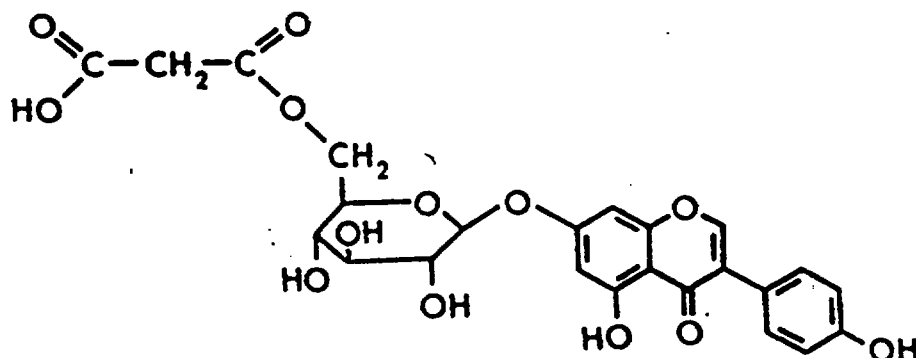
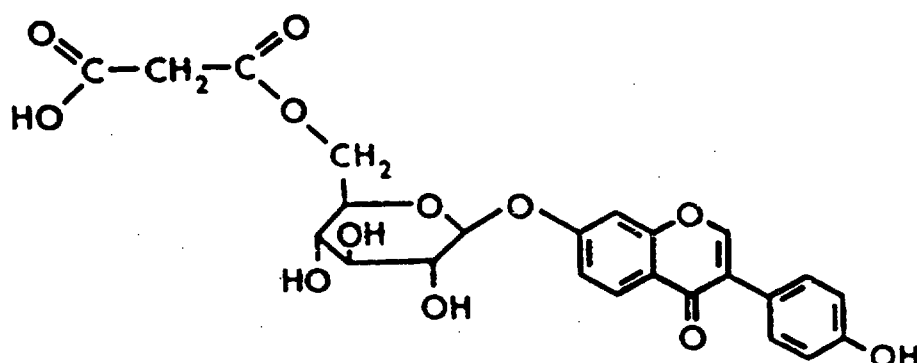


This invention relates to a process for the production of two isoflavones, genistin malonate and daidzin malonate

It is known, for example from the Article by C.M. Francis (J. Sci. Fd. Agric. (1973) 24, 1235), that clover leaves contain genistin which corresponds to the formula:



It is also known, for example from JP 1 146 894 that daidzin malonate, which corresponds to the following formula:



can be isolated from roots or stems of *Pueraria lobata*.

The object of the present invention is to provide a process for simply and economically obtaining these two particular isoflavones in good yields from a food source, namely soya beans.

The process according to the invention is characterized in that ground soya beans are extracted with an alcohol, the crude extract obtained is buffered with an

aqueous buffer solution to a pH value of 6 - 9, the buffer solution containing the crude extract is extracted with a water-immiscible organic solvent, the aqueous phase is recovered and acidified to pH 2.5 - 4, the acidified aqueous phase is extracted with a water-immiscible organic solvent, the organic phase is recovered and neutralized to pH 6.8 - 7.2 and the residual constituents are separated.

In the process according to the invention, ground soya beans preferably having an average particle diameter of 0.5 to 0.8 mm and a maximum diameter of 1.0 to 1.1 mm may be prepared. Before extraction, the ground soya beans may be defatted to remove from the soya the fats which could subsequently interfere with the correct operation of the process. Defatting may be carried out, for example, by refluxing ground soya beans in an organic solvent, such as hexane or petroleum ether. In one particular embodiment, a mixture containing 1 part by weight ground soya beans to 4 - 6 parts by weight n-hexane may be heated with stirring (70 - 90 r.p.m.) for 50 - 70 minutes at a temperature of 60 - 70°C, after which the mixture may be left to cool to room temperature, the ground material may be filtered and then washed with n-hexane and the defatted ground material dried under nitrogen for 10 to 15 hours.

