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(54) Title: A PROCESS FOR ISOLATING PROTEINS FROM SOLID PROTEIN-CONTAINING BIOMASS SELECTED FROM VEGETABLE BIOMASS, ALGAE, SEAWEED AND COMBINATIONS THEREOF

(57) Abstract: The invention relates to a process for isolating proteins from solid protein- containing biomass selected from vegetable biomass, algae, seaweed and combinations thereof, the process comprising the following steps: a) providing a dispersion of the solid protein-containing biomass in an aqueous liquid; b) extracting protein from the biomass by keeping the dispersion at a temperature in the range of from 70 to 150°C during an extraction time; c) separating the dispersion into a protein-comprising extract and protein-depleted biomass; and d) recovering protein from the extract, wherein the pH of the dispersion during extraction step b) is in the range of from 12.3 to 13.5.

A PROCESS FOR ISOLATING PROTEINS FROM SOLID PROTEIN-CONTAINING BIOMASS SELECTED FROM VEGETABLE BIOMASS, ALGAE, SEAWEED AND COMBINATIONS THEREOF

Field of the Invention

The present invention relates to a process for isolating proteins from solid protein-containing biomass selected from vegetable biomass, algae, seaweed and combinations thereof.

Background of the Invention

Protein is present in a relatively high concentration in plant leaves. Depending on the plant species, the protein content in leaves can amount to 15 to 30 wt%, in particular in for example green tea leaves, Jatropha leaves, tobacco leaves, alfalfa leaves, or grass leaves.

Leaf protein concentrates have been proposed as food supplement since the 1960s. Isolation of proteins from leaves is typically carried out by first extracting the protein by extracting comminuted leaves with e.g. water, a phosphate buffer or an alkaline solution, separating the extract from the extracted leaves and then recovering protein from the extract.

Alkaline extraction of proteins from leaves or other vegetable biomass at ambient temperature or moderate temperatures, i.e. up to 50 °C, has been proposed. In *A.E. Ghaly and F.N. Alkoaik, American Journal of Applied Sciences 7 (2010), p. 331-342* for example, protein extraction from leaves at a moisture content of 97% and a pH of 8.5 is disclosed. In *B. Bals and B.E. Dale, Biotechnology and Bioengineering 108 (2011), p. 530-537*, an alkaline extraction in 3% ammonia at 50 °C is disclosed. In *B.E. Dale et al., Biofuels, Bioproducts & Biorefining 3 (2009), p. 219-230*, alkaline extractions at moderate temperature (50 °C) and a pH of up to 10 are mentioned. In *S. Chiesa and E. Gnansounou, Bioresource Technology 102 (2011), p. 427-436*, it is mentioned that extraction is always carried out at relatively low temperatures in order to prevent extensive protein deterioration and that the combination of high temperature and alkaline conditions can irreparably damage the protein in biomass.

Protein extraction yields of alkaline extraction of leaf proteins at low or mild temperature are reported to be typically between 40 and 60 % of the protein present in the starting material.

Summary of the Invention

It has now been found that by extracting proteins from solid protein-containing biomass comprising a cell wall structure, such as vegetable biomass, algae and seaweed, with a liquid at a relatively high temperature and at a relatively high pH during the extraction, a high percentage of the proteins can be extracted and recovered.

Accordingly, the present invention relates to a process for isolating proteins from solid protein-containing biomass selected from vegetable biomass, algae, seaweed and combinations thereof, the process comprising the following steps:

a) providing a dispersion of the solid protein-containing biomass in an aqueous liquid;

b) extracting protein from the biomass by keeping the dispersion at a temperature in the range of from 70 to 150 °C during an extraction time;

c) separating the dispersion into a protein-comprising extract and protein-depleted biomass; and

d) recovering protein from the extract,

wherein the pH of the dispersion during extraction step b) is in the range of from 12.3 to 13.5.

An advantage of the process according to the invention compared to prior art alkaline extraction processes at low or moderate temperature is that a higher protein yield is obtained. Moreover, it appears that, contrary to statements in the prior art, the proteins recovered by the present process still have a relatively high molecular weight and are thus not extensively damaged and deteriorated.

It has further been found that control of the pH of the dispersion during extraction step b) is important to obtain a high yield of proteins with a relatively high molecular weight.

