


Sitosteryl ferulate

(stigmast-5-en-3-ol, 3-(4-hydroxy-3-methoxyphenyl)-2-propenoate, CAS # 4952-28-7)

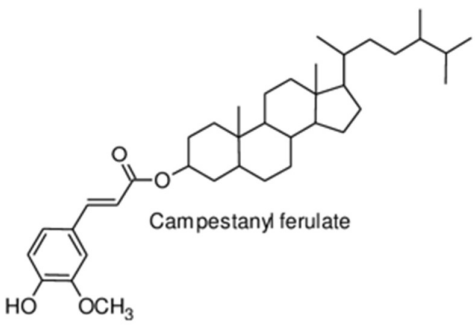


Sitostanyl ferulate

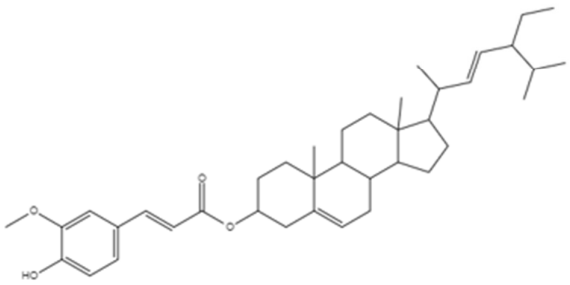


Campesteryl ferulate

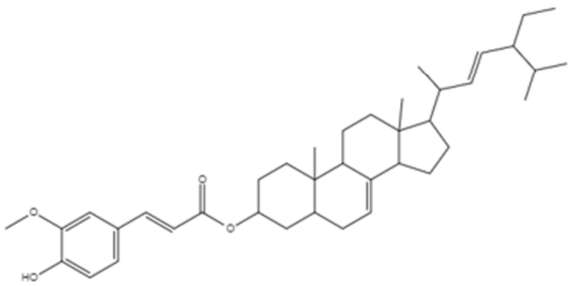
(ergost-5-en-3-ol, 3-(4-hydroxy-3-methoxyphenyl)-2-propenoate, CAS # 20972-07-0)



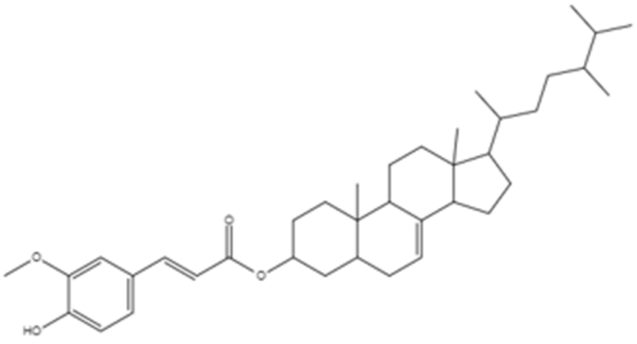
Campestanyl ferulate



Stigmasteryl ferulate



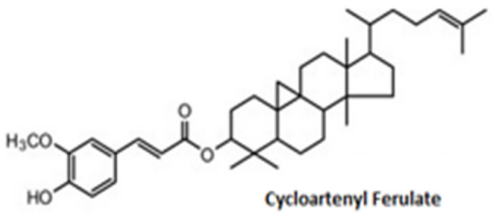
Δ^7-Stigmastenyl ferulate

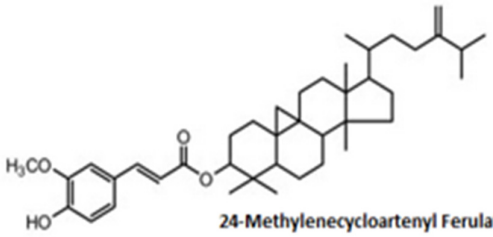


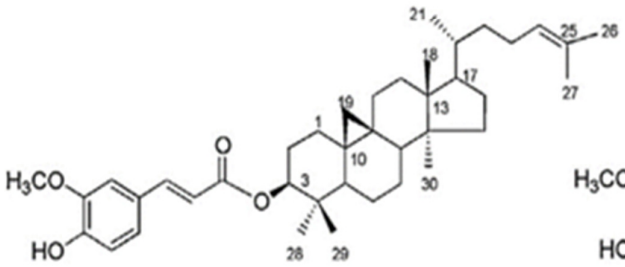
Δ^7-Campestenyl ferulate

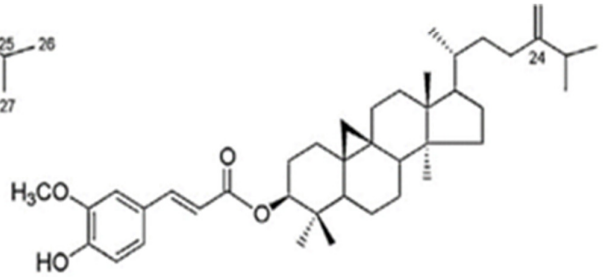
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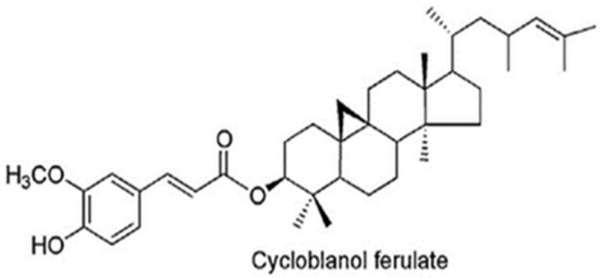
[0015] Examples of ferulic acid esters of triterpenoids (also referred to as triterpene alcohols) that can be found in oryzanol are, but are not limited to:

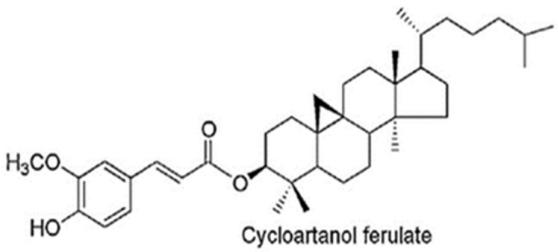


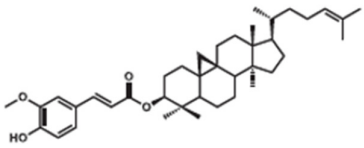


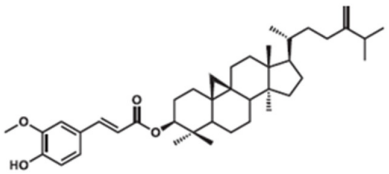


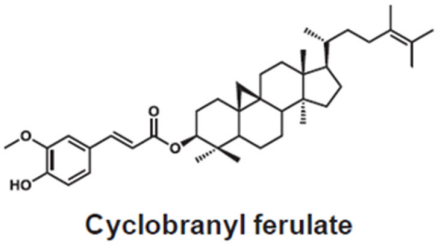


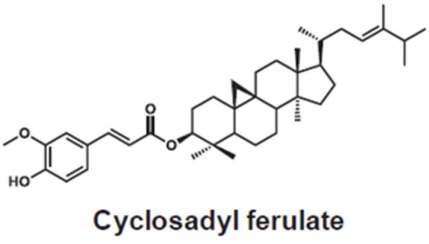












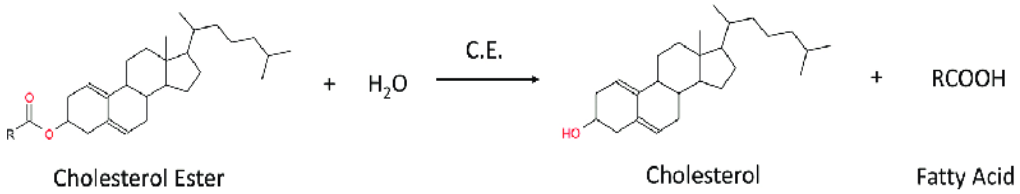
[0016] As used herein, the term “enzyme hydrolytic activity” refers to the ability of an enzyme to catalyse a reaction between a substrate and water as a reactant to cleave a chemical bond. Usually, such