The optionally defatted ground material is then extracted with an alcohol, such as methanol or ethanol for example. This may be done, for example, by refluxing a mixture containing 1 kg defatted ground material and 6 to 8 litres of a 70 - 90% aqueous methanol solution with stirring (70 - 90 r.p.m.) for 50 - 100 minutes at a temperature of 70 - 90°C, hot-filtering the mixture and drying the filtrate, for example under reduced pressure in a rotary evaporator at 25 - 50°C. A crude extract in the form of a brown-coloured solid can be obtained in this way.

The crude extract is then buffered to bring its pH to a value of 6 - 9. To this end, the crude extract may be

suspended or dissolved in a solution of a weak base, such as an aqueous NaHCO_3 solution. This enables the subsequent extraction to be improved, the genistin and the daidzin passing into the organic phase and the desired malonates remaining in the aqueous phase. The buffer solution containing the crude extract is then extracted with a water-immiscible organic solvent, such as butanol or ethyl acetate for example. After extraction, the organic phase mainly contains isoflavones, such as genistin and daidzin, and other constituents while the aqueous phase contains the desired compounds and impurities.

The aqueous phase is acidified to a pH of the order of 2.5 - 4, for example by addition of concentrated, more particularly 5 - 10 N, hydrochloric acid. The acidified aqueous phase is extracted with a water-immiscible organic solvent. It is preferred to use a solvent identical with that used for extraction of the aqueous suspension or solution containing the crude extract, i.e. butanol or ethyl acetate, so that there is no need to use several different solvents, which is always dangerous. The organic phase obtained may then be neutralized by adjusting its pH to a value of 6.8 - 7.2, for example by addition of sodium hydroxide, and dried, for example by evaporation of the solvent under reduced pressure in a rotary evaporator at 25 - 50°C. An impure extract containing the desired compounds is thus obtained in the form of a brown-coloured solid.

The residual constituents may then be separated from this extract, for example by adsorption and filtration chromatography of the impure extract, which also enables any impurities, such as the genistin and/or the daidzin still present, to be removed from the extract. This chromatography may be carried out, for example, in a column containing a gel using an alcohol, such as ethanol or a methanol/water mixture, as eluent. Several fractions containing impurities and several fractions containing a mix-

ture of the desired compounds can be obtained. The fractions of interest may then be subjected to a second chromatography to separate the two desired compounds. To this end, inverse-phase chromatography may be carried out using, for example, a polar column containing in particular octadecyl silane groups attached to silica and an eluent or an eluent gradient of decreasing polarity, such as for example a 10 - 25% solution of methanol, ethanol or acetonitrile. Two fractions each containing a separate compound are obtained in this way. These fractions may be dried, for example in a dry evaporator, under reduced pressure at a temperature of 25 to 50°C. Two products are thus obtained in the form of amorphous solids, one pale yellow in colour and the other white in colour, both of which are soluble in polar solvents, such as water, methanol or ethanol.

These two compounds have been found to exhibit remarkable antioxidant properties, being capable of protecting the fats, vitamins and/or oligoelements present in cosmetic or food products against oxidation.

The present invention is illustrated in more detail by the following preparation example and by the following identification tests for the compounds and activity tests.

Example of preparation of the compounds

i) Defatting

5 kg n-hexane are added to 1 kg ground soya beans having an average particle diameter of 0.8 mm. The mixture is refluxed at a temperature of 65°C for 1 hour with continuous stirring at 80 r.p.m. This mixture is then left to cool to ambient temperature and filtered, the ground material being recovered and dried under nitrogen for 12 hours. The dry ground material is then mixed with another 5 kg n-hexane and refluxed at 65°C for 1 hour with continuous stirring. After cooling and filtration, the

resulting ground material is washed with 1 litre n-hexane. The ground material is dried under nitrogen for 10 hours. Defatted ground soya beans are thus obtained in a quantity of 0.7 kg.