A further advantage of the process according to the invention is that also coagulated protein can be extracted from biomass due to the relatively high alkaline conditions applied during the extraction. Therefore, the process can also suitably be applied for extraction of biomass wherein protein has been concentrated by means of a pretreatment that causes protein coagulation, such as for example biomass that has been

subjected to a heat, acid or alkaline pretreatment step. Thus, biomass with a relatively high protein content can be obtained from which protein can be suitably extracted by means of the process according to the invention. An advantage of biomass with a relatively high protein content is that a protein extract with a higher protein content is obtained. Moreover, since pretreatment steps that cause protein coagulation typically result in killing or deactivation of at least part of the micro-organisms and deactivation of enzymes present in the biomass, the pretreated biomass may be sun-dried without development of an unacceptable odour or excessive microbial degradation of the biomass or autolysis of proteins or other biomass compounds. This is particularly important for biomass with a high moisture content that is harvested and processed in dry and/or climates, such as for example algae, seaweed or leaves of tropical plants such as water hyacinth.

Detailed Description of the Invention

In the process according to the invention proteins are isolated from solid protein-containing biomass by first extracting proteins from the solid biomass by a solid-liquid extraction at a temperature in the range of from 70 to 150 °C and at a pH in the range of from 12.3 to 13.5 to obtain a protein-comprising extract and protein-depleted biomass and then recovering protein from the extract.

In step a) of the process according to the invention, a dispersion of the solid protein-containing biomass with a cell wall structure in an aqueous liquid is provided. Protein is then extracted from the biomass by keeping the dispersion at a temperature in the range of from 70 to 150 °C during an extraction time (extraction step b), wherein the pH of the dispersion during extraction step b) is in the range of from 12.3 to 13.5. After the extraction time, the dispersion is separated into a liquid protein-comprising extract and solid protein-depleted biomass (step c). This may be done by any suitable solid liquid separation known in the art, for example filtration, centrifugation or sedimentation. Protein is then recovered from the extract in a recovery step d).

Any suitable protein-containing biomass selected from vegetable biomass, algae, seaweed and combinations thereof. Preferably, the biomass comprises plant leaves, straw, algae and/or sea weed, more preferably plant leaves and/or straw. Even more preferably, the protein-containing biomass is vegetable biomass, still more plant leaves. Examples of suitable plant leaves are tea leaves, Jatropha leaves, water hyacinth leaves and other tropical leaves, sugar beet leaves, and grass leaves.

It has been found that the process according to the invention is suitable to isolate proteins from biomass comprising intact cell walls. The biomass therefore preferably is biomass comprising intact cell walls.

The biomass may be untreated or pretreated biomass. The process according to the invention is particularly suitable for protein- and water-comprising biomass that has been subjected to a pretreatment wherein the protein has been concentrated by means of coagulation of at least part of the protein followed by removal of at least part of the water, prior to extraction steps a) and b). Such pretreatment may comprise any suitable protein coagulation step and any suitable water removal step. Such steps are well-known in the art. Protein may for example be coagulated by means of a heat treatment, an acid or an alkaline treatment. Water may subsequently be removed by means of for example mechanical pressing, centrifugation and/or drying, for example sun-drying.

Preferably, the biomass is obtained by a pre-treatment comprising heating protein and water-containing biomass at a temperature above 80 °C, preferably in the range of from 80 to 105 °C, to coagulate at least part of the protein. An example of biomass pretreated by a heat treatment that causes protein coagulation are extracted tea leaves, also referred to as tea leaves residue.

Alternatively, the biomass may be pretreated biomass that has been subjected to a mild protein extraction, for example a mild heating step followed by a pressing step, an alkaline extraction at a mild temperature and/or a mild pH . Biomass residue from biorefining processes such as bioethanol manufacturing processes wherein cellulose and hemicelluloses from the biomass are converted into ethanol, may suitably be used as the solid protein-containing biomass in the process according to the invention.

The protein-containing biomass is preferably chopped or otherwise comminuted prior to being extracted in step b). More preferably, the biomass is comminuted such that it has an average particle size in the range of from 1 to 50 mm. Reference herein to particle size is to the largest dimension of the biomass particle. It has been found that, unlike in some prior art protein extraction processes, milling of the biomass is not needed in order to obtain a high protein yield.

