

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
27 March 2008 (27.03.2008)

PCT

(10) International Publication Number  
WO 2008/034180 A1

(51) International Patent Classification:  
C13D 3/16 (2006.01)

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(21) International Application Number:  
PCT/AU2007/001382

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(22) International Filing Date:  
19 September 2007 (19.09.2007)

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(25) Filing Language:  
English

(26) Publication Language:  
English

(30) Priority Data:  
2006905179 19 September 2006 (19.09.2006) AU

Published:

— with international search report

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WO 2008/034180 A1

(54) Title: EXTRACTS DERIVED FROM SUGAR CANE AND A PROCESS FOR THEIR MANUFACTURE

(57) Abstract: An extract derived from sugar cane having GI or burn rate reducing characteristics wherein the extract comprises a mixture of one or more polyphenols, one or more carbohydrates, one or more minerals and one or more organic acids.

## Extracts derived from sugar cane and a process for their manufacture

### Field of the invention

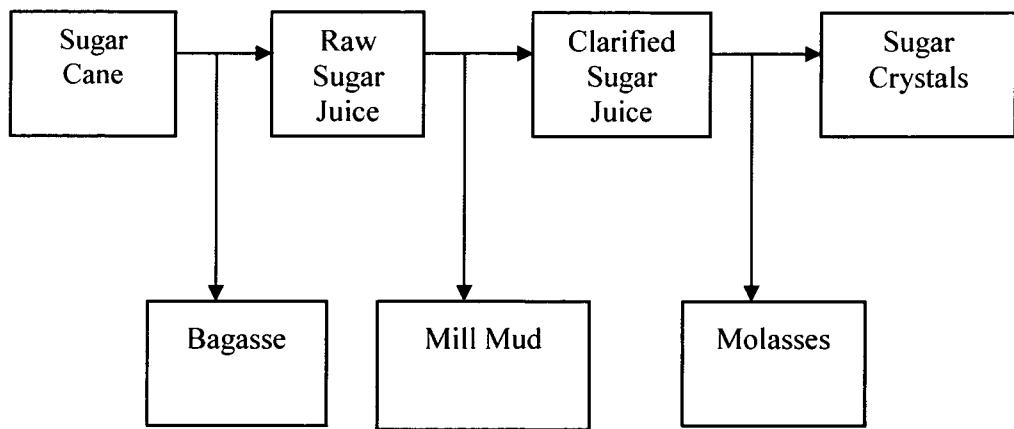
The present invention relates to extracts derived from sugar cane which comprise a mixture of one or more polyphenols, one or more carbohydrates, one or more minerals and one or 5 more organic acids. In addition, the invention relates to a process for refining sugar cane derived extracts as well as other phytochemical extracts.

### Background of the invention

In this specification where a document, act or item of knowledge is referred to or discussed, this reference or discussion is not an admission that the document, act or item of 10 knowledge or any combination thereof was at the priority date, publicly available, known to the public, part of common general knowledge; or known to be relevant to an attempt to solve any problem with which this specification is concerned.

#### *Cane sugar refining*

After being mechanically harvested, sugar cane is transported to a mill and crushed 15 between serrated rollers. The crushed sugar cane is then pressed to extract the raw sugar juice, while the bagasse (leftover fibrous material) is used for fuel. The raw juice is then heated to its boiling point to extract any impurities, then lime and bleaching agents are added and mill mud is removed. The raw juice is further heated under vacuum to concentrate and increase Brix value. The concentrated syrup is seeded to produce bulk 20 sugar crystals and a thick syrup known as molasses. The two are separated by a centrifuge and the molasses waste stream is collected for use as a low-grade animal feedstock. A flow chart of this process appears below.



The sugar refining process thus generates a large number of products including raw juice, bagasse, mill mud, clarified juice, etc.

The bulk sugar crystals from the above process are further refined to produce many  
 5 commercially available sugar products. For example, the further refining may include  
 mixing the bulk sugar crystals with a hot concentrated syrup to soften the outer coating on  
 the crystals. The crystals are then recovered by centrifuge and subsequently dissolved in  
 hot water, a step that is sometimes called affination. This sugar liquor is then further  
 purified by carbonation or phosphatation, filtration, decolourisation and then seeded with  
 10 fine sugar crystals. Once the crystals have grown to the requisite size, the crystals are  
 separated from the syrup by centrifuge, then dried, graded and packaged. There may be  
 several repetitions of recovering sugar crystals from the sugar liquor. The dark sugar syrup  
 which is left after all of the sugar crystals have been recovered is also called molasses.

Almost all of the commercially manufactured sugar is white and granulated. White graded  
 15 sugar is 99.5% sucrose and is made up of crystals averaging 0.6 mm. Caster sugar has an

average crystal size of 0.3 mm. Icing sugar is produced by crushing white sugar in a special mill to produce a fine powder.

There are also a range of non-white sugar products including the following:

(a) Coffee sugar is a large grained, brown flavoursome crystal which is produced using  
5 the syrups left after extracting the white sugar crystals;

(b) Raw sugar is a straw-coloured granulated sugar produced from sucrose syrups  
which contain some residual colour and flavour from the sugar cane plant - it is  
specially selected and handled to ensure a hygienic product;

(c) Golden demerara sugar is a premium raw sugar produced from selected syrups  
10 which imparts a rich caramel taste to food; and

(d) Brown sugar is a flavoursome, fine-grained and moist crystal produced by further  
crystallization of the extracted dark coloured sucrose syrups produced in the  
separation stages of the refining process.

The syrup left after white sugar has been removed is used to make golden syrup and  
15 treacle. These syrups are made in a similar fashion with the difference being that golden  
syrup is decolourised whereas treacle is not.

#### *Cane Sugar Composition*

The composition of cane sugar products and waste streams are complex and quite variable  
- the chemical composition being principally determined by the geographical source of the  
20 sugar cane and the method of processing. For example, Figure 1 sets out the basic  
compositional elements for raw and brown sugar from the following 20 commercial  
sources:

- 1 to 4: Commercial Raw Sugar Brand 1
- 5 to 8: Commercial Brown Sugar Brand 1
- 25 • 9: Coffee Sugar Crystals Brand 1

- 10 and 11: Dark Brown Sugar Brand 1
- 12 to 15: Commercial Raw Sugar Brand 2
- 17: Commercial Raw Sugar Brand 3
- 18: Primary Mill Raw Sugar Sample 1
- 5 • 19: Primary Mill Raw Sugar Sample 2
- 20: Primary Mill Raw Sugar Sample 3

*Refining sugar products*

Molasses and other products of the sugar refining process, especially the thick syrups and juices, are complex mixtures of substances. Typically they are difficult to refine further 10 and there are often substances in the compositions that poison standard separating materials. Molasses and the other thick syrups and juices typically comprise polyphenols, polysaccharides, peptides and proteins, minerals, organic acids, and mono and disaccharides.

Molasses, golden syrup and treacle have been used as a health food since the early 20th 15 century and there have been claims that they are good therapies or cures for a wide range of disorders. However the strong taste make them unpalatable to many people and the high viscosity of treacle and molasses makes them difficult to handle and to incorporate into other foodstuffs. Dark sugars such as brown and light brown sugars can be produced by spraying back molasses onto white refined sugar. Due to the content of reducing sugars, 20 colorants and moisture these finished products clump on storage are hygroscopic which limits commercial and industrial use, and discolour many foods. A sugar with high amounts of molasses and sugar milling waste stream products to promote health with reduced hygroscopic properties, improved flow and lighter colour would be desirable.

The other products of cane sugar refining such as bagasse and mill mud are known to 25 include potentially useful substances but their hitherto intractable nature has meant that they are usually thrown away as waste. It would be desirable to have a method to incorporate these valuable waste stream extracts into finished products including, but not limited to sugar.

The inventors' earlier international patent application no WO 2005/117608 teaches one 30 method of extracting useful extracts from the in-process and waste stream products of

sugar manufacturing. The methods disclosed in that application produce an extract which is high in polyphenols and which is useful for use in methods such as that disclosed in international patent application no PCT/AU2006/000769. However, this extract does not always reflect the complexity of the contents of the raw material.

## 5 List of Abbreviations

BDL	Below Detectable Limit
CE	Catechin Equivalents
DW	Dry Weight
GAE	Gallic Acid Equivalents
GI	Glycaemic Index
IC or ICUMSA	International Commission for Uniform Methods of Sugar Analysis
MF	Microfiltration
N/D	Not Detected
N/T	Not Tested
XAD	Ion exchange separation. Whilst the specification refers to the use of the XAD brand of resins, the invention is not limited to this brand of resin and it is possible to use any brand of resin which can be used in ion exchange separations.

## Summary of the invention

The present invention provides extracts derived from sugar cane and a process for their production.

According to a first aspect of the invention, there is provided an extract derived from sugar cane having GI or burn rate reducing characteristics wherein the extract comprises a mixture of one or more polyphenols, one or more carbohydrates, one or more minerals and one or more organic acids.

The actual level of polyphenols in the extract of sugar cane will depend on the precise method used for its manufacture and the batch of sugar cane used as raw material. For example, an extract produced using XAD after the fractionation by molecular weight

would contain between 15 to 30% polyphenols by weight. In contrast, an extract produced using ultrafiltration and microfiltration (without any XAD) would contain between 1 to 3% by weight of polyphenols.

In one preferred embodiment, the polyphenols in the extract of cane sugar are selected  
5 from the group consisting of p-coumaric acid, ferulic acid, syringic acid, caffeic acid, chlorogenic acid, (-)epicatechin, apigenin, (+)catechin, quercetin, diosmin, rutin and mixtures thereof.

The extract of sugar cane comprises some carbohydrates which improves its taste whilst still maintaining its GI lowering characteristics. Typically, the extract comprises  
10 carbohydrates such as monosaccharides, disaccharides, oligosaccharides and both soluble and insoluble polysaccharides. The extract may also contain xylan derived mono, di, tri and oligosaccharides, such as xylobiose, xylotriose and xylose. The extract may include carbohydrates having GI increasing characteristics such as sucrose and glucose. However, the amount of GI increasing carbohydrates in the extract is not sufficient to detract  
15 significantly from the GI reducing characteristics of the extract as a whole. Further, the extract can comprise some carbohydrates and maintain its usefulness for applications such as body composition redistribution as disclosed in international patent application no PCT/AU2006/000769.

The extract of sugar cane comprises minerals including mineral complexes. Typically, the  
20 minerals are selected from magnesium, potassium, magnesium, calcium and mixtures thereof. Other minerals which may be present include anions such as phosphate, sulphate and chloride.

The extract of sugar cane comprises organic acids. Typically, the organic acids are selected from the group consisting of c-aconitic acid, citric acid, phosphoric acid, gluconic  
25 acid, malic acid, t-aconitic acid, succinic acid, lactic acid and mixtures thereof.

In one preferred embodiment, there is provided an extract derived from sugar cane having GI or burn rate reducing characteristics wherein the extract is a powder which comprises 1.5 to 2.5 wt% of one or more polyphenols, 50 to 80 wt% of one or more carbohydrates, 1 to 3 wt% of one or more minerals and 1 to 3 wt% of one or more organic acids.

30 In another preferred embodiment, there is provided an extract derived from sugar cane having GI or burn rate reducing characteristics wherein the extract is a syrup which comprises 3.5 to 6 g CE/l of one or more polyphenols, 80 to 220 g/l of one or more

carbohydrates, 3 to 5.5 g/l of one or more minerals and 3 to 6 g t-aconitic/l of one or more organic acids.

Typically the extract of the present invention further comprises additional components such as policosanols, phytosterols, lipids, phospholipids, protein, antioxidants, phytosterols 5 such as 1-octacosanol, campesterol, stigmasterol,  $\beta$ -sitosterol, oligosaccharides such as raffinose, 1-kestose, theanderose, 6-kestose, panose, neo-kestose and nystose, aliphatic alcohols, vitamins, gums and neutral and polar lipids, flavonoids (8 subgroups: Flavonols (eg quercetin, kaempferol, myricetin andisorhamnetin); Flavones (eg luteolin, tricin and apigenin); Flavanones (eg hesperetin, naringenin and eriodictyol); Flavan-3-ols (eg 10 catechin, gallicatechin, epicatechin, epigallocatechin, epicatechin 3-gallate, epigallocatechin 3-gallate and theaflavin); Anthocyanidins (eg cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin); Anthocyanosides; Curcuminoids; and Proanthocyanins) and their derivatives, including but not limited to, natural and synthetic conjugates such as glycosides, glucosides, galactosides, galacturonides, ethers, esters, 15 arabinosides, sulphates, phosphates; aldopentoses (xylose, arabinose) aldohexoses (mannose), ketopentoses, ketohexoses (fructose), kestoses, soluble gums, aliphatic alcohols (and complexes), waxes (and complexes), polysaccharides and fibre (soluble and insoluble), oligosaccharides, non-nitrogenous compounds, mineral complexes (organic iron and other minerals), phytochemical complexes (including but not limited to glucosides, 20 glycosides, glycosylates, esters, glucopyranosides etc), chlorophyll, phytosterols (and complexes), phytostanols (and complexes), hydrolysed celluloses and phospholipids. The term "policosanols", within the scope of the present invention, refers to the family of aliphatic alcohols and their derivatives, complexes or analogues which are found naturally in sugar cane. Examples include long chain fatty alcohols such as octacosanol, 25 triacontanol, dotriacontanol, tetracosanol, hexacosanol, tetratriacontanol, hexatriacontanol and docosanol.

In one preferred embodiment, the extract is a syrup derived from molasses and the extract has the following composition:

Component	Range	Preferred
Polyphenols (g CE/l)	3.5-6.0	4-5
Antioxidant (g GAE/l)	1.2-2.4	1.5-1.8
Organic Acids (g/l) (t-aconitic acid)	3-6	4-5
Total Minerals (g/l)	3-5.5	4-5
Carbohydrates (g/l)	80-220	120-170
Color (absorbance at 420 nm)	5-10	6-8
Brix (degrees)	15-27	20-24

The above table refers to a preferred extract as it exits the manufacturing equipment. This extract may be modified prior to its inclusion in commercial products. For example, the

5 levels will change as the extract is concentrated to form a syrup with a higher Brix or a powder. All such concentrated extracts are included within the scope of this invention.

The extract can be derived from any product derived from sugar cane including the sugar cane milling process, the sugar cane refining process to make sugar, and other processes using sugar cane products such as the manufacture of ethanol from molasses as part of the

10 manufacture of rum. The extract can be derived from the raw materials, in-process products, by-products, final products and waste streams. For example, the sugar cane derived product may be the feed stream of raw sugar cane juice, clarified juice and concentrated juice syrup, treacle, molasses (obtained from a primary mill or refinery), golden syrup, brown sugar, bagasse, biodunder, field trash, growing tips, pulp, cane

15 stripings, pith and mill mud. Preferably, the extract is derived from molasses.

In another embodiment, the extract may be a combination of extracts from different sugar cane products. For example, the desired phytochemical profile may be obtained by combining an extract of molasses with an extract of biodunder. All such combined extracts are within the scope of this invention.

The physical characteristics of the extracts of the present invention will depend on their overall chemical composition. Depending on the processing methods applied, the extracts may be concentrated by evaporation generating a syrup, or alternatively, the extract could be fully dried to produce a powder. This ability to prepare extracts having different 5 physical properties increases the commercial utility of the extracts. Depending on their physical characteristics and chemical composition the extracts will be suitable for various uses.

#### ***Method of Preparing the Extracts of the Present Invention***

In a preferred embodiment, the extract is produced using a method which comprises as one 10 of its steps a fractionation by molecular weight and size.

The fractionation by molecular weight maintains in the final extract the natural mixture of phytochemicals that exists in the sugar cane itself. Prior methods used separation processes which removed most of the carbohydrates, minerals and organic acids and the resultant extract was not representative of the natural balance that existed in the sugarcane.

15 The extracts of the present invention are derived from sugar cane product, preferably molasses from the cane sugar refining processes. The extract may be obtained from the sugar cane product by various methods, or combinations of methods, such as:

- solvent and counter-current extraction using non-aqueous or aqueous solvents;
- separation of components falling within a specific molecular weight range by size 20 exclusion processing methods such as gel permeation chromatography or ultrafiltration; and
- separation of the low and high molecular weight components using chromatographic techniques or combinations of techniques such as ion exchange chromatography, hydrophobic chromatography and ion exchange chromatography using fractional elution by stepwise increase in pH or with solvents such as ethanol.

The extracts may be further processed by standard techniques such as microfiltration, reverse osmosis, gel permeation, vacuum evaporation and freeze drying, spray drying and tunnel drying.

*Filtration method*

In a preferred embodiment of the present invention, the extract is prepared by a method comprising the steps of:

- (a) heating a solution of the cane sugar derived product,
- 5 (b) adjusting the solution pH to >7,
- (c) precipitating salts from the solution or matrix,
- (d) separating the precipitate from the solution or matrix, and
- (e) fractioning the solution or matrix by ultrafiltration or similar process to isolate desired extracts.

10 *Gel permeation method*

In a preferred embodiment of the present invention, the extract is prepared by a gel permeation method comprising the steps of:

- (a) heating a solution of the cane sugar derived product,
- (b) adjusting the solution pH to >7,
- 15 (c) precipitating salts from the solution or matrix,
- (d) separating the precipitate from the solution or matrix, and
- (e) passing the solution or matrix down a chromatographic column and collecting eluted fractions.

The preferred conditions for steps (a) to (d) are essentially the same as described in respect 20 of the filtration method. Typically step (e) would be carried out at a temperature of 15 to 25°C, preferably 20 to 25°C.

*Uses of the extract*

The extracts of the present invention represent new products which are economically useful and can be used in a wide variety of applications. The extracts as described herein 25 may be used in a therapeutic capacity in order to treat and/or prevent the many conditions which are considered to be responsive to antioxidants, including but not limited to, cardiovascular disease, atherosclerosis, hypertension, thrombosis, Type II diabetes, obesity, dementia, cancer, HIV aids, degenerative conditions associated with aging, diseases caused by oxidative damage, and changing body composition as described in

international patent application no PCT/AU2006/000769. It is expected that these natural extracts will be better absorbed and may show improved efficacy compared to conventional sources of the components. In populations, which are considered "high-risk" for CVD or any of the oxidation related disorders, it is contemplated that the compositions and foodstuffs to which the extracts are added could be used in primary, secondary and tertiary treatment programs.

The doses of the extracts will vary depending upon, among other factors, the mode of delivery (ie how and into which food, beverage, nutraceutical, cosmaceutical or pharmaceutical the derivatives are ultimately incorporated), the patient size and condition, the result to be achieved, as well as other factors known to those skilled in the art of food additives and medicinal agents. It will also be recognized that the provision of much larger daily doses of the derivatives are not harmful to the animal host, as excess will simply pass through normal excretory channels.

The dose may depend, for example, on the desired reduction in GI. For example, to reduce GI in sugar, the total polyphenol content needs to range from 25-30 mg/100g sugar. The extract according to the invention can be used to prepare the low GI products described in international patent application no WO 2005/117608, including those where the extract is used in conjunction with other GI lowering compounds such as galactose, arabinose, kestose, oligofructose, or cellulose.

The extracts of the present invention may be incorporated directly and without further modification into a food, nutraceutical, cosmaceutical or beverage by techniques such as mixing, infusion, injection, blending, dispersing, emulsifying, conching, extrusion, immersion, spraying, coating and kneading. The extract can be used to impregnate commonly used food ingredients such as fibre (chicory, sugar cane bagasse and other food grade fibres) and flours prepared from a range of cereal sources. Alternatively, these extracts may be applied directly onto a food or into a beverage by the consumer prior to ingestion. These are simple and economical modes of delivery.

The extracts of the present invention may also be incorporated into food products using the delivery systems described in international patent application no PCT/AU2006/000761.

30 *Pharmaceutical and Cosmaceutical Dosage Forms:*

It is contemplated within the scope of the present invention that the extracts of the present invention may be incorporated into various conventional pharmaceutical and

cosmaceutical preparations and dosage forms such as tablets (plain and coated) for use orally, buccally or sublingually, capsules (hard and soft, gelatin, with or without additional coatings), powders, granules (including effervescent granules), pellets, microparticulates, solutions (such as micellar, syrups, elixirs and drops), lozenges, pastilles, ampoules,

5 emulsions, microemulsions, ointments, creams, suppositories, gels, and transdermal patches, other transdermal delivery methods involving active and passive transport, depot preparations, enteral solutions, parenteral preparations, intravenous solutions, modified release dosage forms together with customary excipients and/or diluents and stabilizers.

10 The extracts may also be impregnated, mixed, emulsified, sprayed or coated onto carriers such as cellulose, methycellulose, dextrose, cyclodextrose, cyclodextrin, maltitol, fibre and fibre containing bioactives to improve delivery. Delivery may also be enhanced with a range of surfactants, lipids, complexes, solvents and co-solvent pharmaceutical delivery systems known in the pharmaceutical art to improve bioavailability, absorption and efficacy.

15 *Foods/Beverages/Nutraceuticals:*

As used herein, the term "food" or "food product" includes any edible product, such as but not limited to confectioneries, supplements, snacks (sweet and savoury), cocoa-containing foods, flavours, beverages, dietary supplements and formulations including supplements used in animal health and nutrition. Additional ingredients desired in the resulting food 20 product may be added at any point in the process. In one embodiment of the invention, the extracts are in the form of syrups that can be used as substitutes for regular glucose and high fructose corn syrups from wheat, corn, agave, stevia etc., as a lower Glycemic Index (GI) option.