a cleavage results in a substrate being split into smaller molecules. A hydrolysis reaction may be represented by the reversible chemical equation AB + HOH ⇌ AH + BOH. The reactants other than water, and the products of hydrolysis, can be, but are not limited to, neutral molecules (as in most hydrolyses involving organic compounds), or ionic molecules (as in hydrolyses of salts, acids, and bases). Hydrolysis involving organic compounds can be illustrated by the reaction of water with an ester of a carboxylic acid; all such esters have the general formula RCO-OR', in which R and R' are combining groups (for example, if R and R' both represent the methyl group, CH_3, the ester is methyl acetate). The whole reaction is represented by the following generalised reaction

$$RCO—OR' + H_2O \rightarrow RCO—OH + R'—OH,$$

in which RCO-OH denotes a carboxylic acid, R'-OH denotes an alcohol, and the dashes represent covalent bonds that are broken or formed during the reaction.

[0017] As used herein, the term "cholesterol esterase" (CE; also referred to as bile salt-stimulated esterase or carboxyl ester lipase) refers to a class of enzymes, often found in the pancreas of mammals, that primarily catalyse the hydrolysis of cholesterol esters into cholesterol and their corresponding fatty acids. The following exemplary reaction shows the cleavage of cholesterol ester into cholesterol and a fatty acid with the simultaneous incorporation of H_2O:



[Reaction 1]

[0018] The chemical reaction shown above in reaction 1 illustrates an example of hydrolysis of cholesterol esters by cholesterol esterase to yield, in this instance, a sterol (cholesterol in this example) and a fatty acid. R in this exemplary reaction is defined as an aliphatic side chain, which is either saturated or unsaturated. The term "C.E." refers to cholesterol esterase.

[0019] Enzyme hydrolytic activity can be measured using enzyme assays that measure the rate at which a substrate is depleted, or a product is formed. In one example, cholesterol ester hydrolysis activity refers to the rate at which cholesterol esters are broken down into cholesterol and fatty acids. One example refers to the use of enzymes in breaking down cholesterol esters. Cholesterol ester hydrolysis activity can thus be defined as a rate at which a cholesterol ester is depleted, or cholesterol or fatty acids are formed. Such a rate can be calculated using known mathematical models of enzyme kinetics, for example, but not limited to, Michaelis-Menten kinetics and its respective equation.

[0020] As used herein, the term "esters of phytosterols" refers to a molecule formed by esterification of the hydroxyl group of a phytosterol (also referred to as phytosteroids; which includes phytosterols

as well as stanols) with the carboxylic acid group of an acid. As used herein, the term "steryl ferulates" refers to esters of ferulic acid and phytosterols.

[0021] As used herein, the term "yield of ferulic acid" refers to the amount of ferulic acid resulting from oryzanol hydrolysis.

[0022] Commercially, the preparation of ferulic acid involves oryzanol hydrolysis, with harsh chemicals (i.e. chemicals which are highly reactive and/or cause the reaction to be irreversible) or with enzymes derived or obtained from animals, usually pig and cattle, to release ferulic acids from esters of phytosterols or terpene alcohols. Issues related to the use of animal-derived enzymes are, for example, low yield and purity, both of which can impact the efficiency of the enzymes.

[0023] In the present disclosure, cholesterol esterases were generated by recombinant means, and can therefore be considered to be recombinant proteins. The term "recombinant protein", as used herein, refers to any protein or biologically active portion thereof, that was expressed from an artificially made sequence of nucleic acids. Such artificially made sequence of nucleic acids can comprise a coding sequence of a wild-type or mutant protein of interest; a promoter; a ribosome-binding site; a terminator sequence; or a vector backbone.

[0024] In one example, the cholesterol esterase disclosed herein is a recombinant protein. In one example, the sequence for the recombinant protein as described herein is obtained or derived from a mammal. In one example, the mammal is a Hawaiian monk seal (*Neomonachus schauinslandi*). In another example, the mammal is a European domestic ferret (*Mustela furo*). In another example, the mammal is a Western European hedgehog (*Erinaceus europaeus*). In another example, the mammal is a pig. In another example, the mammal is a cow. As used herein, the terms "Neomonachus esterase", "Mustela esterase", "Erinaceus esterase", "porcine esterase", and "bovine esterase", refer to a recombinant enzyme comprising a cholesterol esterase sequence derived from Hawaiian monk seal (*Neomonachus schauinslandi*), European domestic ferret (*Mustela furo*), Western European hedgehog (*Erinaceus europaeus*), pig, and cow, respectively.

[0025] In one example, the cholesterol esterase disclosed herein comprises parts of, or the whole sequence, of a wild-type sequence of a cholesterol esterase. In one example, the recombinant cholesterol esterase contains the wild-type sequence of the protein selected from the group consisting of *Neomonachus schauinslandi*, *Mustela putorius furo*, and *Erinaceus europaeus.* In another example, the recombinant cholesterol esterase contains a sequence selected from the group consisting of SEQ ID NO: 1, 2, and 3. In the present disclosure, the recombinant cholesterol esterase is selected from the group consisting of SEQ ID NO: 1, 2 and, 3 wherein the recombinant cholesterol esterase has cholesterol ester hydrolysis activity.

[0026] The preference of an enzyme for one specific substrate is defined as its specificity, and the preference for one substrate over another is its selectivity. Although cholesterol esterases primarily hydrolyse cholesterol esters, due to the structural similarity between, for example, steryl ferulates and cholesterol, cholesterol esterases have been found to also be capable of hydrolysing steryl ferulates to

release ferulic acid and its corresponding steroid. In such examples, the steroid can be, but is not limited to triterpene alcohols (triterpenoids) or phytosterols.

[0027] In one example, the recombinant cholesterol esterases preferentially hydrolyses phytosterol esters. In other words, preferentially refers to the recombinant cholesterol esterases' ability to hydrolyse phytosterol esters before, for example, triterpene alcohol esters if both are present.

[0028] The mammalian cholesterol esterases as disclosed herein were expressed in *Pichia pastoris.* The expressed cholesterol esterases were then used in an enzymatic hydrolysis reaction to hydrolyse esters of ferulic acid with phytosterols. Oryzanol, which is a mixture of ferulic acid esters of phytosterol and ferulic acid esters of triterpene alcohols, can be hydrolysed by recombinant enzymes disclosed herein to release phytosterols, triterpene alcohols, or ferulic acid.

[0029] Table 1 below shows a table summarizing the yield of ferulic acid after enzymatic hydrolysis of oryzanol with recombinant cholesterol esterases. The recombinant cholesterol esterases had been derived from *Neomonachus schauinslandi*, *Mustela putorius furo*, and *Erinaceus europaeus,* and have been denoted as Neomonachus esterase, Mustela esterase, and Erinaceus esterase, respectively. From the HPLC results, it was shown that about 30% of total ferulic acid can be recovered by enzymatic hydrolysis.

Table 1 - Yield of ferulic acid obtained with cholesterol esterases disclosed herein

	Yield of ferulic acid (%)	
Mustela esterase	0.25mg	22.3
	0.5mg	25.1
	1.0mg	31.0
Erinaceus esterase	0.25mg	13.7
	0.5mg	27.2
	1.0mg	29.3
Neomonachus esterase	0.25mg	15.3

[0030] Table 2 below shows a table summarising the yield of ferulic acid after enzymatic hydrolysis of oryzanol obtained using cholesterol esterases disclosed in the art (porcine cholesterol esterase, and bovine cholesterol esterase). It is shown that the yield obtained using porcine esterase and bovine esterase is lower than the yield obtained using the cholesterol esterases disclosed herein. The recombinant enzymes as described herein showed a surprising technical effect wherein they were observed to have higher oryzanol hydrolysis activity compared to traditional bovine and porcine cholesterol esterases.