5

ii) Preparation of the crude extract

A mixture containing 150 g defatted ground soya beans as prepared in step ii) and 1 litre of an 80% aqueous methanol solution is prepared. The mixture is refluxed at a temperature of 80°C for 1 hour with continuous stirring at 80 r.p.m. The mixture is then hot-filtered and the filtrate is retained. The ground material is then heated with stirring for another hour at 80°C with 1 litre of 80% methanol. The mixture is hot-filtered and the filtrate obtained is added to the preceding filtrate. The combined filtrate is evaporated to dryness under reduced pressure in a rotary evaporator at a temperature of 40°C. 32.6 g crude extract in the form of a brown-coloured solid are obtained.

iii) Preparation of the impure extract

32.6 g of the crude extract obtained in step ii) are mixed with 150 ml of a 1 M aqueous NaHCO_3 solution. 150 ml n-butanol are added, the two phases are mixed and the aqueous phase is recovered by extraction, the butanol phase mainly containing daidzin and genistin. The aqueous phase is acidified to pH 3 by addition of 6.5 N hydrochloric acid. 150 ml n-butanol are added to the aqueous phase and, after mixing, a first organic phase is recovered. Another 150 ml n-butanol are added to the aqueous phase and, after mixing, a second organic phase is recovered.

The pH of the two organic phases are adjusted to 7 by addition of 1 N NaOH and the two phases are dried under reduced pressure in a rotary evaporator at 40°C. Impure extract in the form of a brown-coloured solid is obtained in respective yields of 2.4 g for the first phase and 1.2

g for the second phase.

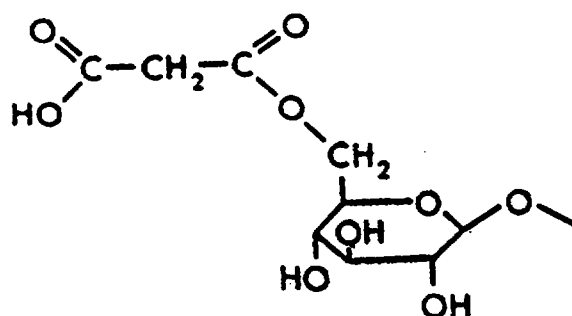
iv) Separation of the constituents

A chromatography column containing a gel (Sephadex LH-20) 3 cm in diameter and 50 cm tall is prepared. 2.4 g of the impure extract obtained in step iii) diluted with ethanol is introduced into the column. The impure extract is eluted with ethanol at a rate of 2 ml per minute. A total of 17 fractions are obtained, 6 of these fractions containing the malonates of genistin and daidzin. These 6 fractions are subjected to a second chromatography in a column containing octadecyl silane groups attached to silica (Lobar RP-18). A 10% aqueous ethanol solution is used as the eluent at a rate of 2 ml per minute. Two fractions each containing a different product are finally obtained. These fractions are dried under reduced pressure in an evaporator at a temperature of 40°C. 25 mg daidzin malonate and 80 mg genistin malonate, both highly pure (92 - 98%), are obtained in the form of amorphous solids, the first white in colour and the second pale yellow in colour.

Identification tests for the genistin malonate

i) Position of the sugar group

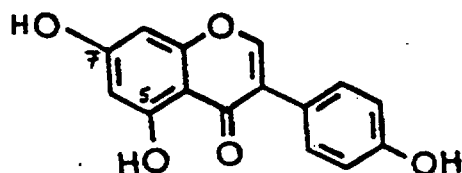
The position of the sugar group having the formula