25 The extracts of the present invention may be incorporated into foods, beverages and nutraceuticals, including, without limitation, the following :

- Dairy Products--such as cheeses, butter, milk and other dairy beverages, spreads and dairy mixes, ice cream and yoghurt;
- Fat-Based Products--such as margarines, spreads, mayonnaise, shortenings, cooking and frying oils and dressings;
- 30 • Cereal-Based Products--comprising grains (for example, bread and pastas) whether these goods are cooked, baked or otherwise processed;

- Confectioneries--such as chocolate, candies, chewing gum, desserts, non-dairy toppings, sorbets, icings and other fillings;
- Sports nutrition products including powders, pre-mixes, juices, energy bars, isotonic drinks and gelatine, starch based or pectin jellies;
- 5     • Beverages--whether hot or cold (coffee, tea, cocoa, cereal, chicory and other plant extract based beverages), alcoholic or non-alcoholic and including colas and other soft drinks, juice drinks, dietary supplement, instant pre-mixes and meal replacement drinks.
- Miscellaneous Products--including eggs and egg products, processed foods such as
- 10    • soups, pre-prepared pastas.

Similarly, food grade ingredients such as soluble fiber (e.g. oligofructosaccharide), insoluble fiber (e.g. sugar cane fiber, oatbran), flour, starch, modified starch, gelatine, or other food, pharmaceutical or cosmetic ingredients impregnated with or containing the extract according to the invention, can produce a unique food ingredient with enhanced 15 levels of polyphenols, policosanols, phytosterols and other phytochemicals derived from sugarcane. Delivery may also be enhanced with a range of surfactants, lipids, emulsifiers, complexes, solvents and co-solvent food and cosmetic delivery systems known in the art to improve dispersion, absorption and efficacy.

The extract according to the invention is particularly useful for increasing the active 20 phytochemical content of coffee products without increasing bitterness. Roasting coffee beans causes the development of the myriad desired flavours of coffee. The roasting also causes the bitter taste of coffee which is related to an increase in the level of the antioxidants chlorogenic acid lactones and phenylindanes. The more that coffee beans are roasted, the more chlorogenic lactones and phenylindanes are produced. The extract of the 25 invention can be used to increase the level of active phytochemicals (such as polyphenols) in coffee products making the coffee products useful for applications such as those disclosed in international patent application no PCT/AU2006/00076.

The present invention includes food products comprising an extract according to the invention alone as the active ingredient or in combination with other active ingredients.

*Low GI products*

In a particularly preferred application the extracts of the present invention may be utilized in a formulation strategy directed to the reduction of GI. The extract of the invention can be used to prepare low GI products, such as those disclosed in international patent 5 application no WO2005/117608, but which have a higher antioxidant content and a better taste than the extracts exemplified in that specification. The extracts according to the present invention in the form of a liquid extract can be sprayed onto standard sugar (whether derived from sugar cane or sugar beets) to produce a low GI sugar. The extracts according to the present invention in the form of a liquid extract can also be sprayed onto 10 other carriers such as flour, starch or fibre thus increasing the levels of bioactives in these food ingredients. The extract used for this application preferably has sufficient sucrose content to assist the extract to adhere to the standard sugar crystals and to minimise 15 impairing the taste of the sugar.

For a low GI product, it is preferable to have low glucose levels. The glucose content of 15 the molasses or other products generated in the sugar processing stream can be reduced using enzymes such as glucose oxidase (GO) which digest glucose. It will be known by those skilled in the art that a combination of glucose oxidase and catalase are typically used to ensure that any hydrogen peroxide is removed, and that the oxygen generated is used by the GO to reduce glucose levels. The method of the invention may also 20 incorporate any other method to reduce glucose in the molasses and other products generated in the processing stream which is then reincorporated in the manufacturing process to reduce the GI of the sugar product or used alone as a food, dietary supplement or pharmaceutical. This may include, but is not limited to, fermentation, or encouragement of glucose digestion through other chemical, and/or thermal reactions prior to, during or 25 after the ultrafiltration and ion exchange processes. Affination syrup and other sugar refinery process or waste streams containing low amounts of reducing sugars are particularly useful sources as feedstocks to produce low GI products. By-products of fermentation and distillation are also useful feedstocks.

The method of the invention may also be used to prepare product for use in the methods 30 and products disclosed in international patent application no WO 2005/117608 or international patent application no PCT/AU2006/00076.

***Refining process***

According to a second aspect of the invention, there is provided a process for producing an extract of sugar cane comprising the steps of:

(a) heating and diluting a sugar cane derived product until the viscosity of the  
5 resulting solution or matrix is less than or equal to about 100 centipoise at a temperature in the range of from 40 to 60°C;

(b)(i) centrifuging the product of step (a);

or alternatively,

(b)(ii) adjusting the pH of the product of step (a) with a base;

10 (c) heating the product of step (b) to a temperature in the range of from 70 to 80°C and then maintaining it in that temperature range for a period of time until a precipitate of insoluble calcium and magnesium salts forms;

(d) removing the precipitate and large particulate matter from the product of step (c);

15 (e) treating the product of step (d) with a fractionation by molecular weight and size to isolate desired extracts.

Preferably, the temperature used in step (a) is about 50°C. Preferably, the viscosity achieved is in the range from 50 to 100 centipoise. Typically, water is used for the dilution in step (a). When the sugar cane derived product is molasses, a ratio of 1:0.8 to 1.2 molasses to water is typically used to provide the desired ratio. The preferred viscosity can 20 also be adjusted by measuring the Brix, in which preferably the range is from 30 to 50 Brix, and more preferably from 35 to 45 Brix.

Preferably, when the pH is adjusted as in step (b)(i), the final pH is in the range of from 7.2 to 9.5. The pH adjustment can be made using any suitable base, such as sodium hydroxide, sodium carbonate ( $Na_2CO_3$ ), potassium carbonate ( $K_2CO_3$ ), bubbling with gaseous carbon 25 dioxide ( $CO_2$ ), or a combination of all the above. Preferably, the pH adjustment occurs using a sodium hydroxide solution.

Preferably, when the diluent is centrifuged, this is carried out using a continuous desludging centrifuge with the supernatant being directly processed through a ceramic or stainless steel membrane (typical 0.10 micron to 1.5 micron, preferably 0.1 to 0.5 micron)

using a pressure of about 4 Bar, a flow rate of approximately 30 to 100 l/hour and at a temperature of 30 to 60°C, preferably 35 to 50°C.

Preferably, the temperature in step (c) is about 75°C. Preferably, the period of time is in the range of from 10 to 30 minutes, more preferably about 20 minutes.

5 Where necessary for the equipment used in step (d), the mixture from step (c) is cooled prior to step (d).

Step (d) can be achieved using any method known to a person skilled in the art for removing a precipitate. Typically it is carried out using a desludging centrifuge or other filtration methods including diatomaceous earth or fine muslin or similar material. For 10 example, the mixture may be passed through a ceramic or stainless steel membrane (0.10 micron to 1.5 micron, preferably 0.1 to 0.5 micron) using a pressure of 4 Bar, a flow rate of 30 to 100 l/hour and at a temperature of 30 to 60°C, preferably 35 to 50°C. The retentate (typically the precipitate from step (d) and large particulate matter) is discarded (typically after washing with water having a pH in the range of from 7.2 to 7.5) and the permeate 15 collected. In some applications, the retentate is not washed but is completely discarded. Alternatively, high speed continuous centrifugation could be used to remove the precipitate and large particulate matter.

In one preferred embodiment, step (e) is undertaken using one or more fractionation filters or membranes selected from the group consisting of ultrafiltration, nanofiltration and 20 mixtures thereof, to isolate desired extracts. The fractionation filters or membranes are known to persons skilled in the art of food processing. For example, the product from step (d) can be treated by passing it through a combination of spirally wound ultrafiltration (UF) membranes with a size exclusion in the range of from 100 kDa to 1 kDa. Preferably from 30 kDa to 50 kDa and/or 1 kDa. Further fractionation can then be achieved with a 0.5 kDa 25 nanofiltration membrane. A person skilled in the art will know that the choice of membrane used will depend on the desired final product, flow rate, pH, pressure, temperature and efficiency across a range of conditions. Typically, step (e) will include diafiltration and both the retentate and permeate will be collected. Typically, step (e) will occur at a temperature in the range of from 30 to 60°C, more typically 35 to 50°C.

30 In another embodiment of the invention, step (e) is undertaken using gel permeation.

In one embodiment of the invention, step (e) is followed by further refining steps which may include ion exchange, hydrophobic chromatography with further ultrafiltration or nanofiltration, gel permeation or reverse osmosis.

The sugar cane derived product used as starting material for this process can be any

5 product derived from sugar cane including products from the sugar cane milling process, the sugar cane refining process to make sugar, and other processes using sugar cane products such as the manufacture of ethanol from molasses as part of the manufacture of rum. The sugar cane derived product can be the raw materials, in-process products, by-products, final products and waste streams. For example, the sugar cane derived product  
10 includes the feed stream of raw sugar cane juice, clarified syrup and concentrated syrup, treacle, molasses (primary mill and refinery), golden syrup, brown sugar, bagasse, biodunder, field trash, cane stripings, pith, growing tips, pulp and mill mud. Preferably, the sugar cane derived product is molasses.

The use of affination syrup, molasses or other process and refining waste products as the

15 starting material, provides a retentate after the use of a 30 kDa membrane that contains large polyphenols, polysaccharides, peptides and proteins, and the permeate will contain smaller polyphenols, minerals, organic acids, and mono, di and polysaccharides. The process of the invention enables the production of a molasses extract containing up to about 90% of the antioxidants contained from the original molasses. The permeate may  
20 then be further treated using ion exchange or hydrophobic resin or further ultrafiltration to produce an extract containing the desired profile of polyphenols, minerals, organic acids and carbohydrates. By using the process of the invention, it is possible to minimise any detrimental effects the resin as the substances which would poison the resin have been substantially reduced.

25 The fractionation filters or membranes are able to separate the material collected in step (e) into a range of functional sugar syrups containing a range of polyphenols as previously specified and different ratios of sucrose, glucose and fructose in combination with a mixture of minerals (eg magnesium and potassium) in ratios similar to that in natural sugar cane juice depending upon the initial feedstock.

30 The syrups collected using the method of the invention can be used as functional sweeteners in beverages, ice creams and iced confections and in confectionery products including but not limited to fondants, fondant cream centres for chocolate products,

truffles, caramels, fudges, gelatin, starch and pectin based gums and jellies and high boiled sweets and toffees. These syrups can also be used in any food application where wheat or corn based glucose syrups or high maltose glucose syrups are used. The fractionation process can be manipulated so that the ratio of monosaccharides to sucrose is sufficient to 5 inhibit sucrose crystallisation in such applications. The syrups while functioning as both sweeteners and crystallisation inhibitors may also deliver additional natural mineral and antioxidant properties to the products.

In one embodiment of the invention, once the fractions are recovered from the method of the invention, they can be further concentrated by a combination of nanofiltration which 10 preferentially removes monovalent anions, reverse osmosis and/or traditional high vacuum evaporation processes. Final solids content of the syrups can range from 65% to 80% w/v with water activities in the range of from 0.2 to 0.3. This further concentration can result in syrups having a shelf life of up to 6 months when stored under suitable conditions such as between 5 and 25°C.

15 The method of the invention may also be used to prepare product for use in the methods and products disclosed in international patent application no WO 2005/117608 or international patent application no PCT/AU2006/00076. International patent application no WO 2005/117608 discloses a method for preparing a low GI product using ultrafiltration with a 300 kDa membrane and solvent extraction, however this process only 20 extracts about 50% of the antioxidants present in the sugar cane product. The method according to the second aspect of the present invention recovers a higher percentage of the antioxidants present in the sugar cane product.

The method according to the invention may be used to refine phytochemical extracts obtained from other sources.

25 According to a third aspect of the invention, there is provided a method for refining a phytochemical extract comprising the steps of:

(a) heating and diluting a phytochemical containing extract until the viscosity of the resulting solution or matrix is less than or equal to about 100 centipoise at a temperature in the range of from 40 to 60°C;

30 (b)(i) centrifuging the product of step (a);

or alternatively,

(b)(ii) adjusting the pH of the product of step (a) with a base;

(c) heating the product of step (b) to a temperature in the range of from 70 to 80°C and then maintaining it in that temperature range for a period of time until a precipitate of insoluble calcium and magnesium salts forms;

5 (d) removing the precipitate and large particulate matter from the product of step (c);

(e) treating the product of step (d) with a fractionation by molecular weight and size to isolate desired extracts.

The phytochemical extract may be sourced from a variety of plant sources from which it is known that phytochemicals such as polyphenols may be extracted. Typical sources 10 include, but are not limited to, cocoa beans, tea waste, pod husks, coffee beans, coffee waste, grape pomace, cereals (eg barley, buckwheat, corn, millets, oats, rice, rye, sorghum, wheat), legumes (eg beans and pulses), nuts (eg almonds, betel nuts, cashew nuts, hazelnuts, peanuts, pecans, walnuts), oilseeds (eg rapeseed, canola, soybeans, borage, cottonseed, evening primrose, flaxseed, sesame seeds, sunflowers, olive oil, palm oil, rice 15 bran oil), fruits (eg berries, drupes, pomes, tropical fruits), vegetables (eg carrots, onions, parsnips, potatoes, beetroot, sweet potato, asparagus, celery, endive, lettuce, spinach, avocado, tomato, pepper), beverages (eg tea, coffee, cocoa, beer, wine, cider) and herbal products (eg Echinacea, ginseng, ginkgo biloba, St John's Wort, valerian, kava kava, saw palmetto, black cohosh, Devil's Claw, goldenseal, hawthorn, ginger, liquorice, milk 20 thistle).

These bioactive rich extracts can be used to enhance the level of bioactives in a range of food products such as fruit juices, and dried and processed fruits. One example of a delivery system which can be used with these extracts is disclosed in international patent application no PCT/AU2006/000761.

25 **Brief description of the figures**

Figure 1 shows the composition of 20 commercial raw and brown sugars.

Figure 2 shows the analysis of the extract obtained after membrane filtration of molasses, Group 1 from Example 4.

Figure 3 shows the analysis of the extract obtained after membrane filtration of molasses, 30 Group 1 from Example 4.

Figure 4 shows the analysis of the extract obtained after membrane filtration of molasses, Group 2 from Example 4.

Figure 5 shows the analysis of the extract obtained after membrane filtration of molasses, Group 2 from Example 4.

5 Figure 6 shows the gel filtration of molasses on Bio-Gel P-2 showing A420 and antioxidant activity for Run 3 from Example 6.

Figure 7 shows the gel filtration of molasses on Bio-Gel P-2 showing A420 and total phenolics for Run 3 from Example 6.

10 Figure 8 shows the gel filtration of molasses on Bio-Gel P-2 showing A420 and sucrose for Run 3 from Example 6.

Figure 9 shows the gel filtration of molasses on Bio-Gel P-2 showing A420 and glucose + fructose for Run 3 from Example 6.

Figure 10 is a photograph of the fractionation of molasses obtained on Bio-Gel P-2 (Pools 1 – 5 from Example 6).

15 Figure 11 shows the gel filtration of molasses on Bio-Gel P-2 showing A420 profiles using formate/acetonitrile buffer pH 5.0 and Tris HCl buffer pH 7.5 from Example 6.

Figure 12 shows the comparison of the UV traces of the extracts from the ethyl acetate extraction (acidic fraction) (black trace) and the SPE extraction (60% MEOH) (red trace) of the XAD-bound sample at 280 nm (a), 320 nm (b) and 370 nm (c) from Example 7.

20 Figure 13 shows the concentrations of selected compounds in the three samples investigated as determined by RP-HPLC with UV detection. Results were obtained by expressing against a *p*-coumaric acid external calibration curve. Individual response factors were not calculated from Example 7.

Figure 14 is a summary of the information produced by the LC-MS experiments for 25 selected compounds from Example 7.

Figure 15 shows the SRM and product-specific MS/MS ion chromatograms showing tricin and tricin-containing compounds detected in the XAD-acid extract from Example 7.

Figure 16 shows the product-specific MS/MS ion chromatogram (parent m/z 299) showing several diosmetin-containing compounds in the XAD-acid extract from Example 7.

30 Figure 17 shows the composition of sugar processing streams Batch 1 from Example 9.

Figure 18 shows the composition of sugar processing streams Batch 2 from Example 9.

Figure 19 shows the composition of buffer tank streams in different batches from Example 9.

Figure 20 shows the composition of mill mud streams in different batches from Example 9.

5 Figure 21 shows the composition of mill mud filtrates in different batches from Example 9.

Figure 22 shows the composition of mill mud extracts in different batches from Example 9.

Figure 23 shows the composition of molasses streams in different batches from Example 9.

Figure 24 shows the composition of raw sugars in different batches from Example 9.

10 Figure 25 shows the composition of fractions of molasses by membrane ultrafiltration from Example 9.

Figure 26 shows the composition of mill mud extracts in different batches from Example 13.

## Examples

Various embodiments/aspects of the invention will now be described with reference to the 15 following non-limiting examples.

### Example 1

In this example, molasses was treated using the method according to the second aspect of the invention.

#### *Method*

20 • Step 1: Molasses was diluted with water in a 1:1 ratio and adjusted to a pH of 7.2 with 5% NaOH solution, and heated to 75°C. The mixture was allowed to stand for 30 minutes at 75°C until a precipitate of Ca and Mg salts formed.

• Step 2: The mixture from step (1) was passed through ceramic or stainless steel membrane with a pore size between 0.1 to 0.5 micron at a temperature between 35 to 50°C and the retentate of insoluble Ca and Mg salts and large particulate matter from the molasses was discarded.

25 • Step 3: The permeate from step (2) was passed through a 30 kDa ultrafiltration membrane at a temperature between 35 to 50°C, and the retentate was diafiltered, and both the retentate (R1) and the permeate (P1) were collected. The retentate

(R1) contained large polyphenols, polysaccharides, peptides and proteins etc and the permeate (P1) contained smaller polyphenols, minerals, organic acids, and mono and disaccharides. The retentate (R1) was placed to one side for further work later.

5     • Step 4: The permeate P1 was passed through a hydrophobic resin and all unbound material was washed off with water. The eluant (E1) was collected and found to contain sugars, organic acids, polyphenols and minerals.

10    • Step 5: The bound material (hydrophobic) was desorbed with ethanol (70% v/v). The recovered desorbed eluant (E2) was found to contain polyphenols, some minerals and minor constituents etc. The ethanol was removed by distillation and condensation and the remaining aqueous concentrate was freeze dried to produce a polyphenol powder (PP1).

15    • Step 6: In a further embodiment, eluant E2 was then passed through a 0.5 kDa ultrafiltration membrane and the retentate (R2) was diafiltered. The recovered product contained polyphenols and was concentrated by freeze drying to generate a powder (PP2).

20    • Step 7: The eluant E1 was passed through a 0.5 kDa ultrafiltration membrane, diafiltered and the retentate R3 collected. The retentate R3 was then concentrated by evaporation generating a polyphenols rich liquid PL1 (which could be used for spraying onto sugar to enrich the antioxidant content and manufacture a low GI sugar according to WO 2005/117608), or alternatively fully dried to produce a powder enriched in polyphenols PP2. The 0.5 kDa permeate P2 was collected and concentrated to recover a sugar syrup SY1 enriched in polyphenols, antioxidants and minerals.

25    • Step 8: In another embodiment the material passing through the initial ceramic or stainless steel membrane was treated on a 0.5 kDa membrane producing a retentate that was diafiltered to produce R3. This retentate was found to contain between 80 to 90% of the original polyphenols and antioxidant from the molasses and 60 to 90% of the sucrose but significantly depleted in fructose, glucose and monovalent cations. This retentate was concentrated by evaporation and also could be used for spraying onto sugar to enrich the antioxidant content and manufacture a low GI sugar according to WO 2005/117608, or alternatively fully dried to produce a

powder enriched in polyphenols PP2. The permeate from this process had a composition very similar to that shown for SY1.

*Results*

Table 1: Composition of Syrup from 30 kDa membrane permeate (P1)

Component	Concentration
Sucrose	420 – 480 g/litre
Glucose	100 – 140 g/litre
Fructose	100 – 140 g/litre
Polyphenols	12 – 16 g/CE/litre
Antioxidants	4 – 5.5 g/GAE/litre
Calcium	2864 mg/l
Magnesium	1510 mg/l
Potassium	12895 mg/l
Sodium	194 mg/l

5

Typically the monosaccharides, glucose and fructose constitute between 25 and 35% of the total sugars of the 30 kDa recovered fractions and exist in a ratio of 68:16:16 (average) for sucrose, glucose and fructose. This ratio is similar to that which occurs in the original molasses but is significantly reduced in colour (about 27 to 30%). The levels of calcium 10 and magnesium are also significantly reduced to that of the original molasses. Only 40-50% of the original molasses polyphenols are recovered in this syrup but they are in a different range and profile (being smaller) than those found in the original molasses.