In step a) of the process according to the invention, a dispersion of solid biomass in an aqueous liquid is provided. The aqueous liquid serves as extraction liquid in alkaline extraction step b). Preferably, the dispersion is provided by providing an aqueous alkaline solution and then mixing the alkaline solution with the biomass to

obtain the dispersion. Alternatively, however, the dispersion may be provided by adding solid alkaline compound and water to the biomass. In case of biomass with a very high moisture content, for example above 50 wt% or above 80 wt%, the dispersion may even be provided by adding solid alkaline compound to the biomass without addition of water.

The pH of the dispersion during extraction step b) is in the range of from 12.3 to 13.5. If the dispersion is provided by mixing an aqueous alkaline solution with the biomass, the alkaline solution may be any alkaline solution that, after mixing with the biomass, may provide a dispersion with such high pH. Preferably, the alkaline solution is a solution of a strong base in water, more preferably a solution of sodium hydroxide and/or potassium hydroxide in water. The solution may further comprise a weaker base such as for example ammonia or calcium hydroxide.

It has been found that the presence of divalent cations such as calcium ions or magnesium ions in the dispersion has a positive effect on the colour of the proteins extracted. Without wishing to be bound to any theory, it is believed that under alkaline extraction conditions as prevailing in step b) of the process according to the invention, biomass compounds such as pigments and polyphenols are extracted and react with protein to form tannins or other compounds or complexes with a brown colour. Calcium or magnesium ions can react or coagulate with some of the coloured/brown compound formed.

Therefore, the dispersion of biomass in alkaline solution preferably comprises calcium and/or magnesium ions. The presence of calcium or magnesium ions in the dispersion may for example be achieved by using biomass that has been pre-treated with a calcium or magnesium salt, for example biomass that has been subjected to a prior extraction with a solution comprising a calcium or magnesium salt. Alternative or additionally, the presence of calcium or magnesium ions in the dispersion is achieved by providing the dispersion by mixing an alkaline solution comprising a calcium or magnesium salt, with the solid biomass and/or by adding a calcium or magnesium salt to the dispersion during step b). The calcium or magnesium salt preferably is calcium hydroxide and/or magnesium hydroxide, more preferably calcium hydroxide. In a preferred embodiment of the process according to the invention, the dispersion provided in step a) is a dispersion of biomass in an aqueous solution of sodium hydroxide and/or potassium hydroxide that further comprises calcium hydroxide.

The pH of the dispersion during extraction step b) is in the range of from 12.3 to 13.5, preferably in the range of from 12.3 to 13.3, during the entire extraction step. Without addition of additional hydroxide or other alkaline compound during the extraction step, the pH of the dispersion may decrease due to biomass components with a buffering capacity, such as for example proteins, pectin or lignin. It is therefore preferred to monitor the pH of the dispersion during extraction step b) and add additional alkaline compound if needed to keep the pH in the range of from 12.3 to 13.5 or within any of the preferred pH ranges.

The concentration of hydroxide in the alkaline solution provided in step a) is not critical as long as the dispersion obtained by mixing the alkaline solution with the solid biomass has a pH of at least 12.3 and not above 13.5 in order to limit protein hydrolysis.

The ratio of volume of the liquid to dry weight of the solid protein-containing biomass in the dispersion may be any suitable ratio. Preferably, the ratio of volume of alkaline solution to weight of protein-containing biomass is in the range of from 0.5 to 50 litres per kilogram, more preferably of from 1 to 40 litres per kilogram, even more preferably of from 2 to 25 litres per kilogram, still more preferably of from 3 to 10 litres per kilogram.

It has been found that the ratio of volume of the liquid to weight of solid biomass is not critical, in the sense that it does not importantly affect the extractability of proteins and the molecular weight of the proteins extracted. In order to maximize the protein concentration in the protein-comprising extract, a low ratio, i.e. below 10 litres per kilogram is preferred. It will be appreciated that the optimum ratio *inter alia* depends on the protein concentration in the biomass.

During extraction step b), the pH of the liquid of the dispersion will decrease. The lower the ratio of liquid to biomass, the stronger this effect. In order to optimise the amount of protein extracted, it is preferred to add additional alkaline compound to the alkaline solution during extraction step b). In particular in case a low ratio of liquid to biomass is applied, i.e. below 10 litres per kilogram dry biomass or more in particular below 5 litres per kilogram dry biomass, it is preferred to add an alkaline compound to the dispersion during step b) in order to keep the pH above 12.3 during the extraction, whilst the initial pH of the dispersion will not exceed 13.5, preferably not exceed 13.3.