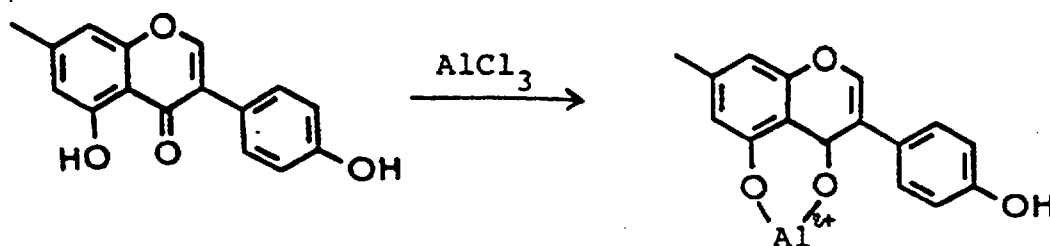
in the genistin malonate molecule is determined by a so-

called shift reaction with aluminium chloride.

The free OH group may be in the 5 position or the 7 position, as shown below, the other position being occupied by the above-mentioned sugar group.



If the OH group is in the 5 position, a complex will be formed in the presence of aluminium chloride in accordance with the following reaction:



Now, the λ_{max} of the complex thus formed is higher by about 10 to 14 nm than the λ_{max} of the genistin malonate. The λ_{max} of the genistin malonate is measured in methanol optionally in the presence of aluminium chloride. The following results are obtained:

- . without AlCl_3 $\lambda_{\text{max}} = 260.1 \text{ nm}$
- . with AlCl_3 $\lambda_{\text{max}} = 270.7 \text{ nm}$

Accordingly, a complex was formed in the presence of aluminium chloride; the free OH group is thus in the 5 position and the sugar group in the 7 position.

ii) Decomposition in alkaline medium

The retention time of malonic acid and genistin malonate, as such or in the presence of sodium hydroxide, is measured by high-performance liquid chromatography (HPLC) at 228 nm. The following results are obtained:

- Malonic acid
One peak is obtained at 2.19 mins.
- 10 - Malonic acid + NaOH
Two peaks are obtained: one at 2.19 mins. corresponding to malonic acid and one at 5.08 mins. corresponding to sodium malonate.
- 15 - Genistin malonate
One peak is obtained at 15.17 mins.
- Genistin malonate + NaOH
Two peaks are obtained: one at 12.80 mins. corresponding to genistin and one at 5.07 mins. corresponding to sodium malonate.
- 20

The genistin malonate is thus decomposed in the presence of sodium hydroxide into sodium malonate and genistin.

25

iii) Infra-red spectrum

The IR spectrum of genistin malonate in the solid state in a concentration of 1% in KBr shows a characteristic band at 1729.2 cm^{-1} corresponding to the carbonyl group of the ester (theoretical value $1735 \pm 10\text{ cm}^{-1}$).

30

iv) Mass spectrum

Mass spectra were prepared by two methods giving a result by deficiency ("negative FAB") and a result by

35

excess ("positive FAB").

The following results are obtained:

- A: molecular peak of the compound (genistin malonate)
- B: peak corresponding to isoflavone glucoside (genistin).
- C: peak corresponding to the basic aglycone (genistein).

Genistin malonate (molecular weight, g)

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	Negative FAB	Positive FAB	Theoretical value
A	517	519	518
B	431	433	432
C	269	271	270

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v) NMR spectrum

The ^{13}C nuclear magnetic resonance spectrum (NMR) of genistin malonate in dimethyl sulfoxide (DMSO-d_6) at 20°C shows the following characteristic signals:

20

	Signal	Multiplicity
	45.4 ppm	CH ₂
	63.1 ppm	CH ₂
	69.7 ppm	CH
5	73.0 ppm	CH
	74.0 ppm	CH
	76.0 ppm	CH
	94.3 ppm	CH
	99.4 ppm	CH
10	99.7 ppm	CH
	106.3 ppm	C
	115.0 ppm	CH (2 carbons)
	120.8 ppm	C
	122.6 ppm	C
15	130.0 ppm	CH (2 carbons)
	154.0 ppm	CH
	157.2 ppm	C
	157.7 ppm	C
	161.7 ppm	C
20	162.5 ppm	C
	168.4 ppm	C
	169.2 ppm	C
	179.9 ppm	C
25		

There are thus 24 carbon atoms, including 3 carbonyl carbons (carboxylic acid and conjugated ketone), 14 aromatic or olefinic carbons (7CH, 7 quaternary), of which 5 are O-substituted, an anomeric carbon CH-OH, 4 O-substituted aliphatic CH, one O-substituted aliphatic CH₂ and one non-O-substituted aliphatic CH₂.