Table 2: Composition of Syrup form 0.5 kDa retentate (PL1)

Component	Concentration
Sucrose	480 – 520 g/litre
Glucose	80 – 120 g/litre
Fructose	80 – 120 g/litre
Polyphenols	16 – 25 g/CE/litre
Antioxidants	5 – 9 g/GAE/litre
Calcium	2003 mg/l
Magnesium	1118 mg/l
Potassium	8002 mg/l
Sodium	114 mg/l

The monosaccharides, glucose and fructose represent between 20 and 30% of the total sugars in the 0.5 kDa retentate PL1.

Table 3: Composition of Syrup from 0.5 kDa permeate (SY1)

Component	Concentration
Sucrose	80 – 120 g/litre
Glucose	280 – 320 g/litre
Fructose	280 – 320 g/litre
Polyphenols	12 – 15 g/CE/litre
Antioxidants	3.5 – 7 g/GAE/litre
Calcium	560 mg/litre
Magnesium	333 mg/litre
Potassium	6526 mg/litre
Sodium	98 mg/litre

5 The monosaccharides, glucose and fructose represent between 80 and 88% of the total sugars in SY1. It is evident from these results, that a high glucose and fructose sugar syrup can be extracted from the molasses. The method of the invention thus enables the production of a valuable sugar syrup which can be used in food products and which obtains further value from a waste product.

10 In an alternative process steps 4 and 5 were omitted, and the 30 kDa permeate (P1) was treated as per step 6 above.

### *Conclusion*

This example clearly demonstrates that a difficult feedstock, such as molasses, can be fractionated by membrane filtration and ion exchange chromatography in order to produce fractions that are both rich in polyphenols and antioxidants in association with varying levels of mono and disaccharides and mono and divalent cations.

By the combination of membrane filtration using 30 kDa and 0.5 kDa membranes, crude molasses from cane or beet can be fractionated into sugar syrups differing in the composition of sucrose, glucose, fructose, minerals, mono and divalent cations and anions such as Cl, SO<sub>4</sub>, PO<sub>4</sub>, organic acids (eg: cis and trans aconitic, citric and malic acids), and polyphenols (phenolic acids, flavonoids, anthocyanins, anthocyanidins etc).

These syrups can be used individually or blended to produce a range of functional sweeteners rich in minerals and polyphenols (antioxidant activity) for use in a wide range of food systems. Using membrane filtration of molasses, products with reduced mineral content free of large particulate matter can be used as feedstock for further fractionation on resins typically used for ion exchange and hydrophobic chromatography. Such feedstock improve the performance (less poisoning and longer usage life) of these resins. Thus further refined and differentiated sweetener syrups and high polyphenol powders can be produced which have wide application in food systems and for use as nutriceuticals.

### **Example 2**

As an example of how the syrups can be blended to produce a different functional sweetener syrup (mono and disaccharides, mono and divalent cations), four parts of the permeate from the 30 kDa filtration and one part of the permeate from 0.5 kDa filtration from Example 1 were mixed to produce a sweetener with a sucrose, glucose, fructose ratio of 5:2:2 compared to the original 30 kDa permeate (ratio 68:16:16) and original 0.5 kDa permeate 1:3:3. Such mixtures will also produce different levels of polyphenols, antioxidants and minerals and will vary in their colour intensity as measured by the absorbance at 420 nm.

### **Example 3**

The 0.5 kDa retentate from Example 1 contained between 80 to 90% of the original polyphenols and antioxidant activity found in the molasses feedstock. After this retentate was concentrated to a polyphenol content of between 20 and 50 g of CE (catechin equivalents) /l, it was sprayed onto the surface of sugar crystals to produce a low GI sugar.

**Example 4**

The process according to the second aspect of the present invention was applied to molasses to illustrate the fractionation capabilities of different membranes.

*Methods*

5 The membrane filtration plant (approximate dimensions 1200 mm x 1200 mm x 1600 mm high) was supplied by GEA Liquid Technologies Australia. The unit was equipped with a stainless steel membrane (pore size 0.1  $\mu\text{m}$ ) and a housing to accommodate spiral-wound membranes (970 mm x 98 mm dia.). The spiral-wound membranes (Syndner 3838) had pore sizes of 0.5, 1.0 and 30 kDa. A spiral-wound reverse osmosis membrane (Dow-  
10 Filmtech 3838) was also used.

15 *Deionised water supply:* Deionised water was provided by an ion exchange system hired from IBC Water and transported from Brisbane. The tri-bed system consisted of a pre-filter and three ion exchange cylinders connected in series: cation, anion and mixed cation/anion exchangers. The cylinders were connected to the mains water supply and deionised water flowed directly to the MF plant.

20 *Molasses:* Hot molasses (70-80°C) was obtained directly from a production tank and was mixed with an equal volume of deionised water. This diluted molasses (80 l) was adjusted to pH 7.5 with 5 M NaOH (900 ml) and heated at 80°C for 20 min in aluminium cans. The MF plant was preheated to 80°C with water which included an added volume of about 15 l and a hold-up volume of about 25 l. The pH-adjusted molasses was then circulated through the plant at 75-80°C before pre-filtration through a 0.1  $\mu\text{m}$  stainless steel membrane. Based on an estimated density of 1.3 g/ml for hot molasses and 40 l water in the plant, the estimated concentration of molasses applied to the pre-filter was about 43% (w/v).

25 Group 1 trials

*Prefilter 0.1  $\mu\text{m}$ :* Diluted molasses (approx 43% (w/v)) was pre-filtered through a 0.1  $\mu\text{m}$  stainless steel membrane at 70°C using a feed pressure of 1 Bar and a recirculation pressure of 3 Bar.

30 *Nanofiltration 0.5 kDa:* The permeate from the 0.1  $\mu\text{m}$  pre-filter was applied to a spiral-wound membrane with a pore size of 0.5 kDa. The filtration was carried out at 40°C using a feed pressure of 20 Bar and a recirculation pressure of 21 Bar. The retentate from this

run was diafiltered with 60 l of deionised water and stored frozen for possible use in coating raw sugar.

*Reverse osmosis:* The nanofiltration permeate was passed through a RO membrane at

40°C using a feed pressure of 25 Bar and a recirculation pressure of 25.5 Bar. The

5 retentate was stored frozen for use in confectionery trials.

#### Group 2 trials

The trials from Group 1 were repeated with an additional membrane step using a 30 kDa

membrane after prefiltration with the 0.1 µm stainless steel membrane. The run with the

30 kDa membrane was carried out 45°C using a feed pressure of 4 Bar and a recirculation

10 pressure of 5.5 Bar. Subsequent steps were as for Group 1. Laboratory samples (100 ml)

were collected were collected from the feedstock, permeates (final and composite) and

retentates. Additional bulk samples were as follows:

#### *List of Bulk Samples*

Group	Bulk sample	Sample volume (l)
1	0.5 kDa permeate (P2)	4
1	0.5 kDa diafiltered retentate	1, 20
1	RO retentate	10
2	30 kDa permeate (P2)	4
2	0.5 kDa diafiltered retentate	1, 15
2	RO retentate	1, 15

15 Samples coded P1 and R1 represented permeate and retentate samples taken at the end of each run. Code P2 represented composite permeate before diafiltration; PW was the composite permeate collected after diafiltration and PWC was the combination of P2 and PW (Figures 2 to 5).

In the figures:

20

- Number before / is membrane cut-off (kDa); /0 is feedstock for that membrane size
- P = permeate; R = retentate; W = post water wash; PWC = composite of total permeate and diafiltration permeate.

- For 0.1 kDa stainless steel membrane, additional volume (+) includes approximately 15 l added water for preheating, and approximately 25 l hold-up volume in plant.
- For non- 0.1 kDa stainless steel membranes, additional volume (+) is approximately 5 l hold-up volume in plant.

*Analyses:* Samples were analysed for the following components: total phenolics, reversed-phase HPLC profiles, antioxidants, mono- and di-saccharides, *cis*- and *trans*-aconitic acids, cations/anions, total solids, ash, conductivity, pH, colour (A<sub>420</sub>) and °Brix. Six samples were analysed for polysaccharides.

#### 10 Components analysed and method of analysis

Component	Method
Total phenolics	Folin-Ciocalteu colorimetric procedure. Results expressed as g catechin equivalents per l.
Polyphenol profile	RP-HPLC, C18 column, (Luna 3 µm, Phenomenex) at 30°C, linear gradient 3-21% acetonitrile for 12 min, 21-60% for 1 min and 21-60% for 3 min. Detection at 214 nm.
Antioxidant activity	ABTS substrate. Results expressed as g gallic acid equivalents per l.
Mono- and di-saccharides	RP-HPLC, NH <sub>2</sub> column at 40°C, isocratic elution with 88% acetonitrile at 1 ml/min for 20 min. R I detection.
<i>cis</i> - and <i>trans</i> -Aconitic acids	Ion-moderated partition HPLC, Aminex column HPX-87H (Bio-Rad). Isocratic elution with 0.004 M H <sub>2</sub> SO <sub>4</sub> at 0.6 ml/min for 40 min at 30°C. UV detection.
Total solids	Vacuum oven at 70°C for 16 h.
Ash	Oven dry 100°C and furnace 550°C 16 h
Conductivity	TPS conductivity meter (model 2102A) fitted with pipette cell. Results expressed as molar NaCl.
Colour (A <sub>420</sub> )	Absorbance at 420 nm on samples diluted in deionised water.
°Brix	ABBE refractometer at 20°C.
Polysaccharides	Measurement of soluble polysaccharides.

Component	Method
	Precipitation of polysaccharides with 100% ethanol, wash with 80% ethanol, digestion of precipitate in 1% sulphuric acid and measurement total reducing sugars by phenol-sulphuric acid at 485 nm with glucose as reference. Results expressed as g polysaccharide per l.
Na, K, Ca, Mg, PO <sub>4</sub> , SO <sub>4</sub>	Inductively coupled plasma – optical emission spectroscopy (ICP-OES) on Varian Vista Pro instrument at UQ.
Cl	Automated colorimetric analyser (Seal AQ2), EPA Method (EPA-124-A)

*Filtration parameters:* Flow rates, temperatures and pressures were recorded for each membrane. Rejection coefficients (r) for each analyte were calculated as follows:

$$r = 1 - C_p/C_R$$

5 where C<sub>P</sub> = concentration of analyte in permeate; C<sub>R</sub> = concentration of analyte in retentate at the end of each run.

Percentage permeation data are calculated as C<sub>P</sub>/concentration of initial feedstock.

### Results

#### Group 1

10 *Plant operation:* The flow rate for the stainless steel membrane was very slow (maximum 21 l/h) even though the operating temperature was at 70°C. It was discovered at the end of Group 1 that the pressure gauges were reading approximately 0.5 Bar too low, resulting in lower flow rates than could have been achieved in the first Group. Pressure levels were adjusted in Group 2. For the 0.5 kDa membrane, flow rates were reasonably high (>40 l/h) 15 for the first 15 min but fell to 20-30 l/h for the remainder of the run. High flow rates (>230 l/h) were achieved with the reverse osmosis membrane.

20 *Composition:* Analytical results for membrane filtration streams from Group 1 are shown in Figures 2 and 3. The original diluted molasses (50% (v/v)) was not sampled. Based on previous data (eg. phenolics, total solids), the recirculated molasses, prior to pre-filtration with 0.1 µm membrane, has an estimated concentration of approximately 43%. There was

little change in most analytes in the pre-filtration permeate. However, colour (A420) was reduced by 13%, with a similar reduction in antioxidant activity. This reduction in antioxidant activity was high compared to Group 2 at 5%. Total phenolics fell by 2% in the permeate, trans-aconitic acid by 8% and polysaccharides by 53%.

5 For nanofiltration, permeations of sucrose (7%) and divalent ions were low, but monosaccharides were high at 60-80%. Permeation of phytochemicals was 15-17% but aconitic acids were less than 2%. The diafiltered retentate (0.5 RW), which could be used for coating raw sugar, had a Brix of 25° and would need to be concentrated at least twice to have adequate solids content for spray drying. The colour of this retentate was black and

10 10 visually comparable to molasses. Reverse osmosis treatment of the 0.5 kDa permeate (0.5/PWC) increased the concentration of most analytes 2-3 fold.

Rejection coefficients calculated for P1 and R1 for each membrane are shown in Table 4. A high coefficient (approaching 1) represents low permeation or high rejection by the membrane, while a low coefficient (approaching zero) represents high permeation or low 15 rejection by the membrane. The results in Table 4 generally reflect previous observations regarding composition of the membrane filtration streams.

Table 4: Rejection coefficients for membranes used in filtration of molasses (Group 1)

Component	Membrane	
	0.1 $\mu$ m stainless steel	0.5 kDa spiral
Total phenolics	0.14	0.90
Antioxidants	0.15	0.86
Fructose	-0.12	0.57
Glucose	0.10	0.47
Sucrose	0.02	0.95
Total sugars	0.01	0.82
<i>cis</i> -aconitic acid	0.05	0.99
<i>trans</i> -aconitic acid	0.15	0.99
Colour (A420)	0.35	0.99
Brix	0.08	0.79
Sodium (Na)	0.02	0.07
Potassium (K)	0.03	0.10
Calcium (Ca)	0.22	0.80

Component	Membrane	
	0.1 µm stainless steel	0.5 kDa spiral
Magnesium (Mg)	0.13	0.76
Iron (Fe)	0.32	0.99
Chloride (Cl)	0.20	-0.51
Phosphate (PO <sub>4</sub> )	0.56	0.96
Sulphate (SO <sub>4</sub> )	0.03	0.89

*RP-HPLC profiles:* The RP-HPLC profiles of the membrane filtration streams for Group 1 were measured. Prefiltration through 0.1 µm had no effect on the polyphenol profile of the permeate. However, for the 0.5 kDa membrane, there was no permeation of polyphenols at 5 hydrophobic end of the profile (> 8min). Reverse osmosis provided a concentration of the components observed in the profile of the 0.5 kDa permeate.

### Group 2

*Plant operation:* The starting molasses for Group 2 was sampled from bulk material which had been standing over the weekend when processing was shut down. The composition of 10 this molasses may have differed slightly from that of Group 1. Average flow rates (after 1.5 h) for the 0.1 µm stainless-steel membrane (were about 65% faster than those of Group 1, but were still too slow at 15 l/h for efficient processing. The fine particles of insoluble calcium/magnesium salts produced by heating at pH 7.5 may have contributed to some 15 membrane fouling. High flow rates (>120 l/h) were achieved for the 30 kDa membrane, for the 0.5 kDa membrane (360 l/h at 5 min) and for the RO membrane (350 l/h).

There was a drop in pH in the 30 kDa streams from pH 7.85 for pre-filtered material to pH 20 5.04 in the recirculated 30 kDa feedstock. As acid detergent was not used in pre-rinsing the 30 kDa membrane, it is assumed that there was a problem with the deionised water supply and that the feedstock was initially recirculated with low pH water. Unfortunately, the pH meter provided malfunctioned in Group 2, so that pH testing of the water supply was not carried out. It is possible that the anion exchange bed may have been exhausted, and failed to neutralise the low pH water from the cation exchanger, in which resin-bound H ions are replaced with hardness cations such as calcium and magnesium.

*Composition:* The compositional data for Group 2 are shown in Figures 4 and 5. As for 25 Group 1, most analytes permeated the 0.1 µm membrane except for polysaccharides (34%

permeation). Permeation through the 30 kDa membrane was 60% for polyphenols and antioxidants, 90% for aconitic acids and greater than 72% for individual sugars.

The 0.5 kDa membrane performed somewhat better than in Group 1, with only 10 $\pm$ 2% permeation of polyphenols/antioxidants. However, permeation of monosaccharides was 5 surprisingly low at 11-12% compared to 59-80% for Group 1. This may have been due to osmotic effects or the use of low pH feedstock (pH 5.3). Sucrose showed 1% permeation by the membrane, and aconitic acids less than 3% permeation. Monovalent cations (Na, K) also showed less permeation than in Group 1. As expected, divalent ions were largely rejected by the 0.5 kDa membrane. A summary of rejection coefficients for Group 2 is 10 shown in Table 5. The incidence of some negative coefficients is due to anomalous results for permeates or retentates.

(a) *RPHPLC profiles*

RP-HPLC profiles of the membrane filtration streams for Group 2 were measured. The 30 kDa permeate showed a notable reduction in the levels of two peaks at the hydrophobic 15 end of the profile (>11 min). As for Group 1, the 0.5 kDa permeate showed a loss of peaks from the hydrophobic region of the profile (after 8 min). Subsequent to this study, an investigation of a lab scale 100 kDa membrane showed a general reduction of peak heights for the permeate, but less reduction at the hydrophobic end than for the 30 kDa membrane. Permeation of antioxidant activity by the 100 kDa permeate (68%) was slightly higher than 20 for the 30 kDa permeate (60%) produced.

Table 5: Rejection coefficients for membranes used in filtration of molasses (Group 2)

Component	Membrane		
	0.1 µm SS	30 kDa spiral	0.5 kDa spiral
Total phenolics	0.16	0.56	0.90
Antioxidants	0.17	0.54	0.87
Fructose	0.20	0.28	0.80
Glucose	-0.15	0.50	0.80
Sucrose	0.03	-0.21	1.0
Total sugars	0.04	0.05	0.95
<i>cis</i> -aconitic acid	0.06	0.20	0.97
<i>trans</i> -aconitic acid	0.11	0.22	0.99
Colour (A420)	0.36	0.86	0.98
Brix	0.06	0.25	0.89
Sodium (Na)	-0.14	0.15	0.25
Potassium (K)	-0.15	0.15	0.28
Calcium (Ca)	0.06	0.31	0.91
Magnesium (Mg)	0.06	0.25	0.93
Iron (Fe)	0.39	0.84	0.97
Chloride (Cl)	0.16	0.31	-4
Phosphate (PO <sub>4</sub> )	0.45	0.56	0.97
Sulphate (SO <sub>4</sub> )	-0.09	0.27	0.99

### Conclusions

Pre-filtering of a 43% (w/v) molasses mix using a 0.1 µm stainless-steel membrane was

5 effective but slow, giving stabilised flow rates of 15 l/h. Most components readily permeated the 0.1 µm membrane except for polysaccharides.

Nanofiltration (0.5 kDa) adequately rejected phenolics, antioxidant activity, sucrose, aconitic acids and divalent ions when using feedstock from 0.1 µm and 30 kDa membranes. However, permeation of monosaccharides by 0.5 kDa was considerably

10 higher when used after the 0.1 µm membrane than after the 30 kDa membrane.

Ultrafiltration (30 kDa) using feedstock from the 0.1 µm membrane showed satisfactory permeation levels for most analytes, with highest rejection for colour at 420 nm (r=0.86)

and iron ( $r = 0.84$ ). Inclusion of a 30 kDa membrane before a 0.5 kDa membrane has the advantages of faster flow rates for subsequent nanofiltration and a reduction of colour in 0.5 kDa retentates. The main disadvantage is the loss of phytochemicals (40%) into the 30 kDa retentate.

5 Reverse osmosis treatment of the 0.5 kDa permeates increased the concentration of most analytes two to three fold in the retentate.

This example demonstrates that a range of membranes and techniques can be used to produce extracts according to the first aspect of the invention which have a variety of characteristics.

10 **Example 5**

Membrane filtration of molasses requires the feedstock to be diluted to 40-60% (w/v) with hot water and pre-filtered or centrifuged to remove the 1-2% (v/v) sediment load. Before proceeding to nanofiltration on a 0.5 kDa membrane, an interim ultrafiltration step is preferred in order to maintain satisfactory flux rates on the nanofiltration membrane and 15 remove polymeric components such as Maillard polymers and polysaccharides. Trials carried out using a 30 kDa spiral-wound membrane after pre-filtration with a 0.1  $\mu\text{m}$  stainless-steel membrane showed high flow rates of 120-150 l/h by the 30 kDa membrane.

The current example investigates whether a 100 kDa membrane offered any advantages in component fractionation compared to a 30 kDa membrane. Specifically, a laboratory scale 20 100 kDa membrane (VivaFlow 50) was tested for its fractionation capabilities using 40% (w/v) molasses at 50°C.

*Methods*

Diluted molasses (40% w/v) was centrifuged at 6000 g for 1 h at 10°C and filtered through a 1.6  $\mu\text{m}$  glass-fibre filter (Whatman GF/A). The diluted molasses (40% w/v) held at 50°C 25 was filtered through a 100 kDa polyethersulphone membrane (107 mm x 84 mm x 25 mm, Vivaflow 50, Sartorius) at 1 Bar and 2.5 Bar in separate experiments using new cassettes. An additional run (Run 2) was carried out on a regenerated membrane at 2 Bar following the first trial at 1 Bar.

*Analyses:* The following tests were carried out on the feedstock, permeates and retentates 30 for each run: total phenolics, antioxidant activity, aconitic acids, polysaccharides, colour (A420) pH, conductivity and HPLC profiles.

### Results

Table 6 shows the changes in colour and composition after filtration through Vivaflow 50 crossflow membrane (100 kDa MWCO). Run 1 was carried out at 1 Bar pressure and Run 3 at 2.5 Bar, using new membranes for each run. The lower pressure in Run 1 was to 5 compensate for leaks in tubing connections. Run 2 used a regenerated membrane following Run 1, however, the supplier advised that membrane reuse was not recommended.