Table 2

	Yield of ferulic acid (%)	
Bovine cholesterol esterase	0.25mg	5.1
	0.5mg	8.3
Porcine cholesterol esterase	0.25mg	13.7
	0.5mg	16.7

[0031] Thus, in one example, the recombinant cholesterol esterase used in the method disclosed herein is but is not limited to, a recombinant cholesterol esterase derived from *Neomonachus schauinslandi, Mustela furo,* or *Erinaceus europaeus*.

[0032] By way of an example, the starting concentration of oryzanol as determined in one of the experiments disclosed herein was 766 µM. After enzymatic hydrolysis with 0.25mg of *Erinaceus europaeus* esterase, the concentration of ferulic acid released was 285 µM. This results in a yield of ferulic acid of about 37.20%. The concentration of oryzanol remaining after the enzymatic hydrolysis step was about 469.5 µM, with about 11.5 µM of oryzanol lost during extraction. This resulted in an extraction yield of oryzanol of about 97.6%. After subsequent alkaline hydrolysis, the concentration of ferulic acid was determined to be about 369.4 µM. Thus, the total yield of ferulic acid obtained from both enzymatic and chemical hydrolysis combined was about 85.43% (about 654.4 µM).

[0033] It will be apparent to a person skilled in the art that the different esterases disclosed herein would have different, yet comparable, efficacies with regard to their ability to hydrolyse oryzanol. In one example, the yield of ferulic acid obtained using *Neomonachus schauinslandi* esterase is around 15%. In another example, the yield of ferulic acid obtained using *Mustela furo* esterase is between 22% to 31%. In another example, the yield of ferulic acid obtained using *Erinaceus europaeus* esterase is between 13.7 to 29.3%. In another example, the yield of ferulic acid obtained using bovine esterase is between 2 to 6%. In another example, the yield of ferulic acid obtained using porcine esterase is between 3 to 6%.

[0034] Such recombinant enzymes, as disclosed herein, provide a sustainable source of non-chemically derived enzymes for oryzanol hydrolysis at an industrial scale.

[0035] The recombinant enzymes as described herein can be used as part of a two-step method for the hydrolysis of oryzanol. The two-step method as described herein allows phytosterols esters and triterpene alcohols esters to be hydrolysed separately. In one example, the first hydrolysis step results in the hydrolysis of phytosterols esters. In one example, the first hydrolysis step is the hydrolysis of compounds that can be hydrolysed using, for example, cholesterol esterases. In another example, the second hydrolysis step results in the hydrolysis of triterpene alcohol esters. Thus, the two-step method as described herein results in the isolation of phytosterols and triterpene alcohols after the first or second hydrolysis steps, respectively, and the isolation of ferulic acids after both hydrolysis steps.

[0036] The first hydrolysis step of the two-step method, as disclosed herein, is performed enzymatically. The first hydrolysis step can be performed using any one or more of the cholesterol esterases described herein.

[0037] The second hydrolysis step of the two-step method, as disclosed herein, is performed chemically. In other words, the second hydrolysis step of the method disclosed herein is performed without the use of enzymes, or in a non-enzymatic manner. In one example, the present disclosure describes a method of hydrolysing oryzanol to obtain ferulic acid, wherein the method comprises: a first

hydrolysis step; and a second hydrolysis step; wherein the first hydrolysis step is enzymatic, and the second hydrolysis step is chemical.

[0038] The first hydrolysis step of the two-step method as disclosed herein, is an enzymatic hydrolysis step that breaks down ferulic acid esters of phytosterols to release ferulic acid and phytosterols. In one example, the first hydrolysis step is performed using a cholesterol esterase which preferentially hydrolyses phytosterol esters. In another example, the first hydrolysis step is performed using a cholesterol esterase, wherein the cholesterol esterase is, but is not limited to, a cholesterol esterase according to SEQ ID Nos 1, 2 and/or, 3.

[0039] The second hydrolysis step of the method disclosed herein is a chemical hydrolysis step which breaks down the remaining ferulic acid esters of triterpene alcohols to release ferulic acid and triterpene alcohols. Two sources of ferulic acids (presented, for example, in percent yield) are produced from these processes, one from enzymatic hydrolysis, and the other from chemical hydrolysis.

[0040] Also contemplated within the scope of the present disclosure is the use of the first and second hydrolysis repeatedly and independently of each other. In other words, in one example, the first hydrolysis step is performed multiple times, after which the second hydrolysis step can be performed one, or multiple times. Thus, in one example, the first hydrolysis step is performed multiple times, followed by the second hydrolysis step being performed once or twice. In another example, the first hydrolysis step is performed once or twice, followed by the second hydrolysis step being performed multiple times. In one example, the first hydrolysis step is performed once, followed by the second hydrolysis step being performed once. In another example, the first hydrolysis step is performed three times, followed by the second hydrolysis step being performed once. In yet another example, the first hydrolysis step is performed once, followed by the second hydrolysis step being performed four times. In one example, the limiting factor of how often a cycle is performed is based on the amount of adduct present. In another example, the first hydrolysis step is performed until the concentration of remaining adduct (i.e. the reagents present prior to the first hydrolysis step) is at most 11% of the original concentration. In other words, the concentration of adduct can be reduced by up to 89%, before being subjected to a second hydrolysis step. In one example, the second hydrolysis step is performed when the concentration of unreacted campesteryl ferulate and sitosteryl ferulate has reached 11% of their original concentration.

[0041] Disclosed herein is a method of extracting ferulic acid. In one example, the method comprises: incubating oryzanol with one or more cholesterol esterases as described herein in a first hydrolysis solution; extracting ferulic acid and phytosterols from the first hydrolysis solution in a first extraction step; subjecting the first hydrolysis solution after extraction of ferulic acid and phytosterols to chemical hydrolysis to obtain a second hydrolysis solution; extracting ferulic acid and triterpene alcohol from the second hydrolysis solution in a second extraction step. In another example, the method of extracting ferulic acid is as disclosed herein, wherein the cholesterol esterase preferentially hydrolyses phytosterol esters.

[0042] In one example, the incubation step is carried out using at least one recombinant enzyme as described herein**.** In another example, the incubation step is carried out using a combination of recombinant enzymes, as described herein. In another example, the recombinant enzyme is a cholesterol esterase as shown in any one of SEQ ID NOs 1, 2 and 3. In one example, the recombinant enzyme comprises the wild-type sequence of the cholesterol esterase from *Neomonachus schauinslandi*. In another example, the recombinant enzyme is as shown in SEQ ID NO: 1. In another example, the recombinant enzyme comprises the wild-type sequence of the cholesterol esterase from *Mustela putorius furo*. In another example, the recombinant enzyme is as shown in SEQ ID NO: 2. In another example, the recombinant enzyme comprises the wild-type sequence of the cholesterol esterase from *Erinaceus europaeus.* In another example, the recombinant enzyme is as shown in SEQ ID NO: 3. In another example, the recombinant enzyme is a combination of the enzymes as shown in SEQ ID Nos 1 and 2. In yet another example, the recombinant enzyme is a combination of the enzymes as shown in SEQ ID Nos 1 and 3. In a further example, the recombinant enzyme is a combination of the enzymes as shown in SEQ ID Nos 2 and 3.