Extraction step b) is carried out at a temperature in the range of from 70 to 150 °C, preferably of from 80 to 130 °C, more preferably of from 80 to 105 °C. At temperatures above 150 °C, substantial protein hydrolysis takes place and it is no longer possible to obtain proteins with a high molecular weight in acceptable amounts.

It will be appreciated that the extraction time will strongly depend on the extraction temperature and the pH during extraction step b). The lower the extraction temperature and the pH, the more extraction time is needed in order to achieve a higher protein yield. Preferably, the extraction time is in the range of from 15 minutes to 30 hours, more preferably of from 1 to 24 hours.

In recovery step d), protein is recovered from the protein-comprising extract obtained in separation step c). Recovery may be done by any means known in the art, preferably by means of precipitation, more preferably acid precipitation, or membrane filtration, more preferably ultrafiltration, size exclusion or ion exchange. Preferably, protein is recovered from the extract by means of precipitation, more preferably acid precipitation.

In separation step c), a protein-depleted biomass is obtained. Such protein-depleted biomass may suitably be used as raw material for the production of bio-ethanol and/or other bio-materials such as lactic acid or biogas.

The invention will be further illustrated by means of the following non-limiting examples.

Examples

Example 1

Green tea leave residue (GTR) was obtained by extracting dried green tea leaves during 45 minutes with water at 80 °C. In a centrifuge tube, an amount of 0.5 grams of GTR was immersed in 20 ml of a solution of 0.1 N sodium hydroxide in water (volume to weight ratio of 40 ml alkaline solution per gram biomass; initial pH of 13). Protein was extracted by placing the tubes in a water bath kept at the desired extraction temperature. During extraction the samples were shaken. The pH during extraction was monitored and did not decrease below a value of 12.3.

After extraction, the samples were centrifuged (17,200 g for 10 minutes) and the supernatants (protein-comprising extracts) were stored at -20 °C prior to analysis.

The protein content of the supernatants was determined by means of Kjeldahl analysis.

Extraction experiments as described above were carried out with GTR at temperatures of 40, 60, 80 and 95 °C. Extraction experiments with Oolong tea residue (dried Oolong tea leaves extracted with water for 45 minutes at 80 °C), Jatropha leaves (ground to 0.1 – 2 mm), road grass (ground to 0.2 – 4 mm), and barley straw (chopped to 0.5 – 5 mm) were carried out as described above at 95 °C for 4 hours. In Table 1, the extraction yields (wt% of protein extracted based on the weight of protein in the biomass) obtained in the different experiments are shown.

Table 1 – Protein extractability for different types of biomass (volume/weight ratio of 40; 0.1 N NaOH). No NaOH addition during the extraction step.

experiment	biomass	extraction temperature (°C)	extraction time (h)	hydroxide consumption (mmol/g biomass)	protein extracted (wt%)
1	GTR	40	4	2.4	30
2	GTR	40	24	3.1	39
3	GTR	60	4	2.9	45
4	GTR	60	24	3.2	69
5	GTR	80	4	3.6	77
6	GTR	80	24	4.0	92
7	GTR	95	1	3.5	76
8	GTR	95	4	3.8	94
9	Oolong tea residue	95	4	4.0	92
10	Jatropha leaves	95	4	3.5	94
11	road grass	95	4	2.5	95
12	barley straw	95	4	1.0	95

The experiments show that at a temperature of 60 °C , almost 70 wt% of the proteins can be extracted, at a temperature of 80 °C or 95 °C even more than 90 wt%.

The molecular weight of the proteins extracted in experiment 8 (GTR, 4h at 95 °C) was determined by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). It appeared that more than 90% of the proteins had a molecular weight above 14 kDa. Approximately 75 wt% of the proteins had a molecular weight of approximately 67 kDa.