Table 6: Composition of molasses after membrane filtration on Sartorius Vivaflow 50 cross-flow cassette with molecular weight cut-off 100 kDa.

10

Run No	Sample	A420	Total phenolics (g/l)	A/Ox activity (g/l)	Cis-Aconitic (g/l)	Trans-Aconitic (g/l)	Poly-sacch. g/l	pH	Conduct. (NaCl, M)
1	Feed	34.4	8.33	2.65	1.80	6.80	10.17	5.58	0.19
	Permeate	17.8	5.89	1.88	1.68	6.46	1.12	5.56	0.21
	Retentate	37.1	8.42	2.59	1.83	6.89	10.64	5.57	0.20
2	Feed	34.7	8.13	2.61	1.78	6.68	10.35	5.58	0.20
	Permeate	10.0	4.91	1.59	1.58	6.04	0.72	5.57	0.21
	Retentate	37.4	8.14	2.71	1.80	6.73	11.04	5.58	0.19
3	Feed	30.3	7.19	2.29	1.53	6.03	9.25	5.64	0.19
	Permeate	11.1	4.90	1.46	1.41	5.68	0.83	5.62	0.20
	Retentate	35.5	7.89	2.38	1.58	6.17	11.43	5.63	0.19

15

For Runs 1 and 3 there was a significant loss in colour in the permeates (48% and 63% respectively). Permeation of phenolics and antioxidants for Run 1 was 71%. For Run 3, which was at slightly higher back pressure, permeation of phenolics and antioxidants was 68% and 64%, respectively. Permeation of cis- and trans-aconitic acids for Runs 1 and 3 was high at 92-95%, while polysaccharides was low at 9-11%. Reversed-phase HPLC profiles of the samples were measured for each run. While peak heights were reduced in the respective permeates, there was no loss of any individual peaks in these samples.

Table 7 shows data collected from membrane filtration trials using approximately 43% molasses feedstock. There was little reduction in phytochemicals or aconitic acids after 0.1  $\mu\text{m}$  filtration. Feedstock for the 30 kDa spiral-wound membrane was considerably diluted by the start-up volume of the filtration plant. The estimated dilution (from reduced 5 component levels in the 30 kDa feedstock) was 1.57 times. This gives an estimated polysaccharide level in the 30 kDa feedstock of 2.2 g/l. Colour loss in the permeate was higher than that for the 100 kDa membrane. Permeation of phytochemicals through the 30 kDa membrane was 60% for phytochemicals, 89% for aconitic acids and 95 for polysaccharides. In these trials, the notable pH drop to 5.0 in the 30 kDa feedstock was 10 possibly due to a failure by the anion exchange cartridge.

Table 7: Composition of molasses after membrane filtration through a 0.1  $\mu\text{m}$  stainless-steel (SS) membrane and a 30 kDa spiral-wound membrane.

Sample	A420	Total phenolics (g/l)	A/Ox activity (g/l)	Cis-Aconitic (g/l)	Trans-Aconitic (g/l)	Poly-sacch. (g/l)	pH	Conduct. (NaCl, M)
Pre 0.1 $\mu\text{m}$ SS membrane	51.4	6.88	2.42	1.59	7.02	10.3	8.1	0.21
Permeate from 0.1 $\mu\text{m}$ SS membrane	40.9	6.84	2.31	1.70	6.73	3.5	7.7	0.22
Feedstock for 30 kDa	20.2	4.6	1.46	1.03	4.24	N/T (est. 2.2)	5.0	0.19
Permeate from 30 kDa	4.8	2.76	0.88	0.93	3.76	0.20	5.3	0.19

### Conclusions

15 This example demonstrated that the permeation of total phenolics and antioxidants using a 100 kDa membrane (64-71%) was slightly higher than that of a 30 kDa membrane (60%). The permeations of aconitic acids (89-95%) and polysaccharides (9-11%) were similar for both membranes, that is, the 100 kDa membrane gave slightly higher permeation of

phytochemicals (phenolics and antioxidants) but similar permeation of aconitic acids and polysaccharides. Colour reduction was greater with the 30 kDa membrane.

Accordingly, a 100 kDa membrane is recommended for improved permeation of phytochemicals but offers no advantage over a 30 kDa membrane for removal of 5 polysaccharides and colour.

### **Example 6**

This example investigates the use of gel permeation to produce extracts according to the first aspect of the invention.

#### ***Methods***

10 Preparative gel filtration on a Bio-Gel P-2 column was used to fractionate diluted molasses (50% w/v) in the molecular weight range of 100 to 1800 daltons (Da). Five molecular weight fractions from six chromatography runs were pooled and freeze dried. The fractions were analysed for antioxidant activity, total phenolics, HPLC profile and sugars.

The molasses was diluted to 50% (w/v) in gel filtration buffer (20 mM ammonium formate 15 pH 5.0 containing 10% acetonitrile) and centrifuged at 6000 *g* for 1 hour at 10°C. The supernatant was filtered through a 1.6 µm GF/A filter (Whatman) and frozen in 30-ml aliquots at -80°C for use in gel filtration chromatography.

#### ***Gel filtration***

A glass column (26 mm x 1000 mm) was packed with Bio-Gel P-2 (BioRad, USA) to a 20 bed height of 910 mm at a flow rate of 60 ml/h. The bed was equilibrated at 30 ml/h at room temperature in 20 mM ammonium formate buffer pH 5.0 containing 10% acetonitrile. Diluted molasses (20 ml of 50% w/v) was applied to the column and 5-ml

fractions were collected. Six gel filtration runs were carried out on a total of 120 ml of diluted molasses. Fractions from the first three runs were analysed for colour (A<sub>420</sub>), total phenolics and antioxidant activity. For the last three runs antioxidant assays were omitted. Fraction volumes were determined gravimetrically by weighing approximately 20 tubes per run and determining the average fraction volume using a density of 1 g/ml.

The gel filtration column was calibrated with three standards: sucrose (360 kDa), NADH (663 kDa) and vitamin B12 (1355 kDa). The distribution coefficient (KDa) for each standard was calculated as KDa = V<sub>e</sub>-V<sub>o</sub>/V<sub>t</sub>-V<sub>o</sub>. The void volume was determined with bovine serum albumin. The fractionation range of Bio-Gel P-2 is 100-1800 Da (BioRad).

10 *Lyophilised bulk fractions:* For each gel filtration run, individual fractions were pooled into five major fractions according to profiles of colour (A<sub>420</sub>), total phenolics and antioxidants. The pooled fractions for each run are shown in Table 15.

Table 8: List of Pooled Fractions for each Run

Run No	Fraction volume (ml)	Pooled fractions				
		Pool 1	Pool 2	Pool 3	Pool 4	Pool 5
1	4.88	34-50	51-66	67-82	83-100	101-120
2	4.74	35-54	55-72	73-83	84-104	105-120
3	4.95	35-52	53-68	69-79	80-100	101-120
4	4.69	35-53	54-71	72-81	82-100	101-120
5	4.52	37-56	57-76	77-87	88-103	104-120
6	4.71	35-51	52-71	72-81	82-99	100-120
Total vol. (ml)		447	435	270	452	470

After six gel filtration runs, the six samples within each pool (Pools 1-5) were combined.

A 10-ml sample was taken from each final pool and the remainder freeze dried.

*Colour (A<sub>420</sub>):* Gel filtration fractions were diluted with ultrapure water (Arium Model 611, Sartorius) and absorbance was read at 420 nm on a Helios $\lambda$  (Unicam) spectrophotometer.

*Total phenolics:* Total phenolics were determined by a Folin-Ciocalteu colorimetric procedure (Kim *et al.*, 2003). To 50  $\mu$ L of diluted sample in a 75-mm test tube was added 650  $\mu$ L of deionised water. Undiluted Folin-Ciocalteu reagent (50  $\mu$ L) was added to each tube. The solution was mixed and allowed to stand for 5 min at room temperature. Finally 10 500  $\mu$ L of 7% Na<sub>2</sub>CO<sub>3</sub> was mixed with the reaction solution and the absorbance at 750 nm was read after 90 min at room temperature. The total phenolics content was expressed in  $\mu$ g catechin equivalents per ml of undiluted sample. Catechin standards were prepared in the range of 0-250  $\mu$ g/ml.

*Antioxidant activity:* Initially, a substrate containing equal volumes of 14 mM ABTS (2, 15 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) and 4.9 mM potassium persulphate was prepared and stored overnight in the dark at room temperature. Prior to the assay, this solution was diluted about 60-fold with ultrapure water and adjusted to give an absorbance at 734 nm of 0.99-1.01. ABTS substrate (1 ml) was preincubated in 75-mm test tubes at 26°C for 5 min in a water bath and 50  $\mu$ L of sample or standard was 20 added. The solution was mixed and held at 26°C for 45 min when absorbance was measured at 734 nm. Antioxidant activity was expressed in  $\mu$ g gallic acid equivalents per ml of undiluted sample. Gallic acid standards were prepared in the range 0-25  $\mu$ g/ml.

*RP-HPLC profiles:* Qualitative fingerprints of molasses extracts were obtained on a Shimadzu system equipped with a system controller (Model SCL-10AVP), dual pumps

(Model LC10-AD), photo diode array (PDA) detector (Model SPD-M10AVP) and Class Vp version 6.14 software for data acquisition and analysis. Samples (10 µl) were eluted at 30°C on a 30 x 4.6 mm Luna 3 µm C18(2) column (Phenomenex). The flow rate was 1.5 ml/min. Mobile phases were: phase A, 0.1% (v/v) trifluoroacetic acid (TFA) in water and phase B, 60% acetonitrile in 0.085% TFA. The gradient profile was 5-35% B for 12 min; 5 35-100% B for 1 min and 100% B for 3 min, 100-5% B for 0.3 min and 5% B re-equilibration for 4.7 min. Eluted peaks were detected by a PDA detector measuring the absorbance spectrum from 200-400 nm at 4 nm wavelength steps and individual channels at 214, 254, 280, 340 and 400 nm, with the 214 nm chromatogram routinely reported. Gel 10 filtration samples were prepared from five lyophilised pools and contained equal concentrations of total phenolics (1 mg catechin equivalents per ml). The sample of molasses used for gel filtration contained 2 mg CE/ml.

*Sugar analysis:* Mono- and disaccharides were analysed by reversed-phase HPLC using a Shimadzu system fitted with a system controller (Model SCL-10AVP), pump (Model LC-15 10ADVP), refractive index detector (Model RID-10A) and Class Vp 6.12 software. Samples (10 µl) were injected into a 5-µm LC-NH2 Supelcosil column (250 mm x 4.6 mm, Phenomenex) operated at 40°C. The mobile phase was 85% acetonitrile and the flow rate was 1 ml/min. Samples were eluted isocratically for 20 min and were analysed in 15 duplicate. Standard curves for glucose, fructose and sucrose were prepared in the range 0.3 to 1.2 mg/ml, using four standard solutions containing the same gravimetric concentrations of the respective sugars. Triplicate injections were made for each standard 20 solution.

*SDS-PAGE:* Electrophoresis by SDS-PAGE was performed on 12% acrylamide gels using the mini-Protean II slab-gel system (BioRad). Lyophilised samples from gel filtration

were dissolved in water (200 mg/ml) and 30  $\mu$ l was digested in an equal volume of loading buffer. A volume of 15  $\mu$ l (1.5 mg of solids) was loaded onto the gel. Electrophoresis was stopped when the bromophenol blue dye front reached the bottom of the gel. The gel was stained in 0.25% Coomassie Blue and scanned on a desktop scanner (Scanjet 5400C, 5 Hewlett Packard).

### **Results**

*Calibration of Bio-Gel P-2:* A calibration curve for determining molecular weights on the Bio-Gel P-2 column was prepared.

#### *Gel filtration profiles*

10 The gel filtration profiles for colour ( $A_{420}$ ), antioxidants and total phenolics from molasses (Run 3) are shown in Figures 6 and 7. The  $A_{420}$  colour profile showed a peak near the void volume of the column and a sharp peak at fraction 62 (MW 832 Da). The absorbance then decreased gradually to baseline. Profiles of antioxidants and total phenolics closely coincided with each other. The first two antioxidant/phenolic peaks coeluted with the  $A_{420}$  peaks. However, a broad antioxidant/phenolic peak at fraction 80 (MW 352) did not correspond to a colour peak. This peak, comprising fractions 69-100 has a molecular weight range of 135-599 Da and may be a mixture of low colour flavonoids and polyphenolic acids.

15

Profiles of sucrose and monosaccharides (glucose + fructose) are shown in Figures 8 and 9 20 respectively. The column was capable of partially resolving sucrose and monosaccharides. Sucrose was eluted on the leading edge of the antioxidant peak (pre fraction 80) and monosaccharides on the tailing edge (post fraction 80). Hence the low colour antioxidant peak contains all the simple sugars of molasses.

For membrane filtration applications, where a low colour antioxidant product is required, it would be necessary to target the molecular weight region below 600 Da. Separation of the antioxidants from the sugars should be possible by ion exclusion chromatography.

*Bulk gel filtration pools*

5 The five pools for each of the six gel filtration runs were thawed and combined before freeze drying. Figure 10 shows the colours of the combined pools (Pools 1-5) prior to freeze drying. Pools 1 and 2 were both very dark; Pool 1 was slightly turbid and Pool 2 was translucent. Pools 3-5 showed decreasing colour from light brown to pale yellow.

Table 9 shows the composition of the combined pools before freeze drying and the average 10 molecular weight range for each pool. From the mass calculations, the low colour antioxidant/phenolic peak (Figures 6 and 7) contained 49% of the antioxidant activity and 50% of the phenolics, respectively. The dark-coloured peak eluting at the void volume (Pool 1) contained 14% of the antioxidant activity, and the dark-coloured sharp peak (Pool 2) contained 28%. Recovery of antioxidant activity from the column was 70%.

15 Table 9: Composition of combined pools from gel filtration on Bio-Gel P-2 prior to freeze drying.

Component	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5
Volume (ml)	437	425	260	442	460
Total solids (g/100 ml)	0.56	1.36	7.3	3.24	0.15
Total phenolics (µg CE/ml)	324	555	761	550	141
Antioxidant activity (µg GAE/ml)	92	183	229	173	49
Fructose (mg/ml)	BDL	BDL	4.0	5.1	BDL
Glucose (mg/ml)	BDL	BDL	5.1	4.2	BDL
Sucrose (mg/ml)	BDL	0.70	50	12	BDL
Total solids (g)	2.45	5.78	18.98	14.32	0.69

Component	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5
Total phenolics (mg CE)	142	236	198	243	65
Antioxidants (mg GAE)	40	78	60	76	23
Average molecular wt range	>1800- 1444	1377- 636	604-373	356-156	150-65

Composition of load molasses: Total solids = 35.6 g/100 ml;  $A_{420} = 43.7$ ;

Total phenolics = 10340  $\mu$ g/ml; Antioxidant activity = 3390  $\mu$ g/ml.

Total volume of molasses for 6 runs = 120 ml.

5 Table 10 shows the composition of combined pools after freeze drying. With respect to physical properties, Pool 1 was a fluffy product and differed considerably from Pool 2 which had a hard crunchy texture. Pools 3 and 4 were crunchy and hygroscopic, and contained 71% and 64% sugars, respectively. Pool 5 was dark and sticky and was difficult to remove from the drying tray resulting in significant loss of product. On a solids basis 10 (mg GAE/g of solids), there was a significant loss in antioxidant activity on freeze drying of Pool 2 (26%) and Pool 5 (34%).

Table 10: Composition of combined pools from gel filtration on Bio-Gel P-2 after freeze drying

Component	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5
Weight of dried product (g)	2.62	5.23	18.17	13.74	0.5
Total phenolics (g/100 g)	5.60	4.1	0.99	1.70	5.42
Antioxidant activity (g/100 g)	1.6	0.99	0.29	0.55	2.1
Fructose (g/100 g)	BDL	BDL	5.0	16	BDL
Glucose (g/100 g)	BDL	BDL	7.1	14	BDL
Sucrose (g/100 g)	BDL	6.7	59	34	BDL
Total phenolics (mg CE)	147	214	180	234	27
Antioxidants (mg GAE)	42	52	53	76	11
Texture	Fluffy	Crunchy	Crunchy	Crunchy	Tacky
Colour	Black	Black	Light brown	Light brown	Black

15 Composition of load molasses: Total solids = 35.6 g/100 ml;  $A_{420} = 43.7$ ;

Total phenolics = 10340  $\mu$ g/ml; Antioxidant activity = 3390  $\mu$ g/ml.

Total volume of molasses for 6 runs = 120 ml.

*RP-HPLC profiles:* Reversed phase HPLC profiles of lyophilised gel filtration pools (profiles b-f) were examined. There were notable differences between all profiles which 5 could be used to characterise the pools. Pool 1 showed a gradual rising profile with only one minor peak. Presumably this sample contains heterogeneous polymeric material which could not be resolved to individual peaks by the HPLC column. Pool 2 represents the molecular weight range 636-1377 Da and includes the sharp peak of dark brown material. This pool showed the most hydrophilic material eluting at less than 1 min, and a number of 10 well-resolved peaks on the gradient. Pools 3 and 4 represent the low-colour antioxidant peak and showed considerable differences between their respective profiles. Pool 5 showed a range of peaks which could represent low molecular weight phenolic acids and a higher molecular weight compounds that have been weakly bound to the column and were not eluted according to their molecular weights. The proportion of hydrophilic material in 15 this pool was low resulting in greater peak heights in the hydrophobic region of the profile. The molasses load sample enables some peaks to be matched up to certain molecular weight ranges in the pools, as well as showing which molasses peaks are weakly bound to the gel and eluted in Pool 5. All samples exhibited a significant peak at 14.5 min which is not relevant to the chromatography, and represents an acetonitrile flush to remove all 20 bound material from the column at the end of the run. Interestingly, this peak which represents the more hydrophobic compounds in molasses, decreased with molecular weight of the pools.

*SDS-PAGE:* Denaturing electrophoresis of the lyophilised pools was used to detect protein material in the extracts. No protein bands were apparent above 14 kDa in the extracts. In 25 Pool 1 (Lane 2) light staining was observed close to the dye front, but it is uncertain if this is stained protein or residual Coomassie Blue from an uneven dye front. Detection of low molecular weight polypeptides (<10 kDa) would require a 16% gel with a Tris-Tricine buffer.

### Conclusion

30 The gel filtration profiles showed that dark molasses colorants measured at 420 nm were eluted in the void volume of the column (>1800 Da) and at 832 Da. Antioxidant activity and total phenolics co-eluted with these two colour peaks. A broad antioxidant/phenolic

peak was eluted between 135 and 599 kDa, but was not associated with a colour peak.

This antioxidant peak contained all the sucrose and monosaccharides. It comprised 49% of the eluted antioxidant activity and 50% of the total phenolics. Consequently, removal of the dark colorants of molasses would approximately halve the antioxidant activity of the

5 product. The dark-coloured polymeric material eluting near the void volume comprised 14% of the eluted antioxidant activity.

The quantities of lyophilised gel filtration pools varied from 0.5 g to 18 g, with high masses obtained for the two pools containing sugars. Recovery of antioxidant activity was greater than 92% in three of the lyophilised pools, but significant losses in antioxidant

10 activity was found in Pools 2 and 5.

HPLC fingerprints of the lyophilised pools showed some distinct differences which could be used to characterise the samples. Protein analysis by denaturing polyacrylamide gel electrophoresis showed an absence of protein material above 14 kDa in all lyophilised samples. Pool 1 showed a trace of a protein stain near the dye front. This could be bound

15 protein associated with hydrolysable tannins

The lyophilised samples will be analysed subsequently for polysaccharides and polyphenol characterisation, and for their ability to inhibit gut enzymes.

The example demonstrates that the colour profiles obtained by the gel filtration of molasses were dependent on the pH and/or composition of the buffer and that a less

20 coloured high antioxidant extract according to the invention can be produced. At pH 7.5, most of the dark colour was eluted at the void volume, while at pH 5.0, a second dark-coloured peak was observed at lower molecular weight. The pH 5.0 buffer contained 10% acetonitrile which could have contributed to a change in the permeation properties of the gel.

25 Such a lower colour high antioxidant extract will be useful as an additive to foods to reduce GI, reduce carcinogenicity or change body composition without interference with the colour or organoleptic properties of the food. Further, a lower colour extract according to the invention is useful in pharmaceutical applications, especially where colour and bitterness are important issues.

### 30 **Example 7**

The levels of 16 phenolic compounds were determined in aqueous acidic and basic fractions of two samples of molasses extracts according to the first aspect of the present

invention, and one sample of sugar sprayed with molasses extract according to the first aspect of the present invention. The phenolic compounds were isolated by ethyl acetate extraction from the samples.

Three samples were received for evaluation. The details of each sample are shown below.

5    Sample 1: XAD bound fraction from molasses (powder)

Sample 2: MF purified fraction from molasses (0.5 kDa retentate from 30 kDa permeate, powder) [MFP]

Sample 3: Low GI sugar

*Methods*

10    *Sample preparation -Reverse Phase Solid Phase Extraction (SPE) clean-up*: A C<sub>18</sub> SPE cartridge (4 ml, 600 mg; Alltech Associates, Deerfield, Ill.) was conditioned with methanol (3 ml) and formic acid (0.05 %, 6 ml). XAD bound fraction (Sample 1) (189.5 mg) was dissolved into aqueous formic acid (0.05 %, 10 ml) in a volumetric flask. An aliquot of this solution (3 ml) was loaded onto the cartridge, washed with formic acid (0.05 %, 5 ml) and 15    was eluted with aqueous methanol (20 % followed by 60% and 100%, 5 ml each) resulting in three fractions. Most brown colour was observed to elute in the 60 % aqueous methanol fraction.