[0043] The starting material (also referred to as an adduct) for the two-step method disclosed herein is, for example, oryzanol. In one example, oryzanol is obtained from seeds of plants of the Poacea family. Other sources of oryzanol include, but are not limited to, oils obtained from *Alpinia galanga*, *Cinnamomum zeylanicum*, *Trigonella foenum-graecum*, *Foeniculum vulgare* and *Myristica fragrans*. In another example, oryzanol is obtained from rice. In one example, oryzanol is obtained from rice bran. In another example, oryzanol was obtained from rice brain oil soapstock.

[0044] As will be appreciated by a person skilled in the art, the duration of the incubation steps in the method disclosed herein will depend on, for example, the quality of the recombinant enzyme used (and thereby the efficacy of the recombinant enzyme). Thus, the more efficient the recombinant enzyme, the shorter the incubation duration can be. Such a duration can also depend on the type and quality of the starting material used.

[0045] As will be appreciated by a person skilled in the art, whether an incubation step as disclosed herein, or an enzyme digestion as disclosed herein, has been carried out for a sufficient length of time will depend on, for example, if release of the desired product can be detected and/or quantified. In one example, the detection and/or quantification of ferulic acid indicates that the incubation step has been performed successfully. In one example, the incubation step is carried out for between 12 to 24 hours. In another example, the incubation step is carried out for at least 12 hours, at least 18 hours, or at least 24 hours. In yet another example, the incubation step is carried out for about 10 hours, about 11 hours, about 12 hours, about 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours, or about 25 hours. In one example, the first hydrolysis step is incubated for 12 to 24 hours.

[0046] As will be appreciated by a person skilled in the art, enzymatic reactions occur under conditions that has been optimised for the enzymatic activity of the specific enzyme used on the reaction. One such

condition is the pH of the solution containing the enzyme. Thus, in one example, the incubation step is carried out at a pH of between 4 to 8, about 4 to 6, about 5 to 7, or about 6 to 8. In another example, the pH of the incubation step is about 4, about 4.5, about 5, about 5.5, about 6, about 6.5, about 7, about 7.5, or about 8. In another example, the pH is about 6.5, about 6.6, about 6.7, or about 6.8. In another example, the first hydrolysis solution has a pH of 6 to 8.

[0047] Another reaction condition is the temperature of the solution containing the enzyme. Thus, in another example, the incubation step is carried out at a temperature range of between 25°C to 45°C, between 25°C to 29°C, between 28°C to 33°C, between 32°C to 38°C, or between 37°C to 45°C. In another example, the incubation step is carried out at a temperature of about 25°C, about 26°C, about 27°C, about 28°C, about 29°C, about 30°C, about 31°C, about 32°C, about 33°C, about 34°C, about 35°C, about 36°C, about 37°C, about 38°C, about 39°C, about 40°C, about 41°C, about 42°C, about 43°C, about 44°C, about 45°C, or about 46°C. In another example, the first incubation step is carried out at 37°C.

[0048] It is well known-in the art that enzymatic reactions occur when an enzyme contacts a substrate. Enzymatic reactions can therefore be facilitated by improving the probability of an enzyme contacting a substrate, such as by agitating the solution containing the enzyme. Thus, in one example, the incubation step can be carried out under agitated conditions. Methods of agitation include, but are not limited to, shaking, or stirring, with any of the devices available to a person skilled in the art and that are capable of inducing a state of agitation in a culture media.

[0049] In one example, the incubation step is carried out with reagents suitable for conducting enzymatic reactions, for example, buffers. In one example, the buffer is sodium phosphate buffer. In one example, sodium chloride is added to the buffer. In another example, co-solvents can be added to the buffer, for example, acetone. In another example, emulsifiers can be added to the buffer, for example, sodium taurocholate. Any other buffers, co-solvents and emulsifiers suitable for use would be readily known to a person skilled in the art.

[0050] In a specific example, the incubation step is carried out in the presence of sodium taurocholate and acetone.

[0051] After enzymatic hydrolysis in the first hydrolysis step, the second hydrolysis step is an alkaline hydrolysis performed under alkaline conditions. Conditions for performing ester hydrolysis by chemical means are well-known by a person of average skill in the art. For example, alkaline hydrolysis can be performed by exposing the solution containing, but is not limited to, sodium hydroxide, or potassium hydroxide.

[0052] In one example, the chemical hydrolysis is performed at a pH of at least 11, or at least 12, or at least 13. In another example, the chemical hydrolysis is performed at a pH of about 10.5, 11, 11.5, 12, 12.5, 13, or 13.5.

[0053] Thus, the two-step method as describe herein comprises a first hydrolysis step from which ferulic acid and phytosterols are obtained from oryzanol, and a second hydrolysis step from which

ferulic acid and triterpene alcohols are obtained from the solution remaining after ferulic acid and phytosterols resulting from the first hydrolysis step had been isolated. In one example, the total yield of ferulic acid from both the first and the second hydrolysis steps combined is between 70% to 90%, between 70% to 80%, between 75% to 85%, between 78% to 88%, or about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79% about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, or about 90%. In one example, the total yield of ferulic acid is between 85% to 86%. In another example, the total yield of ferulic acid is 85%. In yet another example, the total yield of ferulic acid is 86%.

[0054] In order to obtain ferulic acid and phytosterols after the first hydrolysis step, as disclosed herein, methods capable of separating ferulic acid and phytosterols from the remaining solution (or reaction mixture) are required. A person skilled in the art will appreciate that this can be done using methods known in the art. These methods can be, but are not limited to, membrane separation, chromatography, size-exclusion chromatography, flash chromatography, affinity chromatography, high performance liquid chromatography, continuous extraction, distillation, crystallization, filtration, fractionation, and combinations thereof. The same methods can be applied to obtain ferulic acid and triterpene alcohols from the reaction mixture after alkaline hydrolysis. Thus, in one example, the first and/or second extraction step is independently a method selected from the group consisting of membrane separation, chromatography, size-exclusion chromatography, flash chromatography, affinity chromatography, high performance liquid chromatography, continuous extraction, distillation, crystallization, filtration, fractionation, liquid-liquid extraction, solid-phase extraction, and combinations thereof. In another example, solid-phase extraction, or liquid-liquid extraction with a non-polar solvent, can be performed in the first extraction step. In the context of liquid-liquid extraction, in one example, the non-polar solvent is, but is not limited to, ethyl acetate, octane, and hexane. In one example, liquid-liquid extraction is performed, whereby hexane is used to extract any remaining oryzanol and triterpene alcohol from the first hydrolysis solution after incubation. In another example, extraction with isopropyl alcohol can be performed in the second extraction step. In other words, in one example, isopropyl alcohol can be used to extract triterpene alcohols from the second hydrolysis step under alkaline conditions.

[0055] The recombinant cholesterol esterase is obtained using genetic methods which include, for example, use of an expression vector containing a sequence encoding a cholesterol esterase. A person skilled in the art would be able to select suitable vectors and, using techniques well known in the art, introduce a sequence encoding a cholesterol esterase into said expression vector. Exemplary expression vectors are, but are not limited to, pGAP Z A, pGAP Z B, pGAP Z C, pESC, pSGP3, pYES2, pFAi, or pPic9. In one example, the expression vector is pFAi. The expression vectors as described herein can be under the transcriptional control of a promoter, where the promoters can be, AOX1 (P_{AOX1}), or GAP (P_{GAP}). In one example, the promoter is P_{GAP}. The expression vectors described herein can be under the

transcriptional control of a terminator, whereby the terminators can be, but are not limited to, ADH1, CYC1, or AOX1. In one example, the terminator is AOX1 terminator. In one example, the expression vector is under transcriptional control of a promoter and a terminator. In another non-limiting example, the expression vector is under transcriptional control of a promoter and a terminator, wherein the promoter is P_{GAP} and the terminator is AOX1 terminator. In one example, the vector is the vector encoded by SEQ ID NO: 6.

[0056] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that, although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0057] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0058] Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[0059] Other embodiments are within the following claims and non- limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

Extraction of oryzanol from rice bran oil soapstock

[0060] 2 mL of ethyl acetate was added to 2 g of rice bran oil soapstock and the reaction mixture was sonicated with ultrasonication water bath at room temperature. After 10 minutes, the reaction mixture was subjected to gravitational sediment, and the supernatants were collected and filtered. The extraction was repeated by three time, and the filtrates were combined. The filtrates were dried with vacuum concentrator to obtain a powder form of oryzanol.

Strains and plasmids

[0061] E. coli XL1Blue competent cells was utilized for cloning and plasmid amplification while *Pichia pastoris* GS115 was used for expression of five cholesterol esterases. pFAi vector under the transcriptional control of P_{GAP} and AOX1 terminator was employed for cloning and expression of cholesterol esterases. An alpha-mating factor secretion signal peptide from *Saccharomyces cerevisiae* was incorporated upstream of the mature sequence to facilitate secretion into the culture supernatant.

[0062] A vector map of the pFAi vector is shown in Figure 3. The nucleic acid sequence of the pFAi vector as shown in Figure 3 is provided in Table 3.

Construction of recombinant cholesterol esterases in Pichia pastoris GS115 strain

[0063] Five mature cholesterol esterase genes, harbouring two BsaI recognition sites, with GATG as 5' fusion site and TAGC as 3' fusion site, were cloned into pFAi vector using Golden gate cloning. The ligation products were transformed in XL1 Blue cells and positive transformants were selected on LB agar plate supplemented with 25 µg/mL zeocin. After verifying the DNA sequence, pFAi vector containing the desired cholesterol esterase genes were linearized with AvrII for insertion at GAP promoter site, followed by electroporation with electrocompetent GS115 Pichia pastoris strain at 2.0 kV, 200 Ω and 25 µF in 0.2 cm electrode gap cuvettes. His^+ transformant strains with successful genome integration were selected on RDB plate (1 M sorbitol, 2% dextrose, 1.34% Yeast nitrogen base with ammonium sulphate (YNB), 4×10^{-5}% biotin, 0.005% amino acids and 2% agar) after incubation at 30°C for 3 days. Colonies were replica-plated onto BMMY agar plate containing 2% of tributyrin (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5}% biotin and 0.5% methanol (MeOH), 20 g/L agar) to identify the esterase-secreting transformants. Positive esterase-secreting transformants with formation of clear zone on the tributyrin plate were selected for expression in shake flask scale.

[0064] The protein sequence of the cholesterol esterase genes is shown in Table 3.

Expression and purification of recombinant cholesterol esterase

[0065] The GS115 colonies bearing the desired cholesterol esterase gene were grown in 150 mL YPD medium for protein overexpression at 30°C and 220 rpm for 4 days. The cells were then harvested, and the supernatants were concentrated using an Amicon 10 kDa concentrator. To identify the presence of desired His-tagged cholesterol esterases, the concentrated supernatant was subjected for SDS-PAGE analysis (see Fig. 4).

Enzymatic hydrolysis of oryzanol

[0066] A 1.05ml reaction scale was used in the screening of suitable enzyme. 766 µM of oryzanol was incubated in 100 mM sodium phosphate buffer (pH 7.0) containing 786 mM sodium chloride, 8mM sodium taurocholate and 4% (v/v) of acetone. 0.25 mg of cholesterol esterase were added to initiate the reaction and the reaction mixture was incubated at 37°C and 2000 rpm in a thermomixer. After 24 hours, the reaction mixture was subjected to the extraction of ferulic acid, phytosterol, and unreacted oryzanol.

Extraction of ferulic acid, and unreacted oryzanol from enzymatic reaction mixture

[0067] After enzymatic hydrolysis, the reaction mixture was centrifuged, and the supernatant was transferred to another tube. To recover ferulic acid, the supernatant was dried with vacuum concentrator and reconstitute with ethyl acetate. 10 µL of the solution were analysed via RP-HPLC and the amount of ferulic acid obtained was quantified by correlating to a calibration curve.

Chemical hydrolysis of oryzanol and extraction of ferulic acid and triterpene alcohols

[0068] The eluted oryzanol obtained from enzymatic hydrolysis was first dissolved in 1 mL isopropanol, after which 1 mL of 2 M of aqueous NaOH (1:1, v/v) was added into the reaction mixture. The reaction mixture was heated under reflux at 85°C for 10 hours and stirred at 800 rpm. After 8 hours, the reaction mixture was allowed to cool down to room temperature and 200 µL of hexane was added into the reaction mixture. The organic liquid layer was separated from aqueous liquid layer. 500 µl of a 20% of sulphuric acid solution was added into the aqueous liquid phase and pH was adjusted to 3. The aqueous liquid layer was allowed to crystalize overnight and the ferulic acid crystals obtained via filtration. For the recovery of triterpene alcohol, 1.6 mL of water and 80 mg of ammonium sulphate were added into the organic liquid layer and the reaction mixture was allowed to crystalize overnight and performed filtration to obtain triterpene alcohols crystal.

High pressure liquid chromatography (HPLC) for detection of oryzanol and ferulic acid

[0069] A reverse phase (RP) HPLC method using a C18 column was developed to detect oryzanol and a calibration curve was built. Mobile phase A was 0.283 M of phosphoric acid/0.05% TFA and mobile phase B was acetonitrile: methanol (1:1, v: v, 0.05% TFA). The ratio of mobile phase was as follows: 90:10 (A: B) held for 5 minutes, followed by a gradient from 30:70 (A:B) to 0:100 (A:B) for 15 minutes, and held for 25 minutes. Followed by a gradient from 0:100 (A: B) to 70:30 (A: B) for 5 minutes, and lastly, a gradient from 70:30 (A:B) to 30:70 (A:B) for 5 minutes and held for 5 minutes. Injection volume was set at 50 µL with flow rate at 0.8 mL/min and the UV-Vis detector is set a 325 nm for oryzanol detection.

[0070] Another reverse phase HPLC method using a C18 column was developed to detect ferulic acid. Mobile phase A was 0.4% of acetic acid in water and mobile phase B was 100% methanol. The ratio of mobile phase was as follows: 70:30 (A: B) held for 22.5 minutes, followed by a gradient from 70:30 (A: B) to 40:60 (A: B) for 1 minutes, and held for 7 minutes. Followed by a gradient from 40:60 (A: B) to 0:100 (A: B) for 1 minutes and held for 20 minutes. Injection volume was set at 10 µL with flow rate at 1 mL/min. A calibration curve was built with the absorbance monitored at 310 nm.