There are also 16 non-interchangeable protons.

Identification tests for daidzin malonate**i) Decomposition in alkaline medium**

5 The retention time of malonic acid and daidzin malonate, as such or in the presence of sodium hydroxide, is measured by high-performance liquid chromatography (HPLC) at 228 nm. The following results are obtained:

- 10 - Malonic acid
 One peak is obtained at 2.19 mins.
- 15 - Malonic acid + NaOH
 Two peaks are obtained: one at 2.19 mins. corresponding to malonic acid and one at 5.08 mins. corresponding to sodium malonate.
- 20 - Daidzin + NaOH
 One peak is obtained at 8.66 mins.
- 25 - Daidzin malonate
 One peaks is obtained at 13.89 mins.
- 30 - Daidzin malonate + NaOH
 a) Immediately after mixing, one peak is obtained at 11.47 mins., corresponding to daidzin, and one at 8.65 mins., corresponding to that obtained for daidzin in the presence of NaOH,
 b) One hour after mixing, one peak is obtained at 8.65 mins., corresponding to that obtained for daidzin in the presence of NaOH.

iii) Infra-red spectrum

35 The IR spectrum of daidzin malonate in the solid state in a concentration of 1% in KBr shows a characteristic band at 1734.0 cm^{-1} corresponding to the carbonyl group of the

ester (theoretical value $1735 \pm 10 \text{ cm}^{-1}$).

iv) Mass spectrum

5 Mass spectra were prepared by two methods giving a result by deficiency ("negative FAB") and a result by excess ("positive FAB").

The following results are obtained:

- A: molecular peak of the compound (daidzin malonate)
- B: peak corresponding to isoflavone glucoside (daid-
10 zin).
- C: peak corresponding to the basic aglycone (daid-
zein).

Daidzin malonate (molecular weight, g)

15

	Negative FAB	Positive FAB	Theoretical value
A	501	503	502
B	415	417	416
20 C	253	255	254

v) NMR spectrum

25 The ^{13}C nuclear magnetic resonance spectrum (NMR) of daidzin malonate in dimethyl sulfoxide (DMSO- d_6) at 20°C shows the following characteristic signals:

	Signal	Multiplicity
	45.2 ppm	CH ₂
	63.0 ppm	CH ₂
	69.7 ppm	CH
5	73.0 ppm	CH
	74.0 ppm	CH
	76.1 ppm	CH
	99.7 ppm	CH
	103.4 ppm	CH
10	114.9 ppm	CH (2 carbons)
	115.3 ppm	CH
	118.4 ppm	C
	122.0 ppm	C
	123.6 ppm	C
15	126.9 ppm	CH
	129.9 ppm	CH (2 carbons)
	153.2 ppm	CH
	156.8 ppm	C
	157.4 ppm	C
20	161.0 ppm	C
	168.3 ppm	C
	169.1 ppm	C
	174.7 ppm	C

25

There are thus 24 carbon atoms in all, including 3 carbonyl carbons (carboxylic acid and conjugated ketone), 14 aromatic or olefinic carbons (8CH, 6 quaternary), of which 4 are O-substituted, one carbon is anomeric (CH-OH), 4 are O-substituted aliphatic CH's, one is an O-substituted aliphatic CH₂ and one is a non-O-substituted aliphatic CH₂.

30

There are also 17 non-interchangeable protons.

Activity tests for the compounds

1) Qualitative analysis

It is known that β -carotene is oxidized in the presence of linoleic acid by ultraviolet (UV) irradiation. The oxidation is reflected in the decolouration of the β -carotene.

One drop of an aqueous solution of daidzin, genistin, daidzin malonate or genistin malonate is applied to a support plate for thin-layer chromatography. A solution containing 100 mg β -carotene and 1 ml linoleic acid in 100 ml chloroform is then vaporized on the plate. The plate turns yellow. The plate is placed beneath a UV light source and left there until decolouration occurs. It can be seen that, at the places where the genistin and the daidzin were applied, the plate is not decoloured and is still yellow in colour. This is attributable to the fact that the daidzin and the genistin have antioxidant properties which prevent oxidation and, hence, decolouration of the β -carotene. It can also be seen that the plate has the same yellow colour at the places where the malonates of genistin and daidzin were applied. Accordingly, these two compounds also exhibit antioxidant properties.

ii) Quantitative analysis by spectrophotometry

0.01 M solutions of daidzein, genistein, daidzin, genistin, daidzin malonate and genistin malonate in methanol are prepared.

Several solutions of gallic acid in methanol varying in concentration from 0.2 to 20 g l^{-1} are prepared in exactly the same way as standards.

A solution containing 100 mg β -carotene, 1 ml linoleic acid and 100 ml chloroform is also prepared as reactant.

First samples containing 20 μl of the solution to be measured, 60 μl reactant and 2 ml methanol and second

control samples containing 20 μ l of the solution to be measured and 2 ml methanol are prepared.

5 The absorption of the sample in relation to its control sample is measured just after preparation, after which the two samples are exposed to ultraviolet radiation for 12 minutes and their absorption is remeasured.

10 The results obtained for the isoflavones may then be compared with the results obtained for the various solutions of gallic acid, enabling the following equivalences to be defined:

	0.010 M Isoflavone solution	Molar concentration of the gallic acid solution having the same antioxidant power
15	Daidzein	0.020 M
	Daidzin	0.022 M
	Daidzin malonate	0.024 M
20	Genistein	0.028 M
	Genistin	0.027 M
	Genistin malonate	0.029 M

25 It can be seen that the 0.010 M solutions of daidzin or genistin malonate have the same effect as the 0.010 M solutions of daidzin or genistin and the 0.024 M and 0.029 M solutions of gallic acid.

30 iii) Oxidation test

The Rancimat^(R) accelerated oxidation test is used to determine the induction time of chicken fat stabilized by addition of genistin or daidzin malonate.

35 The Rancimat^(R) test comprises passing air through a reaction tube containing a sample of 5 g fat at 100°C and measuring the conductivity of the volatile secondary

products formed during oxidation and entrained with the stream of air. The induction time is graphically determined from the recorded conductivity/time curve by intersection of the tangent to the curve with the time axis.

- 5 The following results are obtained for a chicken fat containing 500 or 1,000 ppm genistin or daidzin malonate or none at all (control):

		Induction time (h)
10	Control	6.95
	Genistin malonate 500 ppm	8.10
	Daidzin malonate 500 ppm	7.68

- 15 The antioxidant effect of genistin malonate and daidzin malonate is thus clearly confirmed.

CLAIMS

1. A process for the production of isoflavones, characterized in that ground soya beans are extracted with an alcohol, the crude extract obtained is buffered with an aqueous buffer solution to a pH value of 6 - 9, the buffer solution containing the crude extract is extracted with a water-immiscible organic solvent, the aqueous phase is recovered and acidified to pH 2.5 - 4, the acidified aqueous phase is extracted with a water-immiscible organic solvent, the organic phase is recovered and neutralized to pH 6.8 - 7.2 and the residual constituents are separated.
2. A process as claimed in claim 1, characterized in that the ground soya beans are extracted with a 70 - 90% aqueous methanol solution by heating for 50 - 100 minutes at 70 - 90°C.
3. A process as claimed in claim 1, characterized in that the water-immiscible organic solvent is butanol.



* C L M S *