*Solvent extraction*: An aliquot of each sample (~200 mg) was dissolved in water (10 ml) that had been acidified (pH 1.6) or basified (pH 9.6). Methyl-4-formyl benzoate (7.4 µg) 20    was added to each solution as an internal standard (ISTD). The mixtures were then extracted with ethyl acetate (2 x 10 ml), the solvent was evaporated under vacuum (40 °C) and the mixtures were reconstituted in aqueous formic acid (0.1%, 5 ml) before subjected to HPLC and LC/MS analysis.

*HPLC analysis*: HPLC was carried out using a Shimadzu system equipped with two high-pressure LC-10ADVP pumps, a SIL-10ADVP autosampler (250 µL sampling loop), a CTO-1-ADVP column oven and a SPD-M10ADVP photodiode array detector (Shimadzu Inc., Rydalmere, NSW, Australia). The column used for the separation of the polyphenols was a Luna C<sub>18</sub>, (4.6 mm i.d x 250 mm length, 5µm particle size, Phenomenex, Lane Cove, NSW, Australia). The mobile phases used for the separation were 2% TFA in water (A) 30    and 0.5% TFA in acetonitrile:water (1:1) (B) under a flow rate of 1 mlmin<sup>-1</sup>. Analytes were eluted using a linear gradient: 20-50% B over 20 min., 50-100% B over 10 min remained

at 100% B for another 10 min. Detection was carried out at 280, 320 and 370 nm. Analytes were identified by comparison of their elution time (and characteristic m/z fragments from LC/MS analysis, Table 2) with those of authentic standards (Sigma-Aldrich, Castle-Hill, NSW, Australia).

5 *LC-MS analysis:* LC-MS analysis was carried out on a Quantum TSQ mass spectrometer (ThermoFinnigan, NSW, Australia) equipped with a quaternary solvent delivery system and an autosampler. An aliquot (10 µl) of each extract investigated was chromatographed on a Ultracarb™ analytical column (2.1 x 150 mm, 5 µm particle size), (Phenomenex, NSW, Australia) which was heated to 30°C in an oven. The mobile phase consisted of 10 0.5% formic acid in water (A) and 0.5% formic acid in acetonitrile/water (1:1)(B) at the rate of 300 µl/min. A linear gradient was used (20% B to 100% B over 19 min). Ions were generated using an electrospray source in the negative mode under conditions set following optimisation using a solution of chlorogenic acid.

### Results

15 As explained below, the XAD sample was found to contain significantly higher levels of 16 selected phenolic compounds than the MFP and low GI sugar sample. Several additional constituents, including tricin- and diosmetin-glycosides, were tentatively identified in the XAD sample by LC-MS/MS experiments.

Extraction approach: An SPE-based extraction of the polyphenolic fraction was initially 20 carried out. As expected from previous findings the 60% methanol fraction contained more polyphenolic compounds (based on UV absorption at 280, 320 and 370 nm) than the other two methanol fractions. However, the UV traces from the SPE extractions exhibited low signal-to-noise ratio.

An alternative approach, based on ethyl acetate extraction, was then used. The resulting 25 HPLC traces (Figure 12) exhibited a greater number of peaks at a significantly higher abundance and better resolution than the traces from the SPE clean-up. Thus, only the ethyl acetate extracts were used for the remainder of the study. Ethyl acetate extraction was carried out on both acidified and basified aqueous solutions of the samples to ensure that as many compounds as possible would be extracted from the samples.

30 *Quantification of selected polyphenols:* Initially, the levels of selected compounds, were quantitatively determined in both acidic and basic extracts of the three samples against methyl-4-formyl benzoate. However, this compound exhibited a strong matrix-related bias

and thus the results obtained from its use were questionable. To eliminate such bias an external calibration curve of p-coumaric acid was established and was used to obtain quantitative results. Each compound was quantified based on its maximum UV absorbance in one of the three wavelengths (280, 320 or 370 nm). No response factors were 5 determined for individual compounds and therefore the results of all compounds (apart from p-coumaric acid) are semi-quantitative in nature. The results obtained for the selected compounds are shown in Figure 13. In the figure,

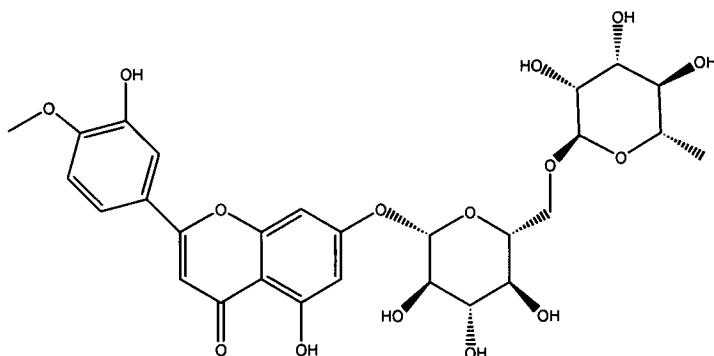
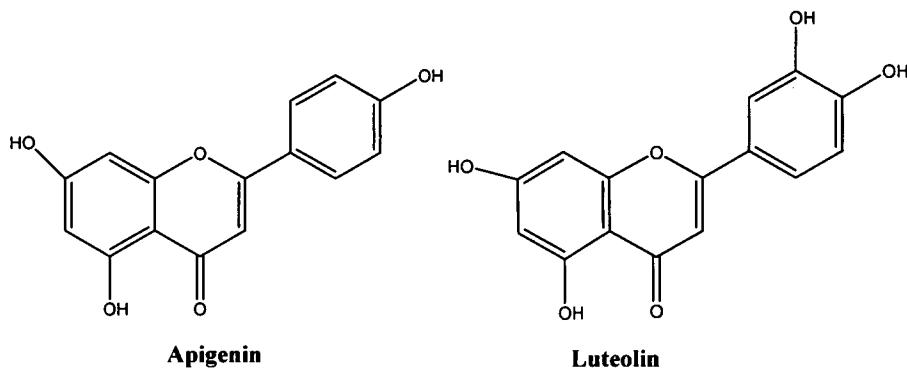
- <sup>a</sup> samples were weighed in the form they were provided. No further treatment was applied prior to extraction.
- 10 • <sup>b</sup> analyses were carried out with enhanced sample-size (~800 mg for MFP and ~1604 mg for sugar)
- <sup>c</sup> trace = less than 0.1 mg/Kg

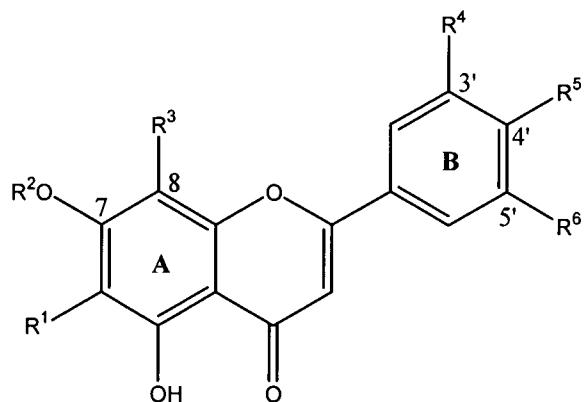
As can be seen, the XAD sample was by far the richest, among all three samples, in these phenolics. Also, the acidic XAD and MFP fractions contained a greater amount of 15 polyphenolic compounds compared to the corresponding basic fractions. This was to be expected, considering that phenolic acids such as coumaric, ferulic and syringic acids have been reported previously as being the major phenolic constituents of sugarcane products. The sugar sample contained the lowest amount of polyphenolic compounds compared to the XAD and MFP samples. However, it was likely that many of the sugar polyphenolics 20 were below the limit of detection of the HPLC method due to the higher content of sugar (per weight unit) in this sample compared with the XAD and MFP samples. To overcome this problem analyses with increased sample size were performed in order to detect additional compounds in the low GI sugar (acidic fraction only). Enhanced sample size analyses were also performed for the MFP sample to increase the number of compounds 25 detected (acidic fraction only).

The presence/absence of selected compounds in some samples (due to time constraints) was confirmed by LC-MS studies (e.g. parent-product reactions, selected reaction monitoring and product-specific reactions). Figure 14 summarises the LC-MS experiments performed for each sample and the information produced for each analyte. In the figure, <sup>a</sup> 30 identification of tricin was based only on MS fragmentation as no authentic reference compound could be obtained and therefore is tentative.

Some compounds shown as ‘not detected’ in Figure 13 were detected in some samples during various LC-MS experiments (e.g. apigenin in XAD-basic and MFP-basic, (-)-catechin gallate in MFP-acid and MFP-basic, luteolin in MFP-acid and MFP-basic). This is possibly due to the better sensitivity of the LC-MS system running in the selected-reaction-monitoring (SRM) mode compared to UV detection employed by the HPLC approach.

A series of LC-MS/MS experiments were carried out on the XAD-acid sample in an attempt to identify as many additional phenolic constituents possible within the timeframe given. Compounds mainly targeted included flavone glycosides previously found in sugarcane extracts, diosmetin (free aglycone of diosmin, already detected in the extracts) and glycosides containing aglycones already confirmed to be present in the extracts (apigenin, tricin, luteolin, quercetin). The results are summarised in Table 11 below. Several of the tentatively identified components have similar structures differing only in the nature of the substituent groups as illustrated by the structures below.





	<b>R<sup>1</sup></b>	<b>R<sup>2</sup></b>	<b>R<sup>3</sup></b>	<b>R<sup>4</sup></b>	<b>R<sup>5</sup></b>	<b>R<sup>6</sup></b>
Tricin-7- <i>O</i> -neohesperoside	H	Glc-Rha	H	OCH <sub>3</sub>	OH	OCH <sub>3</sub>
Luteolin-8- <i>C</i> -(rhamnosylglucoside)	H	H	Glc-Rha	OH	OH	H
Tricin-7- <i>O</i> -glycoside	H	Glc-Rha	H	OCH <sub>3</sub>	OH	OCH <sub>3</sub>
Schaftoside	Glc	H	Ara	H	OH	H
Isoschaftoside	Ara	H	Glc	H	OH	H

Table 11: List of compounds identified from LC-MS/MS experiments in the XAD-acid extract.

<b>Compound</b>	<b>Detected in XAD-acid</b>
Diosmetin	Yes
Myricetin	Yes
Tricin-7- <i>O</i> -neohesperidoside	Yes
Luteolin-8- <i>C</i> -(rhamnosylglucoside)	Yes
Vitexin	No
Tricin	Yes
Orientin	No
Tricin-7- <i>O</i> -glycoside	Yes
Schaftoside	Yes
Isoschaftoside	Yes
4,5-Dimethyl-luteolin-8- <i>C</i> -glucoside	No
Luteolin/Kaempferol glycosides	No
Tricin glycosides	Yes
Apigenin glycosides	No

Compound	Detected in XAD-acid
Diosmetin glycosides	Yes
Quercetin glycosides	No

SRM and product-specific experiments highlighted the presence of additional (to those shown in Table 20) tricin-containing compounds in the XAD-acid extract (Figure 15). However, it appears that diosmetin-containing compounds are the dominating group, at 5 least in the XAD-acid extracts, as several of them were detected during product-specific (m/z 299) analysis (Figure 16).

#### *Conclusion*

The HPLC and LCMS analysis of the XAD and MF powders from sugarcane showed that both contained a unique range of polyphenols and flavonoids quite different in level and 10 mix to that found in other plant sources. They were also different to that found in extracts obtained by other methodology.

As expected the XAD powder had higher levels of the polyphenols compared to the MF powder. The general pattern of polyphenols found in the low GI sugar analysed was also similar to that found in the XAD and MF powders. This reinforced the use of the MF 15 powder in the form of a high Brix syrup as an enrichment spray source to impregnate/coat refined or raw sugar crystals to deliver new functional ingredients with enhanced antioxidant activity and a favourable mineral balance.

These extracts can also be used to produce products having clinical benefits including influencing the GI of foods and modifying body composition. Such a syrup or powder 20 could be used to enrich other food sources such as fibres and flours to deliver similar functional outcomes.

#### **Example 8**

A spray solution suitable for spraying onto raw sugar to convert it to a low GI sugar product was produced from the syrup from the 30 kDa membrane permeate (P1) of 25 Example 1.

The permeate (P1) comprised approximately 4.5g/l polyphenols, 30g/l fructose, 30g/l glucose and 110g/l sucrose, having a Brix value of between 15 and 25.

The storage stability of the permeate was improved by evaporation to 60-70 BRIX, and approximately 4 fold increase in concentration. The composition was thus approximately 18g/l polyphenols, 120g/l fructose, 120g/l glucose and 440g/l sucrose.

This concentrated syrup was sprayed onto a base sugar. The base sugar consisted of (i) 5 crystalline white sugar and (ii) in-process raw sugar from a primary mill or refinery at the fugal stage. The amount of spray solution varied depending upon the initial polyphenol/phytochemical content per kg of the base sugar. Typically between 1 to 12 ml of the concentrated spray syrup was added to each kg of base sugar - the exact amount depending upon the sugar cane variety being processed. For some varieties of cane sugar 10 adequate phytochemical concentration could be achieved with minimal addition of spray syrup (depending upon the amount of reducing sugars remaining and phytochemical concentration). Following the fugal treatment the sugar was dried in a rotating cylindrical sugar drier and upon exit a sample was taken either on-line or off-line for quality assurance/quality control testing to ensure that an adequate polyphenol level is achieved.

15 A similar spraying procedure can be used to impregnate or coat white cane sugar, beet sugar and other carriers such as fibre (eg bagasse) and flour from various cereal sources in order to produce a bioactive enriched product for use as a functional food ingredient.

### **Example 9**

Different processing streams were analysed during the production of raw sugar at a mill 20 and in the case of molasses, compared with extracts prepared according to the ultrafiltration method of the present invention.

*Samples:* Samples were collected from the following processing streams: first extracted juice (FEJ), buffer tank (BT), mill mud filtrate (MMF), mill mud (MM), evaporator supply juice (ESJ), syrup (SYR), molasses (MOL) and raw sugar (RS). The samples were stored 25 frozen and transported to the testing facility.

*Sediment removal:* Liquid samples (juices and mill mud filtrates) were centrifuged at 5000 rpm for 10 min at 5°C and supernatants were vacuum filtered through Whatman No.1 filter papers. Syrups, molasses and raw sugars were diluted to 10%, 10% and 40%, respectively, before sediment removal. Samples for mineral analysis were not treated to remove 30 sediment.

*Mill mud extracts:* A mill mud extract was prepared by homogenising 20 g of mill mud for 1 min in 45 ml of hot (60°C) deionised water, centrifuging at 5000 rpm for 5 min and

decanting the supernatant. The pellet was re-extracted in 45 ml of hot deionised water and the supernatant again collected. The combined extracts were made up to 100 ml with deionised water and vacuum filtered through Whatman No.1 filter papers.

*Analyses:* The following methods of analysis were used:

Component	Method
Total phenolics	Folin-Ciocalteu colorimetric procedure. Results expressed as g catechin equivalents per l.
Polyphenol profile	RP-HPLC, C18 column, (Luna 3 µm, Phenomenex) at 30°C, linear gradient 3-21% acetonitrile for 12 min, 21-60% for 1 min and 21-60% for 3 min. Detection at 214 nm.
Antioxidant activity	ABTS substrate. Results expressed as g gallic acid equivalents per l.
Mono- and di-saccharides	RP-HPLC, NH <sub>2</sub> column at 40°C, isocratic elution with 88% acetonitrile at 1 ml/min for 20 min. R I detection.
<i>cis</i> - and <i>trans</i> -aconitic acids	Ion-moderated partition HPLC, Aminex column HPX-87H (Bio-Rad). Isocratic elution with 0.004 M H <sub>2</sub> SO <sub>4</sub> at 0.6 ml/min for 40 min at 30°C. UV detection. Samples diluted in water pre-injection.
Total solids	Vacuum oven at 70°C for 16 h.
Density	Anton Paar density meter. Readings at 20°C in g/ml.
Total nitrogen	Kjeldahl method using Foss Tecator digestion and distillation units.
Non-protein nitrogen	Sample (2.5 g) made to 50 ml with 12% trichloroacetic acid and filtered. Aliquot (20 ml) analysed by Kjeldahl method.
Fat	Acid hydrolysis followed by Mojonnier extraction.
Polysaccharides	Measurement of soluble polysaccharides (Roberts, 1981). Precipitation of polysaccharides with 100% ethanol, wash with 80% ethanol, digestion of precipitate in 1% sulphuric acid and measurement total

Component	Method
	reducing sugars by phenol-sulphuric acid at 485 nm with glucose as reference. Results expressed as g polysaccharide per l.
Na, K, Ca, Mg, PO <sub>4</sub> , SO <sub>4</sub>	Inductively coupled plasma – optical emission spectroscopy (ICP-OES) on Varian Vista Pro instrument at UQ. Elements were tested on undiluted, unfiltered samples
Cl	Automated colorimetric analyser (Seal AQ2), EPA Method (EPA-124-A)

*Extract*

The molasses was extracted several times using the follow process steps according to the process of the invention:

- 5 (a) heating and diluting the molasses with water until the viscosity of the resulting solution is between 50 and 100 centipoise and between 30 and 50 Brix at a temperature of about 50°C;
- 10 (b) centrifuging the diluent using a continuous desludging centrifuge with the supernatant being directly processed through a ceramic or stainless steel membrane (of 0.1 to 0.5 micron) using a pressure of 4 Bar, a flow rate of approximately 30 to 100 l/hour and at a temperature 35 to 50°C;
- 15 (c) heating the solution of step (b) to 75°C and then maintaining it in that temperature range for about 20 minutes until a precipitate of insoluble calcium and magnesium salts forms;
- (d) separating the precipitate and large particulate matter from the solution produced in step (c) by passing the mixture through a ceramic or stainless steel membrane (of 0.1 to 0.5 micron) using a pressure of 4 Bar, a flow rate of 30 to 100 l/hour and at a temperature of 50°C, the retentate being discarded and the permeate being collected;
- 20 (e) treating the permeate collected from step (d) by passing it though a combination of spirally wound ultrafiltration membranes.

The ultrafiltration membranes were changed each time the process was run. The ultrafiltration membranes had size exclusion ranging from 1000 to 50,000 Dalton. The extracts thus isolated had the compositions set out in Figure 25. In the figure, Perm = Permeate; Ret = Retentate and the data shows percentages of each molasses component in 5 permeate and retentate.

### Results

As illustrated by Figures 17 to 24, the composition of the different processing streams vary widely. Furthermore, the composition of the feed streams varies from batch to batch of sugar cane.

10 In the figures, the following abbreviations are used:

- BT = Buffer tank
- MMF = Mill mud filtrate
- MM = Mill mud
- MME = Mill mud extract (hot water extract, 20 g/100 ml total extract)
- 15 • ESJ = Evaporator supply juice
- SYR = Syrup
- MOL = Molasses
- RS = Raw sugar
- FEJ = First extracted juice. (In Figure 17, the sample is taken from Batch 3. No 20 FEJ sample was provided for Batch 1 or Batch 2.)

### Conclusion

These findings clearly demonstrate the partitioning of bioactive phytochemicals in primary mill sugar cane process streams. Such partitioning indicates that certain streams will be suited for different commercial exploitation of these phytochemicals.

25 Clarified juice, syrup, molasses and mill mud extracts are all potential sources as feedstocks for the recovery of bioactives such as polyphenols/antioxidants, organic acids and minerals. Molasses is the preferred source. Mill mud and bagasse are also useful sources of phytochemicals where a different composition is desired, especially one with higher levels of policosanols and phytosterols but lower levels of carbohydrates.

The five ultrafiltration extracts prepared from the molasses were each used as feed stock for further processing using XAD. The use of these extracts as feedstocks offers the clear benefit that they will minimize the chance of poisoning the XAD resins thus improving the efficiency and useable life of the resins.

### 5 **Example 10**

Three separate extraction processes were carried out as follows to prepare starting materials appropriate for use in the process according to the second aspect of the invention:

1. Fibrated cane tops were dried in a vacuum oven at 40°C of one Group. The dried material was extracted with n-heptane using a soxhlet extractor of rabout 4 hours during which time at least 10 cycles were completed. The extract was dried over anhydrous sodium sulphate and evaporated to dryness to give an oily/waxy material in 1.2% yield, based on dry weight of cane tops.
2. Bagasse was treated in the same manner. The dried material gave an oily/waxy material in 0.65%yield, based on dry weight of bagasse.
- 15 3. Mill mud was treated in the same manner. The dried material gave an oil/waxy material in 6.53% yield, based on dry weight of mill mud.

The three extracts were then subjected to the process of Example 9 to provide three extracts according to the present invention. The extracts were high in polyphenols but low in sugars and would be useful for applications where sugars are not required.

20 The example also demonstrates that a range of feedstocks can be utilized in producing the extracts described as part of the invention.