Example 2

Protein was recovered as follows from supernatants that were obtained by alkaline extraction of green tea residue carried out as described in Example 1. The pH of the supernatants was adjusted to 3.5 by addition of 1N HCl. The acidified supernatants were then kept for 24 hours at 4 °C in order to allow protein to precipitate. Precipitated protein was then collected by centrifuging (17,200 g for 10 minutes) the samples and collecting the solid layer formed. The protein content of the solid layer was determined by means of Kjeldahl analysis. In Table 2 is shown the protein recovery from protein-containing extracts obtained at different extraction conditions. The protein recovery is expressed as wt% protein recovered based on the weight of protein in the protein-containing extract (supernatant obtained in the alkaline extraction step).

Table 2 – Protein recoveries at different alkaline extraction conditions

experiment	biomass	NaOH	T (°C)	T (h)	protein extracted (wt%)	protein recovery (wt%)
13	GTR	0.1	40	4	30	67
14	GTR	0.1	95	4	94	85
15	GTR	0.1	110	2	89	72
16	GTR	0.1	130	2	94	55
17	GTR	0.1	150	2	98	43
18	GTR	0.2	95	2	93	58
19	GTR	0.5	95	2	97	38
20	GTR	1.0	95	2	96	27

Example 3

Six extraction experiments were carried out wherein green tea residue was extracted at 95 °C during 2 hours. The concentration of sodium hydroxide in the initial alkaline solution and the ratio of alkaline solution to biomass were varied. In the first 5 experiments (experiments 21-25), no alkali was added during the extraction. In the last experiment (experiment 26) In the final experiment (experiment 26), a relatively low volume to biomass ratio was used (8 ml of alkaline solution per gram GTR) and the initial concentration of the alkaline solution was 0.1N NaOH. During the extraction step, additional NaOH was added after each 30 minutes extraction time (three additions in total; 1 mmol NaOH per gram GTR in each addition).

After extraction at 95 °C during 2 hours, supernatants were collected and protein precipitated and recovered from the supernatants by acid precipitation at pH 3.5 as described in Example 1. In Table 3, the extraction yields and protein recoveries for the different experiments are shown.

Table 3: Extraction of GTR at 95 °C during 2 hours

experiment	initial alkaline solution (N NaOH)	v/w (ml/g)	addition of NaOH during extraction	protein extracted (wt%)	protein recovery (wt%)
21	0.1	16	No	37	
22	0.1	24	No	67	
23	0.1	32	No	84	85
24	0.1	40	No	88	85
25	0.4	8	No	83	76
26	0.1	8	Yes, three times 1 mmol NaOH per gram GTR	86	86

Comparison of the results of experiments 23, 25 and 26 shows that controlled addition of the alkaline compound (controlled initial pH of the alkaline solution) is important to avoid extensive protein hydrolysis and resulting lower protein recovery.

Example 4

Extraction experiments were carried out with green tea residues using different alkaline solutions: $Ca(OH)_2$ (15 mg/g biomass), NaOH (0.1N), KOH (0.1N), NH_4OH

(0.2N), and a mixed alkaline solution comprising $Ca(OH)_2$ 7.5 mg/g biomass and 0.05N NaOH. For all experiments the ratio of volume of alkaline solution to weight of biomass was 40 ml per gram. In experiments 28-31, extractions were carried out as described in Example 1 at 95 °C for 4 hours. In experiment 27, GTR was first extracted with calcium hydroxide (15 mg $Ca(OH)_2$/g biomass; v/w ratio of 40 ml/g biomass; 2 hours at 95 °C). After centrifugation, the supernatant was collected and protein was precipitated by acid precipitation at pH 3.5 (experiment 27a). The acid precipitate thus obtained was then extracted with 0.1 N NaOH at 95 °C for two hours (exp. 27b) as described in Example 1.

After extraction, the samples were centrifuged (17,200 g for 10 minutes) and the supernatants (protein-comprising extracts) were stored at -20 °C prior to analysis.

The protein content of the supernatants was determined by means of Kjeldahl analysis. The absorbance of the supernatants at 400 and 680nm was determined.

In Table 4, the extraction yields (wt% of protein extracted based on the weight of protein in the biomass) and the absorbance at 400 and 680nm are shown for the different experiments.

The results show that a pre-extraction with calcium oxide followed by an extraction according to the invention at a relatively high pH, advantageously results in a less coloured protein extract. Also the presence of calcium hydroxide in the alkaline extraction solution, together with a strong base to provide for the desired high pH value results in a less coloured extract.

Table 4 - Extraction yields and absorbance of extract at 95 for 4 hours using different alkaline compounds

experiment	Alkaline compound	Concentration of alkaline compound	Initial pH	protein extracted (wt%)	Absorbance	
					400 nm	680 nm
27a[1]	$Ca(OH)_2$	15 mg/g biomass	12.2	32	2.032	0.138
27b[1]	NaOH	0.1 N	13	53	3.328	0.142

28	NaOH	0.1 N	13	94	39.96	3.95
29	KOH	0.1 N	13	95	38.76	3.89
30	NH_4OH	0.2 N	10.2	29	17.78	1.96
31	$Ca(OH)_2$ + NaOH	7.5 mg/g biomass + 0.05 N	12.7	86	19.61	3.11

[1] In experiment 27, an extraction with calcium hydroxide at 95 °C for two hours (experiment 27a) was followed by extraction of the proteins recovered with NaOH at 95 °C for two hours (experiment 27b).

Claims

1. Process for isolating proteins from solid protein-containing biomass selected from vegetable biomass, algae, seaweed and combinations thereof, the process comprising the following steps:

a) providing a dispersion of the solid protein-containing biomass in an aqueous liquid;

b) extracting protein from the biomass by keeping the dispersion at a temperature in the range of from 70 to 150 °C during an extraction time;

c) separating the dispersion into a protein-comprising extract and protein-depleted biomass; and

d) recovering protein from the extract,

wherein the pH of the dispersion during extraction step b) is in the range of from 12.3 to 13.5.

2. A process according to claim 1, wherein the pH of the dispersion during extraction step b) is in the range of from 12.3 to 13.3.

3. A process according to claim 1 or 2, wherein the ratio of liquid and dry weight of protein-containing biomass in the dispersion is in the range of from 1 to 40 litres per kilogram.

4. A process according to any one of the preceding claims, wherein the dispersion is provided in step a) by providing an aqueous alkaline solution and mixing the solid protein-containing vegetable biomass with the alkaline solution to obtain the dispersion.

5. A process according to any one of the preceding claims, wherein the liquid is a solution of sodium hydroxide, potassium hydroxide, or a combination thereof.

6. A process according to claim any one of the preceding claims, wherein the dispersion comprises calcium ions.

7. A process according to claim 6, wherein the liquid comprises calcium hydroxide.

8. A process according to any one of the preceding claims, wherein in step b) the dispersion is kept at a temperature in the range of from 80 to 130 °C.

9. A process according to claim 8, wherein in step b) the dispersion is kept at a temperature in the range of from 80 to 105 °C.

10. A process according to any one of the preceding claims, wherein the protein-containing biomass comprises plant leaves, straw, algae and/or sea weed.

11. A process according to any one of the preceding claims, wherein the protein-containing biomass is vegetable biomass.

12. A process according to any one of the preceding claims, wherein the protein-containing biomass is comminuted biomass having an average particle size in the range of from 1 to 50 mm.

13. A process according to any one of the preceding claims, wherein the protein-containing biomass is obtained by pre-treating protein and water-containing biomass such that at least part of the protein is coagulated and at least part of the water removed.

14. A process according to claim 13, wherein the pre-treating comprises heating protein and water-containing biomass at a temperature in the range of from 80 to 105 °C to coagulate at least part of the protein.

15. A process according to any one of the preceding claims, wherein protein is recovered from the extract by means of precipitation.

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2013/050676

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23J1/00 C07K1/14
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23J C07K A23K C08B

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 066 633 A (GASTINEAU CHARLES ET AL) 3 January 1978 (1978-01-03) column 3, line 30 - column 6, line 3 column 9, line 14 - column 11, line 5; claims 1,13,14,15,16,20,24; examples 2,3	1-15
A	DATABASE WPI Week 197652 Thomson Scientific, London, GB; AN 1976-97035X XP002693837, & JP 51 129776 A (ERNSTER J H) 11 November 1976 (1976-11-11) the whole document	1-15
A	-& JP 51 129776 A (JIYON EICHI ERUNSUTAA) 11 November 1976 (1976-11-11) the whole document	1-15

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

[X] See patent family annex.

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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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1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 624 805 A (LAWHON JAMES T [US]) 25 November 1986 (1986-11-25) column 2, line 33 - column 4, line 34 column 5, line 59 - column 17, line 30; claims 1-18	1-15
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