[0071] Table 3 – Table of sequences used herein:

SEQ ID NO	Name	Sequence
1	*Neomonachus schauinslandi* (Hawaiian monk seal)	MGHLGLVVLGLTCCWAVVSAAKLGAVYTEGGFVEGVNKKLS LFGDYVDIFKGIPFAAPPKALENPQQHPGWQGTLKAKDFKKRC LQATITQDNTYGEEDCLYLNIWVPQGKKEVSRNLPVMIWIYGG GFLMGAGHGANFLSNYLYDGEEIATRGNVIVVTFNYRVGPLG FLSTGDANLPGNYGLRDQHMAIAWVKRNIAAFGGDPDNITIFG ESAGGASVSLQTLSPYNKGLIRRAISQSGVAVCPWAIQKNPLF WAKRIAEKVGCPLDDTSRMAKCLKITDPRALTLAYKLPLAGM EYPVLHYLGFLPVVDGDFIPDDPVKLYANTTDVDYIAGINNMD GHIFASIDMPAINKDKQEVTEEDFYKLVSGLTITKGLRGANATF DIYTEPWAHDTSQETKKKTVVDFETDILFLVPTEFAVAQHRAN AKSAKTYTYVFSHPSRMPTYPKWVGADHADDIQYVFGKPFAS PLGYRTQDRTVSKAMIAYWTNFARTGDPNMGHSAVPTHWDP YTVESGNYLEITKKMDRNSMKQHLRTNYMRYWTLTYHTLPT VTGNGAVPAPPSGDSDAAPAPPSGDSDAAPVPPSEDSEGAQMP IVIGF
2	*Mustela putorius furo* (European domestic ferret)	MGHLGLVVLGLTCCWATVSAAKLGAVYTEGGFVEGVNKKLG LFGDYVDIFKGIPFAAPPKALENPQRHPGWQGTLKAKDFKKRC LQATITQDNTYGAEDCLYLNIWVPQGKKEVSRNLPVMIWIYG GAFLMGSGHGANFLSNYLYDGEEIATRGNVIVVTFNYRVGPL GFLSTGDANLPGNFGLRDQHMAIAWVKRNIAAFGGDPDNITIF GESAGGASVSLQTLSPYNKGLIRRAISQSGVALCPWAIQKNPLF WAKRIAEKVGCPLDDTARMAKCLKVTDPRALTLAYKMPLAG MEYPMLHYLGFLPVIDGDFIPDDPVTLYANTADVDYIAGTNN MDGHIFASIDVPAIDKNNQEVTEEDFYKLVSGLTITKGLRGANT TFDIYTESWALDASQETKKKTVVDLETDILFLVPTEIAVAQHRA NAKSAKTYTYMFSHPSRMPTYPKWVGADHADDLQYVFGKPF ATPLGYRTQDRTVSKTMIAYWTNFARTGDPNIGHSAVPTHWD PYSIESGNYLEITKKMDGHSMKQHLRASYLRYWTLTYEALPTV SGNGAVPAPPSGDSEAAPAPPSGDSEADPAPPSGDSEAAPAPPN GDSEAAPAPHNGDSEAAPAPPSGDSEAAPAPPSGDSEGAQMPV VIGF
3	*Erinaceus europaeus* (Western European hedgehog)	MGHLSLVVVGLTCCLAAVSAAKLGAVYTEGGFVEGVNKKLG LFGDYVDIFKGIPFAAPPKALENPQPHPGWQGTLKAKDFKKRC LQATVTQDDTYGAEDCLYLNIWVPQGKREVSRNLPVMIWIYG GAFLMGSGQGANFLKNYLYDGEEIATRGNVIVVTFNYRVGPL GFLSTGDANLPGNYGLRDQHMAIAWVKRNIAAFGGDPNNITIF GESAGAASVSLQTLSPYNKGLIRRAISQSGVALSPWAIQKNPLF WAKKIAEKVGCPVDDTARLAKCLKVTDPRALTMAYKMPLAG MEYPMLYYLGFLPVVDGDFIPDEPINLYANAADIDYMAGTND MDGHVFSSIDMPAVDKDKQTVTDEDFYKLVKGLTFTKGLQGA QATYDMYTESWAQDSSQEARKKTVVNLETDILFLMPTQTALA QHKSNAKSARTYSYLFSHPTRMPIYPNWVGADHADDLQYVFG KPFSSPLGYRTQDRTVSKAMIAYWTNFARTGDPNMGHSAVPT QWIPYTLENSDYLEINKKINANSVKQHLRANFLRYWTLTYKPL PTVTDEGALPLPPSGDSEAAPVPPSGDSEAAPVPPSGDSEAAPV PPSGDSEAAPVPPSGDSEAAPVPPEISEAVHMPIAIGF
4	Porcine cholesterol esterase	MGSSELVIWCLACCLAAARAAKLGSVYTEGGFVEGVNKKLSL LGDSVDIFKGIPFAAAPKALENPQRHPGWQGTLKAKDFKKRCL QATITQDSTYGDEDCLYLNIWVPQGRKEVSRDLPVMIWIYGGA

		FLMGSGQGANFLSNYLYDGEELATRGNVIVVTFNYRVGPLGF LSTGDANLPGNYGLRDQHMAIAWVKRNIAAFGGDPDNITIFGE SAGGASVSLQTLSPYNKGLIKRAISQSGVALSPWAIQKNPLSW AKTIAEKVGCPMDDTARMARCLKITDPRALTLAYKLPLTKQEF PVVHYLGFIPVVDGDFIPDDPVNLYANAADIDYLAGTNDMDG HLFATVDLPAVDKDKKTITEEDFYKLVSGFTIVKGPRGANITFD VYTASWAQDSSQETKKKTVVDLETDILFLMPTETAVAQHRAN AKSAQTYTYVFAHPSRMPVYPSWYVFGKPFAMIAYWTNFARS GDPNMGGSSVPTHWEPYTLESSKYLEITNKMDSSSMKQDLRTS YLQYWATTYQALPTVADEGAIPVPPTGDSEATPAPPTGDPEAT PAPPTGDSEATPAPPPSDAEVAQMPIVIGF
5	Bovine cholesterol esterase	LGASRLGPSPGCLAVASAAKLGSVYTEGGFVEGVNKKLSLFG DSIDIFKGIPFAAAPKALEKPERHPGWQGTLKAKSFKKRCLQAT LTQDSTYGNEDCLYLNIWVPQGRKEVSHDLPVMIWIYGGAFL MGASQGANFLSNYLYDGEEIATRGNVIVVTFNYRVGPLGFLST GDSNLPGNYGLWDQHMAIAWVKRNIEAFGGDPDNITLFGESA GGASVSLQTLSPYNKGLIKRAISQSGVGLCPWAIQQDPLFWAK RIAEKVGCPVDDTSKMAGCLKITDPRALTLAYKLPLGSTEYPK LHYLSFVPVIDGDFIPDDPVNLYANAADVDYIAGTNDMDGHLF VGMDVPAINSNKQDVTEEDFYKLVSGLTVTKGLRGANATYEV YTEPWAQDSSQETRKKTMVDLETDILFLIPTKIAVAQHKSHAK SANTYTYLFSQPSRMPIYPKWMGADHADDLQYVFGKPFATPL GYRAQDRTVSKAMIAYWTNFARTGDPNTGHSTVPANWDPYT LEDDNYLEINKQMDSNSMKLHLRTNYLQFWTQTYQALPTVTS AGASLLPPEDNSQASPVPPADNSGAPTEPSAGDSEVAQMPVVI GF
6	pFAi vector	TTTTTGTAGAAATGTCTTGGTGTCCTCGTCCAATCAG GTAGCCATCTCTGAAATATCTGGCTCCGTTGCAACTC CGAACGACCTGCTGGCAACGTAAAATTCTCCGGGGT AAAACTTAAATGTGGAGTAATGGAACCAGAAACGTG TCTTCCCTTCTCTCTCCTTCCACCGCCCGTTACCGTCC CTAGGAAATTTTACTCTGCTGGAGAGCTTCTTCTACG GCCCCCTTGCAGCAATGCTCTTCCCAGCATTACGTTG CGGGTAAAACGGAGGTCGTGTACCCGACCTAGCAGC CCAGGGATGGAAAAGTCCCGGCCGTCGCTGGCAATA ATAGCGGGCGGACGCATGTCATGAGATTATTGGAAA CCACCAGAATCGAATATAAAAGGCGAACACCTTTCC CAATTTTGGTTTCTCCTGACCCAAAGACTTTAAATTT AATTTATTTGTCCCTATTTCAATCAATTGAACAACTA TCTATGAGATTTCCTTCAATTTTTACTGCTGTTTTATT CGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACT ACAACAGAAGATGAAACGGCACAAATTCCGGCTGAA GCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCG ATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAA CGGGTTATTGTTTATAAATACTACTATTGCCAGCATT GCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGA GAGGCTGAAGCTGTGATGAGAGACCGAATTCGCGGC CGCTTCTAGAGCAATACGCAAACCGCCTCTCCCCGC GCGTTGGCCGATTCATTAATGCAGCTGGCACGACAG GTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGC AATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAG GCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTG GAATTGTGAGCGGATAACAATTTCACACATACTAGA

		GAAAGAGGAGAAATACTAGATGGCTTCCTCCGAAGA CGTTATCAAAGAGTTCATGCGTTTCAAAGTTCGTATG GAAGGTTCCGTTAACGGTCACGAGTTCGAAATCGAA GGTGAAGGTGAAGGTCGTCCGTACGAAGGTACCCAG ACCGCTAAACTGAAAGTTACCAAAGGTGGTCCGCTG CCGTTCGCTTGGGACATCCTGTCCCCGCAGTTCCAGT ACGGTTCCAAAGCTTACGTTAAACACCCGGCTGACA TCCCGGACTACCTGAAACTGTCCTTCCCGGAAGGTTT CAAATGGGAACGTGTTATGAACTTCGAAGACGGTGG TGTTGTTACCGTTACCCAGGACTCCTCCCTGCAAGAC GGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACC AACTTCCCGTCCGACGGTCCGGTTATGCAGAAAAAA ACCATGGGTTGGGAAGCTTCCACCGAACGTATGTAC CCGGAAGACGGTGCTCTGAAAGGTGAAATCAAAATG CGTCTGAAACTGAAAGACGGTGGTCACTACGACGCT GAAGTTAAAACCACCTACATGGCTAAAAAACCGGTT CAGCTGCCGGGTGCTTACAAAACCGACATCAAACTG GACATCACCTCCCACAACGAAGACTACACCATCGTT GAACAGTACGAACGTGCTGAAGGTCGTCACTCCACC GGTGCTTAATAACGCTGATAGTGCTAGTGTAGATCG CTACTAGAGCCAGGCATCAAATAAAACGAAAGGCTC AGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTT GTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCA CCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAG CGGCCGCTGCAGGGTCTCTTAGCTTTGTAGCCTTAGA CATGACTGTTCCTCAGTTCAAGTTGGGCACTTACGAG AAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGT CAGAATGCCATTTGCCTGAGAGATGCAGGCTTCATTT TTGATACTTTTTTATTTGTAACCTATATAGTATAGGA TTTTTTTTGTCATTTTGTTTCTTCTCGTACGAGCTTGC TCCTGATCAGCCTATCTCGCAGCTGATGAATATCTTG TGGTAGGGGTTTGGGAAAATCATTCGAGTTTGATGTT TTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAA GATTAAGTGAGACTTTCGTTTGTGCGGATCCGCTGGG ATCCTAATGCGGTAGTTTATCACAGTTAAATTGCTAA CGCAGTCAGGCACCGTGTATGAAATCTAACAATGCG CTCATCGTCATCCTCGGCACCGTCACCCTGGATGCTG TAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCCT CTTGCGGGATATCGTCCATTCCGACAGCATCGCCAGT CACTATGGCGTGCTGCTAGCGCTATATGCGTTGATGC AATTTCTATGCGCACCCGTTCTCGGAGCACTGTCCGA CCGCTTTGGCCGCCGCCCAGTCCTGCTCGCTTCGCTA CTTGGAGCCACTATCGACTACGCGATCATGGCGACC ACACCCGTCCTGTGGATCTATCGAATCTAAATGTAAG TTAAAATCTCTAAATAATTAAATAAGTCCCAGTTTCT CCATACGAACCTTAACAGCATTGCGGTGAGCATCTA GACCTTCAACAGCAGCCAGATCCATCACTGCTTGGC CAATATGTTTCAGTCCCTCAGGAGTTACGTCTTGTGA AGTGATGAACTTCTGGAAGGTTGCAGTGTTAACTCC GCTGTATTGACGGGCATATCCGTACGTTGGCAAAGT GTGGTTGGTACCGGAGGAGTAATCTCCACAACTCTCT GGAGAGTAGGCACCAACAAACACAGATCCAGCGTGT TGTACTTGATCAACATAAGAAGAAGCATTCTCGATTT GCAGGATCAAGTGTTCAGGAGCGTACTGATTGGACA TTTCCAAAGCCTGCTCGTAGGTTGCAACCGATAGGGT

		TGTAGAGTGTGCAATACACTTGCGTACAATTTCAACC CTTGGCAACTGCACAGCTTGGTTGTGAACAGCATCTT CAATTCTGGCAAGCTCCTTGTCTGTCATATCGACAGC CAACAGAATCACCTGGGAATCAATACCATGTTCAGC TTGAGACAGAAGGTCTGAGGCAACGAAATCTGGATC AGCGTATTTATCAGCAATAACTAGAACTTCAGAAGG CCCAGCAGGCATGTCAATACTACACAGGGCTGATGT GTCATTTTGAACCATCATCTTGGCAGCAGTAACGAAC TGGTTTCCTGGACCAAATATTTTGTCACACTTAGGAA CAGTTTCTGTTCCGTAAGCCATAGCAGCTACTGCCTG GGCGCCTCCTGCTAGCACGATACACTTAGCACCAAC CTTGTGGGCAACGTAGATGACTTCTGGGGTAAGGGT ACCATCCTTCTTAGGTGGAGATGCAAAAACAATTTCT TTGCAACCAGCAACTTTGGCAGGAACACCCAGCATC AGGGAAGTGGAAGGCAGAATTGCGGTTCCACCAGGA ATATAGAGGCCAACTTTCTCAATAGGTCTTGCAAAA CGAGAGCAGACTACACCAGGGCAAGTCTCAACTTGC AAAGTCTCCGTTAGTTGAGCTTCATGGAATTTCCTGA CGTTATCTATAGAGAGATCAATGGCTCTCTTAACGTT ATCTGGCAATTGCATAAGTTCCTCTGGGAAAGGAGC TTCTAACACAGGTGTCTTCAAAGCGACTCCATCAAAC TTGGCAGTTAGTTCTAAAAGGGCTTTGTCACCATTTT GACGAACATTGTCGACAATTGGTTTGACTAATTCCAT AATCTGTTCCGTTTTCTGGATAGGACGACGAAGGGC ATCTTCAATTTCTTGTGAGGAGGCCTTAGAAACGTCA ATTTTGCACAATTCAATACGACCTTCAGAAGGGACTT CTTTAGGTTTGGATTCTTCTTTAGGTTGTTCCTTGGTG TATCCTGGCTTGGCATCTCCTTTCCTTCTAGTGACCTT TAGGGACTTCATATCCAGGTTTCTCTCCACCTCGTCC AACGTCACACCGTACTTGGCACATCTAACTAATGCA AAATAAAATAAGTCAGCACATTCCCAGGCTATATCT TCCTTGGATTTAGCCTCGGCCAACTCGTCGGCCTCCT CCCTAATTTTAGCGTTCAACAAAACTTCGTCGTCAAA TAACCGTTTGGTATAAGAACCTTCTGGAGCATTGCTC TTACGATCCCACAAGGTGGCTTCCATGGCTCTAAGAC CCTTTGATTGGCCAAAACAGGAAGTGCGTTCCAAGT GACAGAAACCAACACCTGTTTGTTCAACCACAAATT TCAAGCAGTCTCCATCACAATCCAATTCGATACCCAG CAACTTTTGAGTTGCTCCAGATGTAGCACCTTTATAC CACAAACCGTGACGACGAGATTGGTAGACTCCAGTT TGTGTCCTTATAGCCTCCGGAATAGACTTTTTGGACG AGTACACCAGGCCCAACGAGTAATTAGAAGAGTCAG CCACCAAAGTAGTGAATAGACCATCGGGGCGGTCAG TAGTCAAAGACGCCAACAAAATTTCACTGACAGGGA ACTTTTTGACATCTTCAGAAAGTTCGTATTCAGTAGT CAATTGCCGAGCATCAATAATGGGGATTATACCAGA AGCAACAGTGGAAGTCACATCTACCAACTTTGCAGT CTCAGAAAAAGCATAAACAGTTCTACTACCGCCATT AGTGAAACTTTTCAAATCGCCCAGTGGAGAAGAAAA AGGCACAGCGATACTAGCATTAGCGGGCAAGGATGC AACTTTATCAACCAGGGTCCTATAGATAACCCTAGC GCCTGGGATCATCCTTTGGACAACTCTTTCTGCCAAA TCTAGGTCCAAAATCACTTCATTGATACCATTATTGT ACAACTTGAGCAAGTTGTCGATCAGCTCCTCAAATTG GTCCTCTGTAACGGATGACTCAACTTGCACATTAACT

		TGAAGCTCAGTCGATTGAGTGAACTTGATCAGGTTGT GCAGCTGGTCAGCAGCATAGGGAAACACGGCTTTTC CTACCAAACTCAAGGAATTATCAAACTCTGCAACAC TTGCGTATGCAGGTAGCAAGGGAAATGTCATACTTG AAGTCGGACAGTGAGTGTAGTCTTGAGAAATTCTGA AGCCGTATTTTTATTATCAGTGAGTCAGTCATCAGGA GATCCTCTACGCCGGACGCATCGTGGCCGGCATCAC CGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGC CGACATCACCGATGGGGAAGATCGGGCTCGCCACTT CGGGCTCATGAGCGCTTGTTTCGGCGTGGGTATGGTG GCAGGCCCCGTGGCCGGGGGACTGTTGGGCGCCATC TCCTTGCATGCACCATTCCGGAGACTAACCCACACAC CATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTC CAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTT CAAAACACCCAAGCACAGCATACTAAATTTCCCCTC TTTCTTCCTCTAGGGTGTCGTTAATTACCCGTACTAA AGGTTTGGAAAAGAAAAAAAAGACCGCCTCGTTTCT TTTTCTTCGTCGAAAAAGGCAATAAAAATTTTTATCA CGTTTCTTTTTCTTGAAAATTTTTTTTTTTGATTTTTTT CTCTTTCGATGACCTCCCATTGATATTTAAGTTAATA AACGGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTC TTGTTCTATTACAACTTTTTTTACTTCTTGCTCATTAG AAAGAAAGCATAGCAATCTAATCTAAGGGGCGGTGT TGACAATTAATCATCGGCATAGTATATCGGCATAGT ATAATACGACAAGGTGAGGAACTAAACCATGGCCAA GTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGA CGTCGCCGGAGCGGTCGAGTTCTGGACCGACCGGCT CGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGC CGGTGTGGTCCGGGACGACGTGACCCTGTTCATCAG CGCGGTCCAGGACCAGGTGGTGCCGGACAACACCCT GGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTA CGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCG GGACGCCTCCGGGCCGGCCATGACCGAGATCGGCGA GCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCC GGCCGGCAACTGCGTGCACTTCGTGGCCGAGGAGCA GGACTGACACGTCCGACGGCGGCCCACGGGTCCCAG GCCTCGGAGATCCGTCCCCCTTTTCCTTTGTCGATAT CATGTAATTAGTTATGTCACGCTTACATTCACGCCCT CCCCCCACATCCGCTCTAACCGAAAAGGAAGGAGTT AGACAACCTGAAGTCTAGGTCCCTATTTATTTTTTTA TAGTTATGTTAGTATTAAGAACGTTATTTATATTTCA AATTTTTCTTTTTTTTCTGTACAGACGCGTGTACGCAT GTAACATTATACTGAAAACCTTGCTTGAGAAGGTTTT GGGACGCTCGAAGGCTTTAATTTGCAAGCTGGAGAC TAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGA ACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAG GCTCCGCCCCCCTGACGAGCATCACAAAAATCGACG CTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATA AAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTG CGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACC TGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTC TCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAG GTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCC CCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTA TCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCG

		CCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGA GCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAG TGGTGGCCTAACTACGGCTACACTAGAAGGACAGTA TTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCG GAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAA CCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCA GCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAG ATCCTTTGATCTTTTCTACGGGAACG

CLAIMS

1. A method of hydrolysing oryzanol to obtain ferulic acid, wherein the method comprises:
   i. hydrolysing an oryzanol comprising material in a first hydrolysis step; and
   ii. exposing product of i. to a second hydrolysis step;

   wherein the first hydrolysis step is enzymatic, and the second hydrolysis step is chemical.

2. The method of claim 1, wherein the first hydrolysis step is performed using a cholesterol esterase which preferentially hydrolyses phytosterol esters.

3. The method of claim 2, wherein the cholesterol esterase is a recombinant cholesterol esterase.

4. The method of claim 2, wherein the cholesterol esterase comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID Nos 1 to 3.

5. The method of any one of the preceding claims, wherein the first hydrolysis and/or the second hydrolysis step is/are performed multiple times.

6. A method of extracting ferulic acid, wherein the method comprises:
   i. incubating oryzanol with a cholesterol esterase in a first hydrolysis solution;
   ii. extracting ferulic acid and phytosterols from the first hydrolysis solution in a first extraction step;
   iii. subjecting the first hydrolysis solution after extraction of ferulic acid and phytosterols to chemical hydrolysis to obtain a second hydrolysis solution;
   iv. extracting ferulic acid and triterpene alcohol from the second hydrolysis solution in a second extraction step;

   wherein the cholesterol esterase preferentially hydrolyses phytosterol esters.

7. The method of claim 5, wherein the cholesterol esterase is selected from the group consisting of SEQ ID Nos 1, 2 and 3.

8. The method of any one of claims 5 to 6, wherein the first hydrolysis solution has a pH of 4 to 8.

9. The method of any one of claims 5 to 7, wherein the first hydrolysis solution is maintained at between 25°C to 45°C prior to extraction.

10. The method of any one of claims 5 to 8, wherein the first hydrolysis solution is incubated for at least 12 hours.

11. The method of any one of claims 5 to 9, wherein the first extraction step is a method selected from the group consisting of membrane separation, chromatography, size-exclusion chromatography, flash chromatography, affinity chromatography, high performance liquid chromatography, continuous extraction, distillation, filtration, liquid-liquid extraction, solid-phase and fractionation.

12. The method of claim 5 to 10, wherein the second hydrolysis solution has a pH of at least 11.

13. The method of any one of claims 5 to 11, wherein the second extraction step is a method selected from the group consisting of membrane separation, chromatography, size-exclusion chromatography, flash chromatography, affinity chromatography, high performance liquid chromatography, continuous extraction, distillation, filtration, addition of isopropyl alcohol, and fractionation.

14. The method of claim 10, wherein the liquid-liquid extraction is performed using a non-polar solvent.

15. The method of claim 14, wherein the non-polar solvent is selected from the group consisting of isopropyl alcohol, hexane, ethyl acetate, and octane.

16. The method of any one of claims 5 to 14, wherein steps i and ii and/or steps iii and iv are performed multiple times in order.

17. A method of extracting ferulic acid from oryzanol-comprising material, comprising treating the material according to the method of any one of claims 1 to 15 to extract ferulic acid.

18. The method of 16, wherein the oryzanol-comprising material is selected from the group consisting of rice, rice bran, rice brain oil soapstock, and mixtures thereof.