### **Example 11**

The method according to the second aspect of the invention was used to produce an extract according to the invention from biodunder.

Table 12. Composition of biodunder extract

Component	Biodunder
Total solids (g/100 g)	10.4
°Brix	11.4
Density (g/ml)	1.05
Colour (A <sub>420</sub> )	17.8
Conductivity (M NaCl equiv.)	0.25
pH	4.1
Total phenolics (g CE/l)	6.2
Antioxidant activity (g GAE/l)	1.8
Fructose (g/l)	1
Glucose (g/l)	1
Sucrose (g/l)	3
Ash g/100 g	4

### Conclusion

The extract produced in this example was high in polyphenols but low in sugars and would be useful for applications where sugars are not required. The example also demonstrates

5 that biodunder is a useful feedstock for producing extracts according to the invention.

### Example 12

This example demonstrates that molasses from a primary mill has a different composition from a refinery.

Table 13 Composition of molasses from different sources

Component	Mill 1 (Primary Mill)	Mill 2 (Refinery)
Total solids (g/100g)	75.4	84.3
Brix	76	79
Colour (ICU) A420	117 407	58 957
Total phenolics (mg CE/100 g DW)	2842	1258
Antioxidant activity (mg GAE/100 g DW)	864	373
Fructose (g/100 g DW)	9.7	5.2
Glucose (g/100 g DW)	6.0	5.2
Sucrose (g/100 g DW)	38.4	59.0

The example demonstrates that the source of molasses may vary and that this factor needs to be considered in producing the extracts according to the invention.

### Example 13

5 In this example, the composition of mill mud was analysed to demonstrate its potential as a feedstock to prepare extracts according to the invention. The results are in Figure 26.

The aqueous extracts produced in this example were high in polyphenols but low in sugars demonstrating that mill mud would be useful for producing extracts for applications where sugars are not required.

10 **Example 14**

The following table shows the differences in the extracts obtained using the method disclosed in international patent application no WO 2005/117608 compared with the extracts according to the present invention.

Table 14: Composition of extracts made using different processes

Compound	WO 2005/117608 method	MF
Moisture (% wt/wt)	2-5	3-6
Sucrose (%)	0.1-0.2	50-75
Glucose (%)	0.2-0.6	6-15
Fructose (%)	1.2-2.4	6-15
Calcium (mg/g)	6-8	3-4
Magnesium (mg/g)	2-3	1-5-3.0
Potassium (mg/g)	0.2-0.3	8-12
Sodium (mg/g)	0.05-0.07	1-2
Polyphenols (mg CE/g)	180-240	15-25
Antioxidants (mg GAE/g)	50-70	4-7
Trans- aconitic acid (%)	0-0.1	1.5-3.0

Compound	WO 2005/117608 method	MF
Phosphate (mg/g)	n/d	0.2-0.4
Chloride (mg/g)	n/d	1.8-2.5
Sulphate (mg/g)	n/d	10-15

It is important to note that molasses feedstocks vary significantly, particularly in mineral composition, as a result of the variability in the cane being processed and the processing conditions used (particularly in the liming and flocculation step to clarify the mixed juice).

5 The above table shows that the method according to the present invention produces an extract which has a very different composition to that of the extract disclosed in international patent application no WO 2005/117608. This difference is not simply the difference in the molasses feedstock used. The extract according to the invention has a much lower polyphenols content and a much higher content of the other phytochemicals

10 which makes it closer to the natural composition of the sugar cane.

### Example 15

This example compares the extract obtained from biodunder in Example 11 with pools 1 to 4 from Example 6.

Table 15: Comparison of biodunder extract with different fractions of molasses extract

Summary of Results, mg/ Kg					
Target Compounds	Biodunder	Pool 1	Pool 2	Pool 3	Pool 4
Cinnamic Acid	473.60			2.5	16.9
Epicatechin		3.1	6.1	69.1	268.2
Gallic Acid				6.3	
Myrcetin	23.7			1.0	62.9
Protocatechoic Acid		15.2	8.0		
Syringic Acid	58.0	2.7	6.1	68.3	261.9
Vanillic Acid	176.0		4.6	8.5	81.8
Vanillin		5.4		7.5	6.3
Para-hydroxy Benzoic Acid			3.7		28.3
p-Coumaric Acid		5.0			

Summary of Results, mg/ Kg					
Target Compounds	Biodunder	Pool 1	Pool 2	Pool 3	Pool 4
Apigenin	29.5				
Caffeic Acid			0.8	3.1	
Diosmin	19.0				
Ferulic Acid					4.7
Kaempferol	88.0				

The word 'comprising' and forms of the word 'comprising' as used in this description and in the claims does not limit the invention claimed to exclude any variants or additions.

Modifications and improvements to the invention will be readily apparent to those skilled in the art. Such modifications and improvements are intended to be within the scope of this invention.

**The claims defining the invention are as follows:**

- 1 An extract derived from sugar cane having GI or burn rate reducing characteristics wherein the extract comprises a mixture of one or more polyphenols, one or more carbohydrates, one or more minerals and one or more organic acids.
- 2 The extract according to claim 1 wherein the extract is in a powder form and comprises 1.5 to 2.5 wt % of one or more polyphenols, 50 to 80 wt % of one or more carbohydrates, 1 to 3 wt % of one or more minerals and 1 to 3 wt % of one or more organic acids.
- 3 The extract according to claim 1 wherein the extract is in a syrup form and comprises 3.5 to 6 g CE/l of one or more polyphenols, 80 to 220 g/l of one or more carbohydrates, 3 to 5.5 g/l of one or more minerals and 3 to 6 g t-aconitic/l of one or more organic acids.
- 4 The extract according to claim 1 wherein the extract is produced using a method which comprises as one of its steps a fractionation by molecular weight and size.
- 5 The extract according to claim 4 wherein the fractionation by molecular weight is achieved using membrane filtration.
- 6 The extract according to claim 4 wherein the fractionation by molecular weight is achieved using gel permeation.
- 7 The extract according to claim 1 wherein the extract is derived from a sugar cane derived product selected from the group consisting of the feed stream of raw sugar cane juice, clarified syrup, concentrated syrup, treacle, primary mill and refinery molasses, golden syrup, brown sugar, bagasse, biodunder, field trash, cane stripings, pith, growing tips, pulp, mill mud and mixtures thereof.
- 8 A food product comprising an extract according to any one of claims 1 to 7.
- 9 The food product according to claim 8 wherein the amount of the extract is sufficient to lower the glycaemic index of the food product.
- 10 The food product according to claim 8 wherein the food product comprises a material selected from beet sugar, fibres, cereals and mixtures thereof.
- 11 A preparation for use as a pharmaceutical, nutraceutical or cosmeceutical comprising an extract according to any one of claims 1 to 7.

- 12 An extract derived from sugar cane having GI or burn rate reducing characteristics wherein the extract is a powder which comprises 1.5 to 2.5 wt% of one or more polyphenols, 50 to 80 wt% of one or more carbohydrates, 1 to 3 wt% of one or more minerals and 1 to 3 wt% of one or more organic acids.
- 13 An extract derived from sugar cane having GI or burn rate reducing characteristics wherein the extract is a syrup which comprises 3.5 to 6 g CE/l of one or more polyphenols, 80 to 220 g/l of one or more carbohydrates, 3 to 5.5 g/l of one or more minerals and 3 to 6 g t-aconitic/l of one or more organic acids.
- 14 A process for producing an extract derived from sugar cane comprising the steps of:
  - (a) heating and diluting a sugar cane derived product until the viscosity of the resulting solution or matrix is less than or equal to about 100 centipoise at a temperature in the range of from 40 to 60°C;
  - (b)(i) centrifuging the product from step (a);

or alternatively,

  - (b)(ii) adjusting the pH of the product from step (a) with a base;
  - (c) heating the product of step (b) to a temperature in the range of from 70 to 80°C and then maintaining it in that temperature range for a period of time until a precipitate of insoluble calcium and magnesium salts forms;
  - (d) removing the precipitate and large particulate matter from the mixture produced in step (c);
  - (e) treating the product from step (d) with a fractionation by molecular weight and size to isolate desired extracts.
- 15 The process according to claim 14 wherein the sugar cane derived product is selected from the group consisting of the feed stream of raw sugar cane juice, clarified syrup, concentrated syrup, treacle, primary mill and refinery molasses, golden syrup, brown sugar, bagasse, biodunder, field trash, cane stripplings, pith, growing tips, pulp, mill mud and mixtures thereof.
- 16 The process according to claim 14 wherein the temperature used in step (a) is about 50°C.

- 17 The process according to claim 14 wherein the viscosity achieved in step (a) is in the range from 50 to 100 centipoise or 30 to 50 Brix.
- 18 The process according to claim 14 wherein the pH is adjusted in step (b)(i) to a pH in the range of from 7.2 to 9.5.
- 19 The process according to claim 14 wherein the fractionation in step (e) is undertaken using one or more fractionation filters or membranes selected from the group consisting of microfiltration, ultrafiltration, nanofiltration and mixtures thereof.
- 20 The process according to claim 19 wherein step (e) is achieved by passing the product from step (d) though a combination of spirally wound ultrafiltration membranes with a size exclusion in the range of from 100 kDa to 1 kDa.
- 21 The process according to claim 20 wherein the ultrafiltration membranes have a size exclusion in the range from 50 kDa to 1 kDa.
- 22 The process according to claim 14 wherein the fractionation in step (e) is undertaken using gel permeation.
- 23 The process according to claim 14 further comprising step (f) refining the products collected from step (e) with a treatment selected from the group consisting of ion exchange chromatography, hydrophobic chromatography, ultrafiltration, nanofiltration, gel permeation, reverse osmosis and mixtures thereof, to isolate desired extracts.
- 24 The process according to claim 23 wherein step (f) comprises treating the solution collected from step (e) with a 0.5 kDa nanofiltration membrane to isolate desired extracts.
- 25 A method for refining a phytochemical extract comprising the steps of:
  - (a) heating and diluting a phytochemical containing extract until the viscosity of the resulting solution or matrix is less than or equal to about 100 centipoise at a temperature in the range of from 40 to 60°C;
  - (b)(i) centrifuging the product of step (a);  
or alternatively,
  - (b)(ii) adjusting the pH of the product of step (a) with a base;

- (c) heating the product of step (b) to a temperature in the range of from 70 to 80°C and then maintaining it in that temperature range for a period of time until a precipitate of insoluble calcium and magnesium salts forms;
- (d) removing the precipitate and large particulate matter from the product of step (c);
- (e) treating the product of step (d) with a fractionation by molecular weight and size to isolate desired extracts.

26 The process according to claim 25 wherein the phytochemical extract is derived from a source selected from the group consisting of cocoa beans, tea waste, pod husks, coffee beans, coffee waste, grape pomace, cereals, legumes, nuts, oilseeds, fruits, vegetables, beverages and herbal products.

27 A food product comprising an extract produced using the method according to any one of claims 25 and 26.

28 A food product according to claim 27 wherein the food product has a low GI.

FIGURE 1

Component	Sugar sample																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Moisture (g/100 g)	0.01	0.01	0.02	0.80	0.51	1.14	1.64	0.16	1.06	1.54	0.11	0.08	0.20	0.07	0.09	0.07	0.09	0.08	0.78	
Colour (TC units)	803	1200	1040	1080	6640	10790	8430	6700	1990	25290	17710	771	848	1420	776	1070	763	1460	819	4030
Total phenolics (mg CE/ 100 g)	15.9	20.9	17.4	17.1	76.2	98.1	89.1	81.7	27.7	180.3	153.8	15.1	16.2	29.3	13.0	19.4	16.2	28.4	17.7	76.5
Antioxidant activity (mg GAE/100g)	2.5	6.4	5.5	5.3	22.1	26.3	24.4	24.4	8.1	42.3	39.4	4.5	5.5	8.9	4.2	6.0	4.6	10.0	5.9	25.3
Fructose (g/100 g)	0.02	0.03	0.02	0.02	0.42	0.56	0.63	0.30	0.06	0.78	1.50	0.02	0.03	0.07	0.03	0.04	0.03	0.08	0.04	0.33
Glucose (g/100 g)	0.03	0.04	0.03	0.02	0.41	0.44	0.82	0.34	0.05	1.30	1.86	0.02	0.01	0.05	0.02	0.04	0.03	0.10	0.04	0.54
Sucrose (g/100 g)	99.2	98.6	99.1	99.4	97.1	97.0	95.6	96.7	98.3	94.9	93.4	98.7	98.4	98.9	98.8	99.0	99.3	96.4	97.4	95.3
L-aconitic acid (mg/ 100 g)	13.2	19.3	14.4	13.8	48.6	32.9	58.0	80.0	21.6	3.3	24.4	12.6	14.0	28.6	20.3	17.2	6.5	31.9	17.4	84.2
Sodium (mg/kg)	87	23	24	31	43	68	122	86	54	72	75	44	11	24	25	41	15	26	18	51
Potassium (mg/kg)	396	204	176	116	782	948	976	1427	264	636	1054	372	229	626	121	473	275	331	114	1189
Calcium (mg/kg)	356	66	117	46	335	279	410	683	139	242	385	329	42	114	232	52	29	207	5	323
Magnesium (mg/kg)	74	43	33	27	162	159	189	220	68	122	194	28	31	91	27	30	23	45	17	134
Iron (mg/kg)	12	7	10	7	26	13	12	19	18	12	12	8	8	11	15	9	13	11	8	23
Phosphate (mg/kg)	59	17	14	2	16	21	22	33	8	16	20	1	9	10	13	8	8	20	3	47
Sulphate (mg/kg)	349	290	383	401	567	656	648	441	448	449	489	471	533	476	499	586	540	525	599	
Chloride (mg/kg)	52	75	81	32	462	500	513	1081	145	336	621	90	65	242	33	91	51	85	34	419

Figure 2

Sample	Volume (l)	Total phenolics (g CE/l)	Anti-oxidants (g GAE/l)	Fructose (g/l)	Glucose (g/l)	Sucrose (g/l)	Total sugars (g/l)	Total Solids (g/100 g)	Ash (g/100g)	Conductivity (NaCl, M)	A420	pH	Brix
0.1/0	80+	8.72	3.37	25	20	162	207	30.8	3.8	0.22	61.2	7.57	35.0
0.1/R1	34+	10.00	3.48	26	20	172	218	32.9	4.1	0.21	81.4	7.32	39.0
0.1/P1	8.56	2.95	29	18	169	216	30.7	3.8	0.23	53.2	7.29	36.0	
0.1/P2	46	8.40	2.92	25	18	156	199	30.5	3.7	0.23	53.6	7.45	35.0
0.5/0	40+	5.64	1.92	22	12	89	123	20.1	2.5	0.20	31.3	6.77	23.0
0.5/R1	25+	8.40	2.33	30	18	109	157	23.6	2.6	0.19	39.9	6.88	27.0
0.5/RW	6.58	2.36	8.0	4.0	126	134	21.6	1.8	0.05	44.0	6.74	25.0	
0.5/P1	0.84	0.33	13	9.6	5.8	28	5.9	1.6	0.24	0.48	7.24	5.8	
0.5/P2	15	0.64	0.26	11	7.8	2.8	22	4.8	1.5	0.24	0.33	7.15	4.8
0.5/PWC	80	0.42	0.16	7.1	4.5	2.8	14	2.9	0.75	0.12	0.23	7.17	2.9
RO/0	80+	0.42	0.16	7.1	4.5	2.8	14	2.9	0.75	0.12	0.23	7.17	2.9
RO/R1	10+	0.96	0.39	15	11	6.7	32	6.5	1.8	0.28	0.66	7.53	6.7

Figure 3

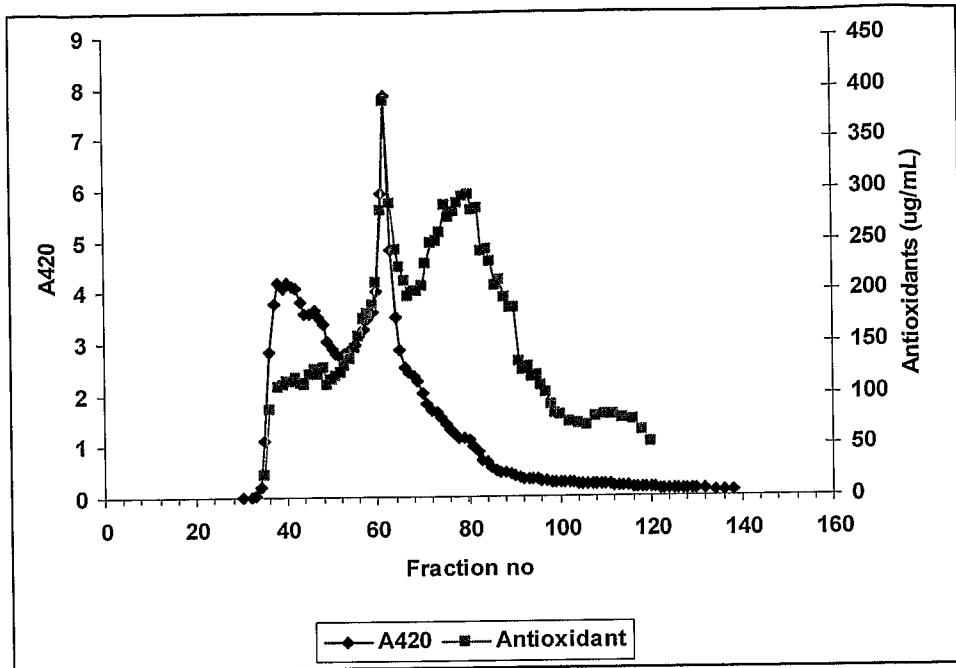
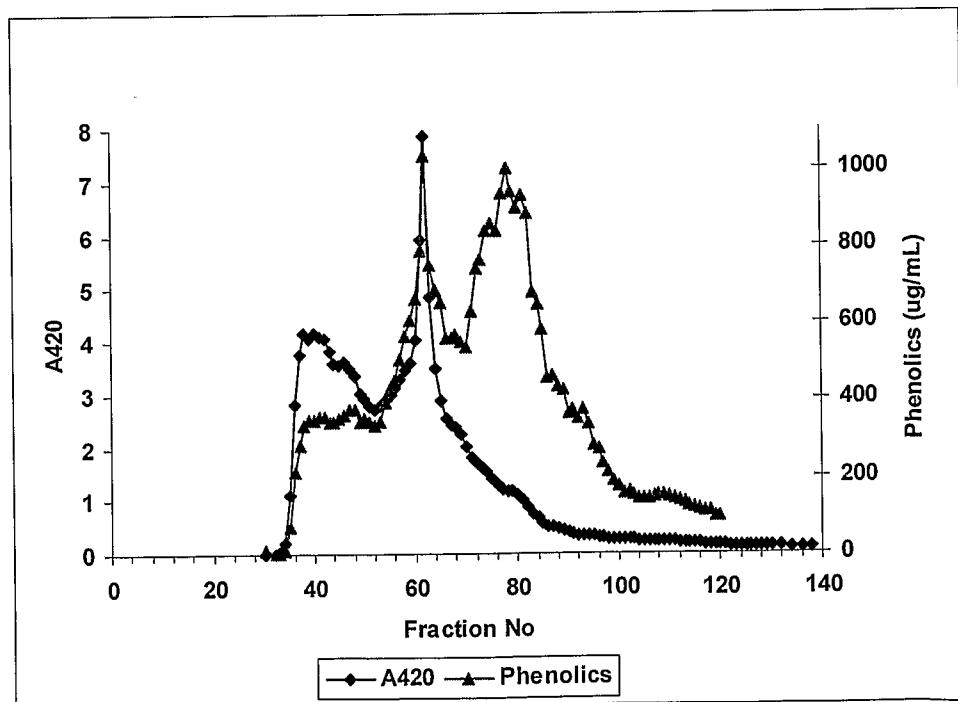
Sample	Volume (l)	Cis- Aconitic (g/l)	Trans-Aconitic (g/l)	Poly- Saccharides (g/l)	Na (mg/l)	K (mg/l)	Ca (mg/l)	Mg (mg/l)	Fe (mg/l)	Cl (mg/l)	PO <sub>4</sub> (mg/l)	SO <sub>4</sub> (mg/l)
0.1/0	80+	2.30	8.22	11.6	1032	7376	1469	661	31.4	4680	147	639.3
0.1/R1	34+	2.38	8.88		1075	7878	1716	732	40.2	5640	223.7	663.2
0.1/P1		2.27	7.57	5.4	1054	7622	1333	640	27.2	4540	98.5	640.6
0.1/P2	46	2.22	7.40		1077	7854	1355	683	33.4	4680	99.2	652.2
0.5/0	40+	1.45	4.87		644	4520	827	425	21.7	2640	62.5	411.4
0.5/R1	25+	1.81	6.10	6.5	674	4706	978	489	26.3	2520	75.0	485.9
0.5/RW		1.81	6.34		588	4465	1680	778	40.8	2652	137.5	780.0
0.5/P1		0.025	0.081		630	4247	200	120	0.2	3800	2.8	52.2
0.5/P2	15	0.013	0.047		479	3178	138	81	0.2	3520	2.0	26.6
0.5/PWC	80	0.012	0.041		267	1730	66	41	0.1	1560	1.1	19.3
RO/0	80+	0.012	0.041		267	1730	66	41	0.1	1560	1.1	19.3
RO/R1	10+	0.030	0.094		630	3800	143	88	0.2	3580	6.9	54.0

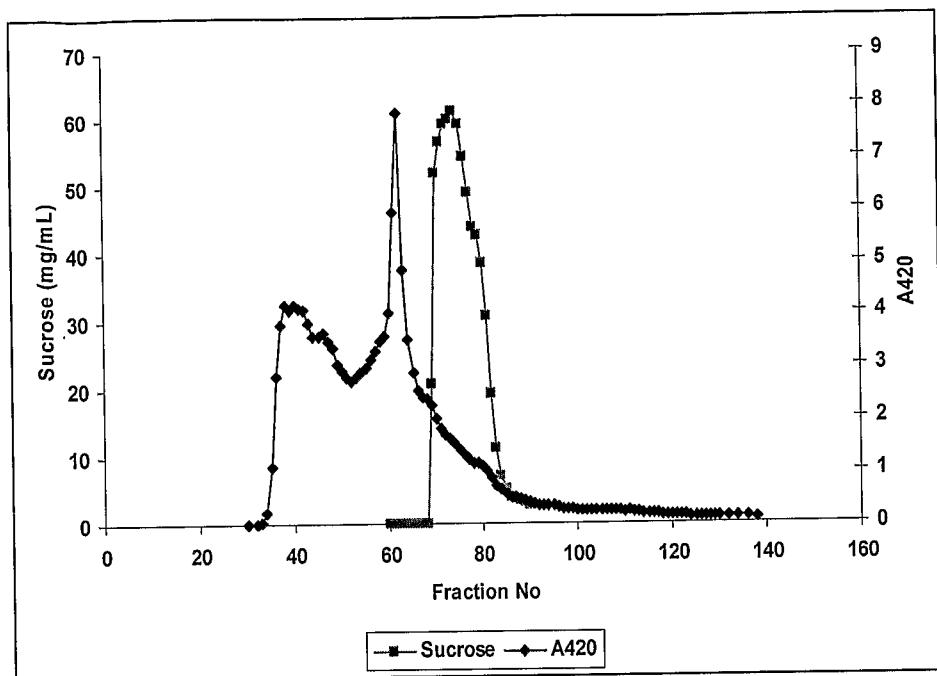
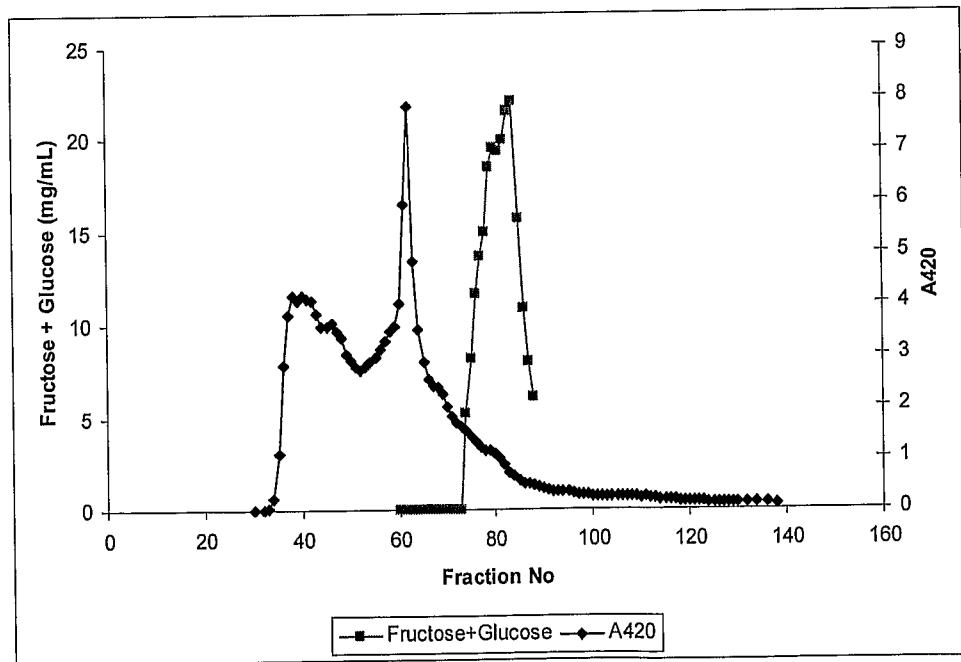
Figure 4

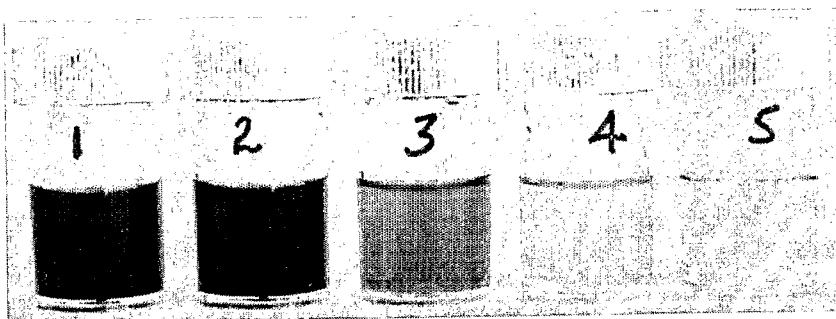
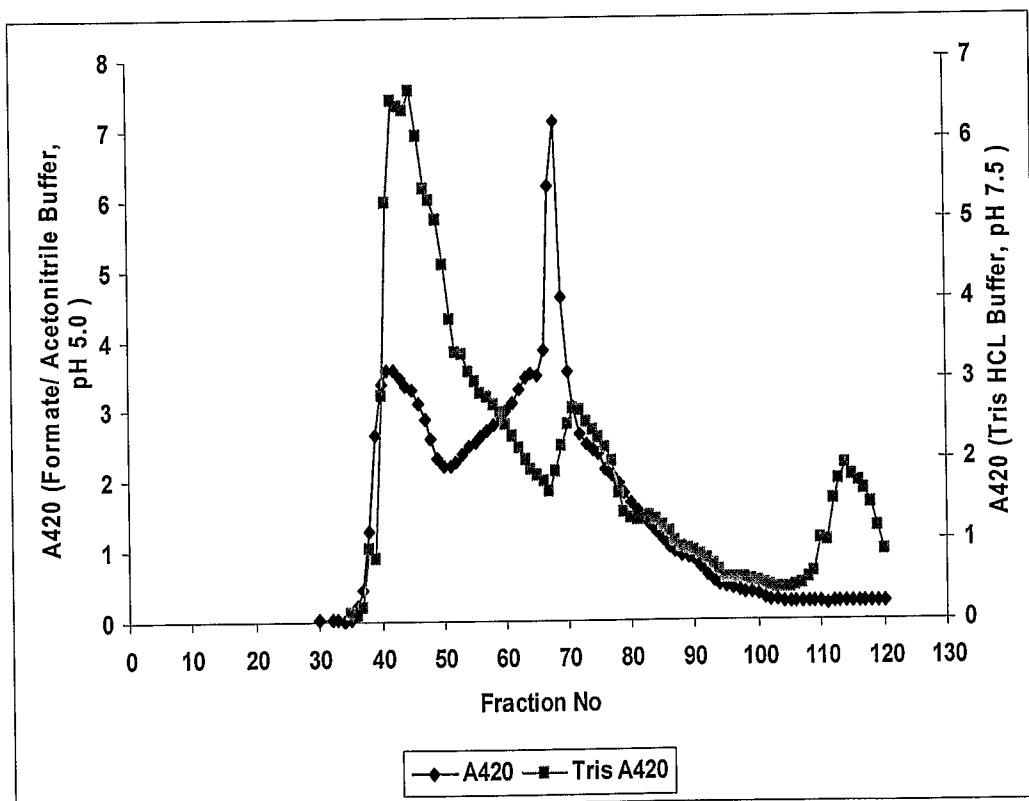
Sample	Volume (l)	Total phenolics (g CE/l)	Anti-oxidants (g GAE/l)	Fructose (g/l)	Glucose (g/l)	Sucrose (g/l)	Total sugars (g/l)	Total Solids (g/100g)	Ash (g/100g)	Conductivity (NaCl, M)	A420	pH	Brix
0.1/0	80+	6.88	2.42	25	21	147	193	26.5	3.1	0.21	51.4	8.07	30.0
0.1/R1	30+	8.10	2.79	30	20	156	206	28.7	3.3	0.20	64.2	7.70	32.0
0.1/P1		6.84	2.31	24	23	151	198	26.8	3.3	0.22	40.9	7.74	30.0
0.1/P2	50	6.18	2.07	25	17	138	180	25.0	3.1	0.21	35.7	7.85	28.0
30/0	50+	4.60	1.46	18	14	68	100	17.2	2.1	0.19	20.2	5.04	19.0
30/R1	10+	6.30	1.91	18	26	61	105	19.4	2.2	0.17	33.7	4.88	21.0
30/P1		2.76	0.88	13	13	74	100	15.4	1.9	0.19	4.79	5.31	15.8
30/P2	40	2.60	0.85	15	16	73	104	15.1	1.9	0.19	4.47	5.34	15.4
0.5/0	36+	2.60	0.85	15	16	73	104	15.1	1.9	0.19	4.47	5.34	15.4
0.5/RW	16+	2.10	0.69	9.0	9.0	63	81	12.0	0.9	0.07	3.80	5.39	12.3
0.5/P1		0.21	0.09	1.8	1.8	0.2	3.8	1.5	0.64	0.11	0.063	5.35	1.4
0.5/P2	20	0.20	0.09	2.0	2.0	0.2	4.2	1.4	0.69	0.10	0.056	5.36	1.3
0.5/PW	70	0.11	0.04	1.0	0.8	BDL	1.8	0.76	0.32	0.05	0.056	5.56	0.7
0.5/PWC	90	0.12	0.05	1.2	1.1	0.1	2.4	0.91	0.44	0.06	0.052	5.48	0.9
RO/0	90+	0.12	0.05	1.2	1.1	0.1	2.4	0.91	0.44	0.06	0.052	5.48	0.9
RO/R1	20+	0.25	0.11	2.5	2.3	0.3	5.1	1.8	0.74	0.12	0.103	5.49	1.8

Figure 5

Sample	Volume (l)	Cis-Aconitic (g/l)	Trans- Aconitic (g/l)	Poly- Saccharides (g/l)	Na (mg/l)	K (mg/l)	Ca (mg/l)	Mg (mg/l)	Fe (mg/l)	Cl (mg/l)	PO <sub>4</sub> (mg/l)	SO <sub>4</sub> (mg/l)
0.1/0	80+	1.59	7.02	10.3	867	5058	1021	502	26.0	3540	96.1	435.0
0.1/R1	30+	1.80	7.53		897	5207	1190	548	35.7	4220	146.4	452.4
0.1/P1		1.70	6.73	3.5	1018	5989	1116	578	21.7	3540	80.3	491.4
0.1/P2	50	1.58	6.31		945	5548	1019	515	18.1	3340	72.1	460.4
30/0	50+	1.03	4.24		692	3971	765	400	15.2	2520	52.9	345.6
30/R2	10+	1.16	4.82		618	3532	724	375	20.9	2680	61.3	324.9
30/P1		0.93	3.76	0.20	526	2988	502	281	3.5	1840	26.8	237.5
30/P2	40	0.88	3.52		583	3288	549	304	3.9	180	28.6	255.3
0.5/0	36+	0.88	3.52		583	3288	549	304	3.9	180	28.6	255.3
0.5/R1	16+	0.77	3.24		229	1290	456	253	3.5	180	26.3	231.4
0.5/P1		0.024	0.027		173	932	42	19	0.1	900	0.7	2.6
0.5/P2	20	0.021	0.024		186	977	44	19	0.1	900	0.9	2.3
0.5/PW	70	0.008	0.021		122	631	27	12	0.1	560	1.5	2.8
0.5/PWC	90	0.009	0.023		141	738	32	15	0.1	760	1.0	1.3
RO/0	90+	0.009	0.023		141	738	32	15	0.1	760	1.0	1.3
RO/R1	20+	0.02	0.045		296	1592	68	31	0.1	1580	0.7	2.1

**Figure 6****Figure 7**

**Figure 8****Figure 9**

**Figure 10****Figure 11**

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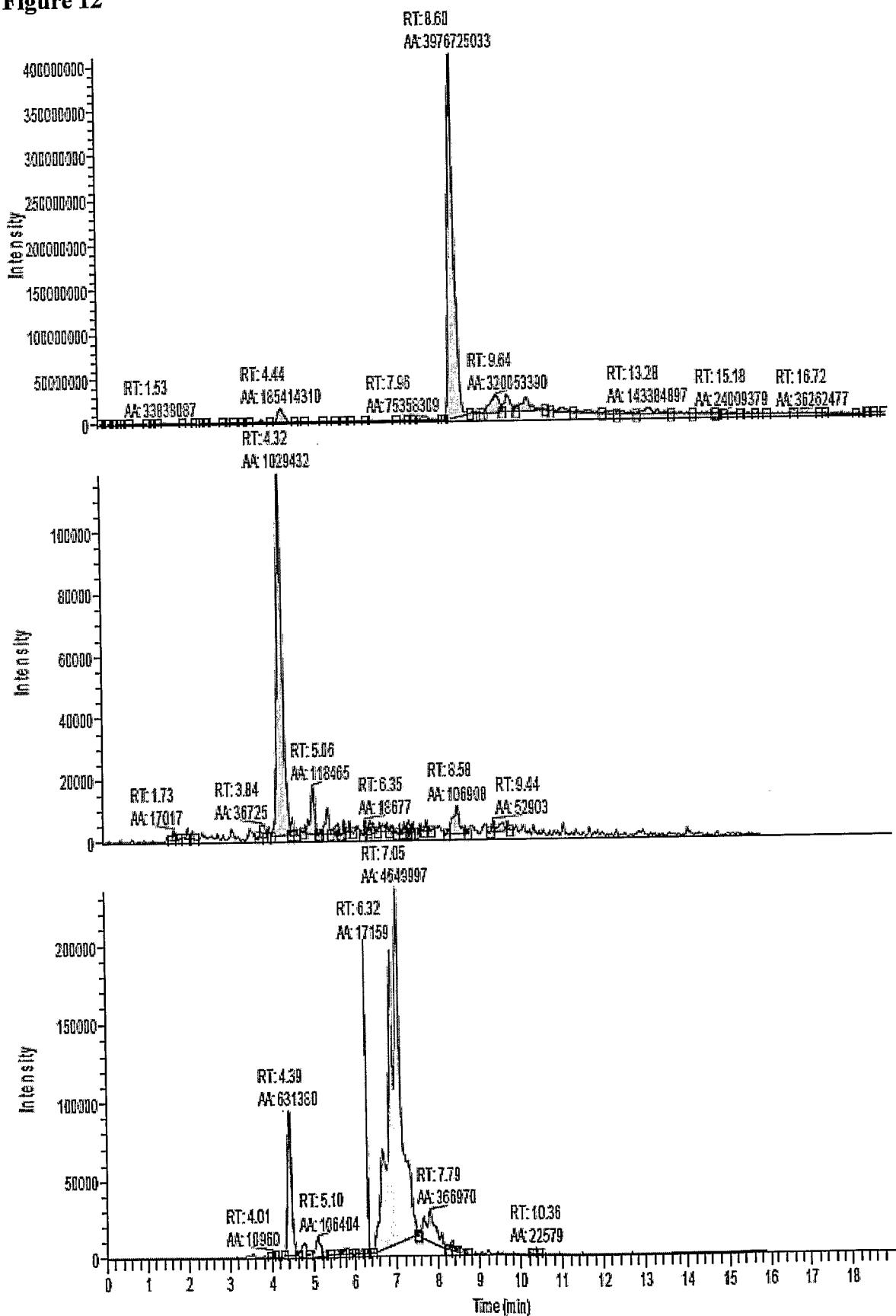
**Figure 12**

Figure 13

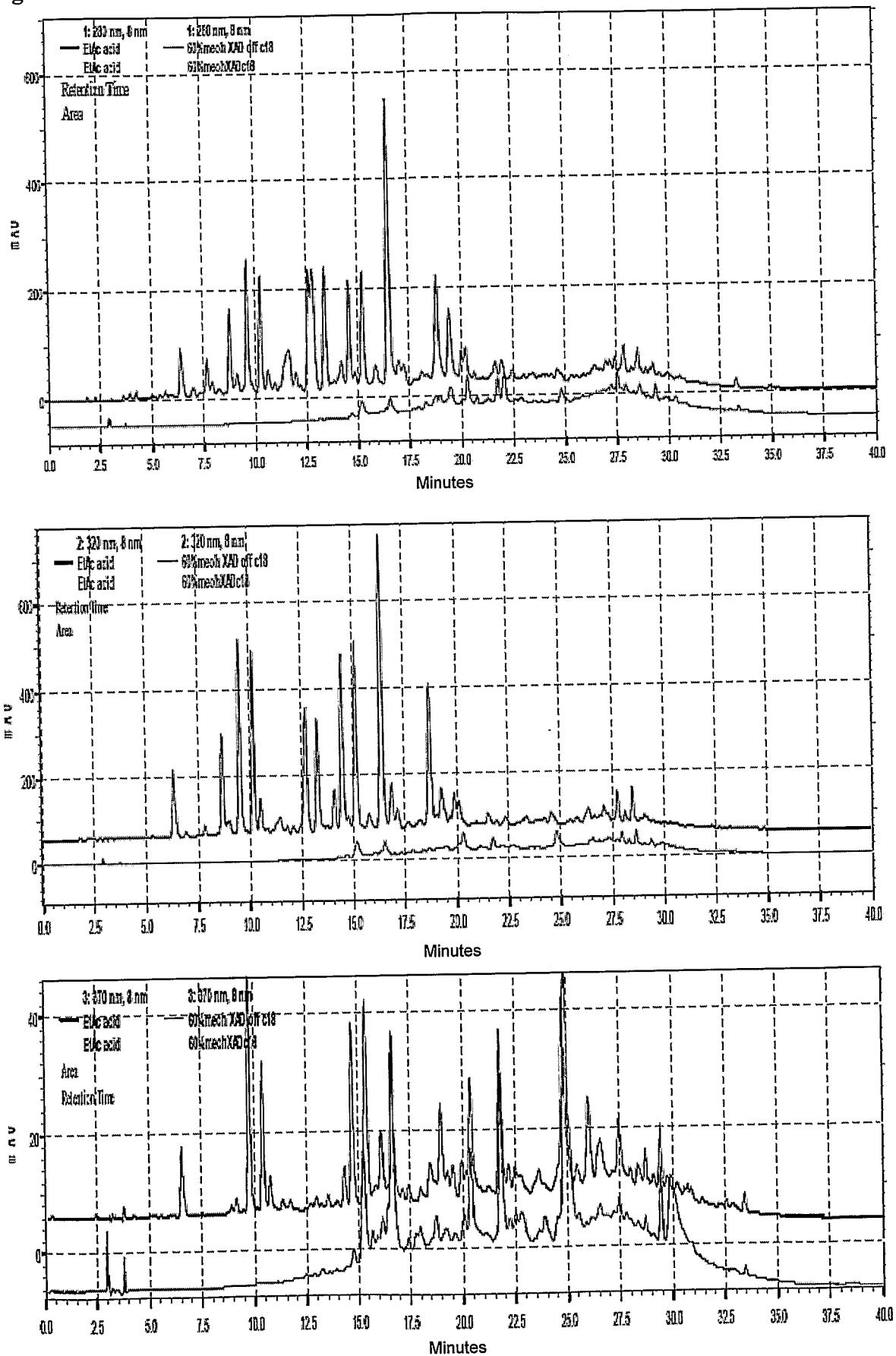
Compound	Sample (fraction) / Amount in sample (mg/Kg) <sup>a</sup>					
	XAD (Acid)	XAD (Basic)	MFP (Acid) <sup>b</sup>	MFP (Basic)	Sugar (Acid) <sup>b</sup>	Sugar (Basic)
Caffeic Acid	168.8	207.4	12.0	9.6	0.6	Not detected
Chlorogenic Acid	368.2	123.1	26.3	2.5	0.7	Not detected
<i>p</i> -Coumaric Acid	1170.9	1253.8	30.9	22.8	2.2	0.2
Ferulic Acid	738.8	724.1	34.7	30.8	0.5	Not detected
Gallic Acid	Not detected	Not detected	4.6	0.8	0.1	Not detected
Syringic Acid	433.5	472.6	85.5	52.3	0.6	Not detected
Vanillic Acid	2.13	Not detected	2.7	Not detected	Trace <sup>c</sup>	Not detected
Apigenin	34.8	Not detected	1.7	Not detected	0.12	Not detected
(+)-Catechin	Not detected	175.2	1.0	7.0	0.22	Not detected
(-)Catechin	Not detected	Not detected	5.9	Not detected	0.40	Not detected
Gallate	Not detected	Not detected	5.9	Not detected	0.40	Not detected
Diosmin	303.6	114.3	2.3	4.1	Trace	Not detected
(-)Epicatechin	168.8	207.4	7.8	6.7	0.4	Not detected
Kaempferol	Not detected	Not detected	0.3	Not detected	Trace	Not detected
Luteolin	18.6	41.3	0.7	Not detected	Trace	Not detected
Quercetin	91.3	137.1	4.7	2.6	Trace	Not detected
Rutin	59.3	59.3	1.8	1.7	Trace	Not detected
<b>Total</b>	<b>3663.8</b>	<b>3515.7</b>	<b>222.8</b>	<b>140.9</b>	<b>6.0</b>	<b>0.2</b>

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Figure 14

	MFP-acid	MFP-base	XAD-acid	XAD-base
	Detected in sample			
Apigenin	Yes	Yes	Yes	Yes
(-)Catechin gallate	Yes	Yes	No	No
Chlorogenic Acid	Yes	Yes	Yes	Yes
Caffeic acid	Yes	Yes	Yes	Yes
Diosmin	Yes	Yes	Yes	Yes
Tricin <sup>a</sup>	Not analysed	Not analysed	Yes	Not analysed
Luteolin	Yes	Yes	Yes	No
Quercetin	Not analysed	Not analysed	Yes	Not analysed
Rutin	Yes	Yes	Not analysed	Not analysed
Syringic acid	Not analysed	Not analysed	Yes	Not analysed
Vanillic acid	Yes	No	Yes	No

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**Figure 15**

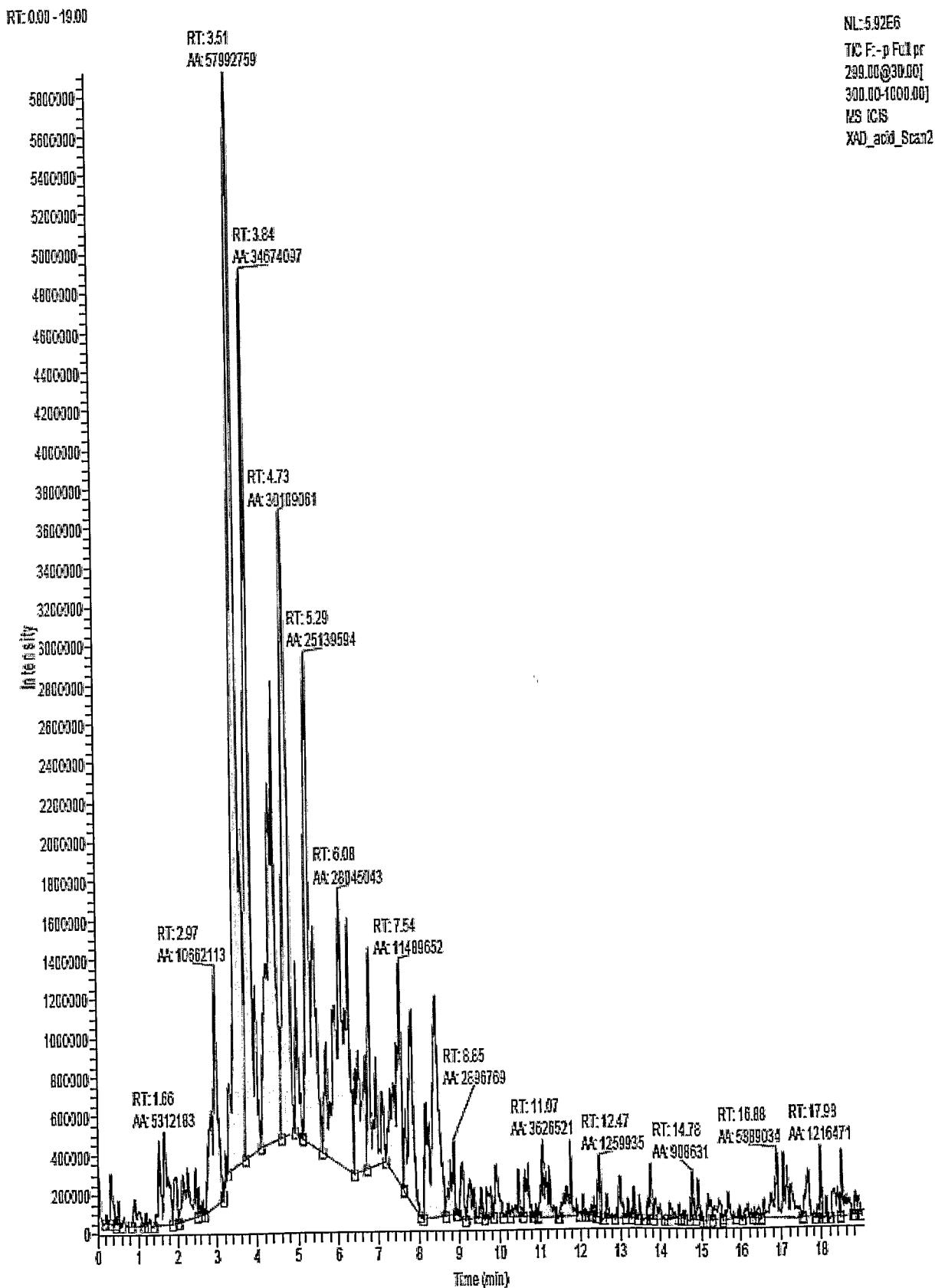
**Figure 16**

Figure 17

Component	Process stream						RS
	FEJ	BT	MMF	MM	ESJ	MOL	
Total solids (g/100g) (filtered)	20.0	14.1	10.7	-	0.29	13.0	7.0
Total solids (g/100g) (unfiltered)	20.4	14.4	11.0	31.2	-	13.0	72.3
Density (g/ml) (filtered)	1.079	1.055	1.044	-	1.000	1.054	1.029
Fructose (g/100g DW) <sup>c</sup>	4.5	3.2	2.0	-	Nil	1.6	2.2
Glucose (g/100 g DW)	4.8	3.8	2.3	-	Nil	1.7	2.1
Sucrose (g/100 g DW)	86	77	76	-	Nil	89	88
Polysaccharides (g/100g DW)	-	0.52	-	4.9	-	-	2.9
Total phenolics (mg catechin equiv/100 g DW)	330	362	402	-	1580	309	391
Antioxidant activity (mg gallic acid equiv/100 g DW)	68.1	75.9	142	-	563	104	115
Fat (g/100 g DW)	-	-	-	3.3	-	-	-
Total N (g/100 g DW)	0.18	0.13	0.14	0.65	0.34	0.11	0.10
Non-protein N (g/100 g DW)	-	-	-	0.07	-	-	0.70
Protein (g/100 g DW) (TN-NPN) x 6.25	-	-	-	3.63	-	-	0.31

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Component	Process stream						RS
	FEJ	BT	MMF	MM	MMF	ESJ	
<b>FA/AA analysis:</b>							
Ca (mg/kg) <sup>d</sup>	45.4	148	542	3547	-	238	1490
Mg (mg/kg)	204	147	185	626	-	111	569
Fe (mg/kg)	127	71.2	142	3958	-	2.49	32.3
K (mg/kg)	1061	880	1290	838	-	777	4122
<b>UQ analysis:</b>							
Ca (mg/kg) <sup>d</sup>		144		3167			8411
Mg		141		721			3221
Na		3.3		26			428
K		857		1446			33679
Cr		0.1		3.6			0
Se		0.3		2.1			3.0
PO4-P		114		1484			676
SO4-S		113		198			3330
Cl		723		30			22716
<i>cis</i> -aconitic acid (mg/100 g DW)		47.5	58.3	n/a	30.7	28.6	170
<i>trans</i> -aconitic acid (mg/100 g DW)		n/a	489	481	n/a	640	515
						1723	1964
							60

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Figure 18

Component	Process stream					
	FEJ	BT	MMF	MM	ESJ	SYR
Total solids (g/100g) (filtered)	-	13.4	13.7	-	0.40	13.5
Total solids (g/100g) (unfiltered)	-	13.8	13.7	23.2	-	13.5
Density (g/ml) (filtered)	-	1.053	1.054	-	1.000	1.053
Fructose (g/100g DW) <sup>c</sup>	-	4.0	1.4	-	Nil	1.5
Glucose (g/100 g DW)	-	4.1	1.3	-	Nil	1.6
Sucrose (g/100 g DW)	-	81	91	-	Nil	89
Polysaccharides (g/100 g DW)	-	0.44	-	6.1	-	-
Total phenolics (mg catechin equiv/100 g DW)	-	349	317	-	1593	296
Antioxidant activity (mg gallic acid equiv/100 g DW)	-	78.0	118	-	514	104
Fat (g/100g DW)	-	-	-	6.2	-	-
Total N (g/100 g DW)	-	0.24	0.18	1.37	0.75	0.16
Non protein N (g/100 g DW)	-	-	-	0.12	-	-
Protein (g/100 g DW)	-	-	-	7.81	-	-
100 (TN-NPN) x 6.25						0.06

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Component	Process stream								
	FEJ	BT	MMF	MM	MME	ESJ	SYR	MOL	RS
<b>FAILA analysis:</b>									
Ca (mg/kg) <sup>d</sup>	-	189	332	6009	-	246	2287	6300	106
Mg (mg/kg)	-	141	134	847	-	102	918	2580	32.0
Fe (mg/kg)	-	71.3	16.1	1734	-	1.73	10.2	154	3.55
K (mg/kg)	-	807	989	621	-	966	9558	23 870	178
<b>UQ analysis:</b>									
Ca (mg/kg) <sup>d</sup>	173		5819				6652	94	
Mg	128		820				2495	31	
Na	3.5		3.9				265	6.7	
K	730		965				26945	255	
Cr	0.1		1.5				0	0.9	
Se	0.5		2.8				4.1	5.3	
PO4-P	101		2962				556	4.7	
SO4-S	115		271				2168	29	
Cl	810		52				19174	28	
<i>cis</i> -aconitic acid (mg/100 g DW)	n/a	39.6	37.2	n/a	50	22.2	126	500	3.7
<i>trans</i> -aconitic acid (mg/100 g DW)	n/a	414	494	n/a	47	482	1966	1688	62.1

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Figure 19

Component	Sample Number						
	1	2	3	4	5	6	7
Total solids (g/100g) (filtered)	14.1	13.4	14.1	13.9	16.7	15.1	17.1
Total solids (g/100g) (unfiltered)	14.4	13.8	14.6	14.3	16.8	15.4	17.2
Density (g/ml) (filtered)	1.055	1.053	1.055	1.054	1.066	1.059	1.067
Fructose (g/100 g DW)	4.5	4.0	3.7	3.7	2.7	2.2	2.0
Glucose (g/100 g DW)	4.8	4.1	4.2	3.9	2.2	2.3	2.2
Sucrose (g/100 g DW)	86	81	81.5	81.0	84.8	82.5	89.7
Polysaccharides (g/100 g DW)	0.52	0.44	0.48	0.46	0.52	0.47	0.35
Total phenolics (mg catechin equiv/100 g DW)	362	349	427	382	414	384	274
Antioxidant activity (mg gallic acid equiv/100 g DW)	76	78	110	85	100	101	72
Total N (g/100 g DW)	0.13	0.24	-	-	-	-	-
cis-aconitic acid (mg/100 g DW)	47.5	39.6	42.8	51	30.7	32	36.2
trans-aconitic acid (mg/100 g DW)	489	414	294	439	351	374	410

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Figure 20

Component	1	2	3	4	5	6	7
Total solids (g/100g)	31.2	23.2	27.0	25.0	24.2	25.7	26.6
Polysaccharides (g/100 g DW)	4.9	6.1	5.9	8.2	9.5	10.8	10.6
Total lipid (g/100 g DW)	3.3	6.2	4.6	5.6	7.4	6.7	7.0

Figure 25

Component	Component fractionation at different pore sizes (%)						
	1000 Da	5000 Da	10 000 Da	30 000 Da	50 000 Da	Perm	
Perm	Ret	Perm	Ret	Perm	Ret	Perm	
Polyphenols	63	41	81	24	72	27	72
Antioxidants	67	39	76	24	69	30	73
Sugars	89	1.1	92	0.55	87	0.59	93
Organic acids	81	1.1	89	0.5	91	0.7	77
Total solids	95	7.6	98	5.0	96	5.9	99

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Figure 21

Component	Sample Number						
	1	2	3	4	5	6	7
Total solids (g/100g) (filtered)	10.7	13.7	13.6	12.4	13.9	14.5	13.0
Total solids (g/100g) (unfiltered)	11.0	13.7	13.7	12.4	13.8	14.5	13.1
Density (g/ml) (filtered)	1.044	1.054	1.054	1.049	1.054	1.057	1.051
Fructose (g/100 g DW)	2.0	1.4	1.4	1.6	1.9	1.7	2.0
Glucose (g/100 g DW)	2.3	1.3	1.5	1.8	1.8	1.8	2.2
Sucrose (g/100 g DW)	76.0	91.0	91.1	91.0	89.9	86.2	84.4
Polysaccharides (g/100 g DW)	-	-	-	-	-	-	-
Total phenolics (mg catechin equiv/100 g DW)	402	317	338	327	326	343	329
Antioxidant activity (mg gallic acid equiv/100 g DW)	142	118	126	131	118	155	135
Total N (g/100 g DW)	0.14	0.18	0.16	0.16	0.14	0.14	0.13
cis-aconitic acid (mg/100 g DW)	58.3	37.2	34	36.5	38.5	39.2	47.9
trans-aconitic acid (mg/100 g DW)	481	494	480	459	466	458	522

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Figure 22

Component	Sample Number						
	1	2	3	4	5	6	7
Total solids (g/100g) (filtered)	0.3	0.4	0.5	0.4	0.4	0.7	0.9
Total solids (g/100g) (unfiltered)	-	-	-	-	-	-	-
Density (g/ml) (filtered)	1.000	1.000	1.001	1.000	1.000	1.002	1.003
Fructose (g/100 g DW)	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Glucose (g/100 g DW)	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Sucrose (g/100 g DW)	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Polysaccharides (g/100 g DW)	-	-	-	-	-	-	-
Total phenolics (mg catechin equiv/100 g DW)	1580	1593	1423	1495	1521	1266	1356
Antioxidant activity (mg gallic acid equiv/100 g DW)	563	514	353	502	616	315	334
Total N (g/100 g DW)	0.34	0.75	0.77	0.45	1.03	0.42	0.33
cis-aconitic acid (mg/100 g DW)	30.7	50	151	51.6	177	147	340
trans-aconitic acid (mg/100 g DW)	640	47	765	409	2450	760	1730

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Figure 23

Component	Sample Number						
	1	2	3	4	5	6	7
Total solids (g/100g) (filtered)	7.7	7.1	7.5	7.9	7.8	7.8	7.7
Total solids (g/100g) (unfiltered)	80.0	73.3	77.8	81.7	80.2	80.6	80.7
Density (g/ml) (filtered)	1.034	1.029	1.031	1.033	1.032	1.032	1.032
Fructose (g/100 g DW)	11.7	12.8	8.9	9.5	13.2	10.8	11.6
Glucose (g/100 g DW)	9.5	9.2	7.5	7.7	9.2	9.3	10.3
Sucrose (g/100 g DW)	42.0	52.0	46.6	45.3	38.2	37.0	38.7
Polysaccharides (g/100 g DW)	2.9	2.4	2.8	3.0	2.9	3.0	3.1
Total phenolics (mg catechin equiv/100 g DW)	2500	2054	2351	2503	2733	2713	2700
Antioxidant activity (mg gallic acid equiv/100 g DW)	726	650	804	864	869	899	873
Total N (g/100 g DW)	0.75	0.68	-	-	-	-	-
cis-aconitic acid (mg/100 g DW)	543	500	597	570	597	589	578
trans-aconitic acid (mg/100 g DW)	1964	1688	1920	2020	1960	2210	2120

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Figure 24

Component	Sample Number						
	1	2	3	4	5	6	7
Total solids (g/100g) (filtered)	35.1	35.2	35.0	35.1	34.9	35.0	35.0
Total solids (g/100g) (unfiltered)	100	100	99.9	99.9	99.5	99.6	99.5
Density (g/ml) (filtered)	1.152	1.151	1.151	1.151	1.151	1.151	1.151
Fructose (g/100 g DW)	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Glucose (g/100 g DW)	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Sucrose (g/100 g DW)	99.3	98.8	99.5	99.4	98.8	99.3	98.9
Polysaccharides (g/100 g DW)	0.053	0.041	0.068	0.044	0.080	0.063	0.091
Total phenolics (mg catechin equiv/100 g DW)	21.1	19.9	22.6	24.8	61.1	43.0	60.0
Antioxidant activity (mg gallic acid equiv/100 g DW)	6.7	6.4	8.3	8.7	21.6	15.5	21.3
Total N (g/100 g DW)	0.01	0.01	-	-	-	-	-
cis-aconitic acid (mg/100 g)	4.1	3.7	4.5	4.1	8.6	7.1	8.3
trans-aconitic acid (mg/100 g)	60	62.1	74	60	177	111	156

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Figure 26

Component	Sample Number								
	1	2	3	4	5	6	7	8	9
Total solids (g/100g)	1.1	1.5	1.4	N/T	0.9	0.8	0.5	0.8	1.2
Brix	1.1	1.4	1.4	N/T	1.0	0.8	0.5	0.8	1.2
Density (g/ml)	1.002	1.003	1.003	N/T	1.002	1.001	1.000	1.001	1.003
Colour (A <sub>420</sub> )	0.5	0.8	0.5	N/T	0.4	0.5	0.4	0.5	0.7
Fructose (g/100 g DW)	0.7	1.0	1.4	N/T	BDL	BDL	BDL	BDL	1.3
Glucose (g/100 g DW)	1.6	1.5	1.3	N/T	BDL	BDL	BDL	BDL	1.2
Sucrose (g/100 g DW)	85.1	80.6	71.0	N/T	88.5	69.3	75.3	71.7	74.7
Total phenolics (mg catechin equiv/100 g DW)	729	629	752	N/T	712	855	985	1093	687
Antioxidant activity (mg gallic acid equiv/100 g DW)	297	318	327	N/T	340	427	403	452	304

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2007/001382

## A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

**C13D 3/16 (2006.01)**

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)

IPC<sup>8</sup>: C13D/-, C13J/-, A23L/-, C09K 015/-

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)

Derwent WPI: IPC<sup>8</sup> as above and ((molass+ or sugar+ or cane+ or sorghum+) and (+phenol+ or +oxidant+ or low or glycemic) and (wast+ or +produc+) and (+filt+ or +frac+ or separate+))

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2005117608 A1 (Horizon Science Pty Ltd) 15 December 2005 Whole Document	1 to 28
A	US 2003/0198694 A1 (Chou) 23 October 2003 Whole Document	1 to 28
A	US 5454875 A (Clarke) 3 October 1995 Whole Document	1 to 28
A	WO 2001/036690 A1 (Tate & Lyle Industries Ltd) 25 May 2001 Whole Document	1 to 28

 Further documents are listed in the continuation of Box C See patent family annex

* Special categories of cited documents:		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search  
08 October 2007Date of mailing of the international search report  
10 OCT 2007Name and mailing address of the ISA/AU  
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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2007/001382

C (Continuation).		DOCUMENTS CONSIDERED TO BE RELEVANT
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 1997/049734 A1 (Societe des Produits Nestle S. A.) 31 December 1997 Whole Document	24 to 28
A	WO 2003/075685 A2 (Council of Sci & Ind Research) 18 September 2003 Whole Document	24 to 28
A	WO 2003/099309 A1 (MD Bioalpha Co Ltd) 4 December 2003 Whole Document	24 to 28
A	EP 1466609 A1 (Archer-Daniels Midland Co) 13 October 2004 Whole Document	24 to 28

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/AU2007/001382

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

2.  Claims Nos.:

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.  Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application; as follows:

See Supplementary Sheet

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2.  As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/AU2007/001382

**Supplemental Box**

(To be used when the space in any of Boxes I to VIII is not sufficient)

**Continuation of Box No: III**

Claims 1 to 13 are directed to an extract derived from sugar cane having reduced GI or burn rate characteristics.

Claims 14 to 24 are directed to a process for producing an extract from sugar cane.

Claims 25 to 28 are directed to a method for refining a phytochemical extract

These groups of claims are not link by a single inventive concept nor is there any common novel element.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2007/001382

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report			Patent Family Member				
WO	2005117608	AU	2005249147	AU	2007100846	CA	2568775
		EP	1781116				
US	2003198694						
US	5454875						
WO	0136690	AU	12902/01	BR	0015571	CA	2390860
		EP	1230401	MX	PA02004621	US	6355110
		US	6406546				
WO	9749734	AU	34361/97	EP	0912614		
WO	03075685	AU	2003209674	CN	1649513	EP	1489924
		US	7070814	US	2004052873	US	2006141080
		ZA	200407968				
WO	03099309	AU	2003231392	AU	2003231394	CA	2486915
		CN	1842341	EP	1507544	KR	2003009166
		KR	2003009176	KR	2005010543	KR	2005011207
		US	2006034951	WO	03099308		
EP	1466609	AU	87879/98	BR	9805069	CA	2249501
		EP	0795553	EP	0906761	HK	1016879
		IL	120409	IL	130611	JP	10023878
		JP	11221048	KR	2001007108	NO	984591
		NZ	332131	US	5702752	US	5792503
		US	6033714	US	6171638	US	6261565
		US	6391308	US	6391309	US	6391310
		US	6395279	US	6399072	US	6509381
		US	6518319	US	6565912	US	6900240
		US	2002168433	US	2002187211	US	2003003168
		US	2003064938	ZA	9808962		

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX