The study & development of an integrated & additive-free waste orange peel biorefinery.

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Abstract

The overall aim of this thesis is to demonstrate how food supply chain waste (FSCW) could be used as a renewable feedstock for a product-focused biorefinery. The volumes of FSCW produced as a result of common food processing operations have been estimated using a new methodology. The latter is based on sold manufactured goods published every year by the European Union (PRODCOM) and highlights the geographical waste hot spots for common food processing operations. The use of this methodology led to the selection of waste orange peel (WOP) as a raw material for the design of an integrated biorefinery. A SWOT analysis was carried out to analyse the opportunity for the design of a WOP valorisation process aimed at the extraction of a maximum of the chemical components, while avoiding the use of acid, additives or pretreatment. The successful development of a microwave biorefinery process centred on the valorisation of WOP was designed based on the integration of microwave assisted *D*-limonene extraction and a low temperature hydrothermal extraction of pectin.

The work carried-out using microwave hydrothermal treatment proved that pectin can be extracted between 100 and 150 °C under acid-less conditions on a 100 mL scale. A competitive molecular weight distribution was obtained for pectin produced at a temperature of 110 and 120 °C using WOP from which *D*-limonene, flavonoids and sugar had been previously extracted. A CPMAS ^{13}C-NMR technique was developed to determine the degree of esterification of pectin. *D*-limonene was extracted under microwave-assisted solvent-less conditions. The optimised conditions studied led to a yield of 1.09 ± 0.18% food grade *D*-limonene with a high reproducibility on a 1 L scale (500 mbars). Finally, the process used also allowed for the isolation and characterisation of flavonoids and monosaccharides. Following ethanol extraction, a mixture of polymethoxy flavonoids (tetra-O-methyl scutellarein, tangeritin, nobiletin and heptamethoxyflavone), hesperidin and sugar monosaccharides were obtained with yields of 5.87%, 0.24% and 18.35% respectively on a wet basis. The later fraction was composed of 74.21% glucose and 25.35% fructose, as determined by quantitative ^{13}C NMR, showing the potential for this fraction as a fermentation feedstock.

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Author's declaration

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Chapter 1
Introduction

L.A. Pfaltzgraff, J.H. Clark, *in Advances in Biorefineries*, eds K. W. Waldron, Woodhead Publishing, 2014, ch. 2, pp. 3-33.

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1 Introduction

Today fossil fuel resources supply 86% of our energy and 96% of organic chemicals.[2] However, they are not renewed in a time interval relevant to our resource utilisation: according to our actual consumption, the future petroleum production is unlikely to meet our society's growing needs. By 2025, our energy demands are expected to increase by 50%.[3] Green chemistry is an area which is attracting increasing interest as it provides unique opportunities for innovation via the use of clean and green technologies, product substitution and especially the use of renewable feedstocks such as dedicated crops or food supply chain by-products for the production of bio-derived chemicals, materials and fuels.[4] This chapter will provide an introduction to the concepts of green chemistry and the biorefinery, the use of waste as a biorefinery feedstock and the role of clean technology such as microwave technology followed by a discussion of the core concepts behind microwave heating in the area of biomass processing.

1.1 Green chemistry and the biorefinery

1.1.1 The 12 green chemistry principles

"Green chemistry is the design of chemical products and processes that reduce or eliminate the use and generation of hazardous substances".[5, 6] The concept emerged 20 years ago with the introduction by Paul T. Anastas and John C. Warner of the 12 principles of green chemistry (see Table 1).

Table 1: The 12 Green Chemistry Principles.[5, 6]

1. Prevention It is better to prevent waste than to treat or clean up waste after it has been created.	**7. Use of Renewable Feedstocks** A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable
2. Atom Economy Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.	**8. Reduce Derivatives** Unnecessary derivatisation (use of blocking groups, protection/ deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.
3. Less Hazardous Chemical Syntheses Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment.	**9. Catalysis** Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
4. Designing Safer Chemicals Chemical products should be designed to effect their desired function while minimizing their toxicity.	**10. Design for Degradation** Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.
5. Safer Solvents and Auxiliaries The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used.	**11. Real-time analysis for Pollution Prevention** Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
6. Design for Energy Efficiency Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.	**12. Inherently Safer Chemistry for Accident Prevention** Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

Efficiency being the key, the subject continues to develop strongly around the following principles in its pursuit of more sustainable processes: [7,8]

- maximum conversion of reactants into a targeted product,
- minimum waste production through improved reaction design,
- the use and production of non-hazardous raw materials and products,
- safer and more energy efficient processes and
- the use of renewable feedstocks.

1.1.2 Green chemistry and Industry

According to these principles, in an ideal case, a reaction would only produce the targeted product. Waste and pollutants would be prevented, improving the reaction yield and reducing losses, thus improving the overall economics of a process. Since our society and industries are governed by increasing efficiency and profit, green chemistry should theoretically fit every manufacturing company's agenda, not only appealing to chemical producers.

Today, the principles of green chemistry are still as meaningful as 20 years ago given the increasing interest this area of research attracts due to concerns over sustainability.[9] The implementation of REACH legislation (Registration, Evaluation, Authorization and restriction of Chemicals or directive EC 1907/2006), ROHS legislation (Restriction of the Use of Certain Hazardous Substances in Electrical and Electronic Equipment or directive 2003/108/EC) together with other initiatives highlighting the hazardous character of some chemicals used in day-to-day consumer products such as the SIN list,[10] are pushing hard for their replacement to avoid harm to human and/or environmental health. However, the substitutes used need to be genuinely safer across the whole life cycle, whilst also being as effective as their unsafe alter-ego. Investing in research and development focused on identifying truly greener substitutions, thus eliminating rushed and weak alternatives which often contain a greater number of formulation components to compensate for the lower performance of a given "greener" formulation. The same applies to the substitution of fossil-derived chemicals with more sustainable bio-derived chemicals. When using renewable feedstocks such as biomass, we have to use clean and efficient synthetic routes, minimising the amount of unwanted by-products and the use of scarce resources (i.e. scarce metals).[11] Water available for

industrial operations is increasingly seen as a scarce resource but its use as a green solvent is increasingly envisaged due to its non- toxicity (amongst other benefits) in comparison to hydrocarbon-based solvents.[12] Nevertheless, contaminated water is difficult, energy consuming and expensive to treat and re-use.

Biodegradability is an important characteristic for "greener" products, but increasing the life-time of a molecule to promote its re-use could be another strategy, allowing for each molecule produced by nature to be used to its full potential.[13]

Re-using and valorising resources available, including waste, is an area gaining in momentum. One of them is food supply chain waste (FSCW), which is a feedstock rich in functionalised molecules.[4] Although it is biodegradable, it should be valorised as a source of renewable chemicals, materials and bio-fuels, leading towards waste minimisation and waste valorisation. Wasting resources should be avoided in any optimised process.

The different strategies highlighted in Figure 1 show how important it is to assess the greenness of a process through every single step, including the upstream and downstream stages of production, raw material employed, its use, end-use and disposal and as a result, guaranteeing the true sustainability of a product.[14]

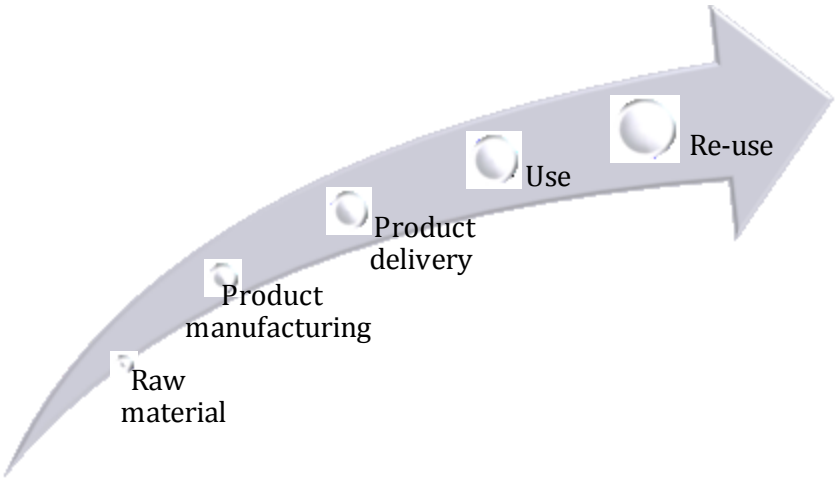


Figure 1: Illustration of a product's life-cycle.

One change can affect several of these steps and it is important to assess a process through its full life cycle however time consuming and data dependent this is. Life cycle assessment can help assess the use of bio-processes versus chemo-processes for example. Many believe bio-processes are preferable to chemo-processes as they

are superior in terms of environmental impact as they use non-toxic enzymes to selectively catalyse a reaction and yield the targeted product. Nielsen *et. al.* have highlighted how the production of enzymes is energy and resource intensive too, showing enzymatic processes are not necessarily the greenest option for the manufacturing of chemicals even though they represent a product selective process.[15]

1.1.3 Drivers for change

Current manufacturing practices are strained by a number of factors, most significant is the increasing price of feedstocks such as oil.[16] This results in increasing costs of energy and petrochemicals. There is also the increasing cost of waste treatment and/or disposal.

The increasing impact of legislation is also affecting almost all aspects of manufacturing supply chains (e.g. what can be made, the supply of raw material, manufacturing, end-use and disposal stages). It now affects the type of process, process steps, emissions, end treatment of waste, meaning every stage of the supply chain of a chemical product now has to be the least polluting possible (i.e. Integrated Pollution Prevention and Control legislation, IPPC).[17] New regulations such as REACH have had an important impact on the industrial sectors like home and personal care products, the pharmaceutical industry and the agricultural sector.

Resource acquisition is a stage in the product life cycle where green chemistry can have a major impact in the future. The use of renewable resources, typically biomass, instead of finite resources is now recognised as more environmentally sound. Biomass is a resource which can be renewed within a time interval which can compete with our resource consumption (as described in Figure 2), biomass being a "biological material derived from living, or recently living organisms".[18] [16]

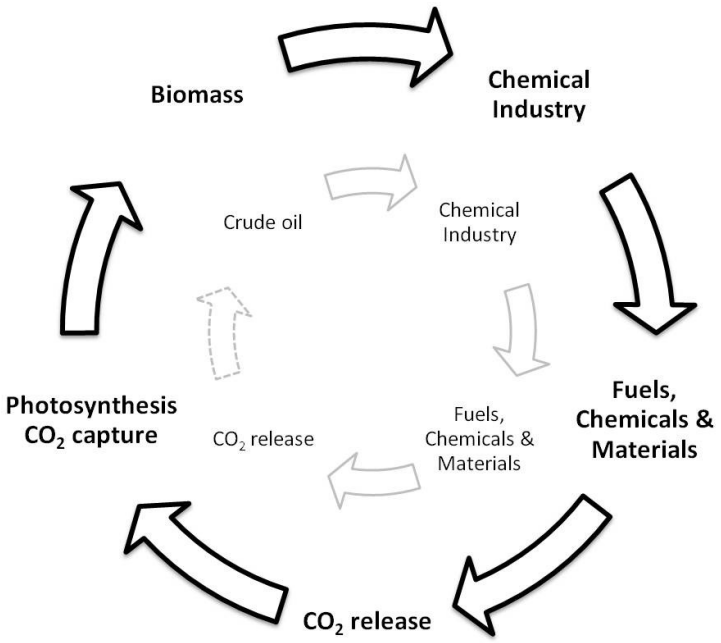


Figure 2: Compared production cycles of chemicals derived from biomass and oil.

The emergence of E.U. standards for bio-based products (Mandate M/429) will, in the near future, embrace life cycle considerations and introduce specifications along the entire supply chain for new and existing products. This should have the effect of further lowering the use of fossil fuel resources in favour of renewable feedstocks such as biomass including bio- wastes.

The public, and consequently the retail sector, have increasingly been made aware of the issues associated with the use of some unsafe chemicals in consumer product formulations (i.e. volatile chlorinated compounds used in dry cleaning, sulfonated surfactants, polybrominated compounds in flame retardants). Manufacturers have been increasingly pressured to produce bio-derived chemicals and reduce the environmental impact of their production. This change in consumer behaviour led to the marketing of a greater number of green and renewable alternatives in many sectors, especially in home and personal care products.

Furthermore, the European Commission proposed further steps for the creation of lead markets in 2006 with the introduction of a new policy aimed at supporting the development of high economic- and societal-value markets. Bio-based products are the subject of one of the identified lead markets and fall into this category for several reasons:[19]

- "use of renewable and expendable resources,

- less dependency on limited and increasingly expensive fossil resources,
- the potential to reduce greenhouse gas emissions (carbon neutral/low carbon impact),
- the potential for sustainable industrial production,
- increased industrial competitiveness through innovative eco-efficient products,
- support to rural development,
- potentially improved population health,"[19] and
- potential for transfer to other regions of the world including the transfer of appropriate technologies discovered and proven in the E.U.

All the above arguments are believed to catalyse the growth of the bio-economy in Europe in the future.

1.1.4 The bio-based economy

A recent study estimates that by 2025, over 15% of the $3 trillion global chemical market will be derived from bio-derived sources.[20] Yet in 2011, the technical feasibility of substituting over 90% of the annual global plastic production (270 million tonnes) by bioplastics had already been highlighted.[21] "In 2005, bio-based products already accounted for 7% of global sales and around €77 billion in value in the chemical sector, with the E.U. industry accounted for approximately 30% of this value."[22] Estimates of the ad-hoc advisory group for bio-based products have identified "polymers & fibres, active pharmaceutical ingredients, cosmetics, organic chemicals and detergents as the most important segments."[19] Overall, the market for bio-derived products has been predicted to be worth €515 billion in 2020 in Europe.[23]

1.1.5 Product-focused biorefinery

It is crucial to ensure that the resources and the process technology used as well as the products made are environmentally acceptable. The 20th century saw the

development of processes designed for the production of energy and organic chemicals based on the oil refinery. The 21st century must see the development of similar processes based on the use of biomass. This is especially relevant as improvements of today's modern formulating-based industries at the production stage of the life-cycle, is restricted (although moving towards renewable energy and zero waste is important and not trivial), the use of renewable feedstocks could offer an important margin for progress, especially for companies, such as fast moving consumer goods manufacturers, keen to dramatically improve the environmental performance (and decrease the CO_2 emissions) of their products.

A biorefinery is an analogue to the current petro-refinery, in the sense it produces energy and chemicals. The major difference lies in the raw material it will use, ranging from biomass to waste. The use of clean technology is another imperative for the biorefinery, ensuring its output(s) are truly sustainable, following LCA analysis. The IEA Bioenergy Task 42 defines biorefining as "the sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals and/or materials) and bioenergy (biofuels, power and/or heat)".[24] Various biorefinery designs varying in size and output number will emerge commercially in the future,[25] taking advantage of flexible technology, helping the concept of a biorefinery to process locally available biomass to its fullest extent in an integrated fuel-chemical-material-power cycle. This will help to improve cost-efficiency, the quality of life of the local population and lowering the environmental impact governed by the three dimensions of sustainability: environmental protection, social progress and economic development (see Figure 3). Flexibility is the key for a successful biorefinery as maximum throughput will only be achieved if they are adapted to the best technology for the feedstock available in a given geographical location.

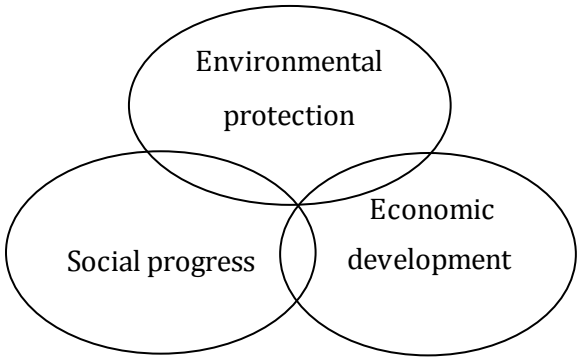


Figure 3: The three cornerstones of sustainability.

The aim is to design an integrated process capable of generating a cost-effective source of energy and chemical feedstocks using biomass as a raw material. The chosen biomass should be available in high quantities and of a low cost, non-food type. Further selection criteria will be highlighted in chapter 2. It should be processed using green chemical technology; ensuring products obtained are truly green as well as sustainable. Technologies used should ideally be flexible enough to accommodate the natural variation of biomass associated with season or variety change.[26] The efficiency of the process needs to be maximal: ideally every out-put has to have a use and a value/market. Practices based on industrial symbiosis looking at re-using the waste produced by one process to feed another one located near-by, converting waste into useful by-products with a marketable value represent an ideal scenario. The aim would be to achieve a zero waste biorefinery capable to compete economically with traditional oil-based refineries. Such a model has successfully been applied in Kalundborg, Denmark. There, a power station, a plasterboard factory, a pharmaceutical plant, an enzyme producer, an oil refinery, a waste company and Kalundborg Municipality exchange steam and water resources.[27]

1.1.6 Technologies used

Adding value to every output of the biorefinery can be achieved by combining several technologies together, using a sequential approach to extract chemicals before biomass is converted to energy.

The main green extraction processes used to extract valuable compounds from biomass include: liquid and supercritical CO_2, ultrasonic or microwave assisted extraction and accelerated extraction.[28] Microwave-assisted extraction is a commercial reality with Crodarom® using this technique to extract purer and more degradation stable plant materials.[29] The extraction can be followed by biochemical or thermochemical processes and internal recycling of energy and waste gaseous effluents. This approach ideally constitutes an economically sound starting point for the design of a biorefinery (Figure 4).

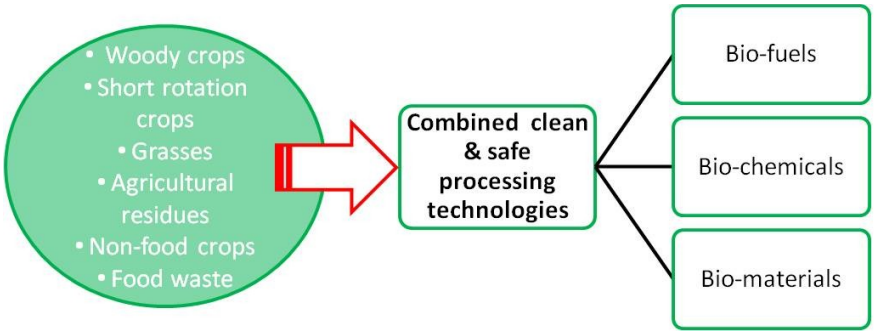


Figure 4: Scheme describing an integrated biorefinery as a mixed feedstock source of chemicals, energy, fuels and materials.

Biomass contains an array of functionalised molecules, many of them having a market value. Compounds such as natural dyes or colorants (i.e. carotenoids), polyphenols, sterols, waxes, nonacosanol or flavonoids (i.e. hesperidin), amino acids and fatty acid derivatives can be extracted selectively using clean extraction techniques prior to the treatment of biomass by biochemical and thermochemical processes. These compounds have known uses in cosmetics, as nutraceuticals or semiochemicals.[13, 30]

Biochemical and thermochemical processes complement each other well, the former being very selective but slow compared to the later. Biochemical processes require low temperatures but pre-treatments are often required (e.g. ammonia fiber expansion or AFEX, dilute acid hydrolysis) to open-up biomass's fibre structure and yield intermediates requiring further downstream processing.[31, 32] Thermochemical processes, which include gasification, pyrolysis and direct combustion (Table 2), operate usually above 500 °C and are much less selective, yielding oils, gas, chars and ash.[33]

Table 2: Most common thermochemical and biochemical processes.

	Process name	Temperature (°C)	Conditions	Product(s)	Application
Thermochemical processes	Gasification	700	Low oxygen level	Syngas (mixture of H_2, CO, CO_2, CH_4)	Fuel or chemical intermediate to ethanol or dimethyl ether or isobutene
	Pyrolysis	300-600	No oxygen	Bio-oil, char and low molecular weight gases	Transportation fuel and chemicals
Biochemical processes	Fermentation	5< T °C <30	Presence of oxygen	Alcohol (e.g. ethanol), organic acids (e.g. succinic acid)	Transportation fuel (e.g. ethanol)
	Anaerobic digestion	30-65	No oxygen	Biogas (CO_2, CH_4)	Production of natural gas (>97% CH_4)

Biomass with a high acid, alkali metal and water content can be difficult to use in conventional thermal treatments. For example, the high water content can render pyrolysis or gasification processes very difficult and the acidity of the feedstock can hinder the use of the pyrolysis oil obtained. Microwave technology for the pyrolysis of straw was shown to improve the quality of bio-oils obtained at lower temperatures (typically below 200 °C), yielding bio-oils with properties comparable to commercial fuel additives with the advantage that the microwave bio-oils have a lower oxygen, alkali, acid and sulphur content.[34]

1.1.7 Waste as a renewable feedstock

Early research on renewable resources focused greatly on plants such as rapeseed, corn or sugar cane. However the controversial competition between food and non-food uses of biomass had an negative effect on crop prices as well as on the public

perception on biofuels.[35] Other sources of biomass are now studied and waste is increasingly considered as another renewable feedstock for the production of bio-derived chemicals, materials and fuels.

In times which increasingly value resource efficiency, waste has become a luxury., The Department of Environment, Food and Rural Affairs (DEFRA) in the UK, has estimated that businesses could save up to £23 billion by re-using resources more efficiently.[36] In the E.U., the Landfill directive or Council Directive 99/31/EC, will drastically reduce the amount of landfill space available as the amount of biodegradable waste sent to landfill will have to reach 35% of 1995's level by 2016 in members countries. As a result, the landfill gate fee has increased from £40-£74 to £90-£110 per tonne (including landfill tax) in the UK between 2009 and 2014.[37-39] Policy makers support alternatives to landfill (e.g. value recovery from waste), especially in the context of achieving a zero waste economy and the vision of the European Bioeconomy 2030 within the context of a resource crisis. [38] To illustrate this argument, it has been estimated that "30% fewer resources [are needed] to produce one Euro or Dollar of GDP than 30 years ago; however, overall resource use is still increasing […] as we consume growing amounts of products and services".[40] As traditional resources such as oil and minerals become scarcer, their availability is likely to be susceptible to highly politicised negotiations and pricing.

Therefore, waste valorisation represents a promising research topic from both an environmental and economic point of view.[41] Current waste management practices should be replaced by strategies allowing the recovery of marketable products for existing or new markets, thus offering added revenues for industry. Valorising waste also has the potential to lower a process's carbon footprint and dependence on fossil fuel resources, increase its efficiency and cost-effectiveness and moving towards 'closed loop manufacturing', one of E.U.'s clear strategies, highlighted in the Europe 2020 strategy document.[42,44] There is a growing recognition that the twin problems of waste management and resource depletion can be solved together through the utilisation of waste as a resource. However, despite the clear benefits, the utilisation of FSCW represents a challenge.[43] A regular and consistent supply chain is important for the successful realisation of a biorefinery based on FSCW. But high accumulative volumes of waste are often only generated over a period of a couple of months per year, potentially affecting the year-round availability of chemicals and materials produced from FSCW.

Although the availability of some FSCW is clearly an advantage regarding security of supply, several limitations exist and need to be taken into account as part of the logistics needed to valorise this resource. FSCW can be/can have:

- a heterogeneous and variable composition (lipids, carbohydrates, proteins),[44]
- fluctuate in volumes available across the year,[44]
- a high water content and[41]
- a low calorific value.[41]

The later characteristics of FSCW can rapidly become a disadvantage when trying to select a suitable technology for valorisation purposes, especially when dealing with high moisture contents. Microwave technology represents an ideal choice given its good interaction with water-rich biomass feedstocks.[45, 46] Biomass can be used wet, by-passing drying operations. The latter is critical given the high costs and energy requirements of drying operations. Proving the suitability of FSCW as a valuable renewable material was one aim of this project. Hence, microwaves have been selected as the core technology for this project.

1.2 Fundamentals of microwave technology

The microwave frequency and the magnetron were first used for radar technology during Second World War. After the end of the war, this technology was used as a source of heat to avoid limitations linked with the use of induction heating (i.e. thermal diffusivity and surface temperature). Microwaves are essentially a high frequency alternating wave produced by the combination of an electrical field and a magnetic field, resulting in an electromagnetic wave. For microwaves, the frequency of this wave must be between 0.3 GHz and 300 GHz.[47] Above 300 GHz, it is the domain of infra red and below 0.3 GHz, it is the domain of radio waves according to the electromagnetic spectrum. The Intercontinental Telecommunications Union limits the frequency of microwave apparatus to 915 and 2450 MHz for industrial, scientific and medical use.[47] The relationship between the energy E and the frequency ν of a microwave is given by the Planck equation and is highlighted below, where h is the Planck constant, c the speed of light and λ is the wavelength.[47]

$$E = h\nu = \frac{hc}{\lambda}$$

1.2.1 Heat production mechanisms under microwave conditions

Heat production within a given substance using microwaves is the result of this substance's dielectric properties. Upon polarisation of a dielectric material following the application of an electric current, the molecule's permanent dipole tries to align itself with the oscillating microwave applied. The high specific frequency used creates a rapid reversal of the electromagnetic wave which is difficult to follow for the permanent dipole. A delay in alignment is observed and energy is lost from the molecule to the surroundings under the form of kinetic energy to re-orient the molecular dipole. This creates heat via friction at the molecular scale of the material.[48] Heat generation is therefore the result of the energy released by the molecule to re-orient its dipolar moment. This heat generating mechanism is called the dipolar polarisation mechanism and is illustrated in Figure 5 for water.[49]

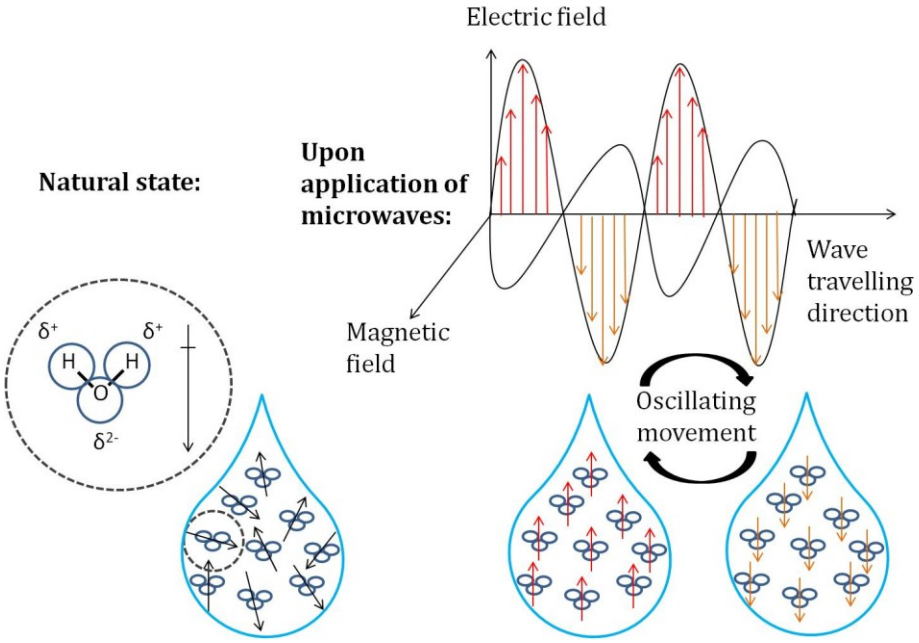


Figure 5: Illustration of the effect of microwaves on a drop of water.

This mechanism can only be applied to polar polarisable substances. The capacity of a substance to be polarised is characterised by its dielectric properties which are summarised in the equation below for the complex dielectric constant ε* in an alternating electrical field.[47]

$$\varepsilon^* = \varepsilon' - j\varepsilon'' = \varepsilon_0\varepsilon' - j\varepsilon_0\varepsilon''$$

In the equation above, ε_0, ε' and ε'' respectively correspond to the dielectric permittivity of the vacuum (which is equal to a constant), the relative dielectric constant and the relative dielectric loss. The relative dielectric constant ε' relates to a material's capability to store electromagnetic energy (i.e. be polarised). ε' is a real number and is a function of the permittivity of free space ε_0.[50] The relative dielectric loss (or loss factor) ε'' relates to a material's capability to convert the stored electromagnetic energy into heat.[48, 50, 51]

They are specific for a material in a given state, microwave frequency and temperature.[50] The ratio $\varepsilon''/\varepsilon'$ is called the loss tangent tan δ or the dissipation factor. It characterises the ability of a substance to absorb the electrical field and convert the electromagnetic energy into heat for a given frequency and temperature.[52] The value of the loss tangent for a given material is proportional to its ability to absorb microwaves.[49] Consequently, as the dielectric loss decreases with an increase in temperature, microwave absorption decreases and penetration depth increases.[52] For glass, a microwave transparent material, tan δ is inferior to 0.01.[53] In the case of water, the dielectric loss of water decreases as temperature increases,[54] increasing the penetration depth of microwaves which is interesting when operating at higher scale. Consequently, it is interesting to note that the value of the loss tangent are inversely proportional to the values of the temperature.[49] Furthermore, the loss tangent is proportional to ion concentration, ion size, microwave frequency and the viscosity of the reaction medium.[52]

Following energy adsorption, the conversion of electromagnetic energy into heat is characterized by the dielectric power absorption P, which is expressed as a function of the relative dielectric loss ε''.

$$P = 2\pi f \varepsilon'' E^2$$

In the equation above, f and E correspond respectively to the applied frequency and strength of the electric field applied to the substances heated. The latter is only applicable to uniform electrical fields.

A different heat generating mechanism has been identified. It applies to ion containing solutions only though. Upon oscillation of the charged ions of the solution following their interaction with microwaves, the incurred displacement of the charged ions generates an electric current. Hence heat is generated within the bulk of the solution as an energy dissipation mechanism. This mechanism is called ion

conduction and in an ion containing solution, its contribution is more important than the one of the dipolar polarisation mechanism.[48, 49, 55]

1.2.1.1 Single and multimode microwaves

Multimode and single mode microwave equipment is available. The choice lies upon design constraints. A single mode microwave relies on the production of a free-standing wave which is directed towards the sample using a waveguide. Care is taken to design the microwave cavity to produce a standing wave (i.e. the incident and the reflected wave resonate producing a standing wave). This makes the position of the sample inside the microwave cavity crucial. In the case of a multimode microwave, a mode deflector is used to produce reflected waves which can either be constructive or destructive. This results in a less homogeneous field of irradiation in the case of multimode microwaves. Hot and cold spots or high and low field intensity spots can then occur, leading to temperature gradients.[56] However this phenomenon can also be observed when using single mode microwaves. Typically, single mode microwave reactors can only be applied to samples of a volume below 50 mL.[49] This is because at 2450 MHz, the wavelength of a single mode (half a wavelength) is of ~ 6 cm, making scale-up of mono-mode microwaves difficult.[57]

1.2.1.2 Temperature control and super-heating

Conventional and microwave heating essentially differ by their temperature profile. Fast heating of the bulk of the targeted material is one of the main advantages of microwave technology.[49] This property is often used under closed vessel conditions, to heat a solvent past its boiling point.[56] "Superheating" has been known to occur in the case of microwave heating: as the temperature increases, the value of the loss tangent increases too, causing the apparent boiling point of a given solvent to increase by up to 28 °C.[55]

Under such conditions, efficient stirring is a necessity to ensure maximum reproducibility. [49] This is to avoid the formation zones of heterogeneous field which would result in the creation of temperature gradients within the sample causing "hot spots" or "super-heating".[52, 58] Additionally, providing temperature control is precise and efficient. Microwaves instantly respond, allowing fast power changes to

achieve constant temperature settings, which is an advantage compared to convection heating for example. Narrow and optimum temperature ranges are hence, easily accessible when using microwave technology. As a result, the "wall effect" observed under conventional heating conditions can be avoided, which is not the case when using conventional heating on a batch process.[56] However, the geometry of the microwave cavity and the algorithm used to control the operational parameters used can cause a certain variability of the results when carrying-out the same experiments using different microwave equipments.[49]

1.2.1.3 Application of microwave technology at larger scale

Achieving precise temperature control is an important area of research when considering microwave technology. The determination of the exact temperature within the bulk of the sample is still discussed within the research community especially as the existence of a non-thermal "microwave effect" is still waiting to be proven. With the combined use of an external and an internal probe (i.e. typically an infra red and a fiber optic probe), a consensus has been reached within the research community.[58] Such a set-up also provides accurate readings in the context of energy consumption measurements, especially when the microwave absorbing properties of the sample change upon heating. However, this solution only provides bulk temperature readings, hence short term localised hot spots would not be detected.

When applied at a larger scale, the efficiency of the energy transfer is critical to ensure the best end-performance, rendering temperature measurement secondary. The measure of reflected energy then becomes essential, helping to determine how much energy is actually absorbed by the system. However, other parameters such as microwave penetration depth or the variation of the sample's viscosity or dielectric properties are to be considered too. Often, microwave constructors will build specific equipment operating at a precise frequency to allow for a maximised energy transfer for a given application.[56] Some benefits are associated with the scale-up of microwave technology. It is known that the penetration depth increases as the dielectric loss decreases, which is interesting at larger scale. However, the penetration depth of the microwaves decreases with the ability of the system to absorb microwaves.[56] Typically, microwaves have a penetration depth of 1.4 cm in water at 25 °C and 5.7 cm at 95 °C (both at 2450 MHz), which needs to be taken into consideration when moving from lab scale to pilot scale. Sairem® has shown the use

of lower frequencies can circumvent low penetration depth beyond lab scale, improving energy efficiency too. Given the influence the microwave frequency, reactor size and geometry as well as the vessel material and size or the use of mono- or multi-mode microwave, optimisation is necessary for each change of scale, to optimise process parameters and minimise energy consumption. Just like for conventional heating, especially as magnetrons are usually designed for a 1000 kg load.[55] However, one can argue that at larger scale the capacity to absorb microwave energy increases with mass for a given loss tangent, and the surface-to-volume ratio decreases minimising energy losses to the surrounding environment. Both factors will have for effect to increase energy efficiency. This however needs to be balanced by the conversion efficiency of electrical energy for example to heat using a magnetron.[56]

1.2.2 Associated benefits of the use of microwaves on food supply chain waste

The use of microwaves instead of conventional thermal heating has increasingly been studied, especially in the last 20 years.[52] Advantages linked with the use of this technology include high heating rates, volumetric and contactless heating as well as a high control of the energy input.[59] In chemistry, microwave heating is believed to offer selective heating as well as rapid heating times.[56, 60] The latter has been recognised as a source of energy savings compared to conventional heating under small scale, closed-vessel conditions, under single mode conditions and when normalised on the basis of moles of product generated.[61] Above 100 mL, multimode microwave reactors have been classified as more energy efficient at increasing scale.[62]

The use of microwave technology was chosen for the particular valorisation of food supply chain waste for the reasons highlighted above. Additionally, this technology has other advantages: it is adaptable for use in continuous processes and scalable, potentially allowing for the design of an integrated conversion process. The technology has known uses in the food industry for sterilising or drying purposes under continuous conditions.[63] In the food industry, the use of microwaves represents an advantage as air is not heated during drying operations for example, creating more selective heating conditions.

More importantly, this technology is not limited by the high water content present in such feedstocks (up to 90%).[64] This is quite the contrary, given the dielectric properties of water (i.e. at 25 °C and 1GHz, ε' and ε'' are of 77 and 5.2)[65]. This results in a good interaction between microwaves and water-based systems,[66] which is important for future industrial scale applications given the importance of WOP drying costs.[67] The criteria cited above show that microwave technology has the potential to allow the valorisation of a pre-consumer type of waste generated on a million tonne scale while using an integrated conversion process.

Regarding biomass processing for chemical and fuel production, thermal decomposition of biomass under anaerobic conditions or pyrolysis, has been studied under microwave conditions, taking advantage of the high heating rates to increase biomass's energy density.[68] Such a process typically produces bio-char, bio-oil and gas (CO, H_2, CO_2 and CH_4).[46] The purpose of this work is to apply microwaves to a specific type of food supply chain waste. Citrus peels will be used as a feedstock to demonstrate the possible use of microwave technology for the extraction of *D*-limonene, polymethoxylated flavonoids, hesperidin, glucose, fructose and pectin from non pre-treated peel. Less extreme conditions will be used compared to microwave pyrolysis, which typically occurs between 200 °C and 380 °C.[46] The work presented in this thesis will use hydrothermal conditions relying on the dielectric properties of as a microwave absorber to extract chemicals from wet biomass below 200 °C, defining the scope of this project.

The next chapter will focus on the characteristics and the availability of food supply chain waste. The use of citrus peel waste as a resource and the opportunities offered by this feedstock in the context of the design of a microwave waste biorefinery will be highlighted in chapter 3.

Chapter 2
The use of FSCW as a feedstock for the production of chemicals. Availability and location in Europe.

L. A. Pfaltzgraff, M. De bruyn, E. C. Cooper, V. Budarin, J. H. Clark, *Green Chemistry*, 2013, **15**, 307-314.

S. K. C. Lin, L. A. Pfaltzgraff, L. Herrero-Davilla, E. B. Mubofu, A. Solhy, J. H. Clark, A. Koutinas, N. Kopsahelis, K. Stamatelatou, F. Dickson, S. Thankappan, M. Zahouilly, R. Brocklesby, R. Luque, *Energy & Environmental Science*, 2012, **6**, 2, 426-464.

Oral presentation given at the COST Network (Action TD1203) Toulouse Working Group 1 Workshop (21st January 2014).

Oral presentation given at the COST Network (Action TD1203) York Management Committee meeting (27th March 2014).

2 The use of FSCW as a feedstock for the production of chemicals. Availability and location in Europe.

Our society currently faces the twin challenges of resource depletion and waste accumulation leading to rapidly escalating raw material costs together with increasingly expensive and restrictive waste disposal legislation. The variety of food processes used in the food and drink industry globally generates FSCW on a multi tonne scale every year. This chapter will demonstrate the opportunity FSCW represents as a chemical feedstock and highlight the advantages of using FSCW as a raw material for the production of chemicals, materials and fuels. A method designed for the estimation of FSCW available in the E.U.-27 from food processing operations will also be developed upon and the main conclusions reached will be presented.

2.1 Use of food supply chain waste (FSCW) as a resource

2.1.1 Definition of the term food supply chain waste

FSCW has been previously defined as "the organic material produced for human consumption that is discarded, lost or degraded. This includes waste arising because of pest degradation or food spoilage."[43] It is estimated that "as much as 50% of the food produced is lost or wasted before and after it reaches the consumer".[69] On a global scale, the latest FAO report suggests that "1.3 billion tonnes per year of food produced for human consumption is [being] lost or wasted", representing a huge environmental, economic and social problem.[70] Our food supply chain has recently been recognised as being inefficient, producing large and accumulative quantities of waste.[71, 72] With "around 89 million metric tonnes of food waste are generated every year in the E.U.-27."[4] 39% is generated by the manufacturing sector, 42% by the household sector (other sectors: 19%). In this case, food waste encompasses domestic, waste produced by individuals in their homes.[73]

The paragraph above highlights one of the first issues encountered when attempting to work with such a type of resource: the definition of FSCW varies from one organisation to another and hence the data reported on the volumes available cannot be compared. In this thesis, FSCW will include all food wastes produced by

the supply chain, excluding food packaging. It will concentrate especially on non-avoidable FSCW and it will include food losses produced as a result of poor stock management at a pre-consumer stage. Agricultural residues and industrial processing waste generated by the food processing industry are examples of types of waste selected for further study here. It would not include household waste, which is very heterogeneous in composition. Criteria will be developed to select suitable types of FSCW for valorisation using clean and green technologies (see section 2.2). Valorisation is defined here as gaining revenue from a co-product traditionally disposed of and/or lost within the value chain or as one with a current low value.

2.1.2 Drivers for the use of FSCW as an alternative carbon feedstock for chemical production

The food supply chain encompasses a wide range of manufacturing processes. The seasonal nature of food production means that large quantities of FSCW, all of which are organic, can accumulate over short periods of time. The latter contain valuable functionalised molecules such as flavonoids, amino acids, proteins, waxes, pigments, lipids, sugars, fibres, biopolymers (cellulose, starch) or fatty acids as shown in Figure 6. These will have uses in all sectors of the chemical industry, including cosmetics, foods, pharmaceuticals, textiles as well as in fuels.[23] An exhaustive publication by Galanakis et. al. lists a large number of examples of sources of FSCW types for given chemical compounds and associated recovery techniques.[74] Functional compounds generated by food processing operations have also been reviewed by Schieber et. al.[75] When using FSCW as a feedstock for 1st generation valorisation strategies, those chemical functionalities are lost or at best, underutilised. First generation strategies include electricity generation (i.e. through anaerobic digestion), fuel generation (e.g. conversion of cellulosic biomass to bio-ethanol) or the production of animal feed. It has recently been reported that the conversion of biomass waste to bulk chemicals for example was nearly 7.5 and 3.5 times more profitable than its conversion to animal feed or transportation fuel respectively, highlighting the marginal value of 1st generation FSCW valorisation strategies.[43, 76]

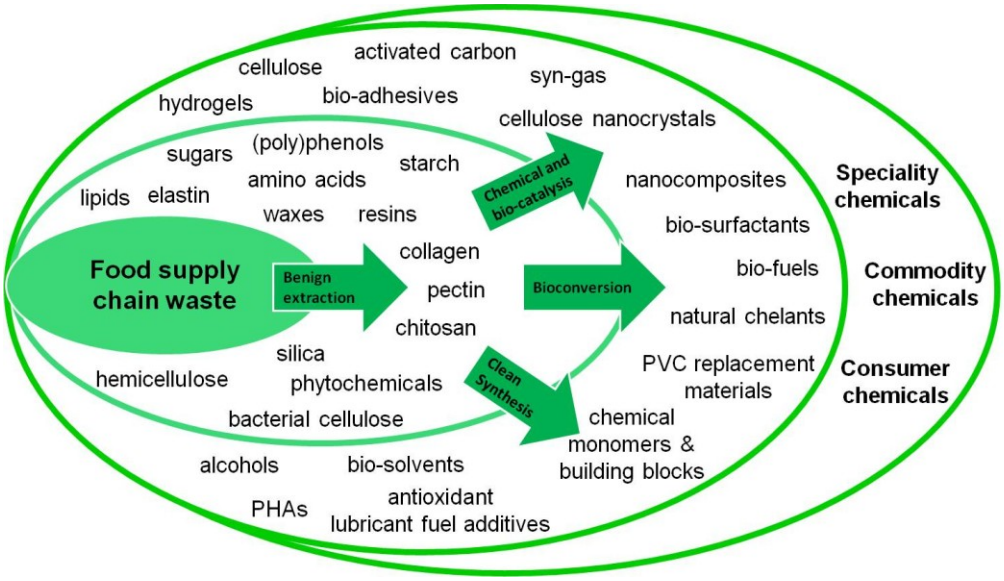


Figure 6: Components present in FSCW and their uses in common end-user products showing how the use of FSCW as a renewable feedstock could have implications in all main sectors of the chemical industry (originally in colour).

Maximising the chemical content extracted from FSCW is even more relevant in the light of the amount of fertilisers, pesticides, energy and water consumed for the production of food.[77] In the U.S.A., the United States Department of Agriculture estimated that the American agricultural sector is responsible for the consumption of 80% of the country's drinking water and occupies 50% of the surface of the usable land. Furthermore, "added to the waste of resources, there are also the consequences of having to manage large quantities of waste."[78] The loss of resources in the form of land, labour, energy needs to be considered as well. The latter have been estimated on a global scale by the Food and Agriculture Organization of the United Nations for wasted edible and non-edible parts of foods.[79] As one example of the amount of resources lost in 2008, 377 tonnes of tomatoes were lost from the food supply chain in the U.S.A., as estimated by Buzby *et. al.*[80] The data they gathered is highlighted in Figure 7.

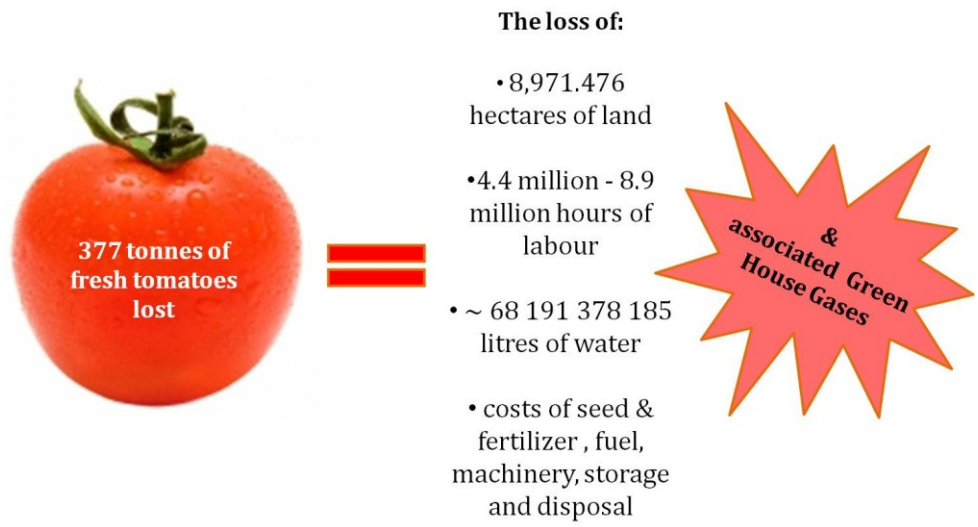


Figure 7: Estimation of the resources lost following the loss of 377 metric tonnes of fresh tomatoes (originally in colour).

The growing demand for environmentally friendly chemicals and fuels together with stricter U.K. and E.U. legislation are encouraging the development of more sophisticated FSCW valorisation strategies, namely 2nd generation FCSW valorisation schemes. The second article of the European Union's funding treaty declares the sustainable use of natural resources as one of the objectives of the E.U.-27's environmental policy.[81] Major drivers include increasingly tighter European regulations and standards, associated with their compliance costs. Recent developments in legislation focus heavily on reducing volumes of waste produced. On a European level, the Integrated Pollution Prevention and Control (IPPC) legislation 96/61/EC and the Landfill directive 1999/31/EC respectively introduce the notion of "polluters pay" and landfill reduction of biodegradable waste.[81] Additionally, the REACH legislation will provide "chemicals manufacturers with an opportunity to provide innovative substitutes based on renewable feedstocks."[82] At the level of the U.K., the Pollution Prevention and Control Act was implemented to fulfil the requirements set by the IPPC and the landfill legislation.[81] The combination of the use of new renewable feedstocks such as FSCW and clean technology will represent another achievement for the creation of a bio-economy. The latter is perfectly aligned with UK's Low Carbon Transition Plan and the Climate Change Act. All are focused on reducing polluting emissions and therefore, waste. The Courtauld commitment (now in its 3rd phase) represents an important development in the area of waste reduction in the U.K. This voluntary commitment has brought together more than 53 companies, all of which aim to reduce the environmental impact of the food retail sector by mainly looking through minimising packaging, the objective

being “a cumulative reduction of 1.1 million tonnes of waste and 2.9 million tonnes of CO_2 emission” by 2015.[83]

The potentially valuable chemical content of FSCW, recent developments in E.U.-27 legislation together with the chemical industry’s shift towards higher sustainability and improve cost-effectiveness, process efficiency and its green credentials make the development of sustainable and innovative strategies for the valorisation of the chemical components present in FSCW an interesting research avenue. Valorisation of FSCW is especially important since the sustainability of the food chain is being questioned in the light of the ever increasing growth of the world’s population. However, despite the clear benefits, the utilisation of FSCW as a biorefinery feedstock is largely unexploited and represents a challenge. These issues will be highlighted in the next paragraph.

2.2 FSCW selection criteria- Food for thoughts

FSCW can be divided into two parts: pre-consumer and post-consumer FSCW. Figure 8 describes the four main points of the FSC and the associated types of FSCW produced.

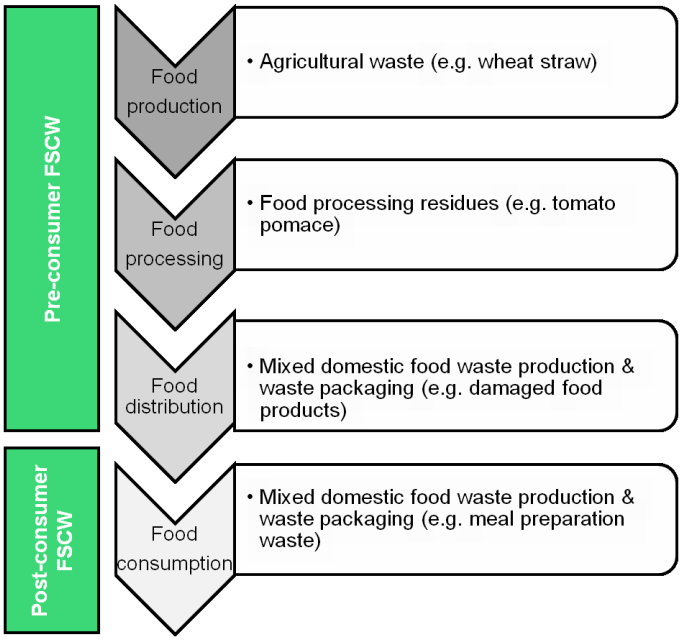


Figure 8: Simplified diagram of the food supply chain highlighting the distinction between pre- and post- consumer FSCW (originally in colour).

Although post-consumer waste is often the most visible, especially on a daily basis from our society's perspective, it is not only difficult to collect and segregate, but also challenging to valorise given its heterogeneous composition.[43] Pre-consumer waste is generated in a more concentrated manner, especially when considering the ever increasing rate of global food production[84] and processing.[43] "More than 70 % of the agricultural goods produced in the E.U.-27 are used to be transformed into food industry products".[85] Consequently, pre-consumer FSCW would display a lower feedstock variability compared to post-consumer FSCW. In terms of logistics, FSCW such as agricultural residues or food processing residues should therefore be preferred for economic valorisation.

Pre-consumer waste, such as food processing residues, represents a growing sector of waste production. In the U.K. for example, food manufacturers generate 4.1 million tonnes of food waste out of the 8.8 million tonnes of waste produced annually along the food supply chain. As a matter of comparison, the catering sector and retailers generate respectively 1.6 and 3.0 million tonnes of waste.[86] The food industry is omnipresent in our society: in 2010, the food and drink generated €953 billion in sales.[87] In comparison, the chemical sector contributes €491 billion in sales to the E.U.-27 economy (excluding pharmaceuticals).[88] Second generation valorisation of FSCW for the production of bio-derived chemicals, materials and fuels could boost the efficiency and competitiveness of two major sectors of the European economy. The food and chemical industry could form a symbiotic relationship instead of competing against each other for the same resources, as highlighted by the food versus fuel debate.[89]

However, several issues are linked with the use of FSCW as a renewable feedstock and their handling needs to be carefully considered. FSCW is mostly of organic nature (although they contain a number of useful inorganic compounds) and are characterised by high associated chemical and biological oxygen demand (COD and BOD)[90] and a high water content.[91] Additionally, it is prone to rapid bacterial contamination[75] and accumulates very rapidly over a short harvesting season, all of which lead to disposal management problems[71, 92] and consequently increasing disposal cost.[38]

These limitations dictate the logistics needed for the efficient production of novel added-value materials, chemicals and fuels from FSCW. The use of transportable continuous processing technology is needed to avoid rapid fermentation/bacterial contamination without the need for stabilisation and pre-treatment (which, to some

extent, could be responsible for loss of functionality). This would also favour decentralised valorisation processes where FSCW is produced, limiting the transport of low value and low density material and reducing operating costs.

The high water content often characterising FSCW should not be an obstacle to 2nd generation valorisation strategies as technologies allowing on-site conversion of waste without resorting to a drying stage exist. Microwave technology has been previously highlighted as a suitable type of technology (see chapter 1). Those technologies should then be coupled with sophisticated extraction techniques capable of selectively removing easily degraded compounds such as carotenoids or flavonoids from the biopolymer matrix that constitutes the bulk of FSCW. Such a combination of technologies is key to the successful integration of FSCW as a raw material next to non food crops for example.

Regardless of the potential efficiency of the valorisation process used, it would be rendered unproductive without careful selection of the type of FSCW used as a raw material. In order to qualify as a feedstock for chemical production, availability is key. Not only in terms of continuation of supply but also in terms of consistency. High, concentrated volumes of FSCW are needed to fit the large production capacity necessary to achieve economy of scale, making the use of in-situ valorisation technology even more important, especially if transport costs can be avoided. Those high volumes of FSCW should preferably occur around the globe (strengthening security of supply at a regional scale) and display the least variable composition (i.e. tomato pomace, wheat straw, rice husks, spent brewer's grain). The available range of extractable chemicals and biopolymers is also important, as the combined production of several marketable components from a single feedstock is essential to guarantee the cost-effectiveness of the process.

Finally, processing operations in the food sector can be classified into two categories: activities processing either animal-derived or plant-derived feedstocks. Fish and meat processing produce waste associated with a high sanitary risk and require strict handling and waste management legislation, making the valorisation of such agro-industrial residues less feasible. On the other hand, plant-derived FSCW represents a lower risk of bacterial and transmissible spongiform encephalopathie contamination and should therefore be favoured for the design of 2nd generation FSCW valorisation strategies.

2.3 Method for the evaluation of FSCW volumes produced by the food sector in Europe

As mentioned earlier, the availability of high volumes of a given type of FSCW is of great importance: the possibility of securing a consistent and regular supply of a given type of FSCW will determine whether industry will start considering this raw material as a viable source of bio-derived chemicals. The drivers responsible for mapping volumes of FSCW available in the E.U.-27 are highlighted in Figure 9.

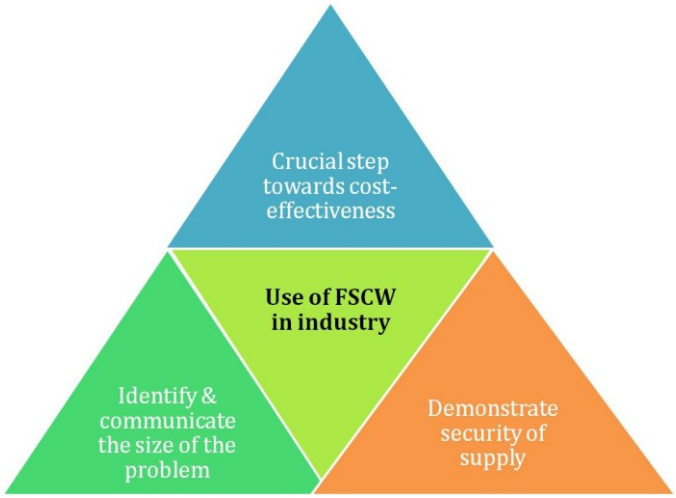


Figure 9: Drivers pushing for the estimation of available volumes of FSCW prior to future use as a renewable feedstock in industry (originally in colour).

Data on the exact amount of FSCW produced by the food supply chain is limited. As an example, no detailed data is issued on an annual basis by the E.U.-27 because of the commercial sensitivity of this information for the food industry. However published data exists, especially at a regional level,[73, 81, 91, 93-97] but discrepancies occur due to variations in the definition of waste and by-products used by the food sector and/or the author of the study for example. This renders a comparison exercise difficult across a geographical region. Published studies are the result of separate initiatives that vary in the methodology used and sometimes in their objectives (e.g. measuring and data gathering are two different endeavours). This will affect the boundaries of the study carried-out.[98] Waste can be defined as edible/inedible food in the retail part of the food supply chain whereas in the processing sector this will come down to whether a given product can enter the value chain or not.[98] The definition chosen for waste, if stated at all, will also

influence whether waste water, surpluses, off-spec material, packaging and/or by-products suitable for animal feed use will be taken into account for example.

As a result of these observations, a methodology has been designed to estimate the volumes of FSCW produced in the E.U.-27 for defined food processing operations, focusing especially on FSCW consistent with the selection criteria developed above. The objective was to obtain a database allowing for the comparison of FSCW volumes available by country with the aim of being able to identify hot spots, or locations concentrating a high volume of a given type of FSCW. Ultimately, a priority list could be obtained by country or for the whole of the E.U.-27, helping to focus the research effort on FSCW generated on an important scale. Such a method can be seen as a selection tool for further cradle-to-cradle assessment, using tools such as life cycle assessment or even techno-economic assessment. This will ensure FSCW will be used in a truly sustainable manner, bringing our society one step closer to a zero waste economy.

2.3.1 Methodology used

The novel methodology developed consisted in estimating FSCW arising following common food processing operations from statistical data. This allowed the utilisation of a large amount of data collected using a similar method, increasing comparability of the final study between countries. The data used in the present study was Prodcom data published by Eurostat. This data source reports sales volumes of manufactured goods for the E.U.-27 every year. Preceding studies in this area of research have used Eurostat data on volumes of waste produced for manufactured goods including food. Inconsistencies in data collection for the Eurostat database are a known issue: each country is free to choose its own method to complete the requested survey, which renders a comparison exercise difficult across countries.[99] The original raw data could not be accessed for Prodcom data. However care has been taken to verify the quality of the data through the associated quality reports issued with Prodcom data.

The objective of this piece of work was to estimate the volumes of FSCW arising for a known for types and food production processes by calculating the difference between the material input and the material output of the process considered. It was assumed the material output was equal to the sold manufactured product, the latter being obtained from Prodcom data.[100] As a result, surplus and “off-spec” goods

produced are not included and have not been accounted as waste per se in the calculations presented here. On the other hand, no distinction is made between edible and inedible production streams here and the data will include by-products and "green wastes".[98] From an ethical point of view, such types of waste streams are avoidable and the priority should be their reduction rather their valorisation. In the U.K., it has been estimated 40% of farmers' crops are wasted due to the implementation of aesthetic criteria for fruit and vegetables by retailers and by bad-weather forecasting.[83]

Prodcom data (or "PROduction COMmunautaire) is used for businesses and marketing analysis, for example to forecast market demand.[101] Prodcom data is obtained by the National Statistical Institutes (NSIs) who conduct a survey of enterprises of 20 employees of more. Survey completeness has been reported to exceed 95%. It includes the volume of production sold during the targeted period for both the domestic and external markets.

A common nomenclature is used for Prodcom, making the data comparable at a European level (i.e. definition of the population, the type of statistical unit, the definitions of the variables, the reporting on each type production).[100-102] Confidential data is suppressed at national level but included as appropriate at the E.U.-27 level to allow the calculations of totals for the whole of the European Union.[100-102] If a Member State's production represents less than 1% of the E.U.-27's total for a given food product, then the data does not need to be collected and the production is reported as zero.[100-102]

Data checks are performed by the Eurostat office. They include value comparisons across countries and over the years. Abnormal variations or questionable accuracy are reported to the declarant country and further explanations and/or corrections are required. This is done by comparing mean value, the minimum/maximum value and the median value for the total production for example. Additionally, the coherence between monthly and annual data is checked, and its coherence with external sources (i.e. foreign trade data).[100-102] Since the methodology presented herein is derived from statistical Prodcom data, the reliability of the methodology depends on the reliability of the underlying statistics. Prodcom data was deemed a reliable source of information which has the advantage of offering a mean of comparison across all E.U.-27 countries.

Eurostat data were used in combination with product percentages obtained from published process flow sheets of common food processing operations. The input

volume was estimated using the product percentage of the process considered found in the literature[103-108] and the amount of product sold. Peer-reviewed publications were preferred when possible to lower the error margin on the results. The input volume was then calculated by dividing the amount of product sold by the product percentage. The concept used is highlighted in Figure 10.

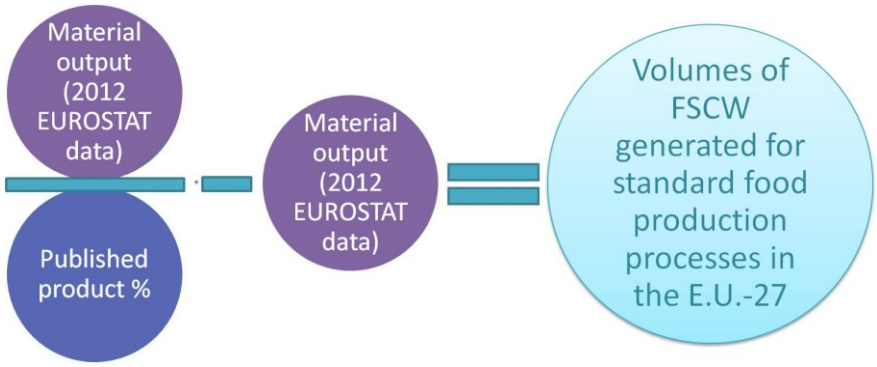


Figure 10: Calculation method used to estimate relative volumes of FSCW produced following food processing operations (originally in colour).

The calculation used is further illustrated using frozen un-concentrated orange juice production in Spain as an example in Figure 11. If 44,450 tonnes of frozen un-concentrated juice are sold annually in Italy, the material input can be calculated by dividing this value by 49.95% which is the product percentage published for the production of frozen un-concentrated juice.[103] The material input is therefore equal to 88,989 tonnes. When subtracting the material input to this value, 44,539 tonnes of FSCW are produced each year in Italy as a result of the production of frozen un-concentrated orange juice.

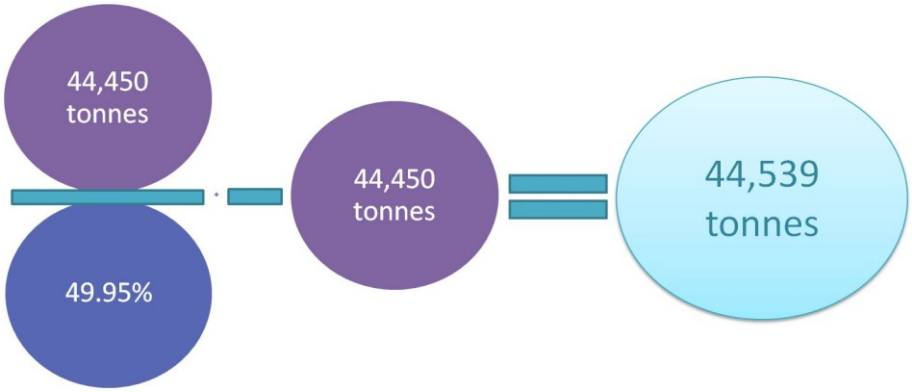


Figure 11: Calculation method used to estimate relative volumes of FSCW generated during the production of frozen un-concentrated orange juice in Italy (originally in colour).

The method allows the comparison of the FSCW data obtained over all the E.U.-27 countries. This is one of the advantages linked with the use of Eurostat data. Furthermore, the database is very detailed, compiled systematically and updated every year, strengthening the ensemble. However, data is missing for some food processing operations, depending on the country considered. Some assumptions still had to be made for some food processes and need to be further refined. This is for example the case for some preserved foods where a 9.32% waste percentage was used for preserved mushrooms and preserved asparagus. It was obtained by averaging the waste percentage obtained for potato and carrot peel waste, since no data was available for those specific cases.

Furthermore, material outputs reported in volume units represent a difficulty in converting to mass since finding precise density data for materials such as citrus pomace can be a challenge. In these specific cases, a density of 1.04 kg/L has been used for the conversion of the output material data from L to kg.[109, 110] This value is valid at 20 °C for 10° Brix according to the data reported in the literature. Additionally, the database does not distinguish between different waste streams for a given processing operation. Given the limitations highlighted above, using this method should be used for relative comparisons only, not as estimations of absolute values.

2.3.2 Main results obtained

The methodology presented above allowed the creation of a database listing the amount of waste generated by country for each processed food type sold in the E.U.-27 for the year 2012. Furthermore, a classification by waste type (cereal, dairy, fruit, vegetable, nut, oil crops, sugar production, bakery, candy and fermented drinks derived wastes) renders the database searchable by waste type in addition to by country and by processed food type.

The combination of the database with Google maps™ allows the identification of "hotspots" for a given FSCW type by country. An example is given in Figure 12 for the production of frozen un-concentrated orange juice. The hotspots shown for both Italy and Germany respectively account for 44,539 tonnes and 26,591 tonnes of waste produced as a result of the orange processing. Such a functionality is interesting when designing a biorefinery for example, as it allows governmental organisations to make informed decisions regarding the geographical location of the

valorisation technology used, minimising transport costs for example for a given resource.

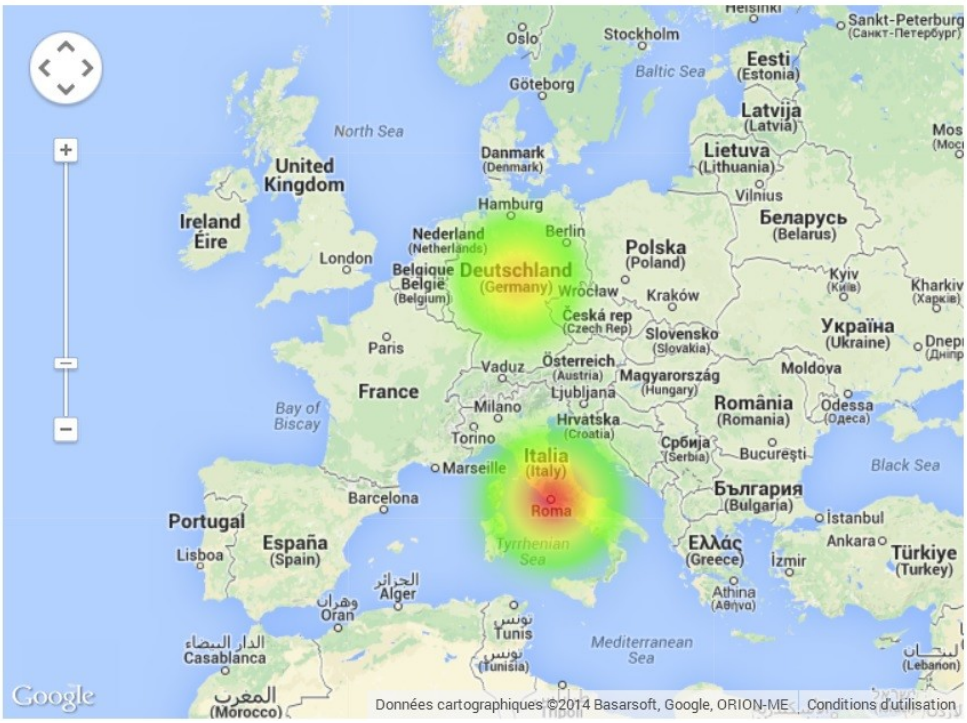


Figure 12: FSCW hotspots identified as a result of the production of frozen un-concentrated orange juice (originally in colour).

The method implemented also allows the creation of FSCW lists by country, ordering food processing operations by increasing amount of associated FSCW produced. The database is also useful to identify the food processing operations generating the highest amount of FSCW within the E.U.-27. A table highlighting the ten food products whose production is the least efficient is shown below as an example. Further developments on the database should allow the identification of food processing operations and associated FSCW produced according to the classification by waste type mentioned on the previous page.

Table 3: The top 10 types of waste generating foods in the E.U.-27 for the year 2012 and associated most important volumes (excluding meat and fish products).

	Type of FSCW	Country	Waste quantity (tonnes)
1.	Refined white cane or beet sugar in solid form	France	26,343,300
2.	Molasses obtained from the extraction or refining of sugar (excluding cane molasses)	Germany	19,868,700
3.	Raw cane and beet sugar in solid form, excluding flavouring & colouring	Poland	10,161,500
4.	Grated, powdered, blue-veined and other non-processed cheese (excluding fresh cheese, whey cheese & curd)	France	7,589,570
5.	Un-ripened or uncured cheese (including whey cheese & curd)	Germany	5,968,260
6.	Refined palm oil and its fractions (excluding chemically modified)	Netherlands	2,832,450
7.	Refined rape, colza or mustard oil and their fractions (excluding chemically modified)	Germany	2,782,180
8.	Potato starch	Germany	2,533,340
9.	Concentrated tomato puree & paste	Italy	2,267,480
10.	Wheat & meslin flour	Germany	1,959,840

In the context of this thesis, the FSCW database generated by the methodology used allowed, for example, the quantification and the identification of the location of orange peel waste within the E.U.-27. A list of the top 5 countries generating the highest amount of FSCW associated to the production of concentrated orange juice n.e.c. (not elsewhere classified) is given in Table 4 to illustrate the results obtained using the methodology reported herein.

Table 4: Quantity of FSCW associated with the production of orange juice n.e.c. in the E.U.-27 for the year 2012.

	Country	FSCW quantity associated with the production of orange juice n.e.c. (tonnes)
1.	Spain	109,128
2.	U.K.	14,433
3.	Greece	7,006
4.	Italy	4,669
5.	Slovenia	1,369

This comparison exercise highlighted an interesting fact: orange juice production is not simply limited to citrus producing countries. In the case of the U.K., which comes in second position, this country produces 13.23% of the quantity produced by Spain (14,433 versus 109,128 tonnes). This observation shows valorisation processes

need to be carefully planned ahead in order to be able to take advantage of all the resources available in all location. This is essential, for the efficient design of 2nd generation FSCW valorisation strategies.

The method presented herein allows the relative quantification of volumes of FSCW produced by country for defined food processing operations. This method solely relies on database consultation (as opposed to database consultation and interviews for example) and offers a common methodology for all European countries. This makes possible the identification of the most problematic areas and the development of appropriate global 2nd generation FSCW valorisation strategies.

Another methodology was designed by the Food and Agriculture Organization of the United Nations. Food balance sheets were designed for each type of food (whether processed or not and including primary commodities) and waste data was included. However they include the amount of waste "lost at all stages between the level at which production is recorded and the household (i.e. during storage, processing and transportation)."[111] This data presents another disadvantage: the waste data is based on assumptions guided by "expert opinion" each country, making the data susceptible to regional variations of the definition of waste and a lack of systematisation.[111]

2.4 Conclusion and future work

FSCW has been identified as an under-utilised source of bio-derived chemicals. Several factors are favouring the development of 2nd generation valorisation schemes exploiting the chemical content of FSCW. The loss of energy, water, fertilisers, land and labour devoted to the production of food for the FSCW produced has been recognised. The environmental impact of waste production and its associated green house gas emissions have been deemed unacceptable and several pieces of legislation have recently been introduced to reduce our society's environmental footprint. As a result an emphasis has been placed on reducing waste and finding re-use strategies for it if possible. FSCW is characterised by a high water content and a variable composition. However, it has been shown that through careful selection of the type of FSCW used, several logistical issues can be eliminated. The selection of plant-derived FSCW occurring in high volumes as a

result of food processing operations represents a source of un-avoidable waste streams which will offer security of supply.

A methodology has been designed based on sold manufactured volumes of food products to evaluate the volumes of FSCW arising by country for common food processing operations. The latter data is systematically compiled and updated every year by the E.U.-27 for all member countries. In the future, the use of such a source of information will allow trends in FSCW generation by year to be obtained. Hopefully the measurement, recording and publication of these trends will act as a further spur for reductions in avoidable food waste and valorisation of unavoidable. As a result, the methodology presented could be considered as a way to monitor progress made in the area of FSCW. Ultimately, the FSCW inventory obtained is the first step towards better understanding the issues arising with the generation of FSCW, further allowing the identification of FSCW hotspots which will represent an opportunity in terms of chemical content and/or volume.

In terms of further work, the application of this methodology should be broadened to the study to agricultural residues based on the same criteria used for FCSW arising from processed food. This would provide a more complete database. Beforehand, the variation of the data should be statistically analysed, multivariant data analysis could be used.

In terms of bigger picture, the database generated could also be linked with data on the properties and the chemical content of the different FSCW streams considered. An online tool could then be developed allowing the matching of a type of waste with the best type of technology available for efficient valorisation according to the chemical content of the FSCW type considered. This concept is illustrated in Figure 13. Such an initiative would provide focus to an emerging research area.

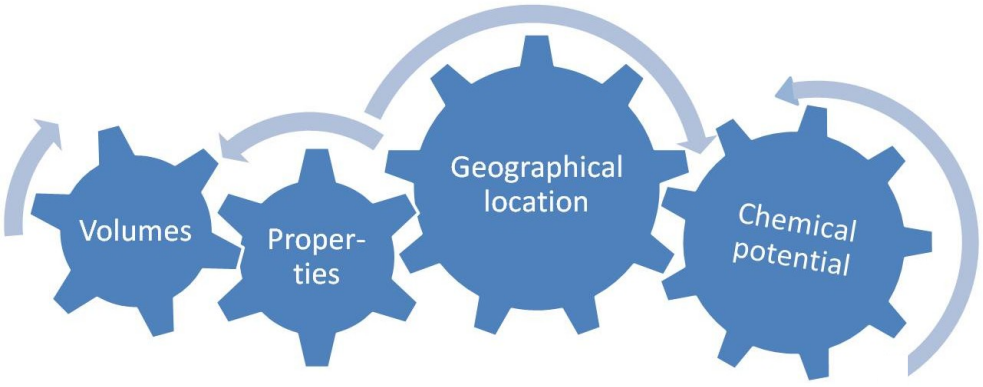


Figure 13: Interconnection of the criteria used to select suitable FSCW types for 2nd generation valorisation.

This present chapter focused on the area of FSCW valorisation as a whole. The following chapter will be dedicated to the description of citrus processing, focusing on orange fruits and existing processing operations. The latter are going to be evaluated and the opportunity for an integrated microwave process for the valorisation of waste orange peel (WOP) produced post juicing will be presented, setting the scope of this thesis.

Chapter 3
The Orange Peel Extraction Company. Opportunity for the valorisation of waste orange peel.

L. A. Pfaltzgraff, M. De bruyn, E. C. Cooper, V. Budarin, J. H. Clark, *Green Chemistry*, 2013, **15**, 307-314.

J. H. Clark, L. A. Pfaltzgraff, V. L. Budarin, A. J. Hunt, M. Gronnow, A. S. Matharu, D. J. Macquarrie and J. R. Sherwood, *Pure & Applied Chemistry Journal*, 2013, **85**, 1625-1631.

J. H. Clark, L. A. Pfaltzgraff, V. Budarin, M. De bruyn, 2013, *Microwave assisted Citrus Waste Biorefinery*, WO2013GB00154 20130404.

M. Balu, V. Budarin, P. S. Shuttleworth, L. A. Pfaltzgraff, K. Waldron, R. Luque and J. H. Clark, *ChemSusChem*, 2012, **5**, 1694–1697.

Oral presentation given at the 5th IUPAC International Conference in Green Chemistry, Durban (18th August 2014).

Oral presentation given at the Future of Food Waste event, Leeds (18th June 2014).

Poster presentation given at the G2C2 event, Cape Town (25th and 26th August 2014).

Poster presentation given at the Food waste in the European food supply chain: challenges and opportunities event, Athens (12th May 2014).

3 The Orange Peel Extraction Company. Opportunity for the valorisation of waste orange peel.

The waste arising as a result of food processing operations is a good example of a pre-consumer waste generated at an important scale, in Europe especially. The scale and rate at which they are produced together with the fact they are putrescible represent a problem for the industry concerned.[75] The processing of fruit and vegetables produces large amounts of wastes from processing activities(washing, peeling, blanching and sterilization processes).[90] Classical waste management strategies include incineration, landfilling or use as cattle feed, but these do not take advantage of the chemical content of these specific types of waste.

Following the presentation of the advantages linked with the use of clean technology (i.e. microwave technology) and by the presentation of the drivers motivating the use of FSCW as a renewable feedstock, this chapter will focus on the valorisation of citrus peel waste (CPW) and, more specifically, on waste orange peel (WOP) obtained from juicing operations. After highlighting the amounts of WOP available following juice processing, this chapter will describe the chemicals present in WOP and their respective use together with the commercial processes that are currently used for their extraction. The assessment of the current situation will lead to the presentation of an integrated waste biorefinery aimed at the sequential extraction of three main components present in WOP, offering an improved process over the current process used.

3.1 Availability of citrus peel waste around the world

3.1.1 Amount of citrus waste produced by the top five producers of orange juice

Citrus fruits are extensively cultivated around the world. They are considered as a commodity product, similar to coffee and tea in terms of international trade. They include oranges, lemons, limes, grapefruits and tangerines. Globally, 51.8 million metric tonnes of orange fruits were produced for the 2013-2014 harvesting season, 40% of which was grown for the sole purpose of being processed into juice.[112] The processing

industry creates a large amount of waste by-product in the form of peel, seeds, rag (the membranes between the citrus segments) and pulp (juice sacs), representing ~ 50-60% of the whole fruit being discarded after juicing.[113] Assuming 50% of the weight of a citrus fruit is discarded in the form of peel when processed,[114] the corresponding amounts of waste orange peel arising from the processing of orange fruits have been estimated based on the latest report published by the United States Department of Agriculture.[112] The data can be found in Table 5. The data reported in the table show that of the top five orange processing nations, three are also the largest orange fruit growing nations. It should be noted that the data does not take into account either "off spec" orange fruits disposed of prior to processing or fruits destined for human consumption but destroyed to regulate the selling price (which is a regular practice in Spain for example). These fruits could also be used for valorisation purposes. The numbers show how citrus peel and more specifically orange peel, which is a by-product of the juicing industry, represent a high volume waste stream occurring on both hemispheres of the globe.

Table 5: Estimated amounts of WOP produced in main citrus producing countries for the 2012-2013 harvesting season.[112]

Country	Amount of orange fruits produced for processing purposes (metric tonnes)	Associated amounts of waste orange peel produced following processing (metric tonnes)
Brazil	10,935,000	5,467,500
U.S.A.	5,423,000	2,711,500
E.U.-27	1,069,000	534,500
Mexico	1,350,000	675,000
China	600,000	300,000
TOTAL:	19,377,000	9,688,500

Through the course of this project, data was also gathered directly from industrial contacts. Recent data obtained in 2013 shows that a Scottish orange juice producer (GetJuiced®) generates up to 14,400 kg of CPW per week and is projecting to reach 86,400 kg of CPW per week by March 2014. Such data is especially relevant as it shows the importance of citrus processing activities in citrus fruits importing countries (i.e. local fresh orange juice production in the U.K. for example). Hence making second generation valorisation strategies relevant to these countries too. Additionally, a smaller country such as Cyprus with a more modest citrus fruit production capacity also has the potential to generate relatively significant

quantities of CPW: two juicers located in Cyprus have been reported to produce every year 8,000-11,000 metric tonnes (sent mainly to animal feed) and 4,500 metric tonnes (3,500 metric tonnes for orange peel and the remaining 1,000 metric tonnes for lemon and grapefruit peel) of CPW respectively.

Furthermore, citrus fruits are grown around the globe, on either side of the equator and even though the harvesting season is fixed in specific locations around the globe,[115] their harvest is spread around the globe throughout the year, ensuring a constant supply of CPW for valorisation purposes.

3.1.2 Process flow sheet for the production of orange juice

After the juice produced, WOP is the second largest output following juicing operations. Figure 14 highlights the general process used for orange juice production together with other outputs. Modifications to this process will occur according to the geographical location of the process. Outputs considered as waste are indicated in red and products entering the value chain are shown in orange. Typically a processor can expect to recover 45-55% of the fruit as juice, 45-55% as peel and 0.2-0.5% as essential oil (shown as *D*-limonene in Figure 14) from the citrus fruit.[116] The amount of water used for washing is very important for this process with a peel:water ratio is fixed to 1:1.[103]

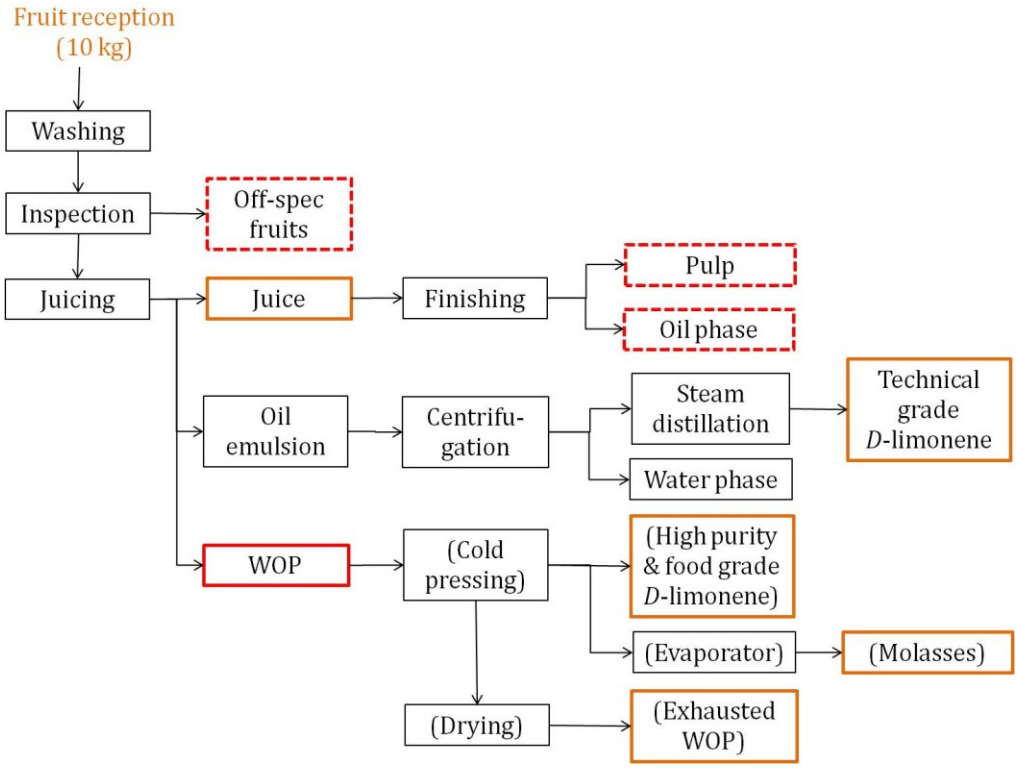


Figure 14: Juice extraction process highlighting common by-products and WOP outputs.[103, 117, 118]

Orange fruits harvested go through a lengthy process for juice extraction. Following fruit grading (including sizing), the fruits are washed and their juice is extracted by pressing. Peel oil is usually recovered as highlighted in the figure above.[116] Molasses and hesperidin can be obtained too, but the inclusion of these steps is not ubiquitous and will depend on the level capital available for investment, the required pay-back period and the juicing equipment used. Therefore the process steps leading to the production of molasses have been put into brackets.[117, 119]

In most cases, following juicing, the juice is finished to remove excess pulp to match required specification and then de-oiled to avoid damaging its organoleptic properties. This oil is not necessarily recovered. *D*-limonene containing oil can be obtained post-juicing by either cold pressing or steam distillation. The two different processes yield respectively a high purity/food grade and a technical grade of *D*-limonene. The latter compound can also be extracted prior to juice extraction by pressing depending on the juicing technology employed.[116] Following the pressing of the peel for the production of high purity/food grade *D*-limonene, molasses can also be obtained. The latter is rich in sucrose and reducing sugars. Molasses finds

applications as animal feed or as fermentation feedstocks for ethanol production for example.[117, 118] The exhausted peel then obtained can be used for animal feed if further dried.

The scale of operation for orange juice production will influence the recovery of additional by-products. Only above 50,000 metric tonnes of fruit processed per harvesting season, has it been estimated profitable to invest into peel drying equipment for animal feed production. Below the 50,000 metric tonnes threshold cited above, the peel has been reported to be disposed of or burnt in the field.[120] In a few cases, further extractions are carried out to produce flavonoids such as hesperidin, however this is not automatic.

According the literature the juicing process would benefit from further improvements: "current [CPW] processing is based upon processing technology that is at least 70 years old. It is largely unprofitable, grossly ineffective, and adds non-combusted fuel oil to the peel mass when fuel oil is used as a direct fire heat source. The process is also a high user of energy and [is] very problematic to operate."[121] Furthermore, "toxic chemical dewatering agents are used in current citrus peel processing operations, use of the end product of dried peel is limited to one product—a toxic cattle feed supplement."[121] Lime is often used for this purpose.[118]

Moreover, the high carbohydrate content of WOP is highly fermentable.[94] The disposal of CPW usually represents a problem at industrial scale. In the U.S.A., citrus processors are not allowed to landfill citrus waste and therefore have to transform it into pelletized animal feed, which is not cost-effective due to the costly drying process needed to lower the moisture level of CPW.[122] The added cost of storage and transport needs to be considered too. Overall, waste treatment represents a significant investment. Transformation of waste into value-added products could potentially allow companies to cut treatment costs, generate additional profits and improve their competitiveness. Moreover, 2nd generation valorisation of FSCW is becoming a necessity as part of the existing sustainable development and environmental protection required by legislation and by the consumer.[123]

3.2 Orange peel waste composition and value

Orange peel is of particular interest given the variety of compounds it contains (see Figure 15). It has been recognised as an interesting source of dietary fiber, natural

antioxidants, food colorants and flavours.[124] *D*-limonene and pectin both have applications in the food and cosmetic industries amongst others; as a fragrance[94] compound and a thickener[125] respectively. The sequential extractions of those compounds for valorisation purposes in the food and chemical sectors amongst others, represents an interesting research avenue, from both academic and economic angles. The main components present in orange peel are summarised in Figure 15 together with their associated weight percentage on a dry basis. WOP can contain up to 80% water.[117, 119] In the following section, the current commercial extraction process used will be described.

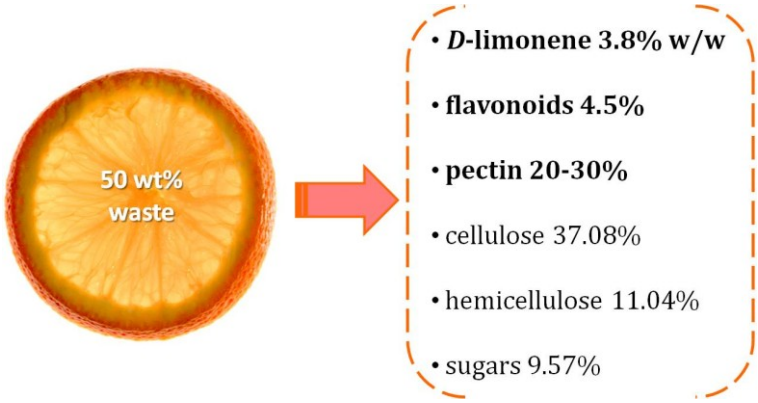


Figure 15: Main compounds of interest present in orange peel and their occurrence (wt% dry basis).[126, 127]

The peel is mainly composed of pectin, hemicellulose and cellulose (50-70%). Lignin typically represents less than 10% of the peel on a dry weight basis.[128] Glucose, fructose, sucrose and xylose constitute the water-soluble fraction of the peel.[117, 118] Of special interest is *D*-limonene, followed by pectin, the flavonoid hesperidin, polymethoxylated flavonoids and sugars which can be used as a fermentation feedstock to access more bio-derived platform molecules.[129]

3.2.1 *D*-limonene and orange oil

D-limonene can be obtained from whole fruits if the harvest is dedicated to oil production or as a by-product of juice production.[119] By *D*-limonene, the material referred to often corresponds in the literature to the peel oil, or essential oil obtained by steam distillation. Figure 16 gives the detailed process flow sheet of the

extraction of this compound by steam distillation. This process will vary according to the geographical location of the operations and the size of the juicing plant.

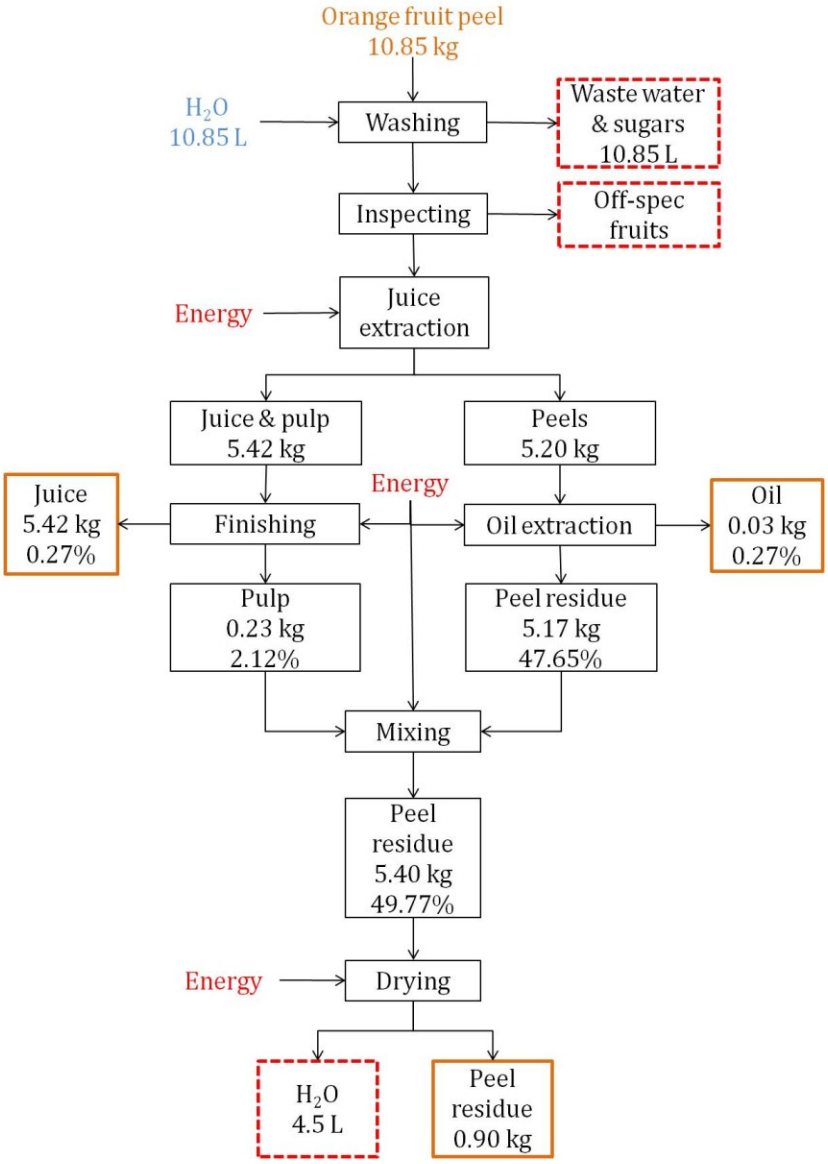


Figure 16: Process flow sheet for the commercial production of *D*-limonene.[103]

Between 50,000 and 70,000 tonnes of *D*-limonene are reported to be produced yearly.[130, 131] The price of *D*-limonene is highly variable depending on supply and demand (i.e. £1.91-6.61/kg for *D*-limonene in 2013).[132] This chemical is currently facing a huge demand. *D*-limonene finds uses as a starting compound for industrially relevant fine chemicals and flavour compounds (e.g. carveol, carvone, α-terpineol, perrillyl alcohol, p-cymene and perillic acid).[94, 133] Another of its uses includes the production of adhesive terpene resins via its polymerisation in toluene using $AlCl_3$.[117] It is also regarded as a

renewable and biodegradable solvent, although its biodegradability is similar to comparable synthetic hydrocarbons. With a Kauri-butanol value of 67, *D*-limonene has good solvating properties.[131, 134] Such properties explain its use in a large variety of consumer products, in addition to its flavour and fragrance properties.[113]

3.2.2 Pectin

Pectin is a complex carbohydrate mainly composed of chains of 1,4-polygalacturonic acid (polygalA) branched with sugar monomers. Its structure, chemical functionalities, properties and uses will be further discussed in chapter 4. This compound is mainly used for its gel forming properties when in water. It is commonly used as a gel forming agent in jams,[135] bakery and sweet food products as well as a stabilizer in dairy drinks.[136] New applications for this natural compound are continually being developed. The latest one concerns the development of an extraction method producing pectic oligosaccharide prebiotics.[137] High methoxy pectin retails for around £10/kg[120] and the global production for this macromolecule has been reported be 35,000 tonnes yearly.[136, 138] The major sources of pectin used in industry include citrus peel (peel residue generated by the extraction of juice and sometimes oil) and apple pomace (juice residue). Lime and lemon peels are sometimes preferred although orange peel has a greater availability and application range. [139] Dry pectin can be obtained in yields of up to 20-30% of the dry weight of the citrus peel.[125] Pectin is not generally a by-product of the juice production industry. It can be extracted from the peel residue following juice extraction, providing the peel is pasteurised (95-98 °C for ~ 10 minutes) and dried rapidly following juice extraction to avoid the degradation of its inherent pectin content by pectolytic enzymes present in the peel (i.e. methylesterase enzymes).[118, 140] Figure 17 highlights the process commercially used for pectin extraction.[1] Pectin is extracted commercially by acid hydrolysis, generating quantities of acidic waste water.[141,116] Pectin production mostly takes place in Europe, Israel and Brazil.[116, 142, 143] The U.S.A. used to manufacture pectin but the acidic and sugar rich effluents produced as a result of the acid hydrolysis process used were deemed to expensive to treat following new regulations issued by the American Environment Protection Agency (E.P.A.) and all pectin manufacturing activities have since ceased in the U.S.A.[144] The acid hydrolysis typically takes place at pH 1-3 and 50-90 °C for up to 12 hours.[1] A yield of 25-30% is generally expected. 10 to 15% of pectin is usually composed of neutral sugars.[140]

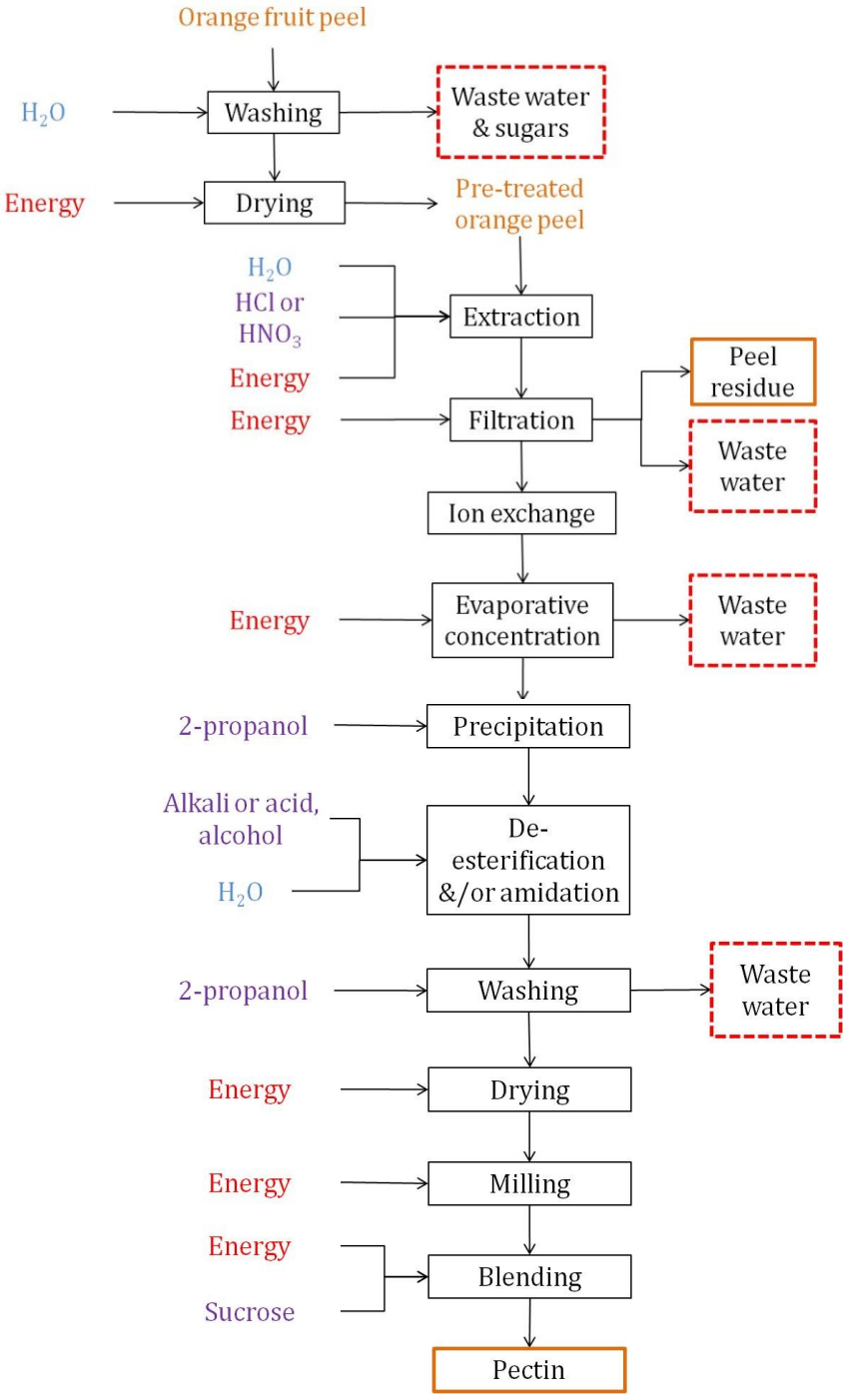


Figure 17: Process flow sheet for the commercial production of pectin via acid hydrolysis.[1]

Several washing and purification stages are needed to remove soluble sugars and acids present in WOP.[145] They make pectin production a wasteful and polluting process, with several different waste streams having to be treated for one process such as acidic waste water. As an example, during the concentration step, between 100 and 170 kg of water need to be removed per kilogram of pectin produced.[1] This is especially the case if aluminium salts are used for the precipitation of pectin.

Overall, the disadvantages associated with the traditional method of production of pectin are:

- the use of a highly perishable feedstock,
- a high energy demand (for the peel drying stage and the acid extraction of pectin),
- the use of environmentally hazardous chemicals,
- the production of acidic effluents and
- the questionable safety of a batch process on a multi litre scale in the light of new developments on continuous processing.

Microwave heating has been used previously in the extraction of pectin from citrus peels essentially to obtain higher quality pectin faster. It has also been shown that microwave heating improves the overall pectin properties including the degree of esterification, molecular weight, viscosity, gel strength and galacturonic acid (galA) content.[146-148] However, all of these methods still involve the use of a strong mineral acid. Herein, microwave assisted production of pectin from WOP under acid-free conditions represents an interesting research avenue.

3.2.3 Flavonoids

The main flavonoids available in citrus peel are hesperidin (main flavonoid in orange peel, 2% dry weight)[149], narirutin, naringin and eriocitrin. Flavonoids are the major source of naturally occurring polyphenols and hence, of antioxidants present in fruits and vegetables. Their polyphenolic skeleton allows them to act as "radical scavengers" due to their hydrogen and electron donating ability and metal chelating ability.[150, 151] Given these properties these compounds have known pharmacological[152] and food applications.[150, 151] Between 1.7 and 2.0% of flavonoids are present in the peel of orange and grapefruits.[149] To date, flavonoids are not routinely recovered at a significant scale commercially. This is due to the absence of an effective extraction protocol.[153] The recovery of hesperidin and polymethoxy flavonoids will be of most interest here.

3.2.3.1 Hesperidin

Hesperidin belongs to the class of citrus derived glycosides. This compound is the most abundant flavanone glycoside found in citrus peel.[153] Between 3.6 and 4.5 kg per tonne of orange peel can be obtained. This equates to a 0.36-0.45% extraction yield.[117, 154] A process flow sheet is given in Figure 18 for the extraction process used for hesperidin according to referenced material. The extraction is carried out at basic pH using calcium hydroxide followed by recrystallisation at pH 4.2-4.7 and 40-45 °C or 55-60 °C and yields hesperidin with a purity of 70-80%. To reach a purity >95%, the recrystalisation is usually repeated. It should be noted that the moisture of the peel can be reduced prior to hesperidin extraction. In this case, a dewatering step is added between the grinding and the extraction steps, generating a great proportion of wastewater effluents.[119, 155, 156] It is known hesperidin needs to be extracted prior to mineral acid extraction of pectin,[155] making the recovery of hesperidin very difficult as a by-product of pectin extraction.

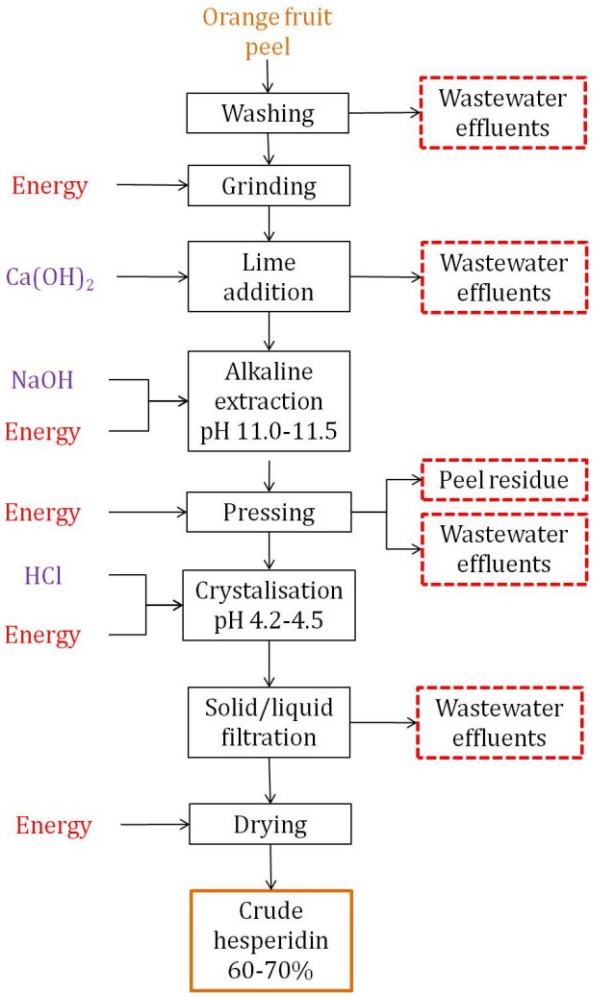


Figure 18: Process flow sheet for the commercial production of hesperidin.[119, 155]

3.2.3.2 Polymethoxylated flavonoids

Polymethoxy flavonoids such as tangeritin or sinensitin are found in citrus peels.[150] At this point, polymethoxylated flavonoids are not commercially extracted at a large scale from citrus fruits. This by-product has only recently attracted interest from the academic community due to amongst others, its antioxidant and anticancer properties. Industrial scale extraction from citrus peels is as yet not established. However, the literature mentions their extraction from molasses by peel maceration with lime with a 0.13% yield.[157] Molasses is the liquid phase resulting from pressing orange peel. They are a result of peel pressing from animal feed from WOP. However, molasses is not produced from WOP in all cases.[158]

3.3 Scope of this thesis

The valorisation of WOP is the centre of a significant body of literature. The potential of this feedstock has long been recognised. Initial research established that WOP could yield dietary fibers,[159] fuel[127] and flavonoids (hesperidin, narirutin, naringin, eritrocin) in addition to the traditional by-products (silage and as a mosquito repellent). It can also be used as a fermentation substrate for single-cell protein production. [94, 117]

First generation biorefineries based on the use of WOP as a feedstock have also been designed. The literature reports methane production from WOP by thermophilic anaerobic digestion,[113,160] and the combined production of bio-ethanol and *D*-limonene and methane by sulphuric acid hydrolysis at 150 °C, The latter has been demonstrated as economically viable when methane is used to fuel the process.[127, 161]

In Florida, scientists have focused on the production of bio-ethanol from WOP,[162] following *D*-limonene extraction (*D*-limonene is detrimental to the activity of the enzymes used for the production of bio-ethanol). The only example of integrated valorisation of WOP was demonstrated in Sweden by Pourbafrani *et. al.* for the combined production of bio-ethanol, methane and limonene.[127] None of the designs mentioned earlier report the recovery of pectin prior to fuel production though.

As a result, the design of a sustainable and integrated process for the production of the highest number of marketable chemicals, materials and/or fuel with maximum efficiency represents an interesting research area, especially if the technology used is safe and transportable. This led to the idea of developing an integrated waste biorefinery capable of producing chemicals from the million tonnes of WOP generated every year globally. The design should have the following attributes:

- avoid the use of a drying and pre-treatment stage
- allow the use of wet biomass
- avoid the use of acid and additives used in the process especially for pectin and hesperidin extraction
- avoid or limit the use of solvent to food grade solvents only and
- allow the valorisation of WOP in-situ.

Microwave processing was chosen as the core technology of the biorefinery as it allowed the use a wet feedstock, by-passing a costly and energy-intensive drying

stage. Additionally, prior work in the area of separate *D*-limonene and pectin extraction using microwaves had been reported but it did not meet all the criteria mentioned above. Furthermore, their sequential extraction under acid-free conditions has not been reported, representing an opportunity for innovation (see Figure 19). This area will be properly discussed in chapter 4. Given the higher selling price of pectin compared to *D*-limonene together with the higher availability of pectin in WOP, the project first focused on the extraction of pectin under acid-free microwave conditions. Especially as it appeared early on that the profitability of the process would depend on the production of pectin and that continued investments by major pectin manufacturers in their processing capabilities.[143, 163]

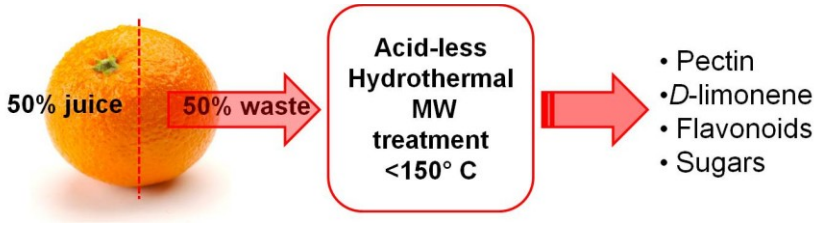


Figure 19: Illustration of the concept of a waste biorefinery centred on the use of WOP and microwave technology.

A brief SWOT analysis (Strengths, Weaknesses, Opportunities and Threats) has been carried-out to specify the desirable features of a waste biorefinery focused on the 2nd generation WOP valorisation. The analysis is given in Figure 20.

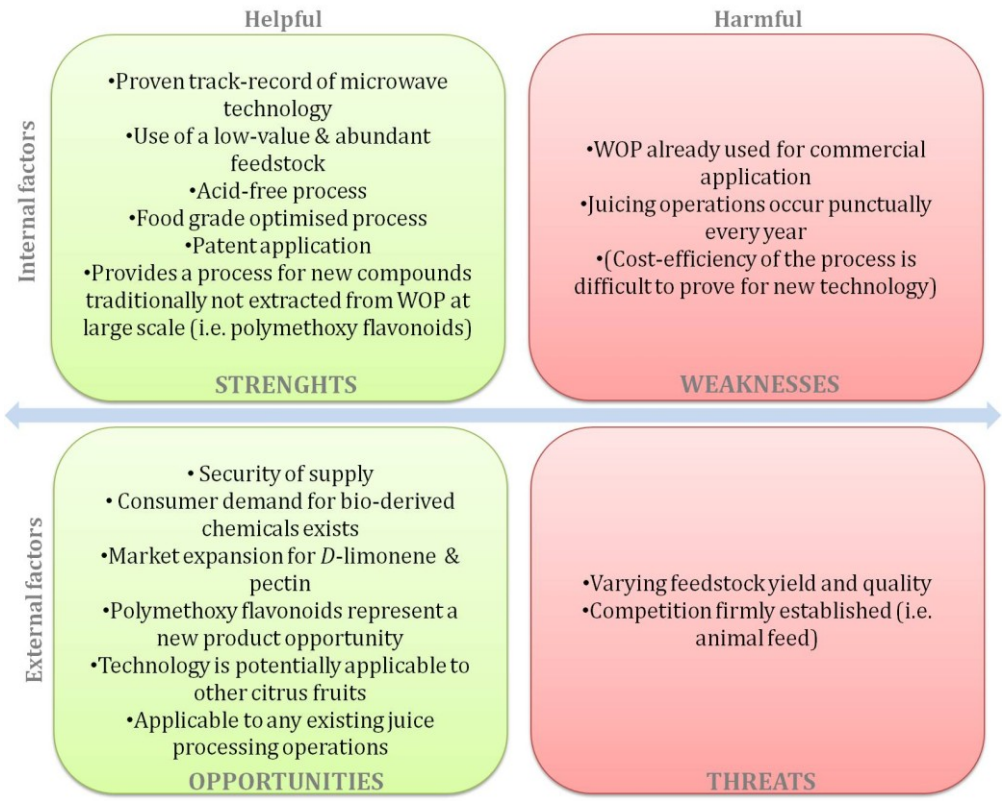


Figure 20: SWOT analysis for the design of a WOP microwave biorefinery.

The analysis of the strengths and opportunities shows that the anticipated growth of the biorefinery could lead to the application of the technology to other processed citrus fruits (i.e. limes and lemons). The analysis shows the weaknesses of the project can be minimised as some of the chemicals targeted already have a commercial value. The opportunity therefore lies in combining juice and *D*-limonene together with pectin production, which are traditionally two different business sectors. Flavonoids and sugar extraction using the same process would represent an additional opportunity, providing all components can be separated using food grade solvents. The main threat to the designed valorisation scheme is the uncertainty in crop yields and quality, influencing the amount of WOP produced and hence, the potential revenue. This last factor renders the diversification of the feedstock used even more important, minimising the influence on the biorefinery revenue of specific crop yields. The threat of the well-established current processes for *D*-limonene and pectin extraction can be minimised if one of them invests in the biorefinery model, or if the stand alone biorefinery has a strong marketing and advertising strategy towards the buyers and legislation (i.e. proven greener credentials, lower waste treatment costs). Therefore, greenhouse gas analysis and full life cycle assessment are as important as a techno-economic assessment of the

biorefinery scheme designed to further validate the strategy imagined and verify the sustainability of the valorisation scheme chosen from cradle-to-grave. Proving the economical feasibility of a biorefinery process is as important as proving its sustainability.

As a result of the SWOT analysis, the scope of this project was determined. The success of the development of a microwave biorefinery process centred on the valorisation of WOP will depend on the:

- successful pectin extraction using a low temperature (below 200 °C) and acid-free hydrothermal treatment,
- successful microwave assisted *D*-limonene extraction under solvent-less conditions,
- integration of both pectin and *D*-limonene extraction to design an integrated microwave process potentially fit for continuous processing and
- extraction of flavonoids and sugars for additional revenue.

The scope of this thesis was therefore to develop a low temperature microwave process allowing for the recovery of a maximum number of compounds present in WOP. This process should be appropriate for in-situ operation at a juicing plant. Pectin production under acid-free conditions was the primary objective and the development of suitable extraction conditions will be discussed in chapter 4. Research on the extraction of *D*-limonene will be discussed in chapter 5. The additional production of compounds such as hesperidin, polymethoxylated flavonoids and sugar monosaccharide was investigated and will be discussed in chapter 6. The entire process developed, namely the OPEC process, has been highlighted in Figure 21.

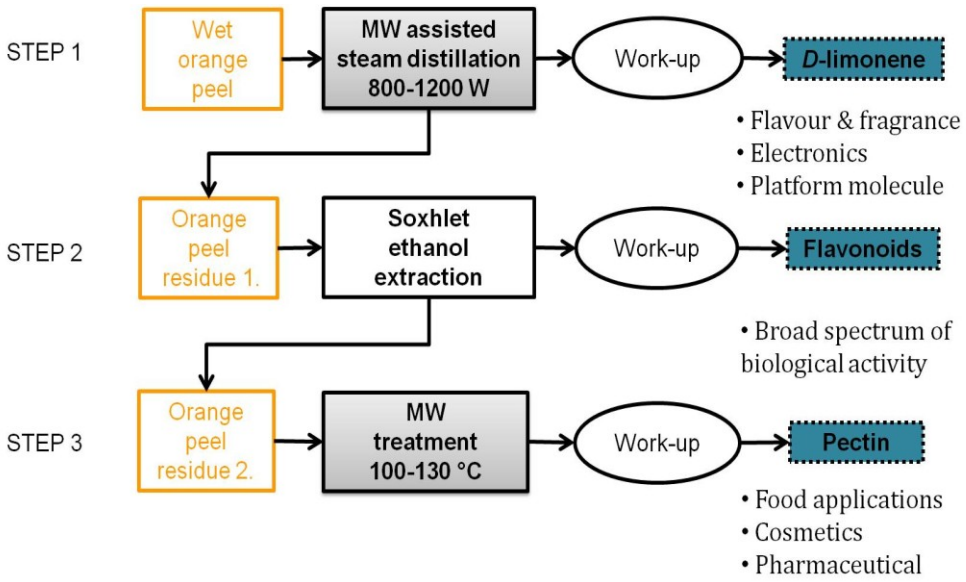


Figure 21: OPEC process used for the valorisation of WOP using acid-free, low temperature microwave hydrothermal condition

Chapter 4
Microwave hydrothermal extraction of pectin from waste orange peel under acid-free conditions

V. L. Budarin, P. S. Shuttleworth, M. De bruyn, T. J. Farmer, M. J. Gronnow, L. A. Pfaltzgraff, D. J. Macquarrie and J. H. Clark, *Catalysis Today*, 2014. Available from: http://dx.doi.org/10.1016/j.cattod.2013.11.058).

L. A. Pfaltzgraff, M. De bruyn, E. C. Cooper, V. Budarin, J. H. Clark, *Green Chemistry*, 2013, **15**, 307-314.

J. H. Clark, L. A. Pfaltzgraff, V. Budarin, M. De bruyn, 2013, *Microwave assisted Citrus Waste Biorefinery*, WO2013GB00154 20130404.

A. M. Balu, V. Budarin, P. S. Shuttleworth, L. A. Pfaltzgraff, K. Waldron, R. Luque and J. H. Clark, *ChemSusChem*, 2012, **5**, 1694–1697.

Oral presentation given at the 5th IUPAC International Conference in Green Chemistry, Durban (18th August 2014).

Oral presentation given at the Future of Food Waste event, Leeds (18th June 2014).

Poster presentation given at the G2C2 event, Cape Town (25th and 26th August 2014).

Poster presentation given at the Food waste in the European food supply chain: challenges and opportunities event, Athens (12th May 2014).

4 Microwave hydrothermal extraction of pectin from waste orange peel under acid free conditions

4.1 Introduction to pectin

Pectin is a structural hetero polysaccharide present in non-woody plant tissues and contributes to cell wall adherence and strength. It is responsible for the "firmness and structure of the plant tissue. It belongs to the primary cell wall structure. It is involved in cellular adhesion and forms highly entangled networks further stabilized by calcium and alkali-labile cross links".[164]

It is a component of protopectin which is naturally present in the cell wall.[139] Figure 22 depicts the chemical structure of pectin.[165, 166] It is mainly composed of chains of D-galacturonic acid (galA) units linked together by α-(1→4) glycosidic bonds. Additionally, the chain of poly α-1,4- D-galacturonic acid (polygalA) showcases neutral sugars such as rhamnose, galactose, arabinose fucose or xylose.[114] The structure of pectin is believed to be organised in four different subunits. Three contain branched sugars grafted on the backbone of polygalA: rhamnogalacturonan I and II (respectively RG I and RG II) and xylogalacturonan (XGA). These are often referred to as the "hairy regions" of pectin. The un-branched backbone of polygalA is called HG or homogalacturonan and is commonly referred to as the "smooth region".[114] These four different subunits form branched blocks organised around the main polygalA chain. A full characterisation of this macromolecule is yet to be resolved.[139] The composition of the pectin extracted will depend on the type and variety of biomass it originates from and the conditions of extraction.[140] The acid hydrolysis extraction process based on the use of mineral acids will yield pectin mainly composed of "neutral sugar free" chains of galacturonic acid.[139, 167] Under E.U. standards, pectin has to contain over 65% galA.[139, 152]

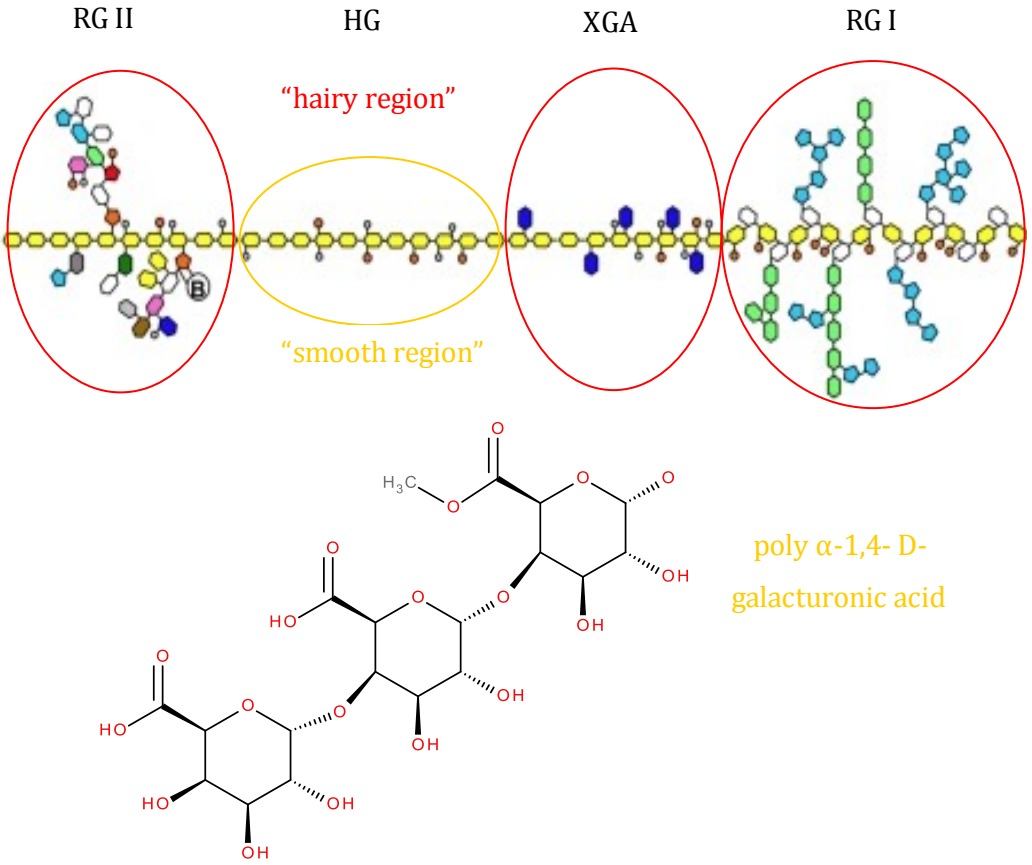


Figure 22: The different structural units of pectin and its components. This diagram was partly published in www.plantphysiol.org and is copyrighted by the American Society of Plant Biologists. It is reproduced with permission (originally in colour).

The ability for pectin to from a gel under controlled conditions is exploited in the food, cosmetic and pharmaceutical sectors. Pectin is used in the food industry as a gelling and thickening agent.[125] It's ability to form a network capable of retaining water is used for jam manufacturing, it acts as a cloud stabilizer in fruit juices and milk drinks and as a fat replacement agent in reduced fat products.[125] Other applications include drug delivery systems in the pharmaceutical industry or for medical adhesives for example.[152] The gel-forming properties of pectin are due to the presence of a carboxyl functional group on the C-6 of galA. This carboxyl group can exist as a free acid or a methyl ester. A 50% DE represents the cut-off point between high and low DE pectins. Below 10% DE, it is called pectic acid.[168] The DE is a particularly important parameter. It is used in the food industry to determine

optimum gel forming conditions. Two types of gel forming mechanisms for pectin are reported: one for high DE pectin (> 50 %) and one for low DE pectin (< 50 %). High DE pectin is typically used in jam and low DE pectin bakery goods. They are summarised in Table 6. Pectin gels at low pH 2.5–3.8 with a high sugar concentration (>55%).[169] There are as many pectin formulations as there are products on the market including pectin in their ingredients list. Pectin's properties for a given application can be "tuned" by pH variation, counter-ions addition, amidation (C_6 position), acetylation (O_2 or/and O_3 position),[170] demethoxylation to optimise its performance for a wide range of applications.[139]

Table 6: Characteristics of different gel forming mechanisms for pectin.

DE (%)	Type of pectin	Gel type	Gel formation mechanism	Setting speed	Setting pH
70-80	High methoxyl content	Acid	Hydrogen bonding & hydrophobic interaction	Rapid set	2.8-3.4
60-65				Slow set	2.8-3.2
<50	Low methoxyl content	Calcium	Inter pectin chain Ca^{2+} ions "bridges" between two pairs of carboxyl groups	Slow set	2.8-6.5

Other characterisation benchmarks and quality parameters include:

- the degree of amidation (for specific applications),[152]
- the degree of acetylation (for specific applications),[152]
- molecular weight,
- neutral sugar content and
- viscosity measurements.

4.2 Occurrence of pectin and traditional extraction of pectin

The yield, quality and gelation properties of pectin varies according to the nature of the biomass it is extracted from. Pectin is commercially extracted from citrus peel, mainly lime and lemon peels as the pectin they produce is known to be of high quality.[171] Dried apple pomace is used too though as well as beet pulp.[152] Table 7 compares the distribution of pectin in the different constituents of oranges and grapefruits.

Table 7: Repartition of the dietary fiber content in parts of oranges and grapefruits (g/100g of fresh material). [117]

	Orange				Grapefruit			
	Peel	**Membrane**	**Juice Sac**	**Seed**	**Peel**	**Membrane**	**Juice Sac**	**Seed**
Pectin	3.7	4.0	4.8	3.0	4.8	4.0	4.9	4.0
Hemicel-lulose	1.8	1.5	1.7	1.6	1.4	1.1	1.5	1.6
Cellulose	3.8	3.5	3.5	6.8	2.6	2.3	1.8	7.0
Lignin	0.3	0.7	1.0	3.2	1.2	0.7	0.9	3.3
Total	9.6	9.7	11.0	14.6	10.0	8.1	9.1	15.9

Pectin is traditionally extracted using acid hydrolysis, generating substantial quantities of acidic waste water.[141] The industrial extraction process involves treating the raw material with hot dilute mineral acids for 3 to 12 hours.[1] Insoluble peel particles are removed from the mixture and the solubilised protopectin is concentrated to 3-5% by vacuum evaporation. Pectin is recovered by precipitation using alcohol or aluminium salts. It is then washed with acidic alcohol. To be sold as a powder, the resulting purified pectin has to be pressed, therewith drying it further until a 6-10% moisture content is reached. It is then finely ground. Pectin can be standardised by blending with sucrose.[1] Dry pectin can be obtained in up to yields of 25-30% of the dry weight of the citrus peel. The many washing and purification stages make pectin production a wasteful and polluting process, with several different waste streams, all needing individual treatment. This is especially true when aluminium salts are used for the precipitation of pectin.[117]

Alternative methods to acid hydrolysis are currently being researched given the disadvantages associated with the traditional process (see chapter 3). Enzymatic extraction is one method being researched. This method has been developed to try to extract pectin with a higher degree of polymerisation and a higher molecular weight, employing enzymes to obtain more controllable hydrolysis conditions with limited success.[172]

4.3 Precedents in acid-based microwave assisted extraction of pectin

Microwave heating has also been investigated to extract pectin from citrus peel under acidic conditions. The use of this technology was primarily aimed at reducing the long extraction times associated with pectin acid hydrolysis. It has also been shown that microwave heating improves pectin properties including the DE molecular weight, viscosity, gel strength and galA content.[146-148] Several publications exist in the area of microwave assisted acid hydrolysis. The most important pieces of work in this specific area are highlighted below.

Fishman *et. al.* has reported that microwave-assisted extraction yields pectin of a higher quality.[173] This author studied microwave heating under pressure (in a closed vessel) to extract pectin from the albedo of oranges. The pectin obtained had a higher average molar mass, size and intrinsic viscosity compared to pectin extracted using conventional heating methods. The DE and the galA content of the pectin obtained were higher too. 20.3% of pectin on a fresh-wet basis was extracted from the albedo. The conditions used were: microwave heating at 630 W, 2450 MHz, pH 2, using hydrochloridric acid as a solvent (albedo:solvent 1:25) and a 3 minutes reaction time in a closed vessel.[148] The same author applied extended this (patented) method to the extraction of pectin from lime flavedo, albedo or pulp. Molar mass, viscosity and the radius of gyration all decreased with time of heating. Interestingly, in this piece of work, the authors concluded that heated dilute acid environments were necessary to extract pectin and that no pectin could be extracted from albedo dispersed in water under microwave conditions alone.[173] Manabe *et. al.* first reported the extraction of pectin from mandarin orange pulp using microwaves in an open vessel.[174] Kratchanova *et. al.* reports that extracting pectin using microwaves results in obtaining pectin in a higher yield and quality (DE, molecular mass and gel strength).[146, 175] This has been attributed to partial disintegration of tissues, hydrolysis of the protopectin and the fast inactivation of the pectolytic enzymes in the raw material.[147] In Kratchanova's work, microwaves were used as a pre-treating method (5, 10 or 15 minutes at 0.45, 0.63 or 0.9 kW) and the pectin was then extracted at 80-82 °C with HCl (0.5 M) to lower the pH to 1.5. The same pH was used under microwave-assisted conditions by Bagherian *et. al.* They studied the influence of the heating time and microwave power on the pectin yield and a maximum yield of 26.3% pectin was obtained at 900 W following a heating time of 6 minutes.[176] At 500 W under the same pH conditions, a 18.23% yield of pectin was

obtained by Guo *et. al.*[177] Microwave assisted extraction of navel orange albedo at 150 °C for 15 minutes yielded 0.8% of pectin on the other hand (1.6% when calculated based on the dry yield of peel).[135] Zhongdong *et. al.* uses the microwaves as a heating method for the extraction of pectin at pH 2 with HCl (85 °C for 4 minutes, 2450 MHz, 1000 W). The author used scanning electron microscopy (SEM) analysis, to demonstrate that microwaves disorganise the orange peel organisation and loosens its structure as the microwaves cause cells to split due to the evaporation of the inherent water content of the peel.[178] A summary of the observations reported in the literature on microwave-assisted extraction of pectin under acidic conditions can be found in Table 8.

Table 8: Reported properties of pectin extracted under acidic microwave conditions.

Pectin origin	Temperature (°C)	MW power (W)	MW heating time (min.)	Yield (%)	galA content (%)	DE (%)	Reference
Orange	80-82	450	20	17.0 g/100 g peel	67.1	72.9	Kratchanova *et. al.*, 2004
Orange albedo	Not measured	630	3	20.2	93	93	Fishman *et. al.*, 2000
Lime albedo	140	Dynamic mode	2.5	6.2 ±0.1	88	66	Fishman *et. al.*, 2006
Navel orange albedo	150	Dynamic mode	15	0.8	-	-	Liu *et. al.*, 2006
Navel orange	80	500	21	18.13 ±0.23	72	82	Guo *et. al.*, 2012
Grapefruit	Not measured	900	10	26.27 ±0.8	68	72	Bagherian *et. al.*, 2011

Results reported in the literature show pectin can be extracted under acidic conditions when using microwaves. Within the context of this work aimed at designing an integrated biorefinery process for the valorisation of WOP, the acid-free extraction of pectin using microwaves was investigated first as microwave assisted extraction of *D*-limonene is already known. Additionally, the cost-

effectiveness of the future biorefinery scheme being developed was considered early on and given the value of pectin compared to *D*-limonene (£10/kg for high-methoxy pectin[40] versus £1.91-6.61/kg for *D*-limonene in 2013),[132] it appeared early on during the project that the profitability of the process would depend on the production of pectin. Given the disadvantages linked with the use of acid hydrolysis for the extraction of pectin, acid-free conditions were investigated with the aim of producing pectin under low temperature hydrothermal conditions. Under such conditions, no acid was used during the extraction or the work-up of pectin. Within the scope of this project, pectin characterisation proved an interesting research avenue. The results obtained using standard methods used in the food industry were not satisfactory. A new application of nuclear magnetic resonance was hence developed as a result. The following section focuses on the analysis of acid-free pectin, highlighting the different techniques used and associated results.

4.4 Characterisation of pectin

Several parameters can be evaluated to determine the quality of the pectin extracted. The main ones are summarised in Table 9 according to their importance. Parameters such as the DE, galA and molecular mass distribution relate to the fine chemical structure of the pectin. They are characteristic of its functionality, and ultimately its applications. Finding analytical techniques available in house was particularly important for this project. Most analytical methods applied for the evaluation of pectin's chemical and functional properties were originally designed for food scientists. The main ones are reported in Table 9.

Table 9: Pectin quality parameters and associated analysis methods.

Priority order	Quality parameter	Indication	Determination method
1	**DE**	The distribution & the pattern of the esterified carboxyl groups has a profound effect on the gelling mechanism & gel properties.[125]	Phenolphthalein double titration,[164, 179] FT-IR,[180-182] ^{13}C NMR (solid and liquid).[183, 184]
2	**galA content**	Provides an indication of purity. A 65% galA is necessary for pectin to be used as a food additive (as E440).[152]	Phenolphthalein double titration,[179] Colorimetric method based on the use of carbazole[185] or *m*-hydroxydiphenyl.[186]
3	**Molecular mass averages & molecular mass distribution**	Molecular weight data allows the determination of the functional behaviour of the pectin macromolecule[187] as well as end-use performance evaluation.[188]	Size exclusion chromatography.[148, 186, 187]
4	**Neutral sugar content**	The relative proportion of neutral sugars relates to the selectivity of the extraction method for 1→4 bonds between galA units.[189] The "kinks" caused by the presence of rhamnose residues within the pectin chains will affect the conformation[190] and consequently the macromolecular properties of the gel formed herein for example.	Gas chromatography-mass spectrometry analysis of trimethylsilylmethyl glycoside derivatives[191] and high Performance Anion Exchange Chromatography.[192]
5	**Intrinsic viscosity**	Relates to the pectin chain length.	Size exclusion chromatography with light scattering detector,[148] viscometer[193] and dynamic oscillatory tests.[194]
6	**Ash content**	For pectin to be used as a food additive (as E440) it needs to contain a maximum of 1% contaminant.[152]	Ashing at 500-600 °C.[195]

The following section will focus on the methods initially chosen to characterise microwave extracted pectin under acid-free conditions. Attenuated Total Reflectance Infra Red (ATR-IR) has first been used to systematically characterise pectin and determine the DE, followed by nuclear magnetic resonance (NMR). Solution and solid state NMR were applied. Gel permeation chromatography was also used to analyse the molecular mass distribution of the obtained pectin samples the results obtained by this technique will be developed in sections 4.5.1.1 and 4.5.4.

4.4.1 ATR-IR characterisation of pectin

ATR-IR was used to rapidly identify signature peaks of acid-free microwave extracted pectin under different conditions. ATR-IR is ideal for routine characterisation as virtually no sample preparation is required. However samples need to be carefully dried to avoid peak interference due to the presence of water. Otherwise an overlap with the bands associated with the carboxyl groups within the 1750-1600 cm^{-1} region of the IR spectra. Hence thorough drying of pectin is very important. Commercial pectin samples derived from citrus fruits were used as a reference. In the case of pectin, transmission Fourier Transform Infra Red (FT-IR) spectroscopy has mainly been used to identify the signals associated with the free carboxyl, which can be identified at ~1620 cm^{-1}, and the esterified carbonyl group at ~1740 cm^{-1}.[196-198] The later data has been obtained using FT-IR where pectin samples have been pressed with KBr. FT-IR is an useful technique to use for pectin characterisation as it has been reported for the determination of the DE. The DE can be determined from the ratio of the peak area of $-COOCH_3$ over the sum of the areas of $-COOCH_3$ and -COOH.[181, 182, 196] It has later been found that these peaks partially overlap. As a result, the quantitative data derived from such procedure has been found to not be reliable (Figure 23).

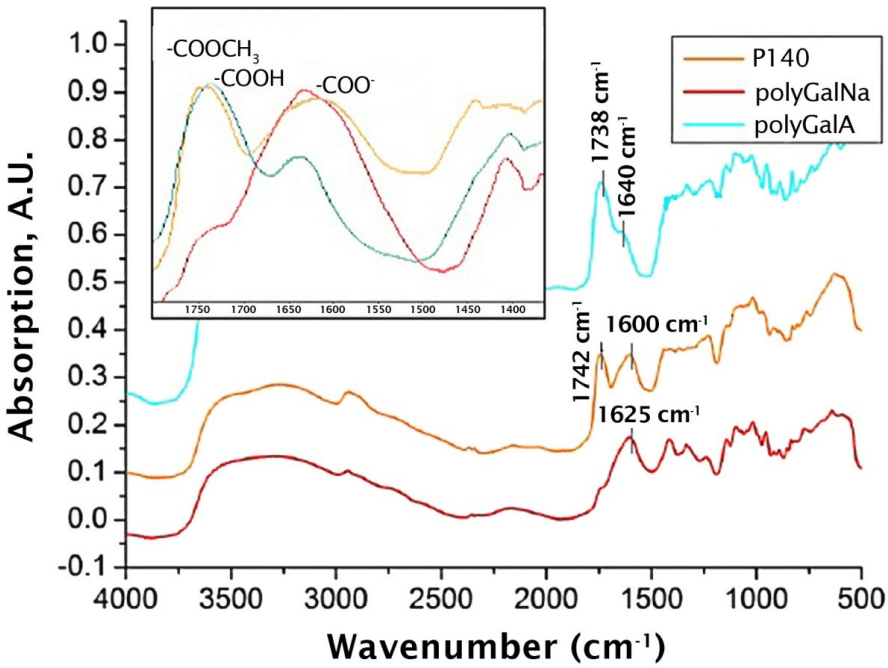


Figure 23: FT-IR spectra of P140, polygalacturonic acid and polygalacturonic acid sodium salt maximised within the range of 1900-800 cm^{-1} (originally in colour).

The esterified and non-esterified carboxylic functions are known to overlap: Kumar *et. al.* reports both functional groups at 1749 cm^{-1}.[199] This has been confirmed by analysing the sodiated form of galA. Figure 24 shows how the ATR-IR analysis of sodiated polygalA displays a peak at ~1625 cm^{-1} which can be attributed to the carboxylate function and not to the free acid. Slightly different ranges have been reported too, where the peaks of the esterified and non-esterified carbonyls of galA appear at 1760-1730 cm^{-1} and 1750 cm^{-1} respectively.[164, 181] This has been attributed to the presence of water, pH and hence, ionic conditions whereby the pectin can be acidified to eliminate the contribution of the carboxylate anion peak and display two distinct peaks for the acid and the ester form of galA.[181] Another strategy has been reported to circumvent this problem. A modified method based on the ratio of the "asymmetric stretching of $-CH_3$ at 1440 cm^{-1} and the galA backbone vibration at 1010 cm^{-1}" has been suggested to by-pass the disadvantage of the latter method.[181] However it is very difficult to attribute a peak with 100% certainty in the fingerprint region of the spectra (1200-800 cm^{-1}).[184] Thus, this method has not been applied here. Another diagnostic peak is the asymmetric stretching of $-CH_3$ (1440 cm^{-1}).[181, 196, 200] Additionally, absorption signals are observed for the ν(CO), ν (CC), γ(OCH) in the ring structure of poly galA chains can be observed ~1010 cm^{-1} (1016 cm^{-1} here).[181] Peaks seen at 3500-3300 cm^{-1} and ~2900 cm^{-1} are commonly attributed to the –OH and the C-H stretch in $-CH_2$ respectively.[197, 199, 201] Care should taken

nonetheless when comparing spectra obtained by transmission IR and ATR-IR since "a relative shift in band intensity and an absolute shift in frequency" can be observed when comparing bands in ATR-IR to transmission IR. A shift of up to 14.1 cm^{-1} has been reported.[202] When comparing the data, a variability might be observed between data reported in the literature and experimental data reported herein. A representative ATR-IR spectrum of a sample obtained at 120 °C following a 10 minutes heating time has been included in Figure 24 for comparison with a non-standardised commercial pectin sample (NS Kelco). The match between the two samples is ideal: all peaks relative to the carboxyl function of galA are present, demonstrating pectin is extracted. Furthermore, the two samples are nearly identical, including the fingerprint region, confirming that the extracted compound is pectin. ATR-IR was therefore used routinely to characterise pectin alongside other techniques for this project.

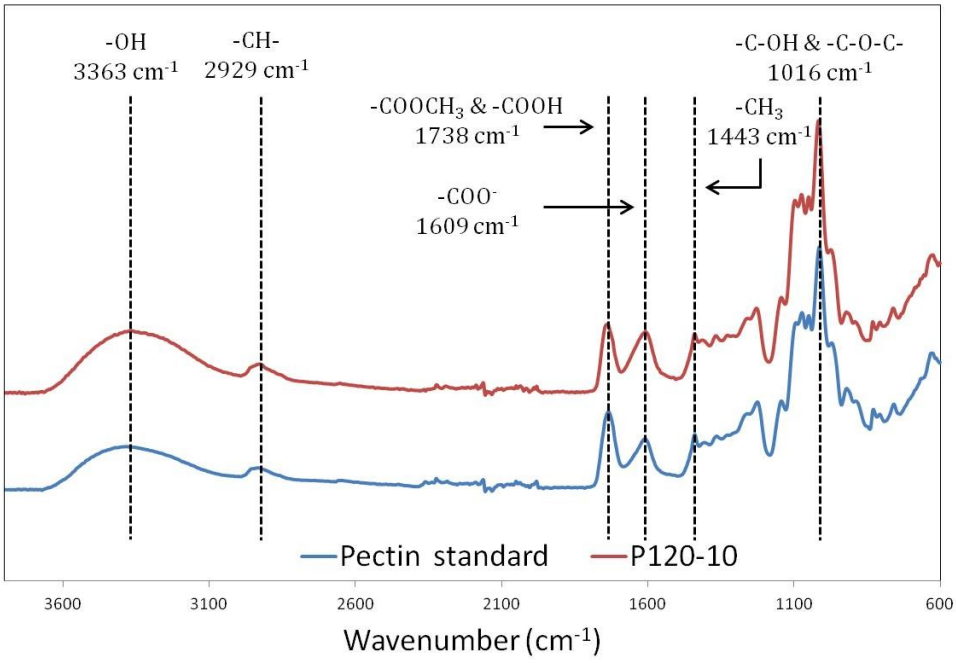


Figure 24: ATR-IR spectra of pectin sample P120-10 and commercial pectin NS Kelco (originally in colour).

4.4.2 Determination of the degree of esterification of pectin by double titration

ATR-IR was used to establish pectin could be extracted under microwave acid-free conditions. However another method had to be used to determine the DE. The DE

and galA content values obtained for microwave extracted pectin under acid-free can be determined via titrimetry using a method reported in the American Foods Chemical Codex. This method is considered a benchmark for the determination of pectin related DE and galA values and its use is standard practice in industry.[179] The protocol used was listed in the Food Chemicals Codex and further optimised by Professor Pete A. Williams, Director of the Centre for Water Soluble Polymers at Glyndwr University.

The reproducibility of the protocol was first assessed and the method was tested on a commercial pectin sample CU 301 (galA 84 %, DE 65 %). The best results obtained are summarised in Table 10. A good reproducibility and an error of 7% on the galA content were achieved at first, reaching a galA of 78.03% and 79.94% for two titrations carried-out in parallel. However upon repetition of the experimental protocol, the error on the galA content did not decrease below 20%. Even if considering an absolute value for the galA and the DE is not realistic, the value should still be comprised within a ± 10% range. It should be noted that although a satisfying error percentage wasn't achieved when trying to reproduce the results first obtained, the galA content was always consistently lower that the value given in the manufacturer's specifications.

Table 10: Summary of the results obtained for a commercial pectin sample using the Food Chemicals Codex method.

	CU 301 (galA 84%, DE 65%)	**CU 301 (galA 84%, DE 65%)**	**Average**
Experimental galA content (mg)	390.22	399.93	395.07
Experimental galA content after acid treatment (%)	78.03	79.94	78.98
Error on galA content (%)	7.11	4.84	5.97
Experimental DE (%)	59.70	62.14	60.92

Several different alterations to the reported method were trialled to eliminate any possible source of error and obtain more consistent values between titration experiments. They are listed below:

- The double titration was done over two days: the back titration was done on the 2nd day instead of 15 minutes after the 1st

titration to see whether the duration of the saponification had an influence on the result obtained.

- The two titrations were done one after the other using a timer to make sure that the dissolution/stirring times were rigorously the same for each sample (upon timing).
- Samples were degassed under nitrogen for up to 1h to remove any CO_2 that might interfere with the result obtained.
- The influence of pectin dissolution times were studied to see whether solubility of pectin and the formation of aggregates might be responsible for the poor results obtained.
- The pectin was never left more than 10 minutes in the 2.7M HCl solution.
- Care was taken to use only very recently boiled water to avoid adding unnecessary CO_2 into the pectin solution.

The protocol modifications listed above have not been found to improve the reproducibility and an alternative method was investigated. The use of nuclear magnetic resonance was investigated next.

4.4.3 Pectin characterisation and determination of the degree of esterification by solution nuclear magnetic resonance (NMR)

4.4.3.1 Characterisation by solution nuclear magnetic resonance

Nuclear magnetic resonance spectroscopy (NMR) is a recognised technique used for the characterisation of chemical compounds, including polymers. This technique is based on the identification of each non-equivalent nucleus according to the behaviour of its electronic environment in a variable magnetic field. A signal is observed if the spin of a nuclei with an uneven number of protons is excited by the application of an electromagnetic radiation of an adequate frequency corresponding to the difference in energy between the relaxed and the excited spin state (the Larmor frequency). The relaxation of the spin causes the emission of a signal which is detected and converted to a chemical shift, generating an NMR spectra after Fourier transform. Figure 25 highlights the concepts used for this technique. It has previously been used for the identification of pectin derived from apple,[193, 203] pumpkin,[184] papaya[183, 193] and citrus fruits[193].

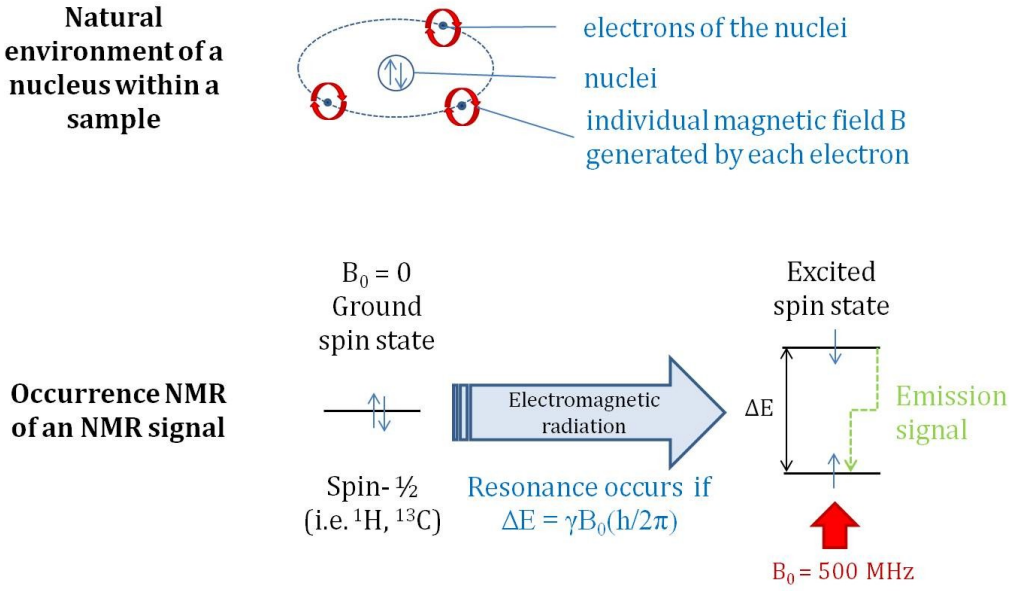


Figure 25: Effect of the application of a magnetic field on the spin of an uneven numbered nucleus (originally in colour).

At first, ^{1}H NMR was explored. This technique is quantitative and provided suitable signals for pectin identification. However, due to the different conformers of sugar monosaccharides present in the side chains, multiple overlapping signals could be detected, rendering spectra interpretation difficult, even in the anomeric protons zone. Given the numbers of protons expected in the sugar region (5.5-3.4 ppm) for rhamnose, xylose, galactose, arabinose and furanose (12 for rhamnose in D_2O for example), a good resolution was difficult to obtain in the region showcasing anomeric protons (5.5- 4.4 ppm) and other protons on the sugar residues (4- 3.4 ppm) at 400 MHz. The use of a 500 MHz spectrometer or a 700 MHz spectrometer combined with the use of long accumulation times and an increase in temperature from 298 K to 318 K and 348 K to see whether the dissolution of pectin could be improved by raising the temperature, did not improve the resolving power. Instead, ^{13}C NMR at 700 MHz was favoured as an alternative to ^{1}H NMR since peak broadness is minimal with this nucleus compared to proton NMR, increasing peak resolution. ^{13}C NMR also has the advantage of potentially allowing the determination of the DE of pectin, providing that the peaks for the –**C**OOCH$_3$ and the –**C**OOH groups are well separated under quantitative acquisition conditions.

700 MHz ^{13}C NMR spectroscopy proved to be useful to unequivocally confirm the presence of pectic polysaccharides. The technique was trialled on an experimental

sample generated at 140 °C under microwave acid-free conditions and a commercial pectin sample. Hereafter, the ^{13}C NMR spectra of commercial orange pectin and P140-10 are respectively denoted as SA (Figure 26) and P140-10 (Figure 27). Following ^{13}C NMR, issues were encountered regarding sample drying. In spite of the extensive drying at 30 °C in a vacuum oven, two clear ethanol peaks are being present for $-CH_2-$ at 58.05 ppm and $-CH_3$ at 17.40 ppm. As a consequence, the $-CH_3$ peak of ethanol is overlapping with the most characteristic rhamnose peak as it appears at lower frequency. This issue will be resolved at a later stage with the use of a freeze-drier to remove all traces of solvent in pectin.

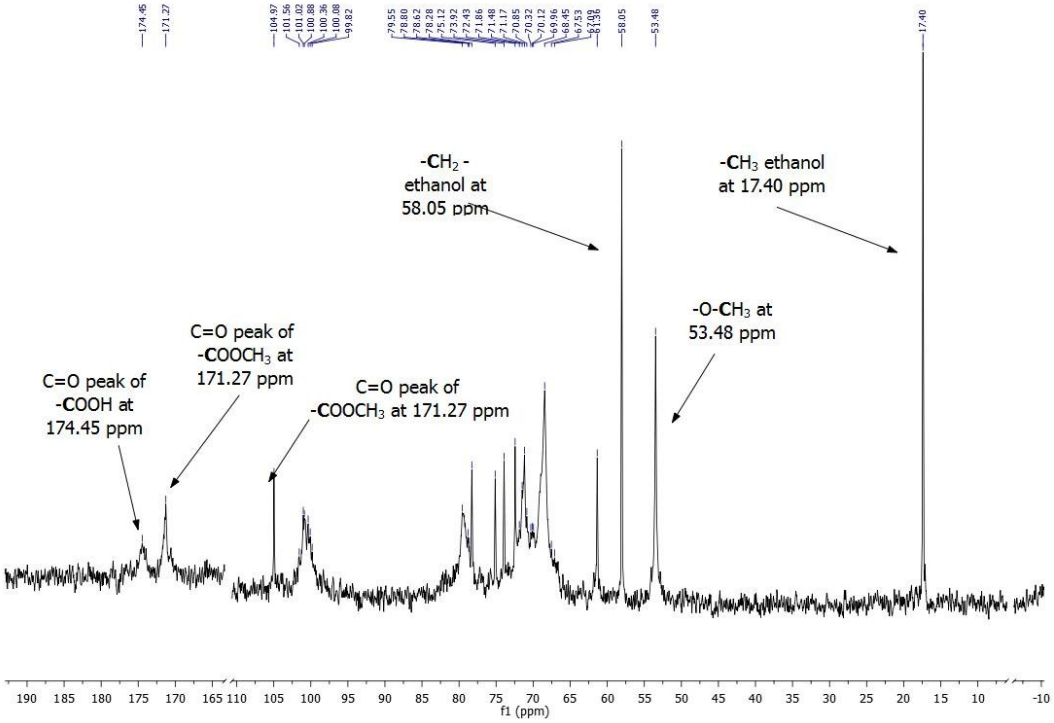


Figure 26: ^{13}C NMR of commercial citrus pectin in D_2O (700 MHz) (originally in colour).

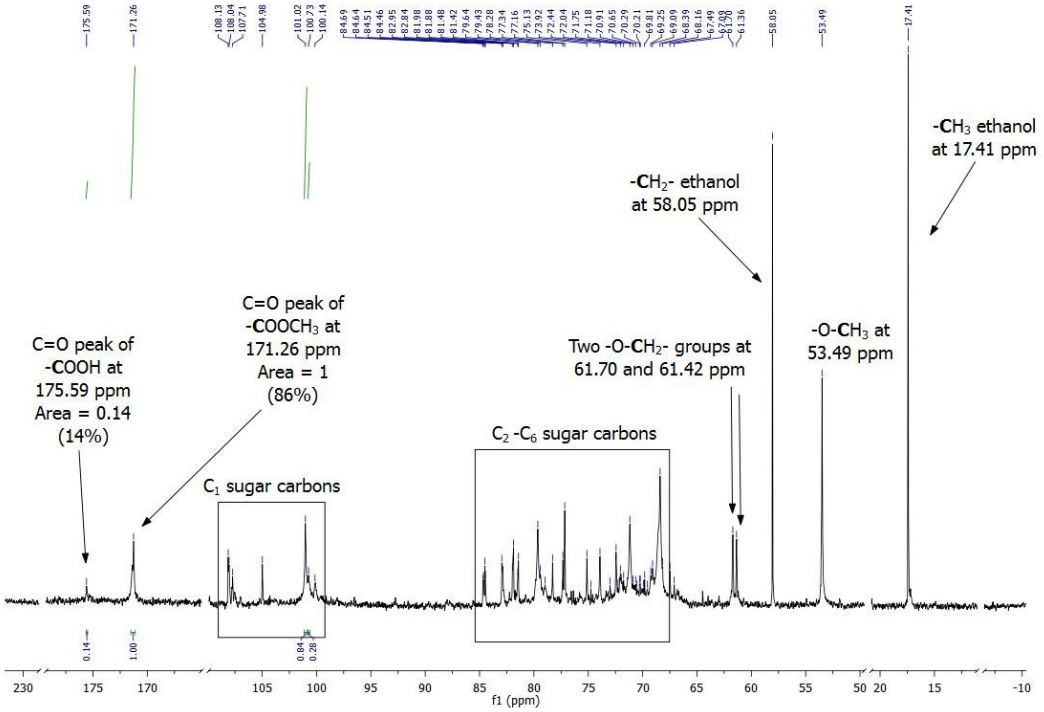


Figure 27: ^{13}C NMR of sample P140-10 in D_2O (700 MHz) (originally in colour).

By comparing the two spectra in Figure 26 and Figure 27, one can confirm pectin is being extracted at 140 °C under acid-free conditions. Several observations strengthen this conclusion. The first one relates to the presence of signals typically associated with the carboxyl function: P140-10 shows two clear peaks at 175.59 and 171.26 ppm. These refer respectively to the free acid carbonyl group and the methyl ester carbonyl. In the SA sample these two peaks show up at 174.45 and 171.27 ppm. This unequivocally establishes the presence of the same methyl ester functionality in both P140-10 and SA (171.26 ↔ 171.27 ppm). Local variations in the pectin are brought forward to explain for the shift at higher frequency of the acid functionality in P140-10, at 175.59 ppm, versus the one in SA, at 174.45 ppm. Our values are in agreement with those reported by Kostalova *et. al.* on pumpkin pectin.[184] Indeed, they report the free acid carbonyl at 175.5 ppm and the methyl esterified one at 171.4 ppm. Westerlund *et. al.* states that the methyl ester carbonyl typically appears around 172.5 ppm and the free acid carbonyl in the 173-177 ppm range.[183] Tamaki *et. al.* lists the methyl ester at 173 ppm and the free acid carbonyl at 177ppm.[201] Similarly for Iacomini *et. al.,* two low file signals at 174.81 and 170.68 ppm have been attributed to the methyl ester and the free acid form of the carbonyl.[204]

Secondly, the signal traditionally attributed to the –O-**C**H_3 group belonging to the ester function was located at 53.49ppm by 135 DEPT ^{13}C NMR (see Figure 28). This compares very well to the shifts reported in the literature for this functionality: Kostalova *et. al.* (53.7 ppm), Tamaki *et. al.* (55.7 ppm), Iacomini *et. al.* at 52.86 ppm and Li *et. al.* at 51.84 ppm.[205] In commercial pectin, the presence of a methyl ester is clearly visible at 53.48 ppm.

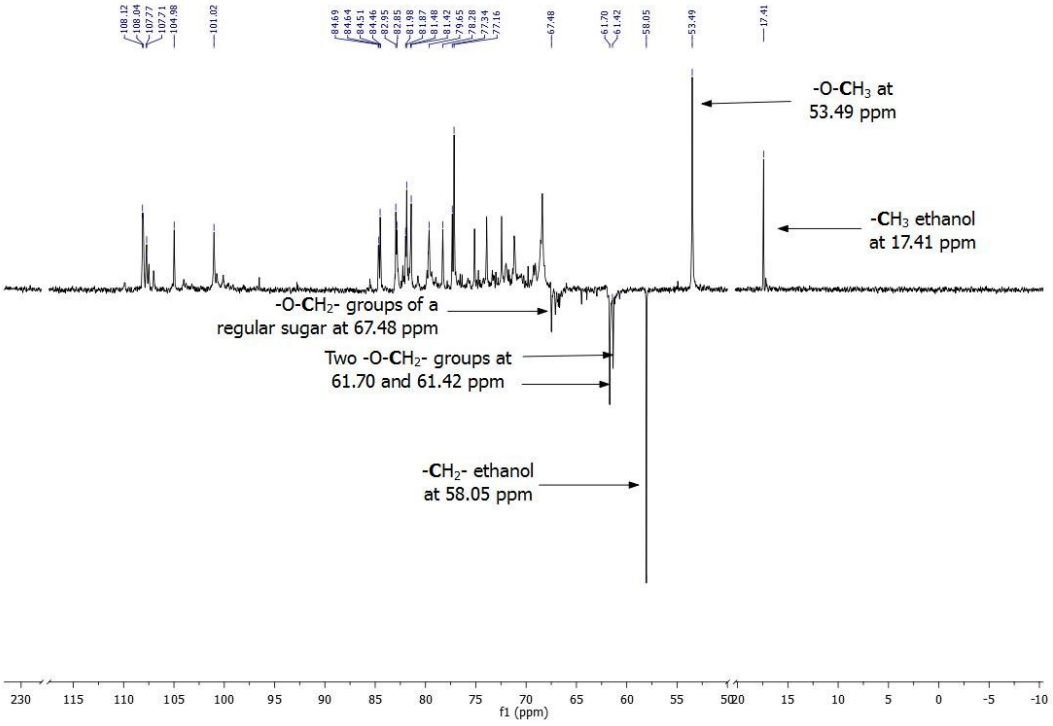


Figure 28: DEPT135 ^{13}C NMR of sample P140-10 in D_2O (700 MHz) (originally in colour).

Thirdly, further analysis of the spectra confirm the presence of the sugar side chains. For mono- and polysaccharide analysis, the anomeric region around 90-110 ppm is particularly informative. P140-10 shows distinct peaks in this region at 108.13, 108.04, 107.71, 104.98, 101.02, 100.73 and 100.14 ppm (see Figure 27). The peaks at 108.13, 108.04, 104.98 and 101.02 are the most pronounced ones. The DEPT 135 spectrum classifies all of them as CH/CH_3 but given their chemical shift, they can only be CH groups (see Figure 28). Commercial pectin shows distinct anomeric carbons at 104.97 ppm and ~100.88 ppm. The main difference thus lies in the existence of the 108 ppm peaks, which have been reported to belong to α-Araf residues.[184] The dominant peak at 101.03 ppm is very close to the one at 100.9 ppm as reported by Kostalova *et. al.* for methyl esterified α-GalpA units. The same authors attributed a peak at 99.9 ppm to the free acid α-GalpA units which is very

close to the 100.11 ppm observed in P140-10. This confirms again the dominant presence of esterified residues of galA.

Additionally, the DEPT 135 spectrum allows us to start attributing the peaks seen in the 60-50 ppm region carbons. They are believed to be the non-anomeric carbons of the neutral sugar side chains of pectin P140-10. The DEPT spectrum classifies them unequivocally as CH_2 carbons. In the O-CH region, a greater number of peaks can be detected for P140-10 than for the commercial pectin sample used.

P140-10 has been acquired quantitatively hereafter allowing for an estimation of the DE. A quantitative sequence was applied with a 10 seconds relaxation time. When comparing the integrations of the carbonyl signals at δ 175.59 (area of 0.14) and 171.26 (area of 1), a DE of ~86% can be determined. Interestingly, Catoire *et al.* states that the C1 of esterified galA can be distinguished from its free acid form. Based on their respective peak integrations of the C_1 of unesterified (100.11 ppm, area of 0.28) and esterified α-GalpA units (101.03 ppm, area of 0.84),[193] a similarly high DE of 78% can be calculated according to the reported work depending on the integration limits used. However, the high signal-to-noise ratio does not allow the determination of an absolute value for the DE. In commercial pectin, even though this spectrum is not run quantitatively, one can clearly see that it has a lower degree of methyl esters compared to P140-10.

It has been observed that pectin has a rather low solubility in H_2O (below 50 mg per NMR sample). The formation of viscous solutions of pectin have been previsouly reported.[206] Some peak broadening can be observed due to the viscous, gel-like behaviour of the solubilised pectin in D_2O. In addition, since the measurements have been done in a quantitative mode, applying a 10 seconds relaxation time instead of a standard 2.5 seconds, a partial precipitation of the sample during the NMR experiment could be observed given the lengthy analysis time required. When taking into account the high signal-to-noise ratio observed when carrying-out such NMR experiments, these phenomena could all be responsible for the difficult determination of the DE with high accuracy. Nevertheless, the results obtained by FT-IR, and ^{13}C NMR all confirm pectin can be extracted under acid-free microwave conditions.

Several methods have been developed over the years for the characterisation of pectin. Earlier tests were based on the ability of hexuronic acids, such as galA , to form chromophores which are detectable by UV-VIS a quantifiable technique.[164]

Figure 29 describes the intermediary species obtained by decarboxylation of D-galacturonic acid and dehydration to a furan with sulphuric acid.[207, 208] The mechanism of the decarboxylation is not known.[209]

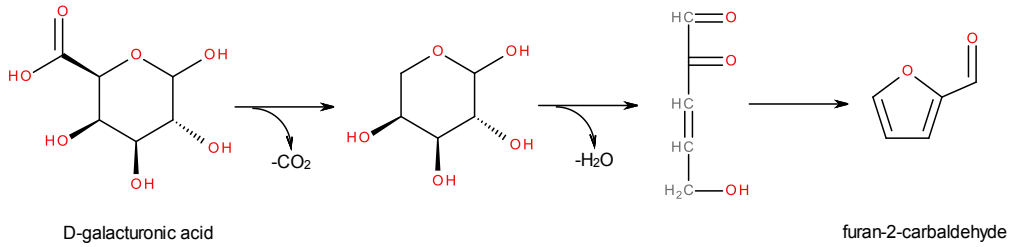


Figure 29: Reaction pathway of D-galacturonic acid to furans under acidic pH (originally in colour).

Carbazole, *m*-hydroxydiphenyl, 2-thiobarbituric are dyes which are known to form chromophores with the acid-hydrolysed products of galA. However, they are non-specific as the total carbohydrate content reacts with the dye (i.e. neutral sugars and cellulose), falsifying the results.[164, 210] Given the potentially high neutral sugar content present in pectin under microwave acid-free conditions (see section 4.5.3), these methods have not been trialled. The double titration of the carboxyl groups before and after saponification of the ester groups of galA has not been conclusive either. Other methods involve the combined detection and quantification of galA together with the neutral sugar content determination by Gas Chromatography-Mass Spectrometry (GC-MS) and High Performance Anion Exchange Chromatography (HPAEC). However both methods rely on the hydrolysis of pectin to galA and saccharide monomers. Pectin can be difficult to "hydrolyse quantitatively when using H_2SO_4 and TFA given the difference in behaviour of sugar monosaccharides and polygalA under such conditions." This is mainly attributed to the difference in bond strength between the galA chains and the neutral sugar chains in acid medium. This can lead to underestimated percentages of monosaccharides and low recovery percentages of galA as the latter is degraded to by-products such as lactones.[192, 210, 211] Additionally, the sample preparation is laborious, prone to human error and time consuming which make these methods difficult to employ systematically for optimisation work. [186] This is particularly true in the case of GC-MS analysis of pectin monosaccharides where incomplete functionalisation can occur during monomer functionalisation.[210] This was an important obstacle as together with the double titration, those methods allow both the determination of the DE and the galA content.

4.4.3.2 Solid state carbon nuclear magnetic resonance analysis

Solid state NMR differs from solution NMR given the anisotropic nature of solids: the absence of molecular tumbling in solids causes peak broadening, resulting in the loss of the structural information obtained with solution NMR. Magic angle spinning is then used to counteract peak broadening by rotating the sample at an angle of 54.74° to the magnetic field applied. Cross polarisation enhancement can additionally be applied to detect the nuclei whose properties render them less detectable. Cross polarisation magic angle spinning (CPMAS) NMR experiments are frequently used to transfer magnetisation from protons to carbons rather than using direct polarisation of the ^{13}C nucleus.[212, 213] The signal intensity observed with solid-state NMR is known to be directly dependent upon the chemical environment. The number and proximity of protons linked to a carbon atom will influence the intensity of its peak. In addition to the proton environment, molecular dynamics and in this case, the rotation of the methyl group will influence peak intensity.[214]

Given solubility issues encountered with pectin in water, quantitative solution ^{13}C NMR was not possible for the determination of the DE given the high signal-to-noise observed then. As a result, solid state ^{13}C NMR was trialled for the determination of the DE of pectin.

Precedents exists on the use of ^{13}C CPMAS for the determination of the DE: [206, 215,216] Synytsya *et. al.* attempted to distinguish between the contribution of the –**C**OOCH$_3$ and -OCO**C**H$_3$ peaks. However, strong evidence is still required to obtain full resolution of the spectra. A later paper has been published using the same method as Synytsya *et. al.* The main issue with the later papers is that the estimation of the DE is based on the integration ratio of the -COO**C**H$_3$ over the one corresponding to the ring carbons C_1-C_5. This does not distinguish between the C_1-C_5 of polygalA and the C_1-C_5 of other sugars, leading to an underestimation of the DE. Furthermore, few of these authors make mention of contact time and if or how this impacts the determination of the DE.

Here it was assumed the DE could be calculated using the integrated intensities of the carboxylate ($Int_{C(O)OR}$) and methoxy (Int_{OCH_3}) signals as highlighted below, where R can correspond to –H, –Na$^+$ or –OCH$_3$ groups.

$$DE_{NMR} = 100 \times \frac{Int_{OCH_3}}{Int_{C(O)OR}}$$

This method presents the advantage of only considering peaks which can only be derived from the presence of polygalA. Hence, the presence of rhamnose for example, would not affect the calculated DE. The chemical shift range used for this experiment were of 165-180 ppm and 50-57 ppm which respectively corresponded to the –**C**(O)OR group and the –O**C**H$_3$ group.

A sample of polygalA was analysed first. The ^{13}C CPMAS spectrum of as-purchased galA is shown in Figure 30. The carboxylic acid signal (~173 ppm) is well resolved from the signals corresponding to the anomeric, C1 carbon (~100 ppm) and the other ring carbons (~65-85). On the other hand, the observed peak integrals (0.72:1:4.1) reveal a significant underrepresentation of the carboxylate carbons due to the absence of directly attached hydrogens, significating a correction factor will be needed.

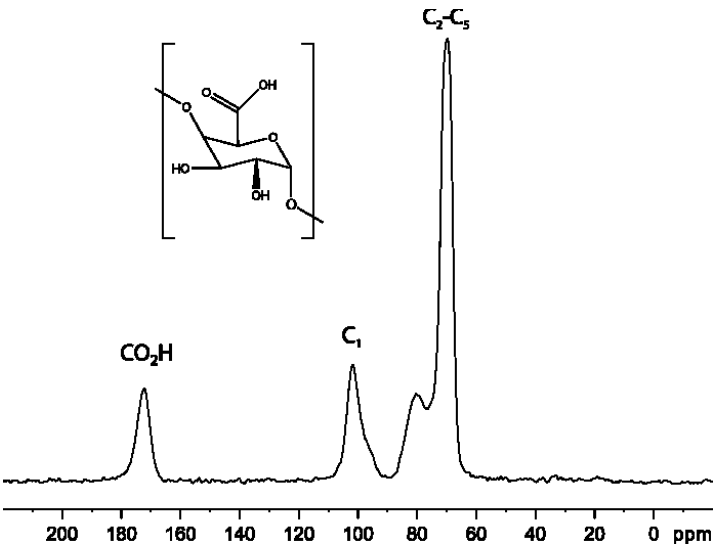


Figure 30: ^{13}C CPMAS spectrum of polygalacturonic acid.

In addition to as-purchased polygalA, a spectrum of polygalA after re-dilution in water and subsequent freeze-drying was acquired; replicating the conditions under which pectin samples were all produced. The two spectra are shown in Figure 31 for comparison. The spectrum of the freeze-dried sample exhibits significantly sharper resonances. The carboxylic acid carbon signal is 480 Hz broad (FWHM) for the as-purchased sample and only 280 Hz after lyophilisation.

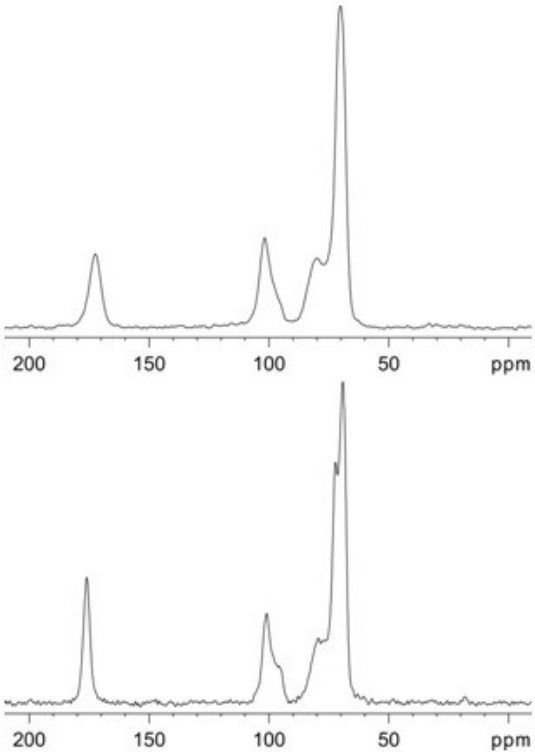


Figure 31: ^{13}C CPMAS spectrum of polygalA as-purchased (above), and after freeze-drying (below).

As a result of the observations made following the analysis of the polygalA sample, five commercial samples of pectin of known DE were analysed to assess the validity of the chosen technique for the determination of the DE of pectin. Their spectra are very similar in appearance to the as-purchased polygalA, except for the presence of an additional resonance at ~54 ppm arising from the -O**C**H_3 of the carboxylic acid groups which are esterified. The spectra of the commercial samples with varying degrees of esterification are shown in Figure 32. The chemical shifts displayed match the ones previously reported. No signal at ~ 20-25 ppm showcase the absence of acetyl groups.[206]

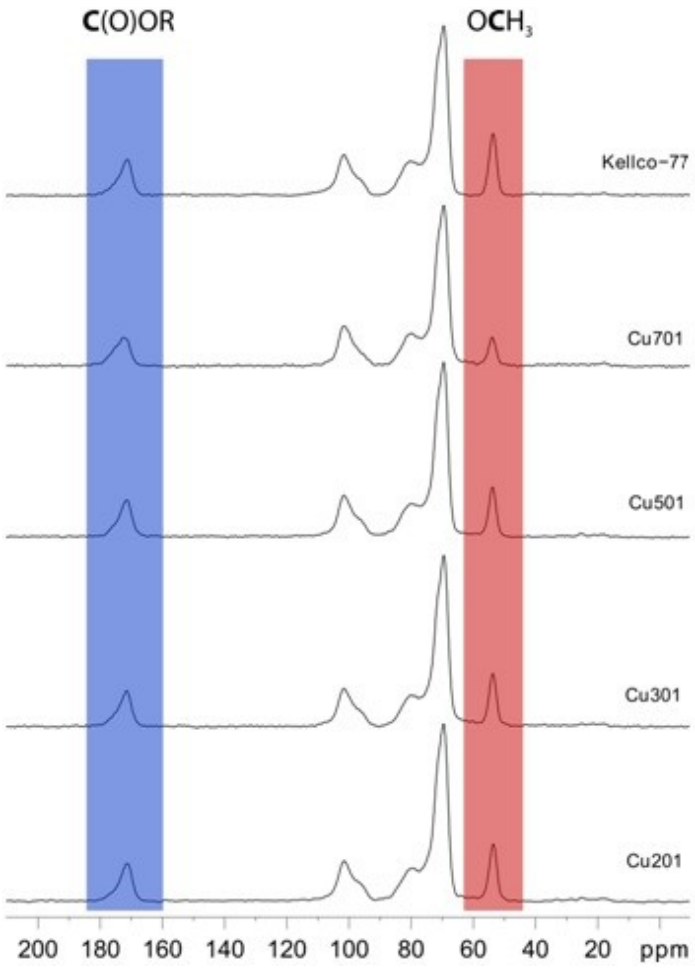


Figure 32: ^{13}C CPMAS spectrum of commercial pectin samples highlighting the carboxylate (δ~172 ppm) and methoxy (δ~53 ppm) carbon signals (originally in colour).

However, as with the galA, we would expect the carboxylate carbon intensity to be under-represented due to the absence of directly linked hydrogen atoms, leading to an over-estimation of the DE. Cross polarisation is known for underestimating the ^{13}C nuclei in carbonyl groups given their low ability to couple with a proton.[217] The mixing time was used to study the influence of ^{1}H-^{13}C coupling on the DE values obtained for commercial pectin. Plots of the measured DEs for the commercial samples versus the DE values provided by the supplier display this trend (see Figure 33 and Figure 34). The best-fit curves to the data yielded slopes of 1.33+0.09 and 1.13+0.07 for the 1 and 2 ms contact times, respectively. These values were subsequently used to correct the behavioural differences of the carbonyl and -OCH_3 environments of C_6. The DE values obtained were corrected such as $DE_{corr} = DE_{NMR}/k$. A correlation factor (R^2 value) of 0.9595 and 0.8125 was obtained for the 1 and 2 ms contact times, respectively. Hence, NMR values obtained for the DE at 1 ms agreed with the values obtained with a conventional method used in the food

industry (i.e. phenolphthalein double titration). The correction factor is used to render the analysis quantitative. This accounts for the absence of protons on the carbonyl group, and for the rotation of the $-CH_3$ group of the ester which interferes with the average dipole-dipole interaction and the cross polarisation process.

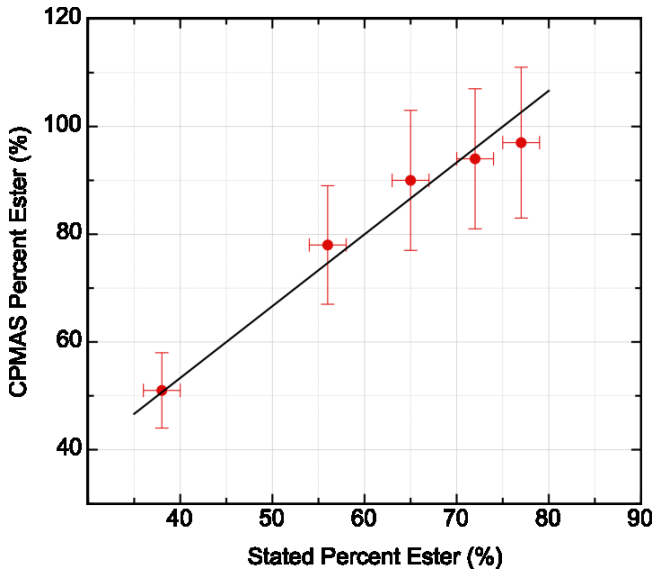


Figure 33: Comparison of the ^{13}C CPMAS determined DE for five commercial standards using a 1 ms ^{1}H-^{13}C mixing time (originally in colour).

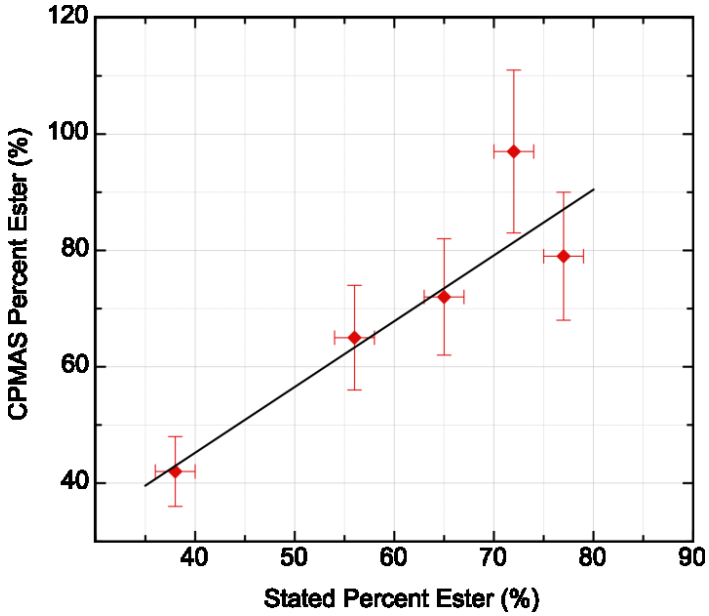


Figure 34: Comparison of the ^{13}C CPMAS determined DE for five commercial standards using a 2 ms ^{1}H-^{13}C mixing time (originally in colour).

The method was applied to a range of pectin experimental samples all obtained using an acid-free hydrothermal microwave process. All NMR spectra were acquired using both a 1 ms and a 2 ms contact time. The DE of these samples were then determined and corrected using polygalA or the commercial samples, generating two different data sets. The difference in the measured DE between the 1ms and 2ms mixing time experiments for each corrector factor used were plotted in Figure 35. The red unfilled bars show the difference in DE between 1 ms and 2 ms for the data sets corrected using the polygalA. The blue filled bars using the scaling factors obtained with the commercial samples.

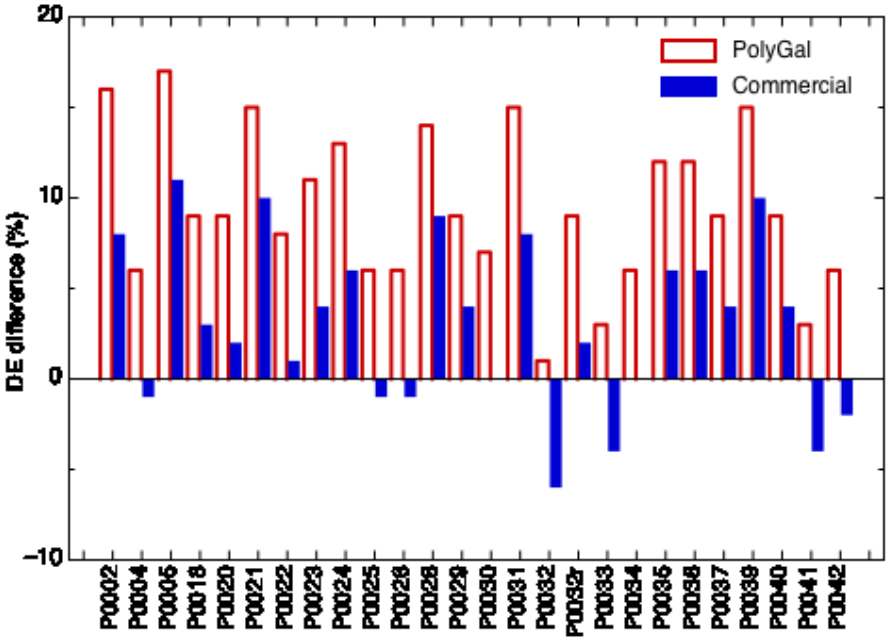


Figure 35: Difference in the measured DE between 1ms and 2ms mixing time (red for polygalA and blue for commercial samples) (originally in colour).

Figure 35 shows how the data set corrected using commercial pectin samples is less prone to a systematic error. In the earlier data set, the difference in DE can reach an absolute value of 17 whereas it decreases to 10 for the later data set. The graph also displays a more random distribution, yielding both positive and negative DE differences. Consequently, the correction factor obtained via the spectra acquisition of five commercial pectin yields less biased DE values than the correction factor obtained via the spectra acquisition of polygalA. As a result, the correction factors using commercial pectin samples will be used.

Regarding the choice of contact time used here onwards, a 1 ms contact time will be used since the linear regression fit gives a correlation factor of 0.9595 which was deemed more acceptable than the correlation factor of 0.8152 obtained for 2 ms

contact time, rendering the results more precise and more reliable. Regarding previous work reported, the correlation factor obtained here is higher than the one reported by Sinitsya *et. al.* (0.901).[206] Besides, the absence of odd points with a contact time of 1 ms further justifies this decision. The results of the application of this method to experimental samples will be discussed in section 4.5.6.

4.5 Hydrothermal microwave extraction of pectin

One of the aims of this project was to improve the extraction of pectin based on previous work reported on microwave-assisted extraction of pectin from CPW. As seen previously, the use of acid produces polluting effluents requiring treatment prior to disposal. This could be avoided by simply using water under microwave hydrothermal conditions. This strategy would benefit from the use elevated temperatures (> 100 °C) under closed vessel conditions. Water is known to dissociate as the temperature increases, between 273 K and 623 K.[218] Figure 36 shows the chemical equation of the dissociation of water. The dissociation constant K_w and pK_w are given for a dilute solution of water (< 10^{-4} M).

$$2\,H_2O \rightleftharpoons H_3O^+ + OH^-$$

$$K_w = [H_3O^+]\,[OH^-]$$

$$pK_w = -\log_{10}(K_w)$$

Figure 36: Water dissociation and associated dissociation constants.

The variation of the pK_w according to the temperatures highlighted in Figure 37. As the pK_w decreases, the equilibrium of the equation shifts to the right and the number of the ions increases, lowering the value of the neutral pH as the temperature increases. The increasing concentration of H_3O^+ and OH^- ions at higher temperatures under closed-vessel conditions could be exploited when extracting pectin under microwave acid-free conditions (i.e. neutral pH conditions).

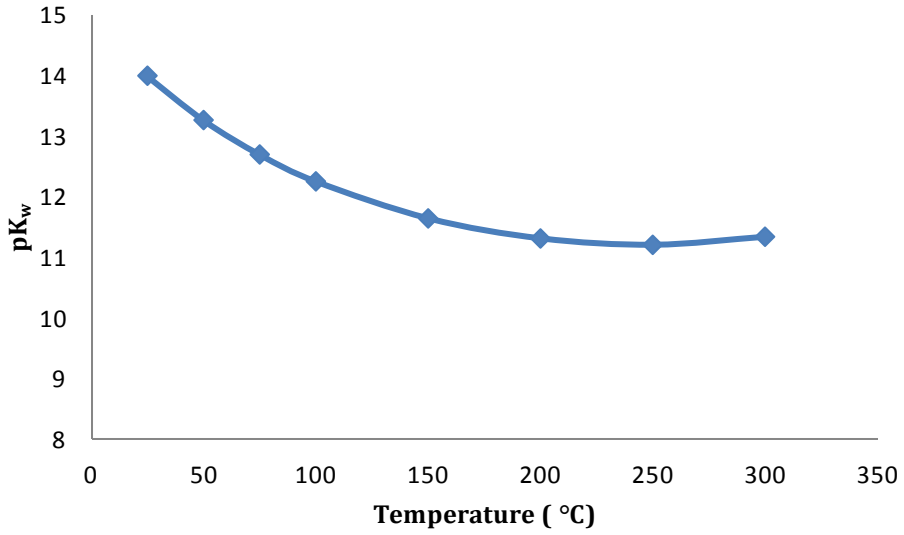


Figure 37: variation of pK_w as a function of temperature at a pressure of 0.1 MPa (originally in colour).[219]

Low temperature hydrothermal microwave treatment of WOP (waste orange peel) under acid-free conditions was investigated as an alternative to conventional acid-mediated pectin extraction methods. No acid (or any other additive) was added for the extraction or the work-up of pectin.

4.5.1 Small scale screening experiments

Screening conditions were first investigated. The effect of the peel to water ratio, temperature and extraction time on the yield of pectin extracted were studied to determine optimised conditions at small scale on the CEM Discovery microwave equipment by M.Sc. student Xin Neh Beh (2010-2011 Green Chemistry MSc cohort). This approach is similar to the one reported by Bagherian *et. al.* The effect of the operational paramaters used on pectin will be studied by GPC analysis. For the next section, pectin experimental samples will be named according to the following code: PAAA-BB where AAA stands for the microwave extraction temperature used (in °C) and BB for the duration of the microwave extraction (in minutes)

The first parameter investigated was the influence of the peel to water ratio (peel:water ratio) on the pectin yield. Experiments were carried out for 10 minutes

extraction time at 180 °C. Ethanol was used as a precipitation solvent since it is renewable and its use is allowed by international food standards.[139] Results can be seen in Figure 38. It was found that the highest yield obtained (8.37%) required a 1:10 peel:water ratio.

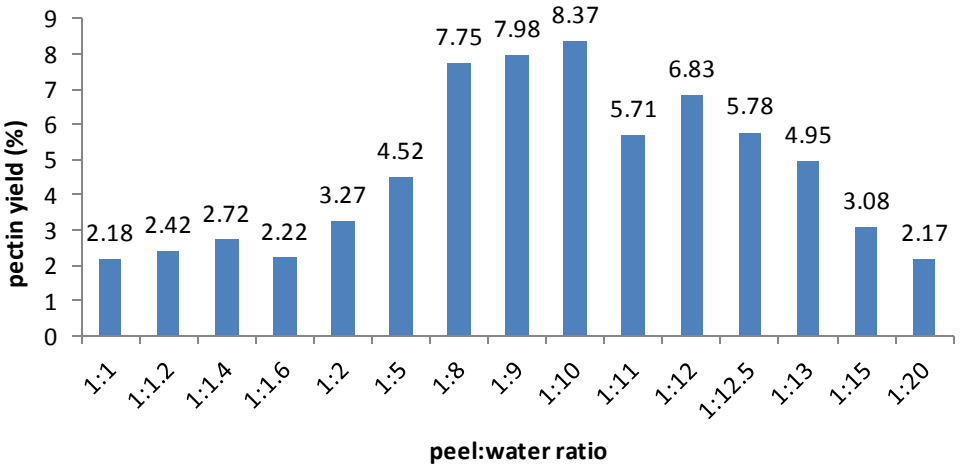


Figure 38: Effect of peel:water ratio on the yield of pectin (% dry mass) extracted at 180 °C for a heating time of 10 minutes (originally in colour).

The extraction efficiency of pectin was also investigated within a 120-200 °C temperature range (Figure 39). The temperature range tested was deliberately taken above 100 °C to benefit from the increased acidity of water under pressure above 100 °C. The highest pectin yield was obtained at 140 °C for a heating time of 10 minutes. Under these conditions, a maximum yield of 20.4% pectin was obtained.

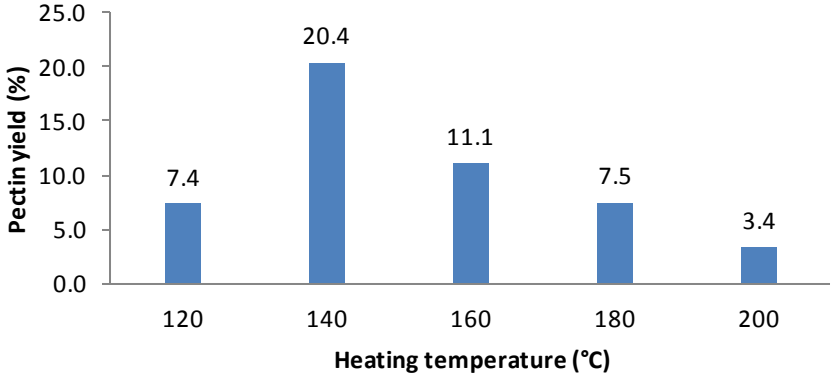


Figure 39: Effect of heating temperature on the yield of pectin extracted (% dry mass) for a heating time of 10 minutes using a 1:10 water:peel ratio (originally in colour).

The effect of the extraction time on the pectin yield was then studied (Figure 40). This experiment was performed at the optimal extraction temperature of 140 °C, as determined earlier. It was found that the pectin yield increased with extraction time. After one minute of heating a pectin yield of ~11% was obtained. Increasing the time of heating to 20 minutes doubled the amount of pectin extracted at 140 °C. A different trend was observed when varying the length of the extraction time at higher temperatures (e.g. 160 and 180 °C). Indeed, increasing the extraction time from 5 to 10 minutes results in a decrease of the pectin yield to 17 and 11% respectively. These results confirm the observations reported by Yeoh *et. al.* whereby pectin degrades during the extraction process when using high temperature, causing the yield of pectin to decrease. Lower heating temperatures are therefore favourable in order to obtain higher yields of pectin while avoiding decomposition during extraction. It is also important to note that the pectin yield obtained in this study is higher than the one obtained under microwave assisted acidic extraction: 5% of pectin were obtained under acidic conditions (1:16 peel:water) versus 7.4% for 10 minutes at 120 °C.[220] In comparison to classic acid hydrolysis, the conditions reported are interesting as traditional pectin extraction generally occurs over several hours. Whereas here, a 10 minutes extraction time only was necessary.

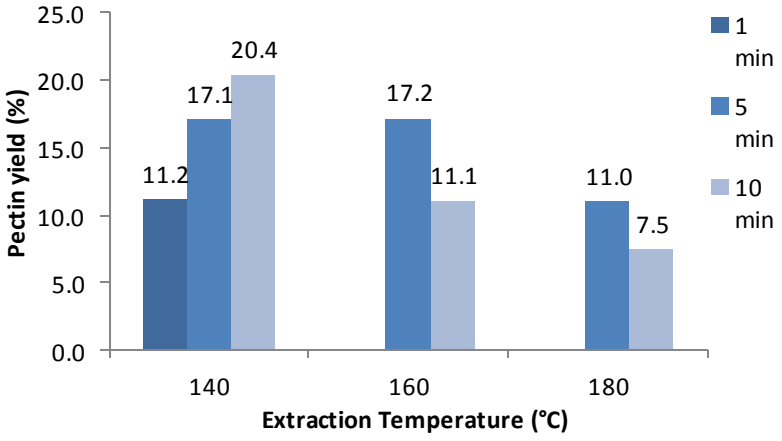


Figure 40: Effect of heating times (1, 5 and 10 minutes) at 140, 160 and 180 °C on the yield of pectin extracted (% dry mass) for a 1:10 water:peel ratio (originally in colour).

The visual aspect of the pectin samples obtained also varied greatly according to the microwave extraction temperature used. Pectin samples obtained at different temperatures showed different physical properties, especially in terms of colour (Figure 41). Pectins obtained at 120 and 140 °C were grey-white. Those obtained at 160, 180 and 200 °C were grey, light brown and dark brown respectively. The brown colouration appearing above 140 °C indicates a degradation process possibly occurring either via pyrrolysis of the OPW or degradation of the pectin extracted by depolymerisation catalysed by the presence of H_3O^+ and OH^- ions produced during the dissociation of water in addition to the acids already present in the peel (kg of dry peel has been reported to contain 90 g of organic acids).[128]

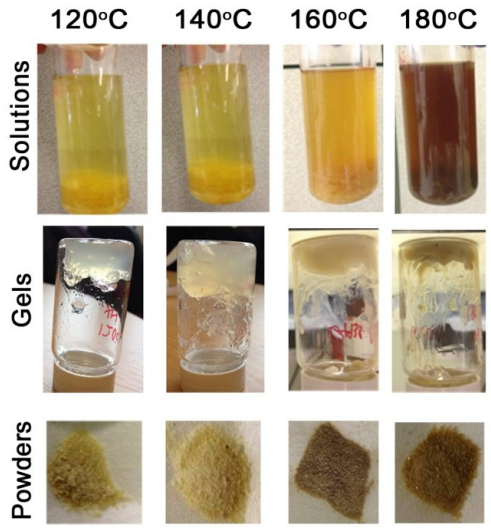


Figure 41: Effect of heating temperature on the visual aspect of pectin samples P120-10, P140-10, P160-10 and P180-10 (originally in colour).

The combined action of heat and acidic pH conditions on carbohydrates can lead to the formation of furan derivatives via the dehydration of the sugar monomers present in pectin. Their subsequent condensation "with themselves or phenolics [present in the peel] produces dark coloured complexes".[186] Alternatively, the brown colouration of the samples could be due to a "browning" reaction at high temperature of the sugars extracted and present in the pectin sample following drying. As an example, *D*-glucose has a boiling point of 146 °C. Sugar monosaccharides could realistically be extracted from WOP alongside pectin under the conditions tested. Both sugars can undergo a conversion to a 1,2-enediol compound which can be dehydrated and form 5-hydromethyl furfural, which is light

brown (see Figure 42).[221] Given the fact that non-coloured additives are preferred in the food industry, microwave heating temperatures up to 140 °C seem to be more favourable to pectin extraction. Regarding gel forming properties, upon addition of water, pectin extracted at 120 and 140 °C showcased gel-like properties.

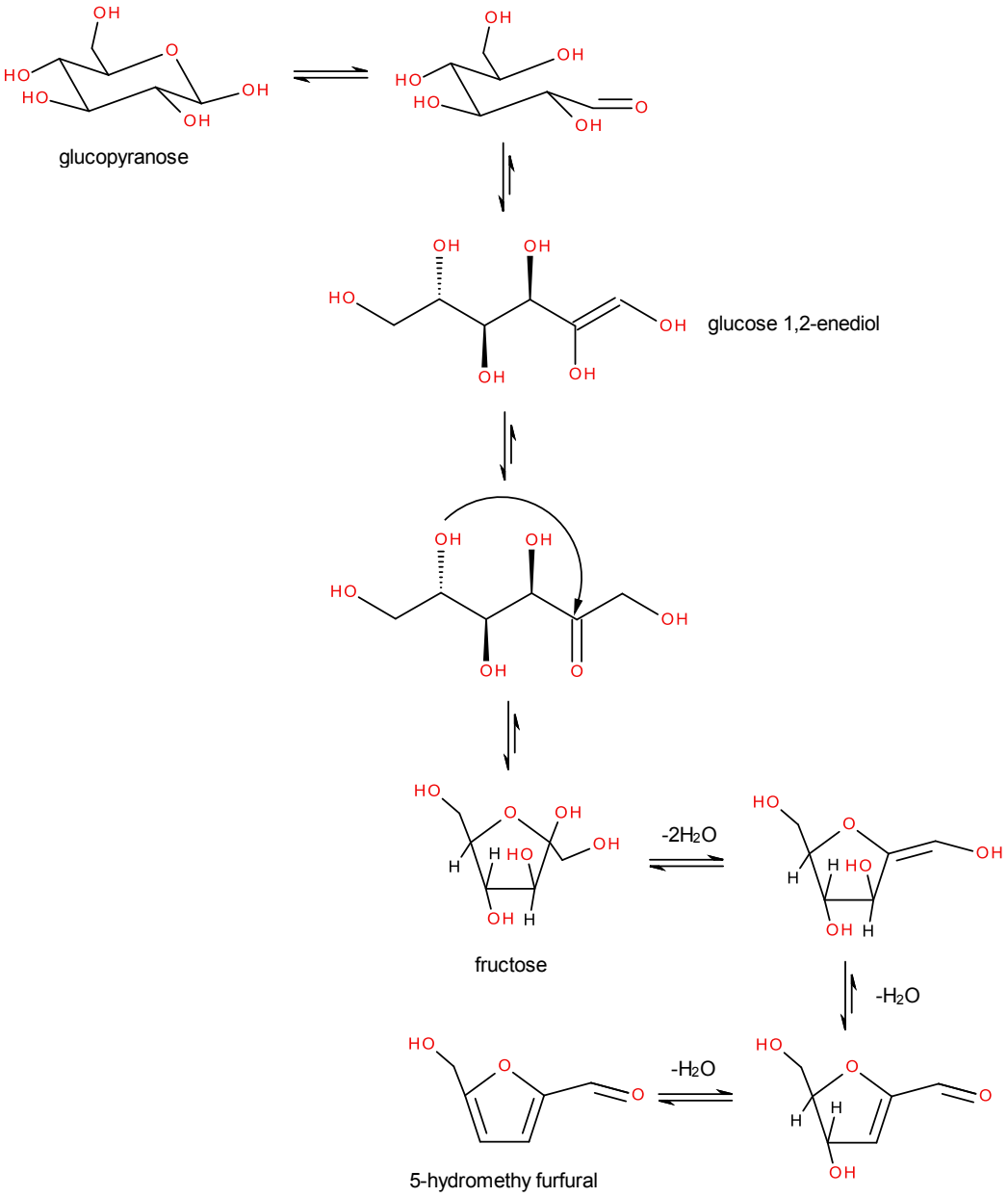


Figure 42: Formation mechanism of 5-hydroxymethyl furfural from glucose (originally in colour).[222]

4.5.1.1 Gel permeation chromatography analysis of pectin

Gel permeation chromatography (GPC), or size exclusion chromatography (SEC), is a chromatographic method based on "the retention and migration of fraction of a heterogeneous, polymeric solute at different rates through a gel matrix, predominantly on the basis of size."[164] This method does not necessitate hydrolysis and/or derivatisation of pectin monomers like for GC-MS or ion exchange chromatography. An illustration of the principle of this analysis method can be found in Figure 43.

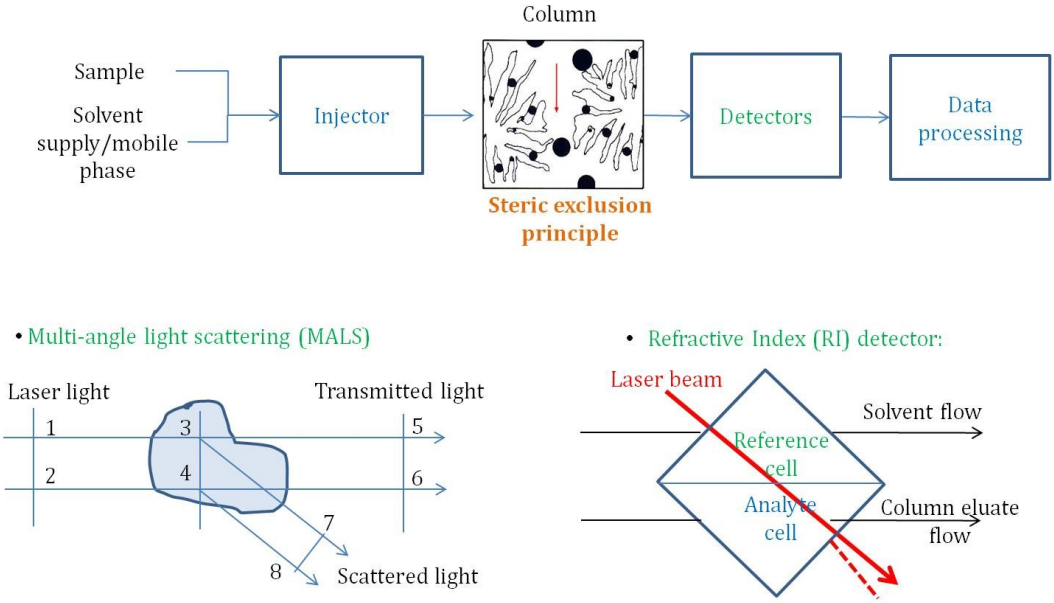


Figure 43: Description of the experimental set-up used for GPC analysis of pectin (originally in colour).

GPC was used in conjunction with a light scattering detector and a refractive index detector to determine the number and weight average molecular weight (Mw, Mn) together with the polydispersity index (PI). This technique is used extensively to check the quality and functional properties of polysaccharides used in food formulation. [187, 223] It is based on the use of the differential index of refraction dn/dc. It expresses the variation of the refractive index with the variation of the concentration and is expressed in mL/g. The value used for pectin was of 0.146 mL/g, as previously reported in the literature.[148] It is constant for a given polymer in a given solvent. It should be noted that if the true value of dn/dc was lower, then the calculated molecular weights would be higher. GPC analysis allows the complete description of the molar mass distribution. The distribution can be characterised by

using the number or the weight average molecular mass as well as by the polydispersity index PI. These parameters can be calculated using the equations below, where N_i is the number of molecules having a molecular weight M_i with $_i$ corresponding to the elution increment for the number average molecular weight M_n and the weight average molecular weight M_w.

$$M_n = \frac{\sum_i N_i M_i}{\sum_i N_i}$$

$$M_w = \frac{\sum_i N_i \, M_i^2}{\sum_i N_i M_i}$$

$$PI = \frac{M_w}{M_n}$$

Microwave extracted pectin under acid-free conditions and commercial citrus derived pectin were compared using this method. It has also been proved to be useful to evaluate the quality of pectin when screening for optimised microwave parameters.

It was found that pectin extracted at 120 °C for 10 minutes (sample P120-10) approached the characteristics determined under the same GPC conditions as commercial pectin. Indeed, the Mw and Mn values obtained for P120-10 are only slightly lower (see Table 11). Interestingly, the polydispersity index was found to be markedly lower for P120-10 compared to commercial pectin (2.8 versus 3.4). However the highest pectin yield was achieved at 140 °C when heating WOP in water for 10 minutes (20.4% for sample P140-10). This is more than double compared to the yield of pectin obtained under the same conditions at 120 °C.

Table 11: Yield, molar weight averages and PI of pectin samples produced under microwave acid-free conditions.

Sample #	Temperature (°C)	Time (min.)	Yield (%)	Mw (Da)	Mn (Da)	PI (Mw/Mn)
P120-10	120	10	7.4	113,000	41,000	2.8
P140-1	140	1	11.2	103,000	26,000	4
P140-5	140	5	17.1	86,500	26,400	3.3
P140-10	140	10	20.4	80,400	20,700	3.9
P160-5	160	5	17.2	56,200	7,440	7.6
P160-10	160	10	11.1	31,000	5,470	5.7
P180-5	180	5	11	22,700	6,830	3.3
P180-10	180	10	8.4	8,860	4,830	1.8
Commercial pectin P9135	N/A	N/A	-	119,000	34,700	3.4

The molecular properties measured show how low temperature hydrothermal microwave heating under acid-free conditions allows the production of pectin with similar properties to commercial pectin. Compared to sample P9135, the Mw of sample P120-10 only differs by 5% and has a narrower molecular weight distribution even though the number of molecules detected is higher than for P9135 with a Mn value of 41,000 Da compared to 34,700 for commercial pectin (see Table 11).

Although the Mw of P120-10 is lower than for P9135, the Mn (which corresponds to the chain length)[194] of P120-10 is higher and the polydispersity of this sample is lower compared to P9135, resulting on a narrower molecular weight distribution for pectin sample P120-10. This could be explained by the processing method used for the collected data. Since Mw is based on M_i^2 of the biopolymers detected after elution, an increase in high or low molecular weight fractions will cause the Mw average calculated to increase, explaining why the Mn of commercial pectin is lower even though the Mw is higher compared to sample P120-10. Additionally, if the pectin extracted is highly branched, the inclusion of neutral sugars such as rhamnose within the galA backbone of pectin is likely going to increase the molecular weight of the pectin chain and will influence values obtained for Mn and Mw as well as their hydrodynamic radius which will influence the separation by size-exclusion chromatography. This principle is illustrated in Figure 44.

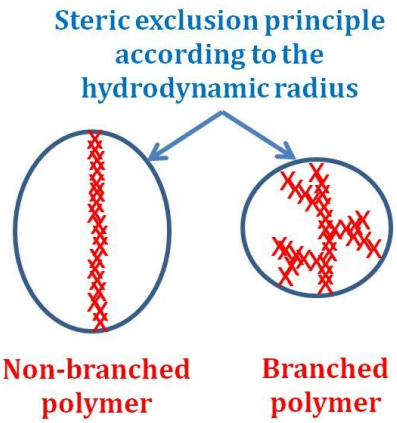


Figure 44: Influence of the level of branching on the hydrodynamic radius (originally in colour).

When studying the influence of the heating temperature between 120 and 160 °C and the variation of the microwave heating time between 1, 5 and 10 minutes, results in the rapid decrease of molecular properties such as Mw, Mn and a rapid increase of the polydispersity. When studying the influence of the heating temperature for a given extraction time of 10 minutes, the Mw and Mn, drop significantly above 120 °C. When using an extraction time of 10 minutes, the Mw drops from 113,000 to 31,000 Da from 120 to 160 °C. Mn values drop from 41,000 to 5,470 Da over the same temperature range. Under the same conditions, the polydispersity increases from 2.8 to 5.7. When studying the influence of the reaction time at 140 °C, at one, five and ten minutes microwave heating. A similar trends is observed for the Mw and the Mn values. From one to 10 minutes, Mn decreases from 103,000 to 80,400. A decrease from 26,000 to 20,700 Da is observed for Mn values. Interestingly, the polydispersity slightly decreases. However the values do not compete with commercial pectin.

Results already published on the influence of microwave assisted pectin extraction confirm these observations. A decrease in molecular mass and DE was reported with increasing extraction temperature.[175] Fishman *et. al.* reports a decrease in Mw, radius of gyration and viscosity of orange peel derived pectin over a reaction time of 2 to 6 minutes (630 W, 1:25 peel:water ratio) under acidic microwave conditions.[148, 224]

When considering all the parameters (yield, molecular weight data, polydispersity, DE, galA content and physical aspect) 120 °C is considered as the best extraction temperature for the microwave-assisted extraction of pectin in water under acid-free conditions (1:10 peel:water ratio and 10 minutes microwave extraction time).

Results have shown this temperature yields pectin which is comparable in quality to commercial pectin. According to GPC analysis, P120-10 compares better with commercial pectin, but the conditions used yield a lower quantity of pectin compared to P140-10. Even though 20.4% pectin was extracted at 140 °C and 7.4% at 120 °C, conditions yielding pectin with a higher molecular weight are likely to be preferred, at least for some applications.

A note of caution should be observed as it was later discovered that the solvent removal method used for pectin drying at small scale wasn't effective. Therefore the pectin yields are to be treated with caution: following the problems observed with pectin drying (see section 4.5.3), there is a strong possibility that the pectin sample produced contained a least 20% of ethanol. However, this should be a systematic error and should not affect how the yields of pectin vary relative to each other, since they were all dried using the same method. Hence the conclusions reached on the best conditions for microwave-assisted pectin extraction under acid-free conditions are still valid. The process flow sheet for this step can be found in Figure 45.

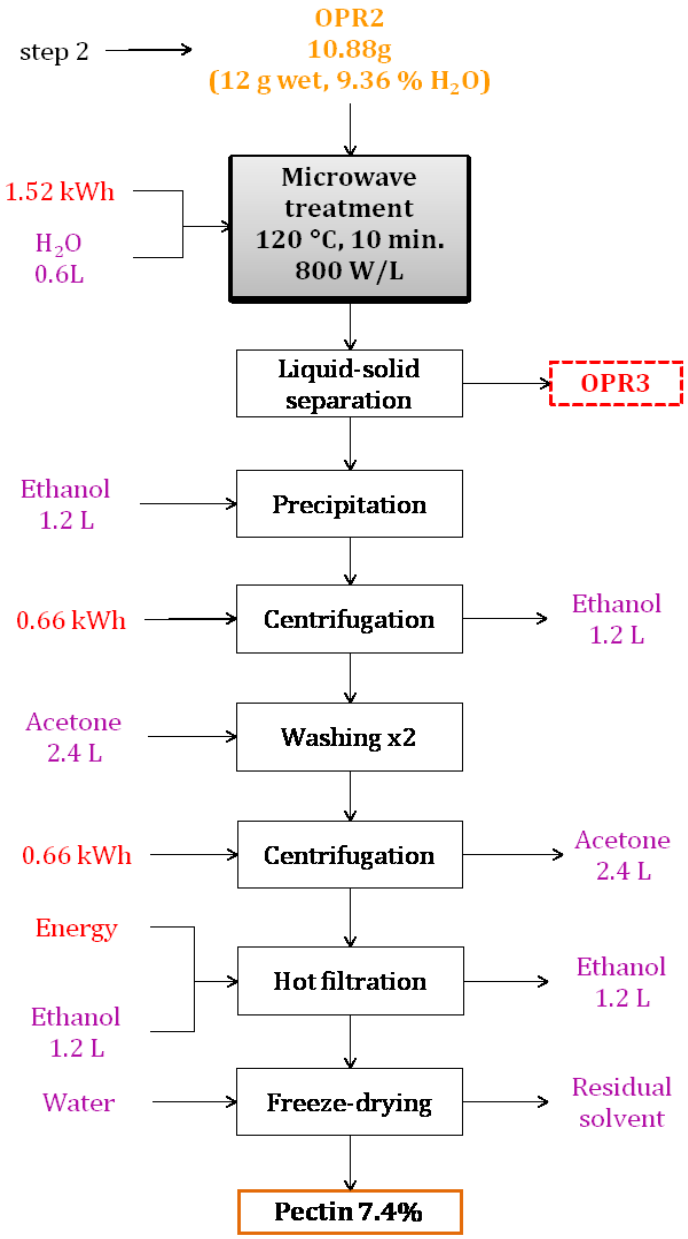


Figure 45: Detailed process flow sheet of step 3
(pectin extraction under microwave acid-free conditions) for sample P120-10.

A mass balance was carried-out for the pectin extraction step (see Table 12). A difference of 27 g was observed between the amount of material coming in and the amount of material coming out. This difference is due to OPR2 being rehydrated during step 3.

Table 12: Mass balance for step 3
(pectin extraction under microwave acid-free conditions) for sample P120-10.

Mass balance based on wet mass for step 3			
IN		**OUT**	
Material	**Mass (g)**	**Material**	**Mass (g)**
OPR2	12	**Pectin**	0.88
		OPR3 (wet)	37.84
H_2O	600	**H_2O**	600
Ethanol	950	**Ethanol**	950
Acetone	1900	**Acetone**	1900
TOTAL	3462	**TOTAL**	3489

Gel formation properties of pectin produced at 120 °C under acid-free conditions will be studied next.

4.5.1.2 Gel formation properties of pectin extracted under acid less conditions

Pectin is especially valuable as a gelling agent. Pectin's main application relies on its gel forming abilities, especially in the food industry. Hence, demonstrating the gel forming properties of pectin extracted under acid-free conditions was an important milestone for the validation of the overall process . Pectin forms a gel in water as a result of the "combination of hydrophobic interactions between the methoxyl functionalities and hydrogen bond formation between carboxylate functionalities and secondary alcohol functionalities".[125, 140, 225] Junction zones are formed and water is trapped in the "pockets" formed (Figure 46). Finding the right balance for the combination of water, pectin, sugar and acid is important. Sucrose is needed to lower the attraction of pectin for water and increase pectin chain interaction.[226,125] However, gelation is only possible if pectin dissolves in water. Pectin solubility

mainly depends of the counter ion's nature, the ionic strength of the cation-anion system and the pH.[125]

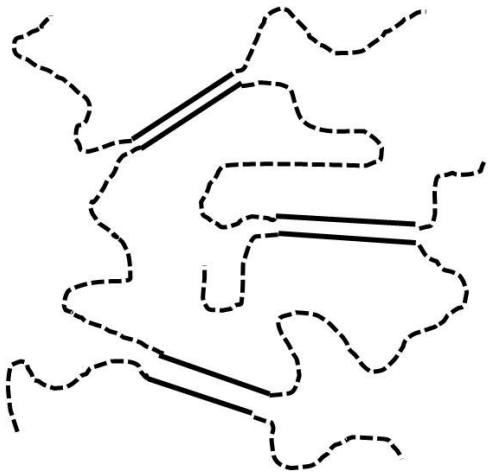


Figure 46: Graphical representation of a 2D view of the junction zones (continuous lines) formed in a pectin gel (adapted).[225, 226]

There are several gelation mechanisms for pectin. All are affected by the DE of pectin (see Table 13). The DE is one of the analytical parameter that allows the prediction of the gelling behaviour. The gelation process results of two types of interactions: for low methoxy pectins, gelation results from ionic interactions via Ca^{2+} ions connecting two carboxyl groups. In high methoxy pectins, hydrogen bonds and hydrophobic interactions are responsible for the gel forming properties.[140] Gelation is influenced by the following factors: soluble solids percentage (SS%), pH, molecular size, DE, arrangements of side chains or "steric fit",[227] charge and density of the pectin macromolecule.[140]

Table 13: Reported gel forming conditions for high & low DE pectin.[224, 225, 228, 229]

Gelling parameters	High DE gelling mechanism	Low DE gelling mechanism
Pectin DE (%)	>50%	<50%
pH	< 3.8	2-6
SS (%)	55-85%	10-70%
Notes	Gelling T° increases with the DE%, the SS% and the M_w.	Gelling T° increases as the DE% decreases, and increases with the concentration of Ca^{2+}. The degree of blockiness is important. Does not require sucrose.

It has previously been established that the microwave extracted pectin herein studied falls into the high DE category 50%. High DE gel conditions were therefore investigated to determine whether pectin produced at 120 °C under acid-free conditions could form a gel. Initial tests carried-out by an industrial collaborator at the Fraunhoffer institute showed that weak gels were formed when comparing samples P0004 and P0005 against commercial pectin.

The standard method used to form a gel has been reported as followed:[225, 230]

- A 1% pectin & 50-70% sucrose solution is prepared.
- The mixture is heated to boiling point.
- A pre-determined quantity of water is evaporated to reach the desired SS%.
- The pH is then adjusted and the gel is left to set at or below room temperature.

A high DE for pectin renders the gel formation delicate. The high number of esterified carbonyl groups lower the opportunity for junction zones to form between the non-esterified carboxyl groups, leaving the formation of the 3D network down to hydrophobic interactions. For example, pectin with a DE of >70%, the creation of water pockets will be rendered more difficult compared to pectin with a DE of 60%. Figure 47 illustrates this concept. HM pectin gel formation is believed to occur via an alignment of molecular helices which are associated to each other through hydrogen bonds forming between non–dissociated carboxyl and

secondary alcohol groups and hydrophobic interactions between methoxy groups.[229]

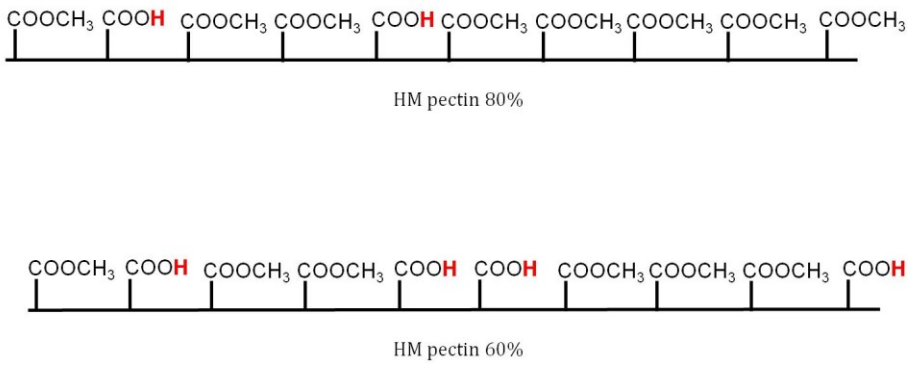


Figure 47: Illustration of the impact of the degree of esterification on the formation of "water-pockets". A 3D network formation is much harder for a DE of 80% for example, as the formation of "water-pockets" is more difficult (originally in colour).

Several solutions have been reported in the literature regarding gel forming conditions for highly esterified pectin (< 75%). The addition of an excess of H^+ ions to lower pH has been suggested in order to depress pectin ionisation[139] (see Figure 48) and improve pectin-pectin interactions. As a result, inter chain interactions are favoured by the absence of negative charges of the carboxylate group at low pH, lowering repulsion forces between pectin chains.[125, 190]

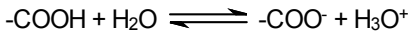


Figure 48: Influence of pH on the dissociation equilibrium of free carboxyl acid functionalities.

Several methods were investigated in order to find the appropriate gelling conditions for microwave extracted pectin under acid-free conditions. Two methods were first tested on commercial pectin (HerbstreiFth & Fox, CU201 and CU 301) with varying degrees of esterification. The strength of the gel formed was assessed qualitatively for all of the methods tested: the pectin/sucrose solution (or jelly mixture) was left to set in a beaker and would successfully pass the test if the gel would remain stuck at the bottom of the beaker when turned upside down. Any gels that were free flowing when the beaker was tilted and turned upside down were

considered a fail. The USA-SAG test was first trialled as an example of a standard method to be applied to high methoxy pectin for rheology measurements.[230, 231,232] Very weak gels were obtained with the USA-SAG method. It led to the use of a method previously reported by MacDougall *et. al.* The later author reported a protocol for high DE pectin requiring the presence of Ca^{2+} in small concentrations (30 µL of a 1M $CaCl_2$ solution).[233] The addition of Ca^{2+} to HM pectin has been reported to facilitate hydrophobic interaction by favouring inter-chain interaction between partly dissociated –COOH groups.[234, 235] The addition of calcium ions has been reported to play an active role in the gelation mechanism observed for block wise distributed pectins but generally only applies to low methoxy pectins.[234] Block-wise distributed pectins are generally extracted from citrus fruits. It should also be noted that Ca^{2+} ions are also believed to be responsible for the binding effect of high DE pectin in the cell wall.[236, 237] The protocol did not require the need for heating, nor the use of acid to lower the pH of the jelly mixture. Again, only weak gels were formed.

Given the precise pH values reported for pectin gel forming conditions, pH control appeared to be an important factor. Decimal point variations of the pH have been reported to affect the gelation and strength of the pectin gel formed.[224, 225, 229] The two previous methods used to test gel forming conditions did not allow precise control of the pH. Lofgren *et. al.* reported the use of a citrate buffer to define with greater precision the conditions used.[234] This protocol was therefore used to also study a broader set of conditions. In addition to the use of citrate buffer of pH 2, 3 and 4, the addition of Ca^{2+} ions and different sucrose content (60% and 70% since 65% is standard)[139] have been tested on a 25 mL scale. Each combination of pH/sucrose content was tested with and without the addition of Ca^{2+} ions as it has been reported to enhance the gelation process via maximisation of the hydrophobic interactions of high DE pectin chains.[235] The percentage of pectin used was kept constant at 0.75%, as reported in the literature, since 0.5-1% pectin is a normal threshold in food formulations containing pectin. All the tests carried out have been summarised together with associated observations in Table 14.

Table 14: Summary of the observations made following the Lofgren protocol to test pectin gel formation conditions.

Gel test #	Pectin (%)	Sucrose (%)	pH	Addition of Ca^{2+}	Gel formation?
PGT15	0.75	60	3	1.2 ml 0.15% $CaCl_2$	No
PGT16	0.75	60	3	-	Yes
PGT17	0.75	70	3	1.2 ml 0.15% $CaCl_2$	Yes
PGT18	0.75	70	3	-	Yes
PGT25	0.75	70	3	-	Yes
PGT23 (repeat of PGT17)	0.75	70	3	1.2 ml 0.2% $CaCl_2$	Yes
PGT24 (repeat of PGT18)	0.75	70	3	-	Yes
PGT19	0.75	60	2	1.2 ml 0.15% $CaCl_2$	No
PGT20	0.75	60	2	-	Yes
PGT21	0.75	70	2	1.2 ml 0.15% $CaCl_2$	No
PGT22	0.75	70	2	-	No
PGT27	0.75	60	4	0.81 ml 0.2% $CaCl_2$	Yes
PGT28	0.75	60	4	-	No
PGT29	0.75	70	4	0.81 ml 0.2% $CaCl_2$	Yes
PGT30	0.75	70	4	-	No

One set of conditions was found to allow successful gel formation. The best conditions were the one applied for PGT18: pH 3 and 70% sucrose. The jelly mixture thickened very rapidly forming a gel as soon as the sucrose was added to the pectin/buffer mixture. Upon calcium addition, PGT17 formed a strong gel too, but at a lower speed than the one observed for PGT18. This indicates that the addition of Ca^{2+} slowed down gel formation at pH 3. The end result can be seen in Figure 49. The results obtained were successfully repeated with gel tests PGT23 and PGT24.

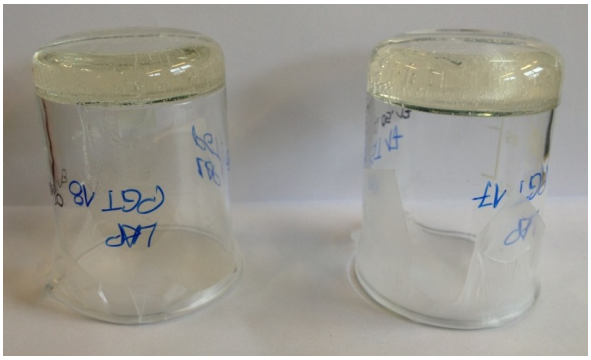


Figure 49: End-result of gel test PGT17 (right) & PGT18 (left) with pectin P0020 using the Lofgren protocol (originally in colour).

Jelly mixtures obtained at pH 3 but with a 60% sucrose performed less well compared to PGT17 and PGT18, forming only slightly thicker pectin solutions indicating a high sucrose content is necessary to form gels with high DE pectins.

The same trends can be observed for jelly mixtures prepared at pH 2. Of the four tests done at pH 2, the one done with 70% sucrose and with no added calcium ions (PGT22), thickened the fastest and poured more slowly than PGT21, which contained Ca^{2+} ions (Figure 50). However the gels formed were not as strong as the ones formed at pH 3 with the same content of sucrose. This might be due to pre-gelling, a phenomena leading to weaker gels, which is sometimes observed for high DE pectin at a lower pH.[139] When lowering the sucrose content to 60% at pH 2, a weak gel was formed (PGT20), confirming the observations obtained at pH 3. The addition of calcium ions under the same conditions led to a weak gel too, confirming the weakening effect Ca^{2+} has on a gel formation, as for pH 3.

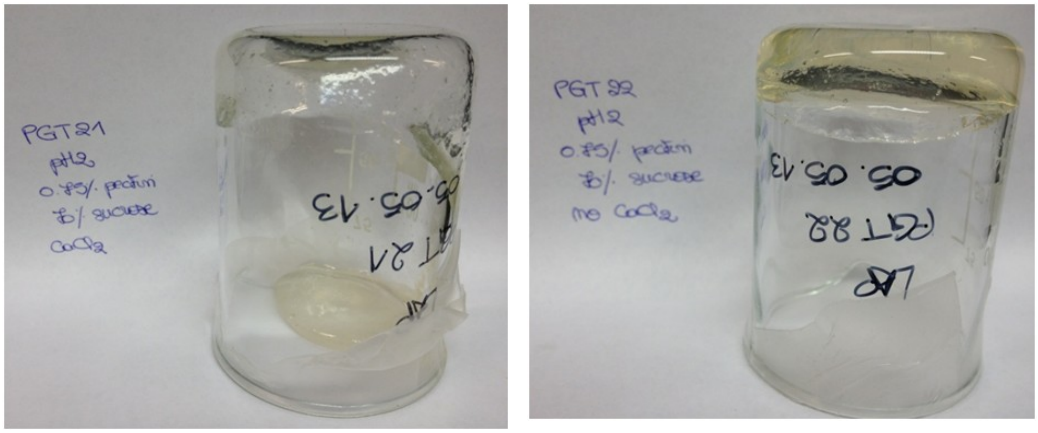


Figure 50: End-result of gel test PGT21 (above) & PGT22 with pectin P0023 using the Lofgren protocol (originally in colour).

The best gel forming conditions seemed to be pH 3 and 70% sucrose (no addition of Ca^{2+} ions). This correlates well with the pKa value of galacturonic acid, which is 3.51:[238] at pH 3, galA will mostly be present as un-dissociated, which is desirable here as it will favour the formation of hydrophobic interactions. Above a pH of 3.51, the –COOH group of the galA will mostly be present in its dissociated form, forming –COO^- anion which will have for effect to repel pectin chains between themselves. The best gel forming conditions were successfully tested on commercial pectin (H&F CU201) as PGT25. The pectin gelled so rapidly after the addition of sucrose that a portion of the jelly mixture could not be transferred in the beaker following heating (Figure 51).

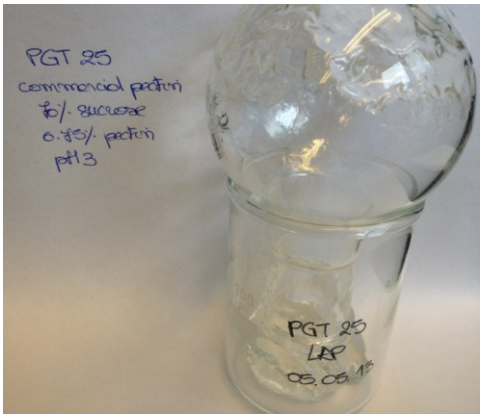


Figure 51: End-result of gel test PGT25 with pectin CU201 using the Lofgren protocol (originally in colour).

The influence of pH was further investigated (to determine how high the pH could be increased until no strong gel was formed. Further tests were done at pH 4. None of the conditions tested at pH 4, allowed the formation of a strong gel after 24 hours at 20 °C, as seen in Figure 52. A slightly stronger gel was formed when using 70% sucrose with the addition of Ca^{2+} ions. This is unusual compared to the results obtained for gel tests PGT17 & PGT2, the addition of calcium weakened the gel compared to PGT18 and PGT22. However after several minutes, the gel would start running down the walls of the beaker. The unsuccessful attempt to gel pectin at pH 4 can be explained by the pKa of 3.51 of galA. The results obtained at pH 4 are in accordance with gelling conditions for high DE reported in the literature, confirming we obtain highly esterified pectin when using a microwave assisted process under acid-free conditions. However the incidence of the DE variation has not been taken

into consideration here as data on the DE of the experimental pectin sample used wasn't available at the time of the experiment. Following the observations made, the gelling process of microwave extracted pectin as described in this report does not seem to be affected by the addition of calcium ions, regardless of the pH of the buffer solution used. This is indicative of a low degree of blockiness as only pectin containing blocks of esterified positions are sensitive to calcium ions.[239] The higher the degree of blockiness, the faster the gel will set (and the higher setting temperature), providing important complementary information.[234] A high degree of blockiness for high DE pectin will provide enhanced protein stabilisation power in acidic dairy based beverages for example.[240]

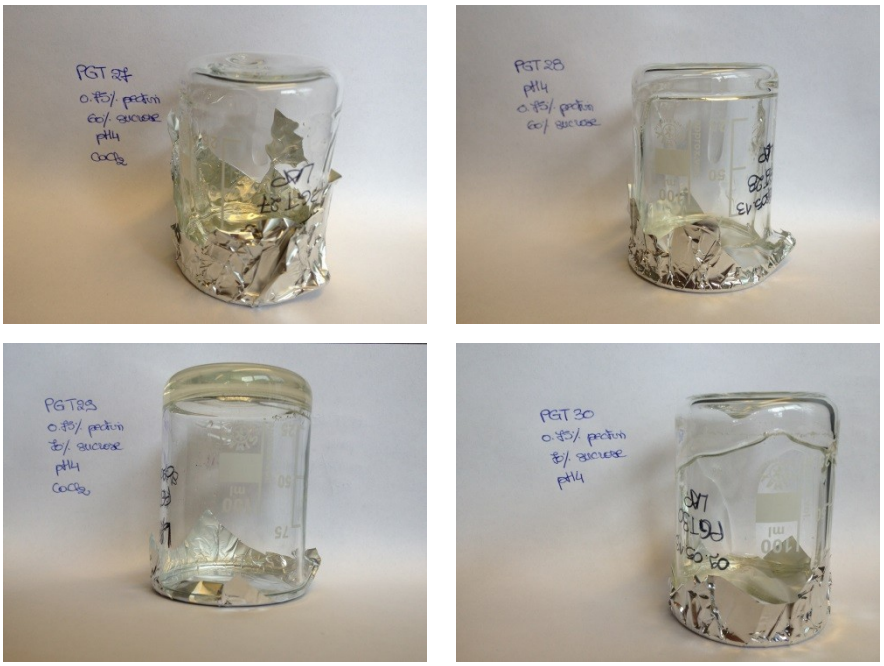


Figure 52: End-result of gel test PGT27, PGT28, PGT29 and PGT30 with pectin P0023 using the Lofgren protocol (originally in colour).

In conclusion, the best gel conditions were found to be pH 3, 70% sucrose and no Ca^{2+} ions. These conditions have been successfully tested on microwave extracted pectin without additional acid and commercial pectin of high DE. Those conditions fit with the ones reported by Voragen *et. al.* and are indicative of a high DE pectin is extracted under our microwave conditions. Whether the presence of citrate in the buffer used to dissolve pectin has had an impact on gel formation is difficult to say. This could be due to citrate polyatomic anions $C_3H_5O(COO)_3^{3-}$ strengthening the gel formed via hydrogen bond formation.

4.5.2 Interaction of microwaves with orange peel waste- study of the reflected power

As part of the preliminary screening experiments done on the microwave assisted extraction of pectin under acid-free conditions, the absorption of microwave power by fresh orange peel was tested. This was important in order to determine the quantity of energy transferred to the sample. It could be determined by calculating the difference between the emitted and the reflected power observed when irradiating a sample. Such an experiment was done with a Sairem Miniflow 200SS microwave equipment which allows the measurement of reflected power (see Figure 53). This equipment is capable of producing a very low controlled power output (0 to 200 W with 1 W power increments) together with the use of a precisely defined frequency range. This is possible due to the use of a solid-state microwave generator as opposed to a magnetron. The equipment uses a fiber optic probe to measure the temperature.

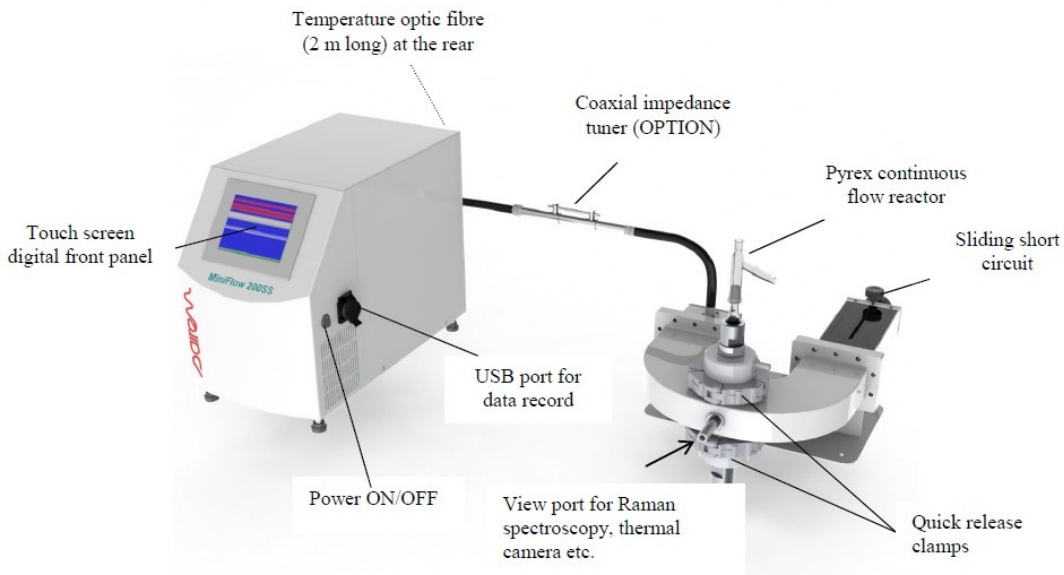


Figure 53: Description of the Sairem MiniFlow 200SS microwave equipment used for the measurement of reflected power (reproduced with the permission of Sairem® SAS) (originally in colour).

This equipment has the particular property of being able to measure the reflected energy for a given experiment via a Calex® PyroUSB 151 infrared sensor, allowing for the estimation of the energy efficiency of a hydrothermal process for example.

The system displays a waveguide can be used to adjust the resonance frequency of a monomode cavity, allowing the generation of a constructive wave, lowering the quantity of reflected energy for a given material being microwaved.

The reflected power was measured on a water sample and orange peel containing water sample. At 100 W, 10 mL of distilled water in a closed vessel were microwaved at 115 °C and 130 °C. In both cases, a reflected power of 15 W was measured. Upon addition of 1 g OPW to the water, the reflected power decreased to from 15 W to 5 W at 115 °C. At 130 °C, the reflected power decreased from 15 W to 7 W. Under the experimental conditions used, the results show how upon addition of orange peel, the system absorbed microwave power better than water. This suggests that for a typical hydrothermal extraction of orange peel, 95% of the microwave energy is effectively absorbed at 115 °C. At 130 °C, it decreases to 93%. This observations implies that microwave energy will be used more efficiently within the lower end of the 100–150 °C range. This is an important point to consider especially when considering scaling-up the process.

The later results are verified by the values of the relative dielectric loss factor ε'' published for water and orange peel. Within the food industry, the dielectric properties of orange peel have been studied. The later together with reported electrical and physical properties of orange peel are summarised in Table 15.[241]

Table 15: Electrical, physical and dielectric properties of orange peel and water corresponding to the temperature expressed in Kelvin (adapted from Birla *et. al.*)[241].

	Thermal conductivity (W/m.K)	**Specific heat C_p (J/Kg.K)**	**Relative dielectric constant ε'**	**Relative dielectric loss factor ε''**
Orange peel	0.40	3300	-0.16T+82.53	3.94T+58.2
Tap water	0.56	4180	-0.48T+84.74	0.33T+11.1

When knowing that ε'' is proportional to the quantity of radio frequency absorbed by the material, the data in the table above illustrates how well WOP will interact with microwaves compared to water, strengthening the results obtained when measuring the reflected power.

The next section will present results produced on a larger scale, using a larger microwave set-up with two independent temperature measuring systems for better

control of the extraction parameters. The results obtained will be compared to several commercial pectin samples and acid hydrolysis extracted pectin.

4.5.3 Big scale hydrothermal microwave extraction of pectin

Working towards a larger scale was an intrinsic goal of the project from day one. The optimised conditions highlighted in section 4.5.1.1 were used as a stepping stone to further test the process using a higher throughput. This meant trialling the acid-free extraction conditions at a lower MMPD, lowering it from 800 W/L to 35 W/L given the increased cavity size of the equipment. The aim of this work was to determine whether hydrothermal microwave-based extraction of pectin under acid-free conditions could be scaled up from a millilitre scale at 800 W/L) to a decilitre scale at 35 W/L. Additionally, pectin samples were generated for a company interested in setting-up a pilot plant sized waste biorefinery (partly in India).

4.5.3.1 Industrial application evaluation

Two pectin samples (duplicate of the same conditions) were therefore generated at a power density of 35 W/L as a proof of principle for scale-up based on the preliminary work. 100 g of pectin were successfully generated at 120 °C (peel:water ratio of 1:10) using again wet defrosted virgin WOP. The temperature holding time was modified from 10 to 20 minutes since the shorter setting wasn't possible on the microwave equipment used at larger scale. A flow diagram of the process can be seen in Figure 54.

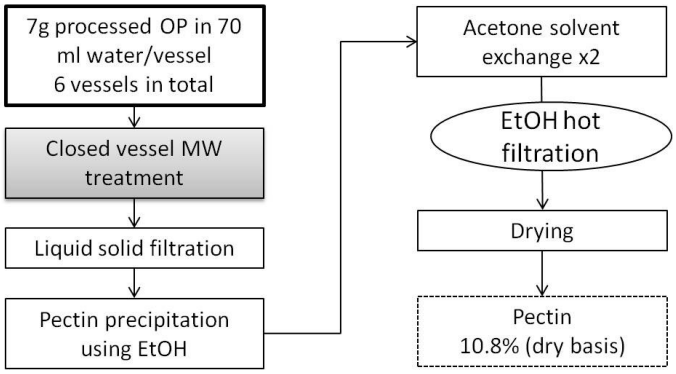


Figure 54: Flow diagram of the process used for microwave-extracted pectin under acid-free conditions.

4.5.3.2 GPC results

The samples sent were evaluated by gel formation tests. Independent gel tests on the samples were carried-out by the Fraunhofer institute revealed that they performed poorly against commercial pectin. The main conclusions reached were the following:

- the pectin was not sufficiently dried and an important fraction of ethanol was still present, hindering solubilisation and therefore gel formation properties of pectin,
- insolubles were present in the microwave extracted pectin (percentage not given),
- in terms of colour, the samples sent were of a darker beige than commercial pectin and this had an impact on the colour and the clarity of pectin in solution,
- the gel strength of commercial pectin was superior (2.7 N) compared to microwave extracted pectin (0.15 N) and
- the viscoelasticity of commercial pectin was superior compared to microwave extracted pectin (10.3 versus 3.6).

Samples P0002 and P0003 were both analysed using GPC to study the variability of the molecular weight distribution between two identical samples. Each value reported is the average of a duplicate analysis. When comparing the number and weight average molar mass (Mn and Mw respectively) and polydispersity (Mw/Mn) between commercial pectin and microwave extracted pectin (see Table 16), higher Mw and Mn values are obtained for both P0002 and P0003 compared to P9135.

Polydispersity is slightly lower for P0002 (2.28 versus 2.83) but decreases to 1.90 for P0003, which is good. Higher Mw and Mn values are obtained for P0003 compared to P0002 but polydispersity of P0003 is lower. Reproducibility between samples P0002 and P0003 is good too although this does not correlate with the elution profile of P0003, indicating a much wider molecular weight dispersion (see Figure 55) for this later sample.

Table 16: Comparison of Mw, Mn and PI (Mw/Mn) of microwave extracted pectin under acid-free conditions using virgin WOP (P0002 & P0003) against commercial pectin.

	Mn (Da)	Mw (Da)	PI (Mw/Mn)
P9135	3.15×10^4	8.93×10^4	2.83
P0002	4.94×10^4	1.10×10^5	2.28
P0003	9.00×10^4	1.68×10^5	1.90

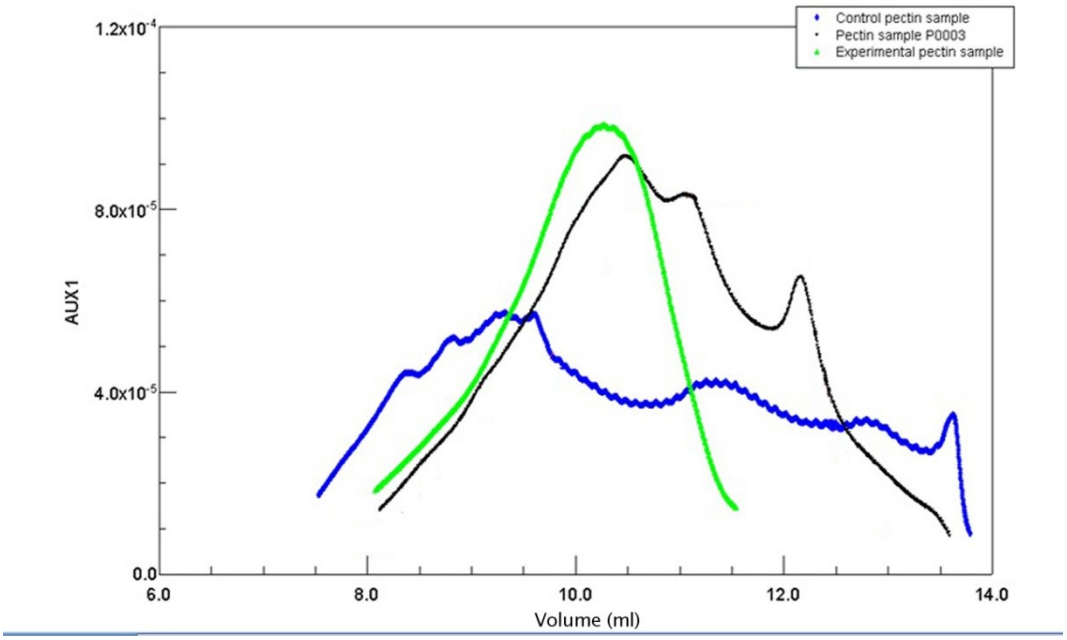


Figure 55: GPC/RI elution profiles of pectin samples P0002 (green), P0003 (black) and P9135 Aldrich citrus derived pectin (blue) (originally in colour).

The analysis of the elution profile in Figure 55 shows that a smooth elution profile is obtained for P0002. This observation shows that P0002 as a markedly narrower molecular mass distribution. This is not the case for commercial pectin, for which the molecular weight distribution is much broader. GPC elution profiles of P0002

and P0003 are different: P0003 displays an intermediate molecular weight distribution. This is attributed to the fact that these concern the analysis of natural polymers. However, the molecular weight distribution of the experimental samples is still narrower than for the standard used (P9135), which has no real pronounced maxima.

No experimental data or protocol was provided by the Fraunhofer Institute regarding the tests done. No information on the grade of pectin used as a control sample could be obtained. Still, several hypotheses could be made as to why the pectin extracted at 35 W/L wasn't comparing well against commercial pectin. The following suggestions will be used for further process optimisation.

- Drying had to be improved:

Washed pectin was dried under vacuum using a rota evaporator followed by further drying under high vacuum. Irrespective of the drying method used, pectin tended to form clumps. It was found that these clumps contained still significant amounts of ethanol inside them. This can be explained based on the amphiphilic nature of pectin.

- The influence of pre-treatment:

Using pre-treated WOP (PWOP) instead of wet defrosted virgin WOP could yield pectin with fewer impurities. The initial idea implied using wet defrosted virgin WOP which was first used for the extraction of *D*-limonene. Additionally, flavonoids could then be extracted from the peel using an acetone soxhlet extraction, stripping the peel from low molecular weight components. This could limit the number of components extracted alongside pectin and limit the beige colouration observed so far. The peel subjected to both extraction could then be used for microwave assisted pectin extraction. This three step process would allow the recovery of additional product streams.

- The influence of microwave maximum power density (MMPD):

The difference in MMPD between the CEM MARS (35 W/L) and the CEM Discovery (800 W/L) could explain difference between the chain length and the neutral sugar content of pectin produced on the CEM MARS and the CEM Discovery. This can be seen by comparing solution NMR spectrum of pectin samples produced under the same conditions on both CEM microwaves. This is especially important as the chain length and the neutral sugar content have an effect on the dissolution and gel forming properties of pectin.[225] The MMPD was calculated on the basis of the

volume of the microwave chamber and the maximum power produced by the magnetron (1800 W for the MARS and 300 W for the Discovery equipment). It was assumed that the MMPD emitted in dynamic mode retained the same ratio between the two different microwave equipments.

- The influence of microwave temperature holding time:

Additionally, the difference in microwave holding time might also have an influence on the quality of pectin. A 20 minute holding time at 120 °C could cause degradation of the pectin chain compared to 10 minutes.

4.5.3.3 Biorefinery process improvements

Changes to the solvent removal stage were done first. Drying was improved by using a freeze-drier to remove residual water and solvent after the precipitation of pectin. This change alone allowed to obtain pectin that was completely white with no trace of brown impurities as seen in Figure 56. According to the manufacturer, freeze-drying yields samples with a moisture content below 1%. This is especially important when ATR-IR characterisation of the pectin samples is used. Additionally, the use of a freeze-drier instead of a heat induced drying method is a plus when drying pectin, as it is a heat labile type of material.[140]

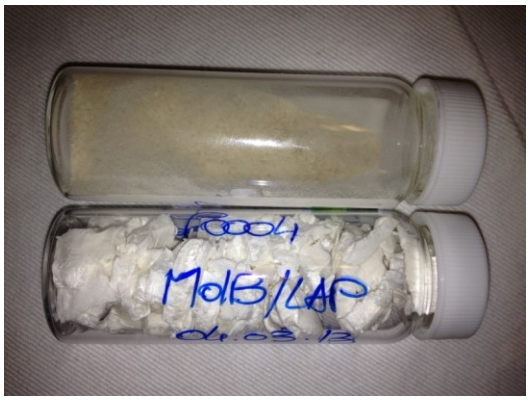


Figure 56: Difference in sample colouration between pectin dried in a rota evaporator (above) and using a freeze-drier (below) (originally in colour).

Following the change of drying technique, further modifications were made to the pectin extraction process. The use of pre-treated WOP was trialled next with the aim

of producing a cleaner form of pectin. Regarding the use of pre-treated orange peel, a first trial was done using *D*-limonene and flavonoid extracted wet defrosted virgin WOP (referred from now on as pre-treated WOP or PWOP). The extraction of *D*-limonene followed by the extraction of flavonoids will be further discussed in chapters 5 and 6. Figure 57 describes the whole process used. The evaluation of the quality of the pectin produced using the modified process was done using GPC analysis.

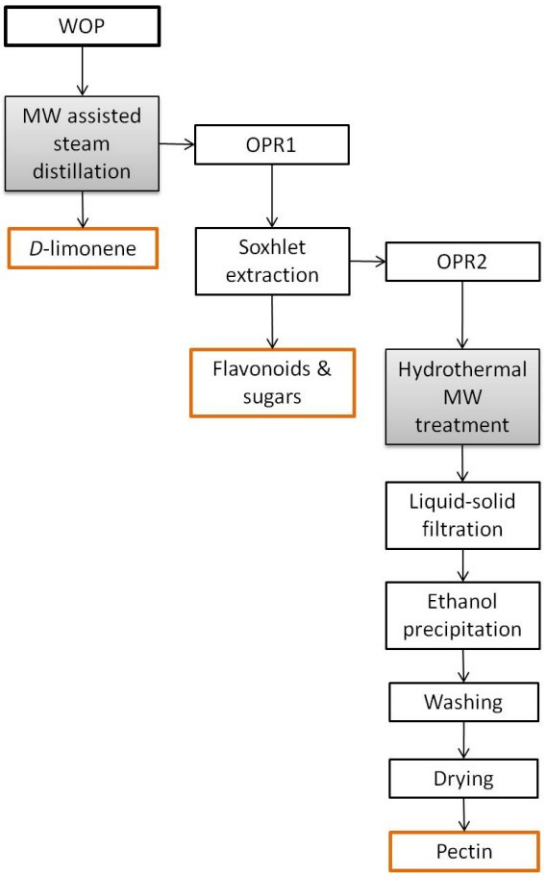


Figure 57: Flow diagram of the OPEC process used for microwave-extracted pectin under acid-free conditions
using *D*-limonene and flavonoid extracted WOP, or PWOP.

Two duplicate samples of pectin have been produced under the same microwave conditions using PWOP: P0004 and P0005. ATR-IR was used to quickly assess whether pectin was actually isolated, judging the presence of signature peaks at 1760-1730, 1750 and 1440 cm^{-1} corresponding respectively to the esterified carbonyl, the non-esterified carbonyl and the methyl group on the esterified carbonyl of galA (Figure 58).[164, 181] The IR spectra confirm pectin has been extracted at 35 W/L and 120 °C.

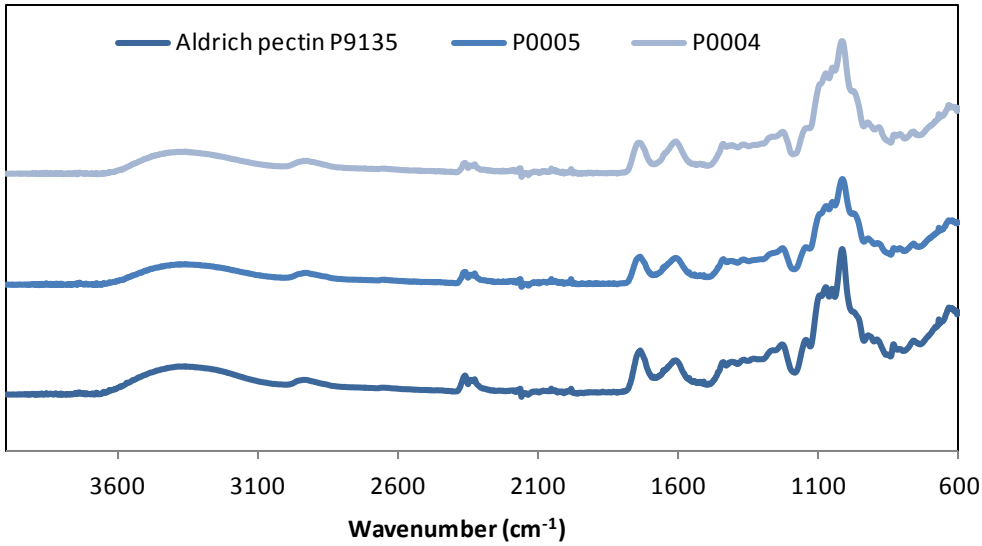


Figure 58: ATR-IR spectra of pectin samples P0004, P0005 and commercial pectin P9135 (Sigma-Aldrich) (originally in colour).

Both samples have also been subjected to the same GPC analysis (same method and equipment) and the results are reported in Table 17. Each value reported is the average of a duplicate analysis.

Table 17: Influence of peel pre-treatment on Mw, Mn and PI of microwave extracted pectin under acid-free conditions against commercial pectin.

	Biomass used	Mn (Da)	Mw (Da)	PI (Mw/Mn)
P9135	-	3.15×10^4	8.93×10^4	2.83
P0002	WOP	4.94×10^4	1.10×10^5	2.28
P0003	WOP	9.00×10^4	1.68×10^5	1.90
P0004	PWOP	1.43×10^5	2.29×10^5	1.62
P0005	PWOP	1.31×10^5	2.23×10^5	1.71

When focusing on the uniformity and the narrower distribution of molar mass, samples P0004 and P005 are clearly of better quality than P0002 and P0003. The latter samples have a higher weight and number molecular weight than all the other samples (commercial pectin included) and a lower polydispersity, both of which are

highly desirable for pectin. Additionally, the reproducibility between P0004 and P0005 is enhanced compared to the P0002 and P0003 when using PWOP compared to WOP for both the M_w, M_n and M_w/M_n values. The molecular weight distribution diagram in Figure 59 confirms this observation.

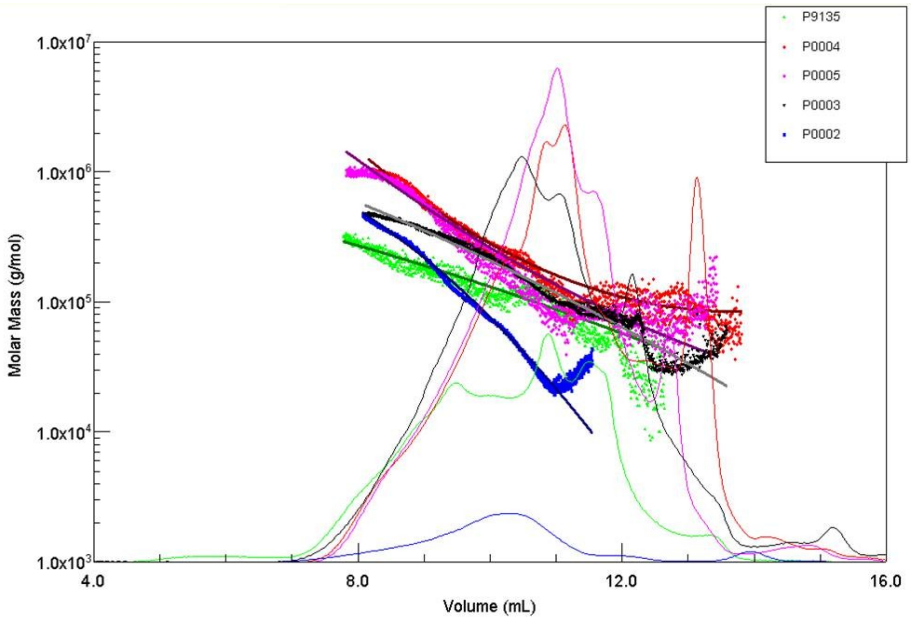


Figure 59: Molecular mass distribution of MW extracted pectin under acid-free conditions against commercial citrus-derived pectin using virgin WOP (P0002 & P0003) and PWOP (P0004 & P0005) (originally in colour).

The effect of MMPD was studied next. Based on observations made on the work of a visiting student (Sebastian Keeß, Autumn term 2012), differences in the sugar region of the ^{13}C NMR spectra could be observed between samples generated at 35 and 800 W/L. The spectra obtained when using quantitative solution ^{13}C NMR in D_2O, showed a clear difference the neutral sugar region. Figure 60 displays two ^{13}C NMR spectra corresponding to pectin extracted from PWOP at 800 W/L (sample P0018, A.) and 35 W/L (sample P0004, B.). Notable differences between the two spectra can be observed in the neutral sugar chains region (110-65 ppm).[184] When comparing the ratio between the peak observed for the C1 of the galA unit (99.89 ppm) and the anomeric carbons of neutral sugars (103-107 ppm), an indication of the neutral sugar content can be obtain relatively to the galA content.

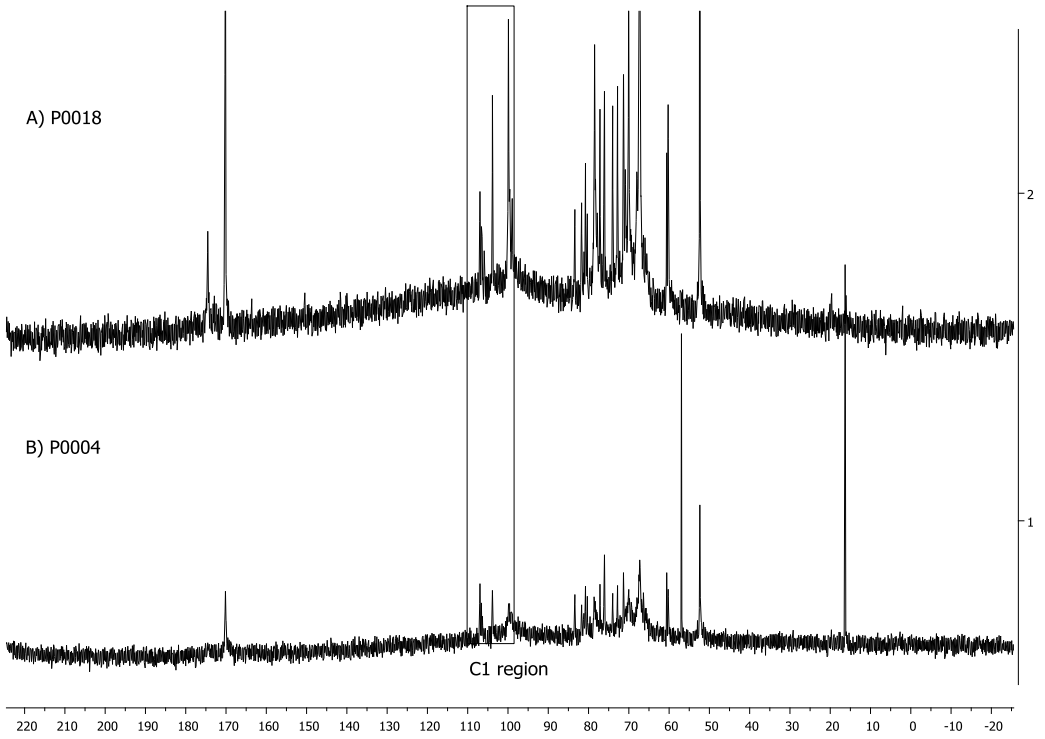


Figure 60: ^{13}C NMR spectra of samples P0018 (800 W/L) and P0004 (35 W/L) obtained in D_2O (500 MHz).

According to the spectra in Figure 60, a higher proportion of neutral sugar content is observable in sample P0018. Given that gel formation properties of pectin also depend on the steric fit of the pectin chains around the neutral sugar side chains,[225, 242] MMPD will have an influence on the properties of the pectin extracted. A higher proportion of neutral sugars could definitely be responsible for the observed enhanced dissolution of P0018 in D_2O, improving the resolution of the spectra obtained. Three extraction parameters could explain the lower content of neutral sugars in P0004: the lower MMPD of the microwave equipment used, the temperature holding time (10 versus 20 minutes) and/or the difference in microwave extraction temperature used (120 °C versus 140 °C).

A variation of MMPD from 35 W/L to 800 W/L could allow the extraction of pectin chains containing a lower proportion of neutral sugar monomers, only extracting the "smooth" section of the pectin chain as present in the cell wall (see Figure 22). Bonds between the galA units and the neutral sugar units are known to be less acid-resistant than bonds between two galA units.[125, 189] If the variation of MMPD can affect the proportion of neutral sugars embedded in the pectin extracted, this would represent an opportunity to fine-tune the fine chemical structure of the extracted pectin, affecting the gel formation properties of pectin and its characteristics in food

product formulations. In the case of pectin, water solubility of natural polysaccharides tends to increase with the degree of chain irregularity or branching and the increase in molecular weight.[194, 221] One way of checking the variation of neutral sugars would be to characterise and quantify the amount of neutral sugars by GC-MS (Gas Chromatography-Mass Spectrometry) or by HPAEC-PAD (High Performance Anion Exchange Chromatography-Pulsed Amperometric Detection).[192, 243]

4.5.4 Optimisation of pectin extraction at big scale

4.5.4.1 Influence of extraction temperature, pre-treatment and MMPD on the molecular weight distribution of pectin

GPC results obtained on pectin extracted at various temperatures at 800 W/L have shown pectin extraction at 120 °C yielded a product which competes with commercial pectin. The influence of MMPD could potentially be responsible for the higher proportion of neutral sugar chains present in pectin extracted at 35 W/L, explaining its lower solubility. As a result, GPC and monosaccharide analysis (MSA) were used to assess the pectin obtained at 800 W/L and 35 W/L to verify this hypothesis. The GPC results obtained are summarised in Table 18. With the exception of samples P0018 each set of conditions trialled was duplicated and the average molecular weight distribution data is reported.

The effect of several operational parameters has been studied alongside MMDP. In order to complete the study, the effect of the variation of the microwave temperature and the effect of pre-treatment were studied next alongside the MMPD. The variation of the Mw, Mn and PI according to those parameters can be seen in Table 18. Two samples of commercial pectin obtained from two different pectin manufacturers were used to highlight the spread of the molecular weight distribution from one manufacturer to another.

Microwave extraction temperature was the first factor to be studied on samples generated at 120 °C and 140 °C with a MMPD of 800 W/L. Samples corresponding to these conditions are P0018, P0020 and P0023. Mw and Mn values for P0018 at 140 °C are the lower ones observed for the samples analysed, showing how a 20 °C increase in the extraction temperature negatively impacts the molecular mass distribution. P0018 has the highest polydispersity of all samples analysed, reaching a value of 3.69, which is over three times the polydispersity of the commercial pectin sample CU201. The best microwave extraction temperature to be selected therefore

is 120 °C, as highlighted before. This is in agreement with the literature: a weight average molecular weight comprised between 10^4 and 10^5 Da has previously been reported.[221]

When excluding the polydispersity of sample P0018, the highest polydispersity reached by pectin samples analysed were of 2.54 and 3.33 for samples P0021 and P0022 respectively. These were obtained following conventional pectin extraction at pH 1.5 at 81 °C with HCl, using WOP (P0021) and PWOP (P0022). In comparison to acid hydrolysis, microwave extraction yields pectin with a lower PI. Furthermore Mn values for both P0020 and P0021 are lower than commercial pectin samples obtained under similar conditions. Thus, the results suggest that the pectin obtained under acid-free microwave conditions is superior to pectin extracted using acid hydrolysis.

Table 18: Summary of the GPC results obtained for pectin samples obtained under different microwave conditions.

Pectin sample	Biomass used	MMPD (W/L)	Temperature (° C)	Yield (%)	Mw (Da)	Mn (Da)	PI (Mw/Mn)
Commercial pectin CU201	N/A	N/A	N/A	N/A	232,250	198,840	1.17
Sigma Aldrich commercial pectin	N/A	N/A	N/A	N/A	125,600	100,100	1.26
Acid-free microwave extraction							
P0002	WOP	35	120	14.03	110,000	494,000	2.28
P0003	WOP	35	120	6.31	168,000	90,000	1.90
Average	***N/A***	***N/A***	***N/A***	***10.17***	***139,000***	***292,000***	***2.09***
Standard deviation	*N/A*	*N/A*	*N/A*	*5.45*	*41,012*	*285,671*	*0.27*
P0004	PWOP	35	120	32.22	229,300	142,500	1.62
P0005	PWOP	35	120	-	223,100	130,800	1.71
Average	***N/A***	***N/A***	***N/A***	***N/A***	***226,200***	***136,650***	***1.66***
Standard deviation	*N/A*	*N/A*	*N/A*	*N/A*	*4,384*	*8,273*	*0.06*
P0018	PWOP	800	140	19.2	55,945	80,610	3.69
P0020	PWOP	800	120	7.46	225,200	95,645	2.35
P0023	PWOP	800	120	5.93	223,650	113,050	1.98
Average	***N/A***	***N/A***	***N/A***	***6.70***	***224,425***	***104,348***	***2.17***
Standard deviation	*N/A*	*N/A*	*N/A*	*1.08*	*1,096*	*12,307*	*0.27*
Acid hydrolysis							
P0021	WOP	N/A	81	2.12	299,650	118,200	2.54
P0039	WOP	N/A	81	6.59	394,000	365,450	1.14
P0040	WOP	N/A	81	4.37	430,450	378,600	1.14
Average	***N/A***	***N/A***	***N/A***	**4.36**	**374,700**	**287,417**	**1.61**
Standard deviation	*N/A*	*N/A*	*N/A*	2.23	67,502	146,693	0.81
P0022	PWOP	N/A	81	6.4	127,900	38,465	3.33
P0026	PWOP	N/A	81	7.18	420,300	383,350	1.10
P0038	PWOP	N/A	81	2.8	395,450	337,550	1.17
Average	***N/A***	***N/A***	***N/A***	**5.46**	**314,550**	**253,122**	**1.87**
Standard deviation	*N/A*	*N/A*	*N/A*	2.34	162,120	187,303	1.27

The influence of the pre-treatment of orange peel on a big scale was investigated next. At 35 W/L, the comparison between samples P0002 & P0003, obtained with WOP, and samples P0004 & P0005 obtained with PWOP shows how the reproducibility of the GPC results is enhanced when using as a raw material PWOP. The standard deviation on the average of Mw, Mn and PI decreases drastically when extracting pectin from PWOP. As an example, the standard deviation of Mn decreases from 285,671 to 8,273 Da when using PWOP. Whether this is entirely due to the pre-treatment step is difficult to fully establish even though the pectin samples have been produced in the same microwave equipment, at the same temperature and using the same raw material. The poor reproducibility between samples P0002 & P0003 does not allow to draw conclusions on the effect of WOP pre-treatment on the specific values of Mw and Mn. However it should be highlighted that pectin samples obtained from PWOP at 120 °C (excluding P0018) display a lower polydispersity, whether extracted at 35 or 800 W/L. Overall, the pre-treatment step consisting of extracting *D*-limonene and flavonoids from the WOP prior to pectin extraction does not seem to be detrimental to the properties of the pectin. As the use of PWOP allows the separation of a greater number of compounds compared to WOP, valorising this raw material to its full potential, this conclusion represents an important milestone for this project aiming at designing a resource-focused microwave biorefinery.

The influence of the MMPD on the molecular weight was studied next at 120 °C. When comparing the sample sets P0004 & P0005 (35 W/L) against P0020 & P0021 (800 W/L), the average Mw is slightly higher for samples obtained at 35 W/L. The same applies for the average Mn value, although a greater variation of the values obtained for Mn between the two sets of samples is observed. Additionally, a lower polydispersity is obtained for at 35 W/L.

GPC analysis was also used to compare pectin samples obtained at 120 °C and 35 W/L with two different commercial pectin samples. The average Mw value is situated within the same range as the Mw value obtained for sample CU201 (226,200 versus 232,250). The corresponding Mn values for these same samples are much lower though (136,650 versus 198,840), indicating the obtention of shorter but more branched chains. Regarding the PI, low values have been attained: the average PI at 120 °C is of 1.66. This is an encouraging results because the typical polydispersity of the standards analysed is of ~ 1.2 and the PI of natural polysaccharides is usually comprised between 1.5-2.0.[194] At 800 W/L and under the same temperature conditions, the average Mn value is lower, reaching 104,348 with

an average PI of 2.17. Consequently, the results imply that the pectin obtained at 35 W/L posses a more uniform molecular weight distribution compared to pectin obtained at 800 W/L. The combined use of low MMPD microwave extraction conditions at 120 °C with the use of PWOP can yield a greater number of pectin chains of a uniform molecular weight distribution.

This is advantageous as the CEM MARS microwave equipment has many advantages over the CEM Discovery: their vessels are three times larger, making the production of a couple of grams of pectin easier and less time consuming. It has both an IR and a fiber optic probe to measure the heating temperature, making the control of experimental conditions more precise, as recommended in the literature.[58] Additionally, when using the dynamic power mode, the power input of the CEM Discovery is much less controllable below 50 W. This conclusion will later be verified against the results obtained by monosaccharide analysis. Furthermore, the galA content analysis should be useful to check whether lower molecular weight values are due to a loss of neutral sugar chains from the hairy regions within the pectin chain for example.

Pectin yields recovered are generally low for our experiments: comparatively, Bagherian *et. al.* have reported pectin yields reaching 26.27% under "MW-assisted acid hydrolysis" type conditions at 900 W for a six minutes microwave treatment.[244] Further improvements of the yields could be achieved by using extraction temperature of 130 °C.

4.5.4.2 Temperature optimisation of pectin extraction on a big scale

After studying the influence of the MMPD and the effect of using pre-treated peel, a thorough investigation of yields of pectin extracted according to the microwave holding temperature was deemed necessary. Having established that 35 W/L represented the best MMPD, tests at 100, 110, 120 and 130 °C were carried out to extract pectin from PWOP. Triplicates were carried-out for each temperature except at 100 °C and the average molecular weight distribution data is discussed here (see Table 19).

Table 19: Influence of the microwave temperature on the molecular weight distribution of pectin
generated from PWOP at 35 W/L under acid-free conditions.

Pectin sample	Temperature (°C)	Yield (%)	Mw (Da)	Mn (Da)	PI (Mw/Mn)
Commercial pectin CU201	-	-	232,250	198,840	1.17
Sigma Aldrich commercial pectin	-	-	125,600	100,100	1.26
Acid-free microwave extraction					
P0028	100	0.98	289,650	139,050	2.09
P0029	100	1.5	317,350	212,400	1.50
Average	***N/A***	***1.24***	***303,500***	***175,725***	***1.80***
STDEV	*N/A*	*0.37*	*19,587*	*51,866*	*0.42*
P0031	110	5.89	267,750	159,900	1.62
P0032	110	6.1	249,550	135,700	1.85
P0034	110	5.87	238,450	152,400	1.57
Average	***N/A***	***5.95***	***251,917***	***149,333***	***1.68***
STDEV	*N/A*	*0.13*	*14,793*	*12,388*	*0.15*
P0004	120	32.22	229,300	142,500	1.62
P0005	120	-	223,100	130,800	1.71
P0024	120	21.69	284,950	109,100	2.61
P0033	120	11.11	265,650	97,720	2.72
Average	***N/A***	***21.67***	***250,750***	***120,030***	***2.16***
STDEV	*N/A*	*10.55*	*29,531*	*20,315*	*0.58*
P0035	130	18.41	205,450	48,030	4.28
P0036	130	15.24	172,600	35,725	4.83
P0037	130	14.25	140,150	35,480	3.95
Average	***N/A***	***15.97***	***172,733***	***39,745***	***4.35***
STDEV	*N/A*	*2.17*	*32,650.20*	*7,176*	*0.44*

The variation of the yield of pectin obtained according to the extraction temperature follows similar trends at 35 W/L and 800 W/L. In section 4.5.1., the yield of pectin was seen to drop above 140 °C. At 35 W/L the yield decreases from 21.67% at 120 °C to 15.97% at 130 °C. Although the yield obtained at 130 °C is the second highest of the serie, the PI of pectin is very high under such conditions, rendering such conditions unsuitable for the obtention of pectin with a narrow molecular weight distribution.

When comparing samples produced at 110 °C and 120 °C, the same reasoning can be applied in the light of the results obtained: a significantly higher yield of pectin is obtained at 120 °C however the PI of 2.16 is still too high compared to commercial pectin. When considering the need to obtain pectin with a yield important enough to compete with commercial process alongside quality parameters, pectin obtained at 110 °C displays the best molecular weight distribution. It has a Mw and a Mn of 250,750 and 120,030 respectively. The PI is the lowest obtained for the extraction temperatures tested (1.68). These samples also present the lowest standard deviation on the Mw, Mn and PI values determined. Hence, 110 °C should be the preferred temperature used for further work in this area.

4.5.5 Monosaccharide analysis of pectin

A method to analyse and quantify the neutral and acid sugars of pectin is GC-EI-MS on depolymerised pectin. Six pectin samples have been analysed using this technique (see Table 20). Specific attention was given to the analysis of rhamnose, arabinose, galactose and galacturonic acid since they are the main components constituting pectin side chains (see Figure 61).[125] The results reported have not been repeated and should be considered as preliminary work only.

Table 20: Pectin samples analysed by monosaccharide analysis.

Pectin sample	Extraction conditions
Commercial pectin CU201	N/A
Non-standardised (NS) commercial pectin (Kelco)	N/A
P0004	120 °C, 35 W/L, WOP, 1:10 (peel:water)
P0021	81 °C, pH 1 , PWOP, 1:10 (dry peel:water)
P0022	81 °C, pH 1 , PWOP, 1:10 (dry peel:water)
P0020	120 °C, 800 W/L, PWOP, 1:10 (peel:water)
P0023	120 °C, 800 W/L, PWOP, 1:10 (peel:water)

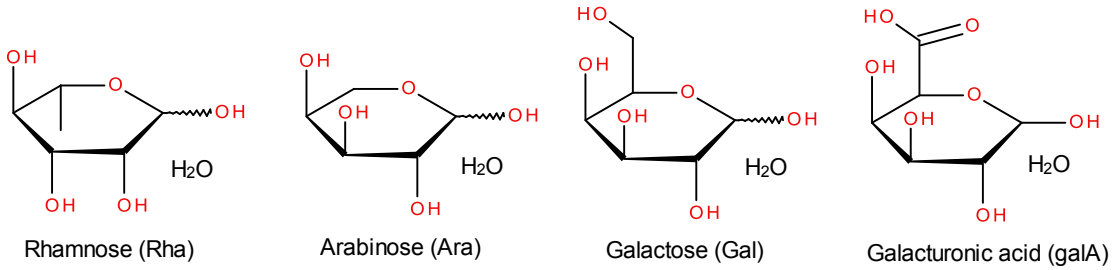


Figure 61: Structure of the sugar monomers considered for monosaccharide analysis (originally in colour).

The results obtained for the MSA analysis are summarised in Table 21. Two commercial pectin samples of known galA content have been subjected to MSA to assess the reliability of the method. For sample CU201, the reported galA content of the commercial sample is of 88%. The analysis carried out determined a galA content of 92.89% which is slightly above the usual ± 5% error. For the NS Kelco sample, a galA content of 85.98% was obtained. The manufacturer reports a galA content of 82.6 %, which is within the ±5% error range, validating the results obtained for the MSA.

Table 21: Summary of the pectin characteristics and associated results obtained by MSA.

Pectin sample	Biomass used	MMPD (W/L)	galA content (w./w.%)	Neutral sugar content (w./w. %)				Total monosaccharide content (w./w. %)
				Ara (%)	Gal (%)	Rha (%)	Total neutral sugar content (%)	
H&F pectin CU 201	N/A	N/A	92.89	0.90	5.46	0.75	7.11	100
NS Kelco pectin	N/A	N/A	85.98	5.17	7.88	0.97	14.02	100
Acid-free microwave extraction								
P0004	PWOP	35	75.20	13.12	10.50	1.18	24.80	100
P0020	PWOP	800	9.44	15.71	9.44	0.96	26.11	35.55
P0023	PWOP	800	79.18	12.06	7.73	1.03	20.82	100
Acid hydrolysis								
P0021	WOP	N/A	65.75	9.61	23.68	0.96	24.25	100
P0022	PWOP	N/A	78.07	4.45	16.12	1.36	21.93	100

Regarding the NS content, NS Kelco pectin should contain a much lower amount of neutral sugars since it is not standardised with sucrose, unlike standardised pectin such as the H&F CU201. Standardised pectin samples can contain up to 50% sucrose (typically 25-30%).[120] Samples could be re-run following hydrolysis in an acidified methanol solution, which should remove the sugar fillers.

Regarding the analysis of experimental samples, initial results indicate that the reproducibility of the galA content between samples generated under the same conditions seems poor (see results for samples P0020 & P0023). All samples display a similar neutral sugar content comprised between 24.80% and 26.11%. Compared to commercial samples, the later value are higher than the neutral sugar content of 7.11 % and 14.02 % obtained for CU201 and NS Kelco respectively. More precisely, when considering samples CU201 and P0004, the later has a neutral sugar content almost 3.5 times higher than CU201. However, the MSA data also shows pectin is predominantly composed of galA. When excluding the value obtained for P0020, the galA content of microwave extracted pectin samples is consistently measured above

65%. Regarding the influence of MMPD on the galA and the neutral sugar content, a 5.25 % difference is observed between the galA content of samples P0004 and P0023. This difference seems too low to explain any difference in properties between the two samples obtained respectively at 35 W/L and 800 W/L. However, if the samples are re-run to account for a greater number of monosaccharides (i.e. D-apiose), a potential decrease of the galA content could then explain a difference in properties and MSA could be used to explain the difference in properties between pectin extracted at 35 or 800 W/L.

The galA content measured seems to be slightly higher for the pectin extracted at 800 W/L (P0023) compared to pectin extracted at 35 W/L (P0004). Additionally, the galA content measured for the two microwave extracted pectin samples (P0004 & P0023) is above the legal requirement (65%). However no conclusion can be reached on the MMPD according to MSA data as further samples need to be run.

A comparison exercise between the galA and the neutral sugar content obtained herein and data available in the literature has been done (see Table 22). The highest galA content has been achieved for sample P0023, reaching 79.18% which is within the range of the results reported in Table 22, showing a lower MMPD is preferable to achieve a higher galA content.

Table 22: Reported results in the literature on MSA of citrus peel residues.

Biomass extracted	Extraction conditions (roughly)	galA (%)	Neutral sugar (%)	Reference
Orange peel	MW pre-treated, HCl extracted (120min)	73.2	-	[175]
Orange albedo	MW heating under pressure (2.5 min), HCl extraction	96	4.2	[148]
Citrus peel walls	Water extraction	80.1	19.9	[245]
Orange peel	Water extraction	4.52	33.91	[189]
Lemon peel	Water extraction	1.68	53.07	[189]

In conclusion, the galA contents obtained for P0004 and P0023 are encouraging, even though they are slightly lower than the ones found in the literature for acid-mediated pectin extractions using microwaves as a method of pretreatment.

4.5.6 DE determination using ^{13}C CPMAS

Using CPMAS ^{13}C NMR, the DE of pectin produced under acid-free microwave conditions at 35 W/L was determined. The results are given in Table 23. When considering the samples obtained by microwave extraction, virtually no variation of the DE can be observed when increasing the microwave holding temperature from 100 to 130 °C. A DE of 68, 66, 69 and 69% has respectively been obtained at 100, 110, 120 and 130 °C. When looking at the DE values obtained for pectin extracted by acid hydrolysis, little change can be observed with the values previously reported for microwave extraction. Whether WOP or PWOP is used, a DE of 68% and 71% respectively has been determined. The data does not appear to be inconsistent: a measure of the accuracy of the method has carried-out, and an error of ± 10% was determined, which was deemed reasonable, especially when compared to traditional methods used (i.e. phenolphthalein titration). Given the results obtained, the method designed using the integral ratio $Int_{OCH_3}/Int_{C(O)OR}$, this method proves to be fairly robust.

Table 23: DE of pectin extracted under acid-free microwave conditions (35 W/L) and by acid hydrolysis determined by CPMAS ^{13}C NMR.

	Biomass used	Temperature (°C)	DE (%)	Error (%)
Acid-free microwave extraction				
P0028	PWOP	100	74	11
P0029	PWOP	100	62	10
Average			**68**	
P0031	PWOP	110	78	12
P0032	PWOP	110	61	9
P0032r	PWOP	110	62	10
P0034	PWOP	110	63	10
Average			**66**	
P0004	PWOP	120	64	10
P0005	PWOP	120	75	12
P0024	PWOP	120	70	11
P0033	PWOP	120	67	10
Average			**69**	
P0035	PWOP	130	74	11
P0036	PWOP	130	69	11
P0037	PWOP	130	63	10
Average			**69**	
Acid hydrolysis				
P0021	WOP	81	70	11
P0039	WOP	81	70	11
P0040	WOP	81	63	10
Average			**68**	
P0022	PWOP	81	68	11
P0026	PWOP	81	73	11
Average			**71**	

The DE of pectin extracted using acid hydrolysis on both WOP and PWOP has also been carried-out. Very similar DE have been reached. In the case acid hydrolysis extraction of pectin on WOP an average DE of 68 has been obtained. In the case of PWOP, an average DE of 71 has been determined. Given the accuracy of the calibration curve previously reported in section 4.4.3.2, it was concluded that the results can be used as a good indication of the DE of pectin extracted under acid-free conditions.

4.6 Conclusion & further work

In this chapter, the possibility to extract pectin under acid-less conditions using microwaves has been proven below 150 °C. This has been demonstrated on a 10 mL and a 100 mL scale, showing the process is scalable. Pectin has been successfully characterised by ATR-IR, solution and solid state ^{13}C NMR. Gel forming properties of pectin have been demonstrated, validating the "proof-of-concept" work for the design of a waste biorefinery based on the use of WOP.

The results obtained show that microwave-assisted extraction of pectin in water only is possible, offering competitive molecular weight distribution as analysed by GPC when compared against commercial standards.

The utilisation of a clean and safe technology for the production of pectin, avoiding acidic waste water effluents is of particular interest. Especially as "environmentally friendly processing without any use of chemicals [acids in this case] can positively influence the consumer" when faced with the decision to buy a product over another.[246] The method reported here would allow for the elimination of acidic wastewater treatment, rendering it safer and potentially less harmful to the environment. No dewatering agent such as lime is used either.

Based on observations made using GPC analysis, microwave holding temperatures of 110 or 120 °C are ideal depending on the required yield and quality of pectin for a given type of application. These have been determined following use of a 35 W/L MMPD on peel previously used for *D*-limonene and flavonoid extraction. The added advantage of this method is the combined production of *D*-limonene and flavonoids as separate product streams prior to pectin extraction. This pretreatment do not seem to affect the properties and the quality of the pectin extracted, filling in the conditions pre-determined in chapter 3 according to the SWOT analysis. The later two product streams and associated production steps will be discussed separately in chapter 5 and 6 respectively.

Hence, the conditions reported herein for pectin extraction can be very valuable for the production of high methoxy pectin and, based on results obtained following the development of a new technique for the determination of the DE. Following previous results reported in the literature, a CPMAS ^{13}C-NMR technique was developed. This technique allowed a fairly accurate determination of the DE when compared to commercial samples of known DE. In the light of the literature review reported in

this chapter on previous methods used in food science to quantify pectin quality, it is believed CPMAS ^{13}C-NMR offers a reliable and reasonably quick alternative which offers good accuracy and limiting human error compared to previous methods highlighted.

Several improvements and process modifications should be done to complete the work reported in this chapter. Thinking chronologically, when considering the production stage first, the work-up of pectin could be improved by testing the properties of pectin obtained when using ethanol only for precipitation and washing. So far acetone has been used for washing following precipitation. But in order for pectin's use to be approved in food products, ethanol and water are the only solvents this material can come into contact with.

Further work can be done on the gel-forming properties of pectin. It should be noted that the results of the gel tests have only been assessed qualitatively. Rheology measurements should be done to quantitatively assess gel strength via the determination of visco-elastic properties for example. Gel strength can be assessed using a texture analyser, allowing the determination of the storage modulus G' and the loss modulus G'' by small amplitude oscillatory rheological tests.[247] Additionally, the gelling point of the pectin produced under acid-free conditions, should be checked especially as "the gelation temperature of pectins depends not only on their botanical source, manufacturing conditions, molecular and materials properties but also on their gel preparation procedure and cooling conditions".[225] This shows how many parameters need to be considered to know exact gel forming conditions for a given type of pectin.

Regarding the characterisation of the DE and the galA acid content of pectin, the results obtained by CPMAS 13C-NMR need to be confirmed using another technique based on a different principle. An example could be the quantification of the amount of CO_2 produced during for different pectin samples of varying DE by thermogravimetric analysis coupled by IR (TG-IR).

Regarding the application of pectin in food formulation, performance studies of pectin produced under acid-free conditions should be carried-out in collaboration with industry.

Chapter 5
Microwave assisted extraction of *D*-limonene

V. L. Budarin, P. S. Shuttleworth, M. De bruyn, T. J. Farmer, M. J. Gronnow, L. A. Pfaltzgraff, D. J. Macquarrie and J. H. Clark, *Catalysis Today*, 2014. Available from: http://dx.doi.org/10.1016/j.cattod.2013.11.058).

L. A. Pfaltzgraff, M. De bruyn, E. C. Cooper, V. Budarin, J. H. Clark, *Green Chemistry*, 2013, **15**, 307-314.

J. H. Clark, L. A. Pfaltzgraff, V. L. Budarin, A. J. Hunt, M. Gronnow, A. S. Matharu, D. J. Macquarrie and J. R. Sherwood, *Pure & Applied Chemistry Journal*, 2013, **85**, 1625-1631.

J. H. Clark, L. A. Pfaltzgraff, V. Budarin, M. De bruyn, 2013, *Microwave assisted Citrus Waste Biorefinery*, WO2013GB00154 20130404.

A. M. Balu, V. Budarin, P. S. Shuttleworth, L. A. Pfaltzgraff, K. Waldron, R. Luque and J. H. Clark, *ChemSusChem*, 2012, **5**, 1694–1697.

Oral presentation given at the 5th IUPAC International Conference in Green Chemistry, Durban (18th August 2014).

Oral presentation given at the Future of Food Waste event, Leeds (18th June 2014).

Poster presentation given at the G2C2 event, Cape Town (25th and 26th August 2014).

Poster presentation given at the Food waste in the European food supply chain: challenges and opportunities event, Athens (12th May 2014).

5 Microwave-assisted extraction of *D*-limonene

Citrus peel is known for secreting a substance, commonly known as citrus peel essential oil. The main use of essential oils in the European Union is as flavour enhancers in foods, in perfumes and in pharmaceutical applications. Steam distillation is used on a commercial scale for its extraction.[248, 249] The main component of orange oil is *D*-limonene (83-97% by weight), a non-oxygenated monoterpene made from the combination of two isoprene units.[250] The present chapter aims to describe the work done on microwave-assisted extraction of *D*-limonene as part of an integrated microwave biorefinery. Two types of extraction have been trialled:

- Open vessel microwave-assisted extraction of *D*-limonene
- Solvent-less open vessel microwave-assisted extraction of *D*-limonene.

The extraction of *D*-limonene is the first step in the OPEC process described in this thesis. Yields of *D*-limonene will be compared between the different experimental set-ups mentioned above. The extraction of *D*-limonene using microwave technology will be studied with the aim of reaching a high purity extract under solvent-less conditions. The *D*-limonene yield obtained for optimised solvent-less open vessel microwave-assisted extraction under vacuum will be compared against *D*-limonene extracted using conventional steam distillation and the composition of the *D*-limonene containing extract will also be highlighted.

5.1 Open vessel microwave-assisted extraction of *D*-limonene

D-limonene ($C_{10}H_{16}$) is naturally present in over 300 essential oils, and especially in citrus fruit peels, in which it is the main component. It has been used for over 50 years as a flavour and fragrance component and is now increasingly applied as a solvent in industry given its good degreasing properties. The degreasing agents are used for cleaning electrical circuits for example. *D*-limonene is usually obtained as a by-product of citrus juice production in Brazil and Florida.[251]

Conventional solvent extraction was studied first to establish a baseline for future experiments. This piece of work was designed as a proof of concept. The aim was to

obtain a significant volume of *D*-limonene at lab scale: 100 mL of *D*-limonene of a given purity was to be sent for analysis to an industrial collaborator. Figure 62 A, B and C show the set-up used for *D*-limonene extraction at a three litre scale as well as the extract obtained. More specifically, hexane was used to extract *D*-limonene at a three litre scale. Hexane was selected as an extraction solvent to both guarantee a maximum solubility of *D*-limonene, a cyclic terpene, and an easy recovery due to its low boiling point. Interestingly, *D*-limonene has previously been reported as an alternative to hexane for rice bran oil extraction, demonstrating *D*-limonene's excellent oil solubility properties.[252] Cause for concern exists over the use of hexane for several reasons and a suitable alternative is actively being researched in academia.[253]

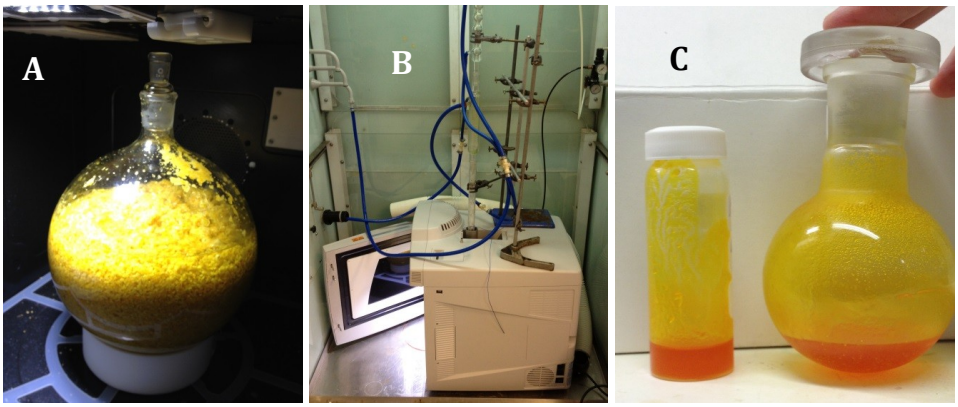


Figure 62: A) Three litre round-bottom flask containing macerated orange peel in hexane; B) CEM MARS microwave equipment used (open vessel configuration); C) obtained *D*-limonene containing extract (originally in colour).

Following the conditions described, a 1.52% yield of *D*-limonene was determined on a wet basis was obtained when working at this scale. Steam distillation of the resulting orange oil allowed for the recovery of *D*-limonene (Figure 63). A ^{13}C NMR spectrum confirmed its identity (see experimental chapter).

Figure 63: Steam distillation set-up used for *D*-limonene purification (originally in colour).

Following purification of the *D*-limonene obtained by hexane extraction, the formation of an orange precipitate was observed during the steam distillation. Using ElectroSpray Ionisation (ESI) mass spectrometry it was found to consist of 4 different polymethoxyflavones; these being tangeritin, nobiletin, tetra-O-methylscutellarein and heptamethoxyflavone (see Figure 64) which precipitated in water as *D*-limonene was removed by steam distillation. ^{1}H NMR was used to identify functional groups indicative of the nature of the extract obtained. Since the detected polymethoxy flavonoids (PMFs) are present in a mixture, ^{1}H NMR spectrum could not be decisively interpreted but does show several very strong aromatic methoxy signal at ~3.97 ppm (see Figure 65). Further analysis of this spectrum will be provided in chapter 6 which will specifically focus on the isolation and characterisation of orange peel related flavonoids. Aromatic methoxy groups are characteristic of all the above mentioned polymethoxyflavones. PMFs are a subclass of flavonoids. Citrus-derived flavonoids, "especially polymethoxyflavones (PMFs), have been of particular interest because many of these flavonoids exhibit a broad spectrum of biological activity."[254] However, few reports exist on their pharmaceutical activities, especially *in vivo*. This is mainly due to their high trading price: as an example, 3,5,6,7,8,3',4'-heptamethoxyflavone reached a value of $300/mg.[255]

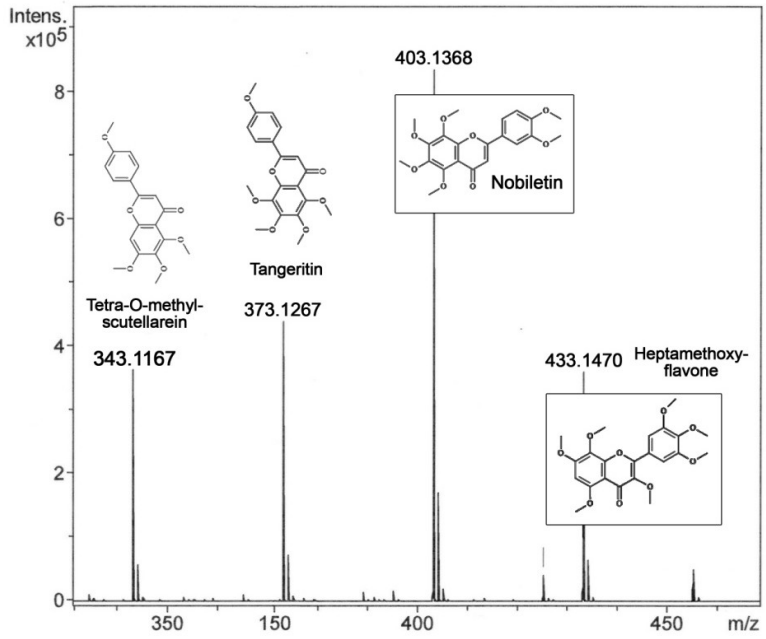


Figure 64: ESI spectrum of the precipitate formed in the aqueous phase of the orange oil extracted using hexane following purification by steam distillation.

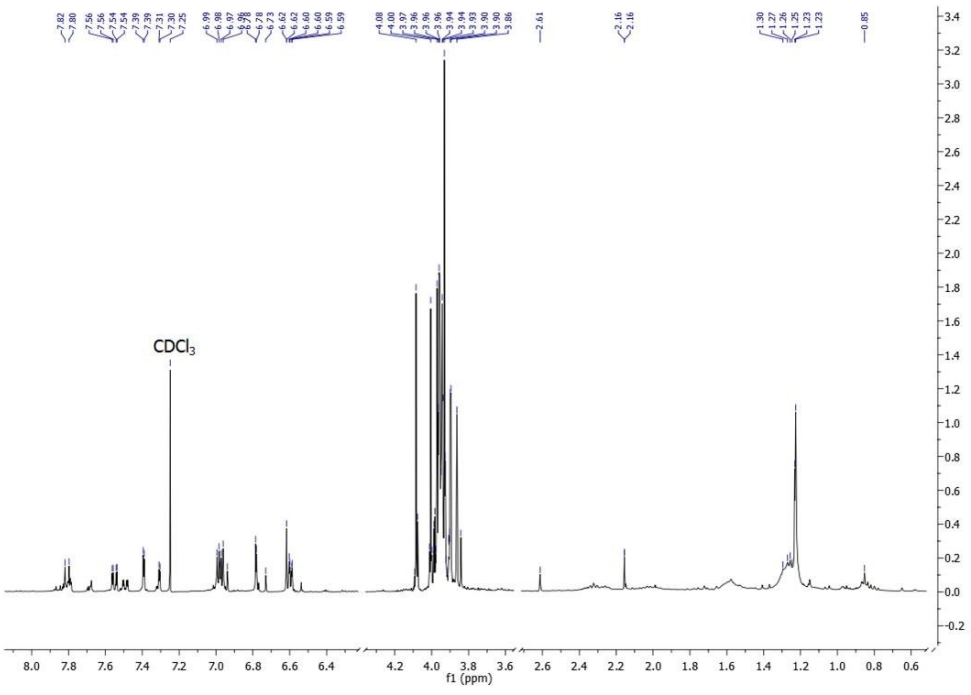


Figure 65: ^{1}H NMR of the acetone soluble, water insoluble orange oil precipitate obtained following *D*-limonene purification by steam distillation (originally in colour).

The high value of the flavonoids detected motivated the modification of the overall OPEC process to recover them using a dedicated step. Based on the difference in

volatility between *D*-limonene (176 °C) and PMFs (~550 °C), it was thought *D*-limonene could be recovered using a microwave-based steam distillation step. The *D*-limonene extracted peel (OPR1) could then be used for the extraction of flavonoids in a separate step using a soxhlet extraction, as previously mentioned in chapter 4. As well as allowing for the recovery of the said flavonoids, this step "stripped" the peel of low molecular weight organic compounds, facilitating the work-up of pectin extracted thereafter. The recovery, separation and characterisation of the extracted flavonoids will be discussed in further depth in the next chapter.

Further work was done in an attempt to phase out the use of hexane for the extraction of *D*-limonene. Hexane is known as a neurotoxin and is classified as a hazardous air pollutant according to the Environmental Protection Agency (EPA).[256, 257] Preliminary work and optimisation of the aforementioned microwave-assisted extraction technique for *D*-limonene will be developed in the following paragraphs. The results will be compared against *D*-limonene extracted by steam distillation.

5.2 Open vessel solvent-less microwave-assisted extraction of *D*-limonene

5.2.1 Preliminary work

Hexane based extraction of *D*-limonene in the microwave yielded 1.52% of *D*-limonene. Given the risks associated with hexane, the research effort shifted to solvent-less extraction technique. With the latter in mind, trials were first done microwave steam distillation. This technique is known for its application on the extraction of essential oils: results on lavender, rosemary, lemon oils have been reported amongst others.[258-260] Microwave steam distillation is known to be faster due to a more efficient heating process, fast temperature control and reduced thermal gradients via a fast interaction of the water with the microwaves given its high dielectric constant.[64] Up to three fold reductions in extraction time have been reported for the extraction of lemon essential oil using microwave assisted steam distillation (MASD) compared to steam distillation.[258]

MASD was tested by Kai Shute (visiting student for the October-November 2012 period) with defrosted WOP on the Milestone ROTOSYNTH microwave. All oil yields are quoted on a wet basis. Two experimental parameters were evaluated. First the

peel:water ratio (1:1, 1:0.5 and 1:0) and secondly, the duration of the microwave treatment (1200 W for 6 minutes followed by 800 W for 10, 15, 20, 25 or 30 minutes). The influence of the peel:water ratio was studied first.

Figure 66 illustrates the obtained results. The data shows how the yield of oil collected is inversely proportional to the amount of water used. The maximum yield of oil is reached with a peel:water ratio of 1:0.5 (over the range tested). Under these conditions, 0.41% of oil was obtained using a microwave power of 100 W.

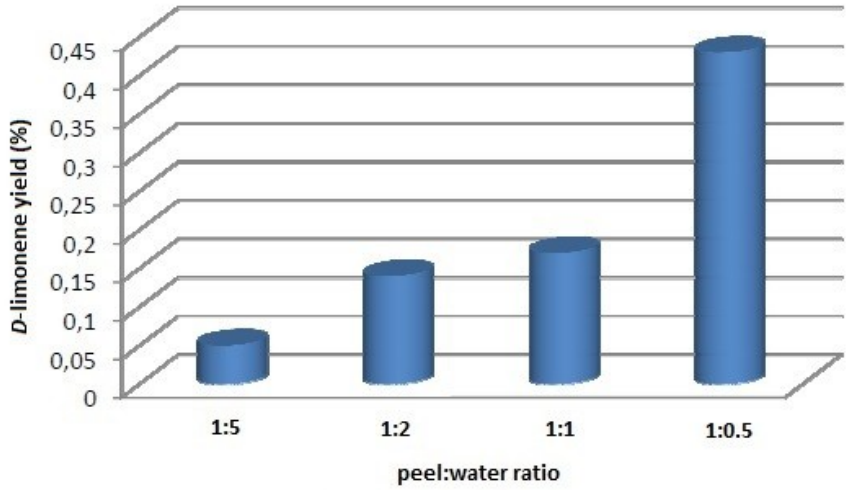


Figure 66: Oil yield collected by MASD as a function of the peel:water used at 100 W (originally in colour).

The influence of the time of extraction for MASD was also investigated for a peel:water ratio of 1:1 (

Figure 67) and 1:0.5 (Figure 68). By varying the extraction times, it was clear the extraction yield reached a plateau after 25 minutes. In both cases, the oil yield increased with the time of extraction until it reached 25 minutes. Extraction times over 25 minutes led to pyrolysis as by then all the free water had been removed thus heating predominantly the biomass components. After 25 minutes, a smell of burning and a brown colouration appeared on the peel present in the vessel. The maximum yields obtained are very similar in both cases: 0.94% and 0.96% were achieved with a peel:water ratio of 1:1 and 1:0.5 respectively. The latter results represent a two folds improvement over the 0.41% yield obtained previously at 100 W. The results show that the peel:water ratio has a minimal influence on the yield of oil obtained. Consequently, MASD of WOP alone was trialled, without any additions of water. The experiment proved to be successful: a 1.08% oil yield was attained under solvent-less conditions. Although the use of water was already a good

alternative to hexane in terms of reducing the toxicity, flammability, VOC emissions and handling hazards associated with this hydrocarbon solvent (especially at an industrial scale), the use of water as a solvent presents different challenges. The recovery of this solvent is difficult due to its high heat capacity and boiling point. This has the effect of rendering the use of water as a solvent "costly and energy-intensive".[261] In 1996, it was even reported that in Florida, many citrus-processing plants "have avoided recovering cold pressed oil totally or partially, because of the significant impact this has on waste water treatment investments."[262]

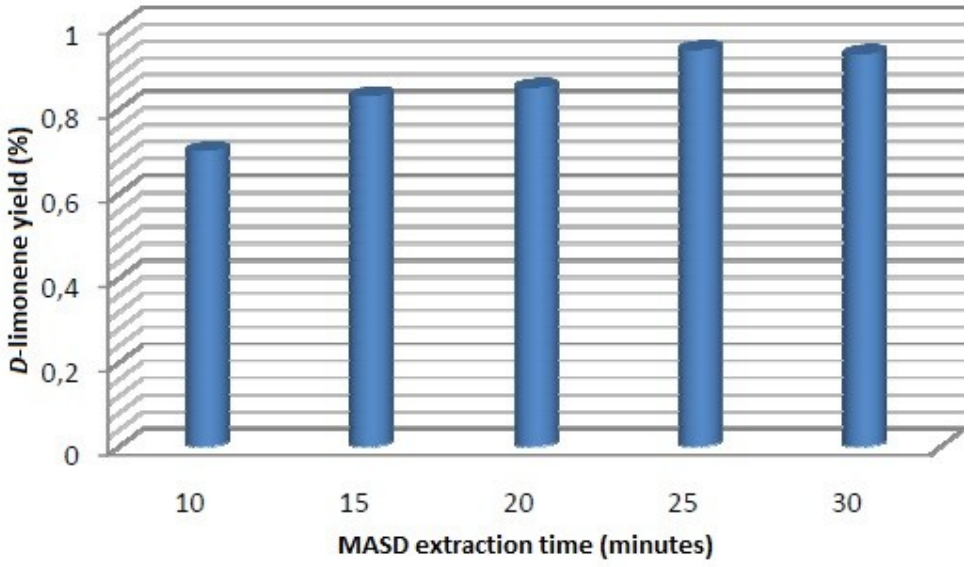


Figure 67: Oil yield collected by MASD as a function of the extraction time used for a peel:water ratio of 1:1 (originally in colour).

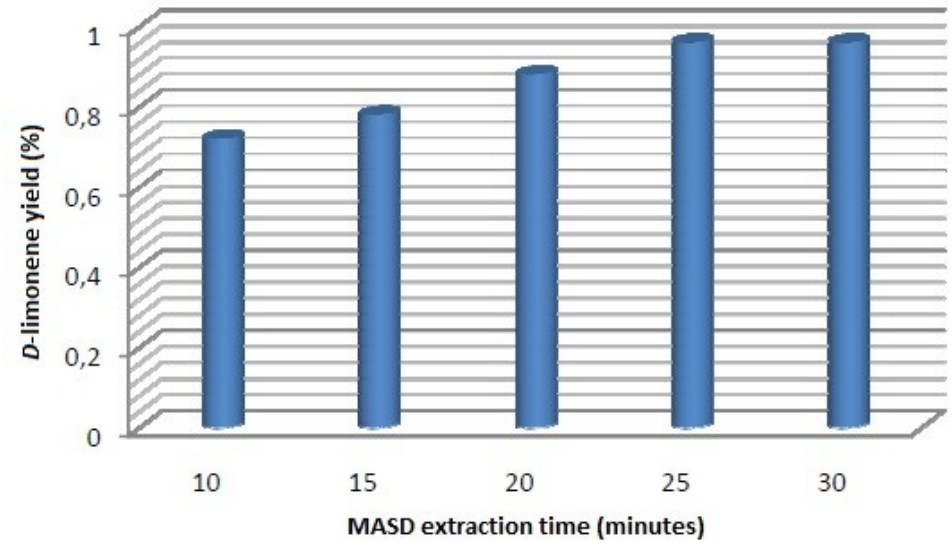


Figure 68: Oil yield collected by MASD as a function of the extraction time used for a peel:water ratio of 1:0.5 (originally in colour).

5.2.2 Solvent-less extraction of *D*-limonene under microwave conditions at 100 mbars

Based on the observations and good results obtained, for the MASD extraction of citrus oil under solvent-less conditions, the use of this technique under vacuum was thought of as a way to further increase the yield of *D*-limonene collected. Such a technique has been previously used by Budarin *et. al.* to fractionate the volatiles produced during microwave activation of wheat straw.[34] Here, the main objective was to further lower the boiling points of compounds traditionally found in essential oils (200-300 °C)[263] and trap a maximum of organic compounds extracted in the round-bottom flask, avoiding their loss in the pump traps. The experimental set-up is highlighted in Figure 69 A, B and C).

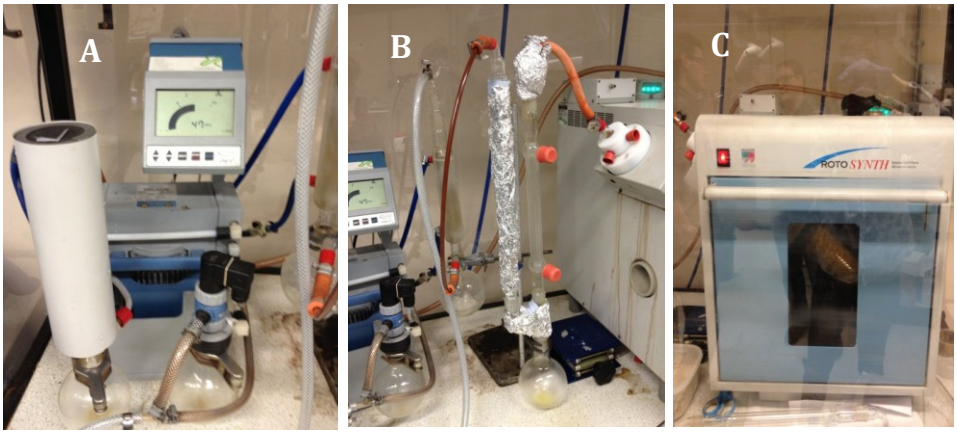


Figure 69: Pictures of the *D*-limonene extraction set-up
A) pump; B) extract condenser system; C) microwave equipment (Milestone RotoSYNTH) (originally in colour).

For a first experiment, 2569.11 g of WOP were used and a vacuum of 100 mbars on average was applied during the microwave-assisted extraction. The WOP had a moisture content of 18.23%. The energy consumption was measured using a power meter for each run: on average 0.91 kWh were consumed by the whole system. This includes the microwave unit, the chiller and the vacuum pump. The maximum temperature reached at 1200 W was of 58 °C and 61 °C when lowering the microwave power to 800 W (see power ramp used for the extraction in the experimental section). The whole process for this step is described in Figure 70.

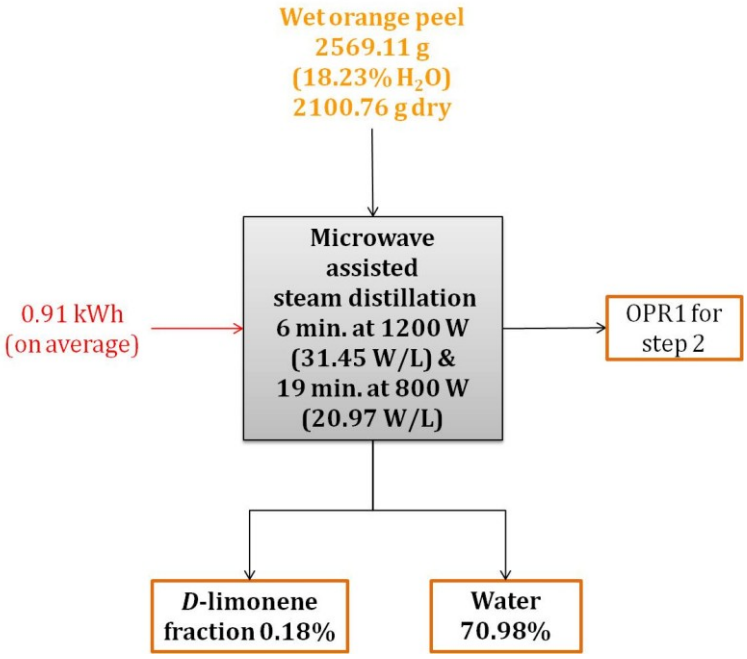


Figure 70: Detailed process flow sheet of step 1 (*D*-limonene extraction DL002 at 100 mbars) (originally in colour).

D-limonene was quantified using GC-FID, employing ethyl benzene as an internal standard (IS). The concentration range used was of 0.07- 0.20 molar. The details of the analysis can be found in Table 24.

Table 24: GC-FID data for the calculation of the *D*-limonene concentration extracted with a response factor of k = 0.8377 (sample DL002) at 100 mbars.

	Boiling point (°C)	**Retention time (min.)**	**Peak area**	**Concentration (mol/L)**
Ethyl benzene (IS) $C_6H_5CH_2CH_3$	136	4.84	3859.8	0.094
***D*-limonene $C_{10}H_{16}$**	176	8.23	2985.8	0.08

The conditions trialled allowed the recovery of 2.96 g of *D*-limonene per litre of liquid phase. Since 1528.23 g of liquid phase has been collected, 4.52 g of *D*-limonene has been extracted. The wet yield therefore amounts to 0.18% (0.22% on a dry basis). On a wet basis, 70.98% of the original weight of the WOP was collected under the form of water. The latter will also contain volatile components other than

D-limonene; these have not be quantified or characterised as this wasn't defined as part of the scope of this project.

In order to verify no gases were lost following the extraction, a mass balance was done for this step (see Table 25). A difference of 195.18 g was observed between the amount of material coming in and the amount of material coming out. The GC-FID chromatogram does not display many other compounds other than ethyl benzene and *D*-limonene (see experimental section). Since *D*-limonene has a non-negligible volatility, the difference in mass might be due to a high portion of volatiles being left in the vacuum traps by the high vacuum used. The yield of *D*-limonene extracted could therefore be improved by decreasing the vacuum applied to the extraction vessel, leaving room for optimisation.

Table 25: Mass balance for step 1 (*D*-limonene extraction DL002 at 100 mbars).

IN		OUT	
Material	**Mass (g)**	**Material**	**Mass (g)**
OP (wet basis, 18.23% H_20)	2569.11	*D*-limonene	4.52
		OPR1	843.22
		H_20	1526.19
TOTAL	**2569.11**	**TOTAL**	**2373.93**

The experiment was repeated a second time for reproducibility purposes (sample DL001). A wet yield of 0.16% (0.22% on a dry basis) was obtained.

Both yields are the same on a dry basis, demonstrating the good reproducibility of the experiment under the conditions trialled. In order to further improve the yields obtained, a lower vacuum (i.e. 500 mbars instead of a vacuum of 100 mbars) could be used. A higher yield would also allow for an easier collection of the organic layer by liquid-liquid separation. This is especially important on a larger scale when solvent extraction can be avoided, rendering the process safer and more-environmentally friendly.

Generally, current processing removes a relatively small amount of citrus oil from the peel prior to juicing operations. Less than ~15% of the total available quantity is removed this way.[264] Eventually it should be possible to extract a greater portion of *D*-limonene. Orange essential oil contains between 83 and 90% *D*-limonene.[265] It has been estimated that 5 kg of oil can be recovered from 1000 kg of oranges (wet waste). This corresponds to a yield of 0.5% (on a wet basis) for the essential oil that could therefore be recovered.[113, 117] In a worst case scenario, a minimal yield of

0.42% (on a wet basis) of *D*-limonene is expected when accounting for a 83% *D*-limonene content. The yields obtained at 100 mbars are far off the minimal yield calculated above for steam distillation of the peel used for juice production. Hence, the decrease of the vacuum used represents a promising change in the experimental conditions used and will be tested next.

5.2.3 Optimisation of MASD at 500 mbars

Following the results obtained at 100 mbars for the microwave-assisted extraction of *D*-limonene under solvent-less conditions, further experiments were carried-out using a 500 mbars vacuum with the aim of increasing the yield of *D*-limonene extracted with improved reproducibility. Extractions were performed in triplicates except at 100 mbars. The yields were determined by GC-FID, using a correlation factor of 0.791 for a *D*-limonene concentration ranging from 0.004 molar to 0.101 molar. Additionally, the results obtained were compared to steam distillation. All yields obtained have been summarised in Table 26.

Table 26: *D*-limonene extraction conditions and associated yield (*D*-limonene was abbreviated to DL here).

Extraction conditions	Sample ID	WOP water content (%)	Mass OP used (g) wet	Mass OP used (g) dry	Mass DL extracted (g)	Dry yield DL (%)	Average dry DL yield (%)	Standard deviation on dry DL yield	Wet yield DL (%)	Average wet DL yield (%)	Standard deviation on wet DL yield
Microwave 100 mbars	DL001	17.13	1734.56	1437.43	2.81	0.20	0.20	0.01	0.16	0.17	0.01
	DL002	17.13	2569.11	2129.02	4.52	0.21			0.18		
Microwave 500 mbars	DL003	19.95	3589.56	2873.44	32.52	1.13	1.28	0.25	0.91	1.03	0.20
	DL004	19.30	942.89	760.91	11.92	1.57			1.26		
	DL005	19.30	970.50	783.19	8.86	1.13			0.91		
Steam distillation	SD1	20.59	100.00	79.41	0.82	1.03	0.61	0.37	0.82	0.49	0.29
	SD2	20.59	100.00	79.41	0.27	0.34			0.27		
	SD3	20.59	100.00	79.41	0.37	0.47			0.37		

In comparison with values found in the literature for orange peel, Ferhat *et. al.* report yields of essential oil of 0.42 ± 0.02% and 0.39 ± 0.02% for microwave assisted extraction and hydro distillation respectively,[64] both of which are much lower than the *D*-limonene 1.03% yield obtained for the experiment done under a 500 mbars vacuum. Regarding the recovery of lemon essential oil by cold pressing and steam distillation, yields of 0.05 ± 0.01% and 0.21 ± 0.01% have been reported.[258]

Although interesting, the results obtained at 500 mbars should be put in perspective with the ones previously obtained at atmospheric pressure. The latter conditions yielded 1.08% of *D*-limonene which does not represent an improvement compared to when a vacuum of 500 mbars is used (1.03%). However it does still demonstrates how a microwave-assisted extraction can be an improvement over the traditional extraction method in terms of reproducibility but also in terms of process speed. A microwave-assisted extraction of WOP required half the time of a steam distillation for over five times the quantity of peel extracted (Figure 71). Additionally, no solvent was required.

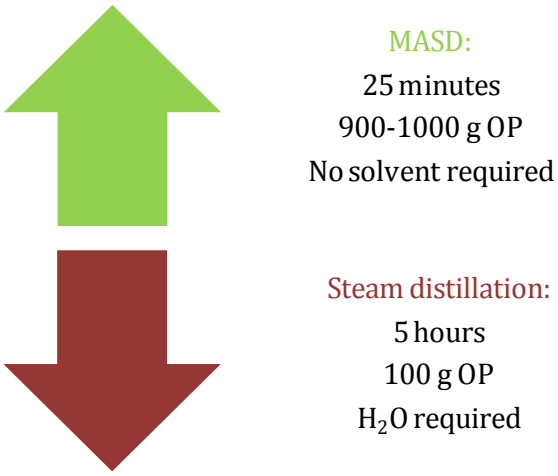


Figure 71: Advantages of MASD over classic steam distillation (originally in colour).

On the other hand, if we compare MASD at 500 mbars against microwave-assisted extraction in hexane, the yields of *D*-limonene obtained in hexane are still higher by 32.68% (1.53% versus 1.03%). However the lower MASD yield could be outweighed again by the fact that no solvent is be used at all, cutting out CAPEX costs on solvent recovery and use when applying the process at commercial scale.

Table 27 summarises results reported in the literature on microwave-assisted extraction of citrus oil from fruits such as oranges, lemons and limes with a focus on microwave solvent-less extraction. Under solvent-less conditions, the yield of 1.03% is situated in the higher end of the results reported, even though the peel used for the experiments reported herein has been extracted post-juicing. A yield of 1.6% is reported for lemon peel by Bousbia *et. al.* This is a very high yield and compared to other values reported for lemons, and seems to be unrealistically high. However, the variety of the fruits used and the maturity of the fruit at the time of extraction can have an important influence on the yields of oil collected, as shown by the data reported by Bousbia *et. al.* for lemons. It is not however the sole factor influencing yields. From the data shown in Table 27, it seems clear that the high yield of 1.03% is mainly due to the high microwave power applied during the extraction. Compared to the work of Ferhat *et. al.* where a power of 200 W is used (500 W for Bousbia *et. al.*), this factor seems to be determinant.

Table 27: Summary of *D*-limonene yields reported in the literature for microwave assisted extraction techniques applied to varied citrus fruits (solvent-less conditions for 1.-3., solvent-less microwave hydro diffusion & gravity for 4.-9. and microwave steam diffusion for 10. and 11.)

	Biomass used	**Biomass initial moisture content (%)**	**Extraction conditions**	***D*-limonene containing fraction yield (%)**	***D*-limonene content (%)**	**Reference**
1.	Orange fruits of different variety collected post-juicing	19.52 (average taken)	6 min. at 1200 W & 19 min. at 800 W at 500 mbars	1.03 ± 0.20	-	Own experimental results
2.	Orange (*Citrus sinensis* L. Osbeck) Valencia late.	90	200 W for 30 min. at atmospheric pressure.	0.42 ± 0.02	76.7	Ferhat *et. al.* (2006)
3.	Lemon (Citrus limon (L.)	-	200 W for 30 min. at atmospheric pressure.	0.24 ± 0.01	69.65	Ferhat *et. al.* (2007)
4.	Lime (*Citrus aurantifolia*) Swing	-	500 W for 15 min. at atmospheric pressure.	0.8	60.56	Bousbia *et. al.* (2009)
5.	Lemon (*Citrus limon* L.) Eureka	-	500 W for 15 min. at atmospheric pressure.	0.7	69.65	Bousbia *et. al.* (2009)
6.	Lemon (*Citrus limon* L.) Villa Franca	-	500 W for 15 min. at atmospheric pressure.	1.6	60.56	Bousbia *et. al.* (2009)
7.	Orange (*Citrus sensis* L.) Tarocco	-	500 W for 15 min. at atmospheric pressure.	1.2	95.19	Bousbia *et. al.* (2009)
8.	Orange (*Citrus sensis* L.) Valencia late	-	500 W for 15 min. at atmospheric pressure.	1.0	94.64	Bousbia *et. al.* (2009)
9.	Orange (*Citrus sensis* L.) Washington Naval	-	500 W for 15 min. at atmospheric pressure.	0.9	95.20	Bousbia *et. al.* (2009)
10.	Orange (*Citrus sinensis* L. Osbeck) Valencia late collected post juicing.	90	200 W at atmospheric pressure with a mass flow rate of steam of 25 g/min for 12 min.	1.54	94.88	Farhat *et. al.* (2011)
11.	Orange (*Citrus sinensis* L. Osbeck) Valencia late collected post juicing.	75	200 W at atmospheric pressure with a mass flow rate of steam of 14 g/min for 6 min.	5.43 (dry basis)	96.20	Sahraoui *et. al.* (2011)

Unfortunately, a thorough comparison between the experiments highlighted in Table 27 is not possible given the differences, in microwave set-ups, power, extraction times and peel moisture content. The same applies to *D*-limonene content originally present in the biomass. The table also details results obtained by microwave steam diffusion. It is interesting to note that in this case, yields of recovered oil are much higher. But whether the experiment is done under solvent-less conditions is debatable given the use of a steam generator which is maintained to temperature using microwaves. From an environmental standpoint, the use of this technique is subject to discussion as a higher quantity of water will have to be treated and extra energy is required to produce steam prior to microwave extraction.

The extraction mechanism of oil from CPW under MASD has previously been reported by Ferhat *et. al*. The heating of the inherent water content of the peel caused the oil glands present in the peel to burst, releasing the oil as the water contained in the peel is vaporised.[64, 258, 266] This was proven by cytology analysis of fragments of peel stained with acid-Schiff's reagent under the microscope.[123, 258, 266] Kinetic studies have shown microwave treatment of the peel under microwave steam diffusion facilitates oil extraction.[123, 267]

In terms of energy consumption, steam distillation consumes 4.32 kWh over 5 hours on average. For MASD at 500 mbars, 0.93 kWh are consumed over 26 minutes. This corresponds to 0.014 and 0.036 kWh per minute for steam distillation and MASD respectively. As a result, MASD is over two times more energy intensive. However MASD is over ten times faster, even when working with a volume of WOP nearly 10 times larger. It should be noted that there is not a big difference between the energy consumption between 100 mbars and 500 mbars, which is interesting.

Finally, the mass balance of the optimised conditions developed for *D*-limonene extraction has been determined (see Table 28).

Table 28: Mass balance for step 1 (*D*-limonene extraction DL003 at 500 W, 4 microwave runs in total).

IN		OUT	
Material	**Mass (g)**	**Material**	**Mass (g)**
OP (wet basis, 19.95% H_20)	3589.68	*D*-limonene	32.52
		OPR1	1846.78
		H_20	1710.38
TOTAL	**3589.68**	**TOTAL**	**3589.68**

Figure 72 describes step 1 at 500 mbars together with the energy consumption. The mass balance shows that the amount of material coming in is equal to the amount of material coming out. This is an improvement over the MASD at 100 mbars and allowed the resolution of the loss of material in the pump traps.

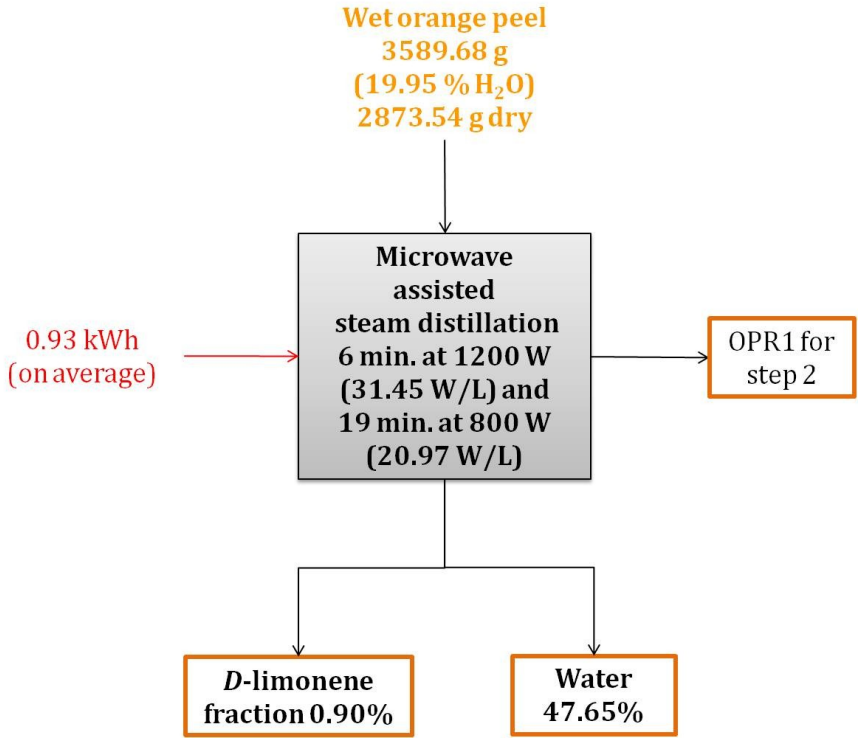


Figure 72: Detailed process flow sheet of step 1 (*D*-limonene extraction DL003 at 500 mbars) (originally in colour).

5.2.4 GC-MS analysis and comparison between the extracts obtained at 100 and 500 mbars against steam distillation

The terpene composition of each *D*-limonene fraction was determined for every extraction condition tested. This was done by GC-MS analysis for the extracts generated at 500 mbars and 100 mbars as well as for *D*-limonene obtained by steam distillation of WOP. GC-TOF was used to determine the grade of limonene produced

and see which other volatiles were extracted. This technique is routinely used. "Most of the commercialized essential oils are chemotyped by gas chromatography and mass spectrometry analysis."[249]

The latter could be used to verify whether the use of microwave technology has any influence on the composition of the *D*-limonene extract produced. *D*-limonene is classified into three categories depending on the extraction method used: high purity, food grade and technical grade. The technical grade "is often un-standardised and therefore *D*-limonene contents may vary from 60%-90%." High purity and food grade extracts respectively have to attain a *D*-limonene content superior or equal to 98.5% and 95% as determined by HPLC analysis. The latter two are obtained by cold pressing. In practical terms, following cold pressing of peels, more *D*-limonene can be further extracted by steam distillation, yielding the technical grade.[268,269] All three grades differ by price, with the food grade reaching up to a 19% premium. Providing that the right grade can be produced, MASD has the potential to replace both cold pressing and steam distillation, generating a competitive advantage with a cost-effective microwave process that gives a high yield and high purity *D*-limonene.

The GC-TOF analysis was run in collaboration with Tony Larson (CNAP, department of biology at the University of York). The use of this technique was aimed at estimating the relative percentage of *D*-limonene present alongside other major components present. Elucidating the detailed composition of all three extracts wasn't within the scope of the project. However the content variation of the major terpene components will be estimated.

Three samples were analysed using GC-TOF: DL002, DL004 and SD1. DL002 and DL004 were respectively generated at 100 mbars and a 500 mbars under MASD conditions. SD1 is the result of a steam distillation. Each sample was run three times and average values are reported. The *D*-limonene content was compared first between samples. The analysis technique chosen established that *D*-limonene is the main component of the three extracts. The highest *D*-limonene content was obtained for sample DL004 (95.38% ± 0.80), followed by SD1 (84.27% ± 10.10) and DL002 (42.44% ± 15.53). The higher *D*-limonene content value obtained for DL004 compares well with previous work reported on MASD on orange peel. Farhat *et. al.* and Sahraoui *et. al.* respectively report a *D*-limonene content of 94.88% and 96.20%.[266] Bousbia *et. al.* obtained a *D*-limonene content of 94.64% by microwave hydrodiffusion.[270] All the latter authors report *D*-limonene as the main component of the extract obtained under microwave conditions. More importantly, the results obtained show that under optimised microwave conditions at 500 mbars, the *D*-

limonene fraction obtained falls into the food grade category. This is interesting as traditionally, food grade *D*-limonene is obtained by cold pressing and only technical grade *D*-limonene (the lowest grade of *D*-limonene) is obtained under heating (i.e. steam distillation).

Regarding the variation of *D*-limonene between samples DL002 and DL004, the much higher *D*-limonene content obtained for DL004 shows how the use of a moderate vacuum improves the *D*-limonene content. This confirms the hypothesis that the high vacuum setting affects negatively the *D*-limonene content.

Alongside *D*-limonene, other compounds have been identified by using corresponding standards. The content in α-myrcene , α- and ß- pinene, *p*-cymene, α-terpineol and linalool have been estimated using standards of the latter compounds alongside a standard of *D*-limonene. The latter components have all been identified in the literature as the main terpene constituents present in orange oil. Other compounds detected were reported as unknown. The percentage peak areas corresponding to each standard have been reported in Table 29.

The method was applied to samples DL004 and SD1. The results obtained show that, when excluding the unknown components, the fraction of oxygenated compounds represents 0.16% of the terpene content in DL004. In sample SD1, this value almost doubles, reaching 0.31%. This difference in the quantity of oxygenated terpene will impart a different scent profile to the oil. Furthermore, the monoterpene content is significantly different between sample DL004 and sample SD1. In the former it reaches 96.96% and 85.76% for the latter sample. Overall, the results obtained show that under optimised conditions, MASD yields a limonene fraction that has a characteristically different composition profile including a higher proportion of monoterpenes over oxygenated terpenes. From the standards run, none of the fractions differ in composition regarding the main terpene content.

Table 29: GC-TOF results obtained for samples DL004 and SD1.

	Extraction ion chromatogram peak area (%)	
	DL004	SD1
Monoterpenes	**96.96**	**85.76**
D-limonene	95.38	84.27
α-myrcene	0.91	0.74
α-pinene	0.44	0.39
ß-pinene	0.20	0.21
p-cymene	0.02	0.15
Oxygenated monoterpenes	**0.16**	**0.26**
linalool	0.15	0.15
α-terpineol	0.01	0.11
Total:	**97.12**	**86.02**
Other compounds:	**2.88**	**13.98**

The *D*-limonene content is much higher in the case of MASD, establishing MASD as a more efficient extraction technique in comparison to steam distillation. This observation was initially confirmed by carrying out a mass and an energy balance on step 1.

5.3 Conclusion & further work

MASD of WOP under solvent-less conditions was proven as an effective method for the extraction of *D*-limonene. No drying or pre-treatment of the WOP prior to extraction was needed. Under optimised conditions at 500 mbars, a 1.09% yield of *D*-limonene was achieved, with a standard deviation of ± 0.18% on the average yield. In comparison, steam distillation and MASD at 100 mbars yielded respectively only 0.49% and 0.17% *D*-limonene. Good reproducibility was also demonstrated at a 500 mbars and a scale of 1 L. In this chapter, it has been demonstrated that microwave technology presents distinct advantages for the fast and reproducible production process of food grade *D*-limonene. The work presented in this chapter shows MASD under solvent-less conditions is reproducible and has the potential to be scaled-up, eliminating the need for solvent, offering lower energy consumption and greater purity compared to traditional steam distillation. The results obtained show how the first step of the OPEC process can be successfully carried-out at a

scale of up to 1 litre, fulfilling one of the aim of this project, as determined in chapter 3.

A huge challenge remains though: some progress still needs to be done on the isolation and purification method for *D*-limonene. At this point, it was detected and quantified following solvent extraction. This is a disadvantage knowing the extraction can be done under solvent-less conditions. Solvent-less conditions are especially useful to avoid the presence of solvent residues in the extract. This is not negligible for an extract used in food or cosmetic products, as the composition of those consumer products is heavily legislated. The absence of residues can also have the potential to increase the value of the extract obtained. Thinking ahead, the cost-efficiency of a process can also be increased by the use of a solvent-less process since solvent removal can represent an important CAPEX investment for the scaled-up plant in terms of equipment and later in terms of operation costs for energy-consumption. If the isolation issue is resolved, microwave technology will have the potential to carry out "full reproducible extractions [...] in seconds and minutes with high reproducibility, reducing the consumption of solvent [and the risks associated with their use] simplifying manipulations and work-up, [...] and eliminating post-treatment of waste water."[259] The influence of lower powers on *D*-limonene yields should also be further investigated as in the case of lavender oil extraction by microwave assisted steam diffusion, there was little difference between maximum yields obtained at 200 W or 400 W (~4.4%), even in terms of speed of extraction.[259]

In order to further gain information on the application of *D*-limonene in consumer products, MASD extracted D-limonene should be tested independently in food products as a flavour or as a fragrance in cosmetic products. Regarding its use as a fragrance component, the difference in scent might be due to the presence of *L*-limonene, which has a scent which is closer to pine than to citrus. The ratio between (*S*)-limonene (*L*-limonene) and (*R*)-limonene (*D*-limonene) could be determined by chiral chromatography for example. The presence of *L*-limonene could also explain the slight difference in scent noticed for the *D*-limonene fraction extracted according to the method reported herein.[120]

Finally, in order to thoroughly assess the scalability of step 1, the reproducibility of the developed *D*-limonene MASD extraction method should be trialled on fresh waste orange peel as opposed to defrosted WOP. This is especially important as the latter will have lost a great quantity of water following freezing-defreezing. This might influence the yield of *D*-limonene recovered. Such tests will also represent a good opportunity to study the variability introduced by different feedstock history.

It would also be interesting to verify the *D*-limonene yield varies as a function of the orange variety used. Additionally, it would be interesting to see whether the use of a lower power for MASD can improve reproducibility.

Chapter 6
Flavonoid and sugar extraction

L. A. Pfaltzgraff, M. De bruyn, E. C. Cooper, V. Budarin, J. H. Clark, *Green Chemistry*, 2013, **15**, 307-314.

J. H. Clark, L. A. Pfaltzgraff, V. Budarin, M. De bruyn, 2013, *Microwave assisted Citrus Waste Biorefinery*, WO2013GB00154 20130404.

Oral presentation given at the 5th IUPAC International Conference in Green Chemistry, Durban (18th August 2014).

Oral presentation given at the Future of Food Waste event, Leeds (18th June 2014).

Poster presentation given at the G2C2 event, Cape Town (25th and 26th August 2014).

Poster presentation given at the Food waste in the European food supply chain: challenges and opportunities event, Athens (12th May 2014).

6 Flavonoids and sugar extraction

6.1 Introduction to flavonoids

Alongside *D*-limonene, CPW contains other valuable components. Flavonoids are secondary plant metabolites present in the peel and leaves of fruits.[271] They are part of a large group of naturally present polyphenolic compounds with anti-cancer, antiatherogenic, antimicrobial and anti-inflammatory properties.[150] Citrus peel is a known source of flavonoids: the flavonoid content of an industrial by-product from sweet orange peel has been reported to vary from 4.50% to 11.00% by dry weight.[126] They have physiological and pharmaceutical properties which imparts notable health benefits, mainly associated with their antioxidant activity. [272, 273] Flavonoids not only differ by the nature of their B ring but also by their substituents which can be hydrolxyl or methoxyl groups. When methoxy substituents are present on the benzo-γ-pyrone ring (C_6-C_3-C_6), the flavonoids fall into the sub-class of polymethoxyflavonoids (PMFs) or polymethoxy flavones. These compounds are often found in citrus fruits.[274, 275] Structurally, they are characterised by two aromatic rings connected by a pyrone ring (see Figure 73).

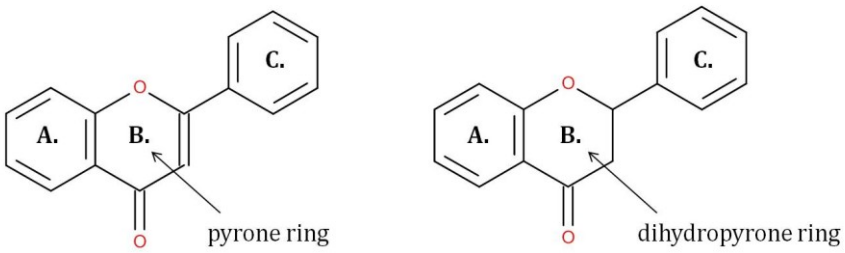


Figure 73: Basic chemical structures of flavonoids distinguishing flavones from flavanones.

In biomass, the function of flavonoids ranges from pigment source, to UV and insect protection.[276] The main flavonoids (or flavanone glycoside) available in citrus are hesperidin, narirutin, naringin and eriocitrin (see chemical structures in Figure 74).

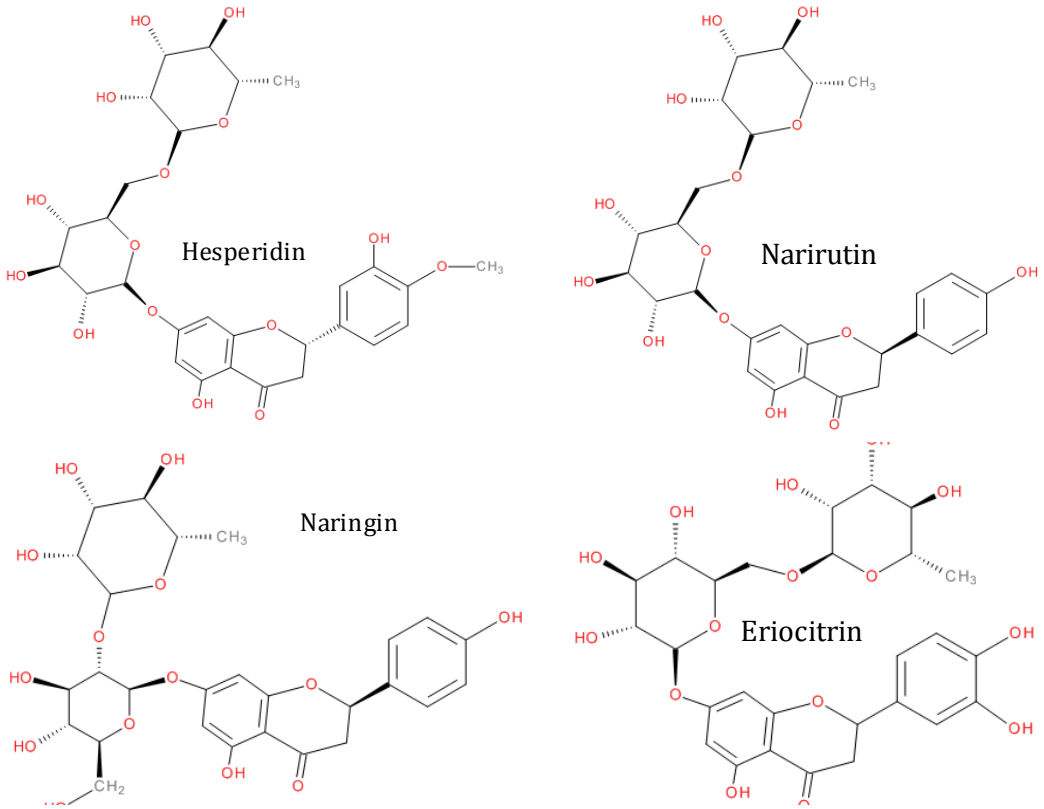


Figure 74: Chemical structures of some of the most common flavonoids found in citrus fruits (hesperidin, narirutin, naringin and eriocitrin) (originally in colour).

Citrus fruit peel also contains PMFs, which are known for their interesting medicinal properties.[277-279] However, their pharmacokinetic properties have rarely been studied, due to their high cost (see chapter 5).[255] This chapter will be centred on step 2 of the OPEC process: the extraction of the PMF molecules previously detected when purifying a fraction of *D*-limonene, as seen in chapter 5. It will highlight the optimum extraction method selected, the solvent used as well as the isolation and the characterisation of PMFs alongside with other molecules that are co-extracted from OPR1 (*D*-limonene extracted peel). Hesperidin was extracted together with a monosaccharide containing fraction. Both have been characterised and the sugar fraction has been quantified using ^{13}C NMR.

As part of the first round of experiments carried-out for an industrial collaborator (the production of 100 mL of *D*-limonene using microwave-assisted extraction), the formation of a precipitate in water was observed following purification by steam distillation of solvent extracted orange oil. Using ESI mass spectrometry, the mixture was found to consist of 4 different PMF compounds namely tangeritin, nobiletin, tetra-O-methylscutellarein and heptamethoxyflavone (see chapter 5). The ^{1}H NMR spectrum of the mixture could not be used to precisely identify each PMF detected by ESI. However, the presence of peaks between 3.8 and 4.2 ppm is indicative of aromatic

methyl ethers (Figure 75). Peaks corresponding to aromatic protons can also be seen in the 6-7.5 ppm region. This observation instantly rules out compounds such as ß-carotene, as the presence of alkene conjugated protons could not be detected between 5.3 and 5.8 ppm, even though aliphatic –CH- signals can be detected ~ 1.25 ppm. The peak seen at 2.16 ppm is due to residual ethanol being present in the sample.

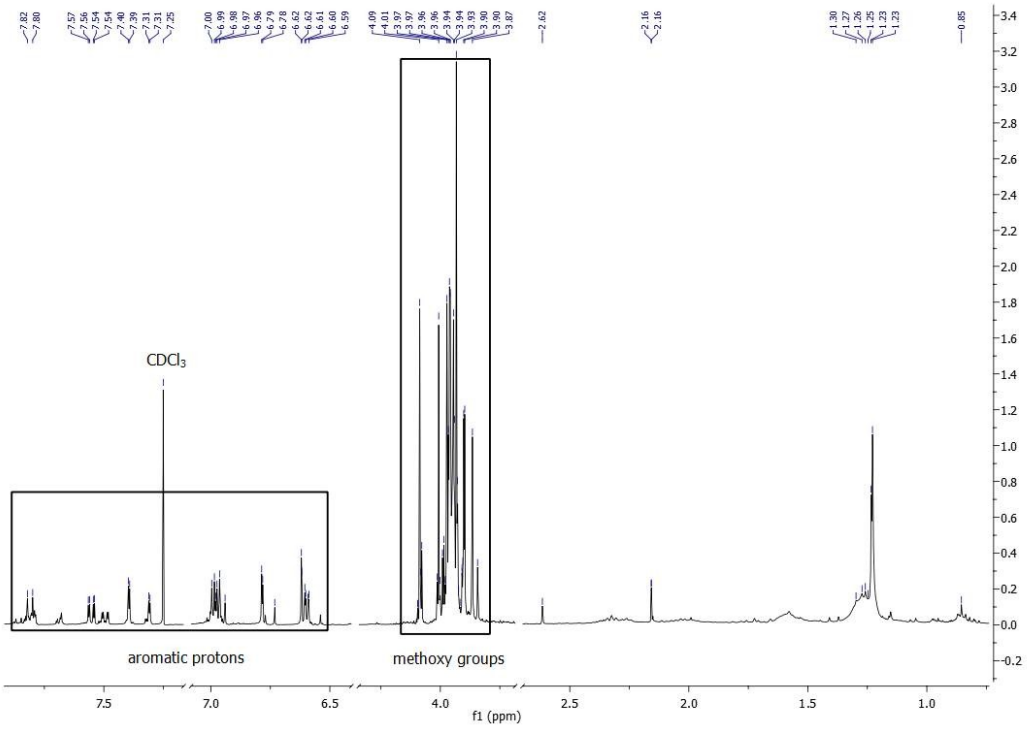


Figure 75: Detail of the methoxy group region of the ^{1}H NMR spectra (400 MHz, $CDCl_3$) of the flavonoid precipitate isolated following *D*-limonene purification by steam distillation (originally in colour).

This, together with soft ionisation mass spectrometry data, confirms the presence of PMFs as opposed to carotenes, another common component present in orange peel. Tangeretin, heptamethoxyflavone, nobiletin, heptamethoxyflavone and sinensitin are amongst the major PMFs found in citrus fruits and can be extracted "using non polar solvents such as hexane and polar solvents such as water, ethanol and methanol."[280] Their structures are given in Figure 76.

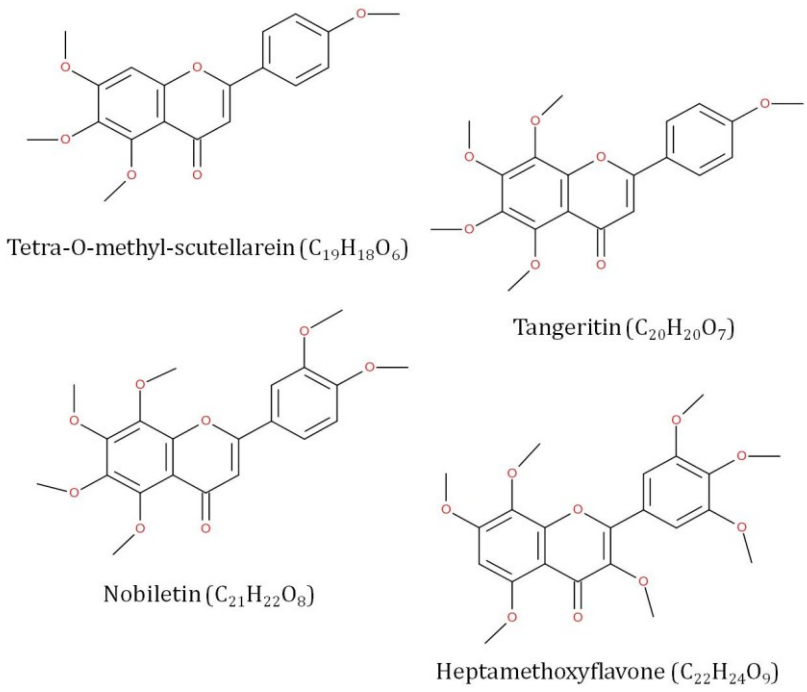


Figure 76: Chemical structures of tetra-O-methylscutellarein, tangeritin, nobiletin and heptamethoxyflavone (originally in colour).

Following the results obtained after sending the second set of pectin samples for an industrial collaborator (see chapter 4 section 4.6.), the decision was taken to include a soxhlet extraction step to extract the PMFs detected. The soxhlet extraction was to be added to the process between *D*-limonene extraction (step 1) and pectin extraction (step 3). This appeared to represent an opportunity to extract the PMFs detected by ESI and NMR, adding another product stream to the process. A soxhlet extraction was chosen as it would have the double advantage of extracting flavonoids and removing acetone soluble low molecular weight substances such as monosaccharides present in the peel following *D*-limonene extraction. This strategy was chosen knowing that pre-treating peel prior to pectin extraction using a washing step with 96% alcohol to "remove alcohol-soluble low molecular weight substances and for inactivation of endogenous enzymes" has been previously reported.[189] Acetone was first chosen as an extraction solvent as the precipitate obtained previously displayed the best solubility in acetone and its low boiling point, ensuring good recovery of the solvent following extraction.

6.2 Soxhlet extraction technique

Soxhlet extraction is a well known continuous "solid-liquid extraction" technique used to transfer a given compound in the liquid phase, allowing for its characterisation and/or quantification. It was developed in 1879 and has since become the standard extraction method for solid-liquid extraction against which new extraction techniques are evaluated.[281] Although it is an established and effective continuous extraction method, the volumes of solvent and energy consumed have motivated further improvements to this technique over the years.[281] The combination of soxhlet extraction together with microwaves or ultra-sounds have been reported.[281]

A soxhlet relies on the siphon to operate continuously: upon heating, as the condensed solvent vapour is collected in the extraction chamber, the level of solvent rises up to the point when it reaches the over-flow level of the siphon. The extraction chamber then empties itself, releasing the extract in the bulk of the solvent. Once the solvent reaches its boiling point again, the same cycle repeats itself until the heating is turned off, exposing the sample to fresh solvent. Figure 77 depicts the equipment used for a conventional soxhlet extraction.

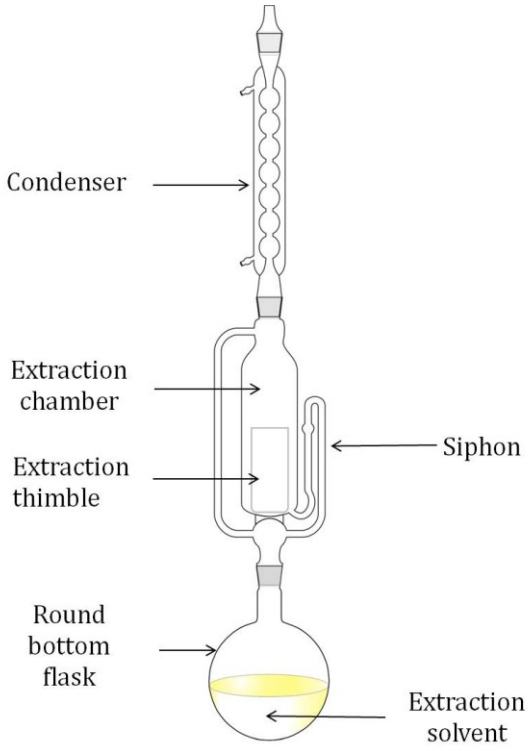


Figure 77: Conventional soxhlet extractor (originally in colour).

This extraction method was chosen here to extract the PMFs detected as well as other low molecular weight components with the hope of simplifying the purification process of pectin. It was also selected for the high throughput of biomass this technique can handle.

6.3 Acetone soxhlet extraction

The extraction was carried out on a 3 L scale using the set-up represented in Figure 77 with acetone as an extraction solvent and the orange peel residue OPR1 (*D*-limonene extracted peel). Following acetone removal, a thick orange paste was obtained. Solubilisation tests of the obtained extract were done in different solvents with the aim to determine a selective and efficient work-up procedure for the PMFs. Water, acetone and ethanol were all tested first. The outcome of each solvent solubility test was assessed by ESI. The composition of the different extracts could then be quickly determined to understand the distribution of compounds in the different solvents tested according to their solubility properties. Care was taken to select renewable solvents which could be easily removed, bearing in mind toxicity and biocompatibility issues. A work-up method was then designed following two main observations: PMFs were solubilised in ethanol, a renewable food grade solvent which allowed for a white precipitate to form. Figure 78 shows a picture of the precipitate in ethanol.

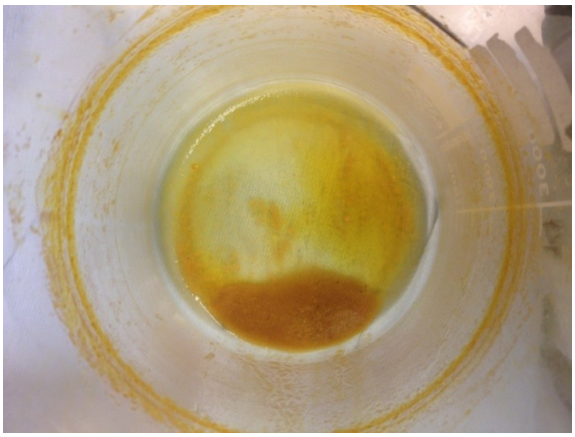


Figure 78: Ethanol precipitate observed following isolation of the PMF mixture (originally in colour).

By using ATR-IR spectroscopy, the white solid was identified as hesperidin. The exact characterisation of this compound will be discussed in section 6.5.2. A solubility test using water also revealed that monosaccharides contained in the extract could be separated by filtration as the rest of the mixture is water insoluble. Since flavonoids have a limited solubility in cold water, the latter solvent was used first before solubilisation in ethanol to remove monosaccharides from the water-insoluble flavonoid mixture. Water removal was done using a freeze-drier and yielded a brown, rock hard solid. The solid was later identified as a mixture of fructose and glucose by ^{13}C NMR (see 6.5.3). The whole process used is highlighted in Figure 79 along with every product stream obtained and their associated work-up. The work-up procedure designed allowed isolation of the mixture of four PMF compounds, hesperidin and a mixture of sugars with yields of 2.22%, 0.14% and 4.34% respectively on a wet basis. On a dry basis, the yields for the four PMF compounds, hesperidin and a mixture of sugars with yields of 2.32%, 0.15% and 4.52% respectively. The characterisation of the obtained products will be discussed in section 6.5. The experiment was repeated and the same products were obtained.

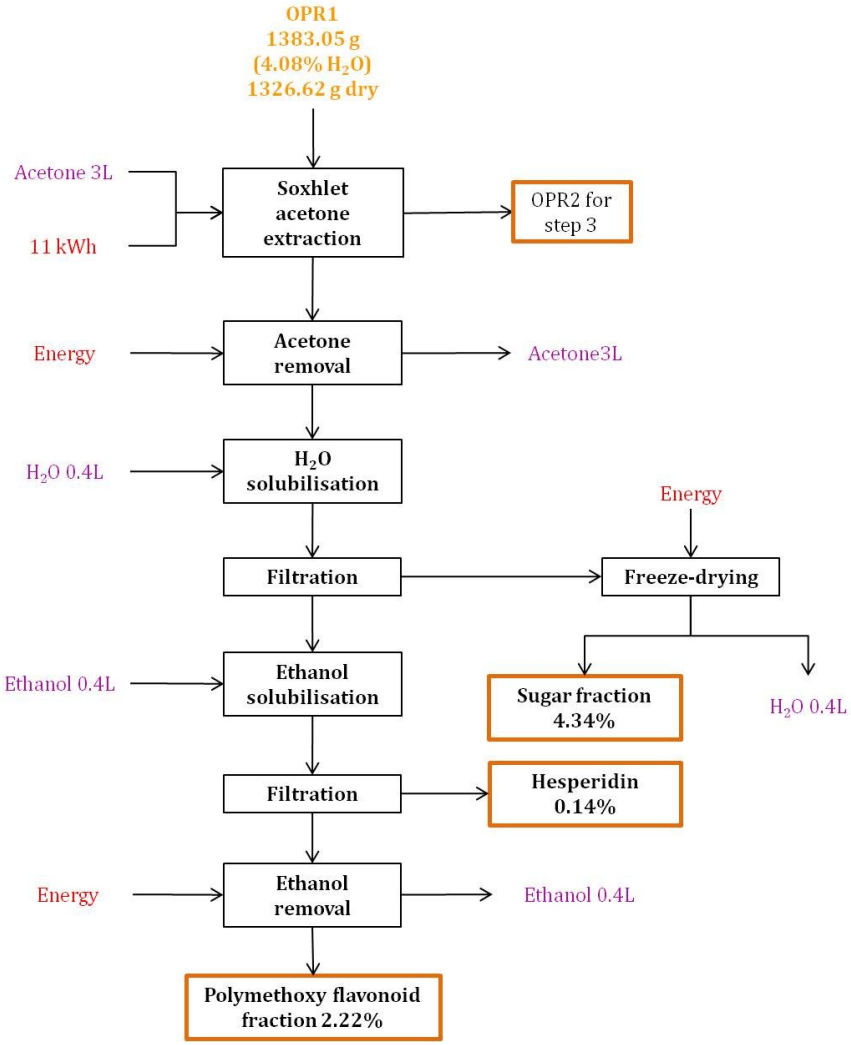


Figure 79: Detailed process flow sheet of step 2
(flavonoids extraction using an acetone soxhlet, experiment SO1) (originally in colour).

A mass balance was carried-out for the flavonoid extraction step (see Table 30). A difference of 132.79 g was observed between the material input and the material output. This difference corresponds to 1.88% of the total mass materials going in for step 2, which is negligible when working on a 3 L scale. The difference in mass was most probably due to human error.

Table 30: Mass balance for step 2 (flavonoids extraction SO1).

IN		OUT	
Material	**Mass (g)**	**Material**	**Mass (g)**
OPR1	1383.05	Polymethoxy flavonoids	30.75
		Sugar fraction	60.01
		Hesperidin	2
		OPR2	1157.5
Acetone	2373	Acetone	2373
H_2O	400	H_2O	400
Ethanol	316	Ethanol	316
TOTAL	4472.05	TOTAL	4339.26

Marin *et. al.* reports peel produced as a result of orange juice production contains up to 4.50% of flavonoids (dry weight) following HPLC analysis (value obtained post-juicing).[126] This value seems high when knowing that processing operations have been reported to decrease the flavonoid content by up to 50% (i.e. leaching by water).[272]

Alternatively, Manthey *et. al.* have reported a flavonoid content of 1.7-2% on dry basis for orange and grapefruit peel.[149] This value seems more reasonable following the results obtained by acetone soxhlet extraction. Nevertheless, the results obtained show there is still scope for improvement. A possible solvent change might improve extracted yields but could also lower the selectivity of the method for the targeted PMFs.

6.4 Ethanol soxhlet extraction

The results obtained when stripping OPR1 in an acetone soxhlet led to one main modification for step 2. Acetone was replaced by ethanol to enhance the yield of PMFs recovered and eliminate the use of a non-food grade solvent. The latter is especially important as PMFs and pectin cannot be in contact with a non-food grade solvent given their targeted applications in the food or the pharmaceutical sector. This change was further motivated by the use of ethanol for the work-up of the

soxhlet extract, reducing the number of solvents needed. Methanol has been reported as an extraction solvent for PMFs but ethanol was preferred as it is less toxic and more easily bio-derived.

The use of ethanol for PMF extraction is known: 75% ethanol in water and 100% ethanol were reported to give 100 % extraction efficiency for PMFs for example.[280] Using such a solvent combination might change the nature of the extract though. When using acetone the PMF and the hesperidin are easily separated using liquid-solid extraction with renewable solvents such as water and ethanol. This could change if phenolics such as galic, vanillic or *p*-coumaric acid are also extracted, complicating the work-up protocol.

In addition to the change of solvent, it was decided to use OPR2 obtained following the extraction of *D*-limonene at 500 mbars whereas OPR2 obtained from a 100 mbars extraction was used in both cases for the acetone extraction. Although this represents two process modifications instead of one, making it difficult to attribute the change in yields to either parameter, this decision has been taken as it would allow a more precise picture of the optimised OPEC process to be drawn within the time frame given. Figure 80 shows the soxhlet equipment used for the extraction when using ethanol.

Figure 80: Ethanol soxhlet (originally in colour).

The change in solvent and the use of OPR1 extracted at 500 mbars gave better results than previously observed. The same three different types of products were isolated when using ethanol as an extraction solvent. The mixture of four PMF compounds, hesperidin and a mixture of sugars were obtained with yields of 5.40%, 0.24% and 18.35% respectively on a wet basis. On a dry basis, the yields for the four PMF compounds, hesperidin and a mixture of sugars with yields of 6.15%, 0.26% and 19.92% respectively. On a dry basis, the yields obtained with ethanol represent an outstanding improvement by a factor of 2.6 for the PMF compounds, 1.73 for hesperidin and 4.4 for the sugars compared with the results obtained with acetone. A table highlighting all yields obtained for every product stream in both acetone and ethanol is given below.

Table 31: Summary of the yields obtained for all product fractions following acetone and ethanol soxhlet extraction on a dry and wet basis.

Product stream	**Yields given on a wet basis (%)**			**Yields given on a dry basis (%)**		
	PMFs	Hesperidin	Sugars	PMFs	Hesperidin	Sugars
Acetone	2.22	0.14	4.34	2.32	0.15	4.52
Ethanol	5.40	0.24	18.35	6.15	0.26	19.92

The characterisation of those compounds will be discussed in section 6.5. A photo of the extract can be seen in Figure 81.

Figure 81: PMF fraction obtained following ethanol soxhlet extraction (originally in colour).

The results obtained with ethanol show that the change of solvent did not affect the type of products extracted: the nature of the fractions isolated is consistent between the two solvents with yields significantly higher than for acetone. Figure 82 details the process used.

Little literature can be found on extraction yields of PMFs from citrus peels. Most of the reported data in this area focuses on flavonoid detection and separation as they are often found as part of the molasses obtained following juicing operations.[149, 157] When their extraction is investigated, a mixture of flavonoids is often reported and the research effort focuses on their identification and separation. When reported, quantitative analysis is expressed in the form of a total phenolic content in ppm or as concentrations. This links with the research done on the activity and the flavonoid composition in juice products.[272] Although the yield of polymethoxy flavonoids seems low, it has been reported that 1.3 kg of total polymethoxylated flavones can be produced from 1000 kg of 65° brix Valencia orange molasses, which corresponding to a 0.13% yield. In this context, the wet yield of 5.87% obtained for PMFs represents an important achievement over the traditional method extraction (lime maceration of the peel).[157]

Additionally, soxhlet extraction allows for the production of PMFs without the use of lime, potentially lowering the environmental impact of the production of this class of compounds. Regarding previous reported soxhlet extractions of sweet orange peel: Lee *et. al.* reported the extraction of PMFs using 85% aqueous ethanol for four hours. Under these conditions, 14.34 mg of nobiletin and tangeritin were obtained per gram of peel, strengthening the work reported here.[274] It seems the MASD of *D*-limonene prior to PMF extraction by an ethanol soxhlet has a positive influence of the amount of PMFs extracted when comparing it to the conventional method and conventional soxhlet extraction.

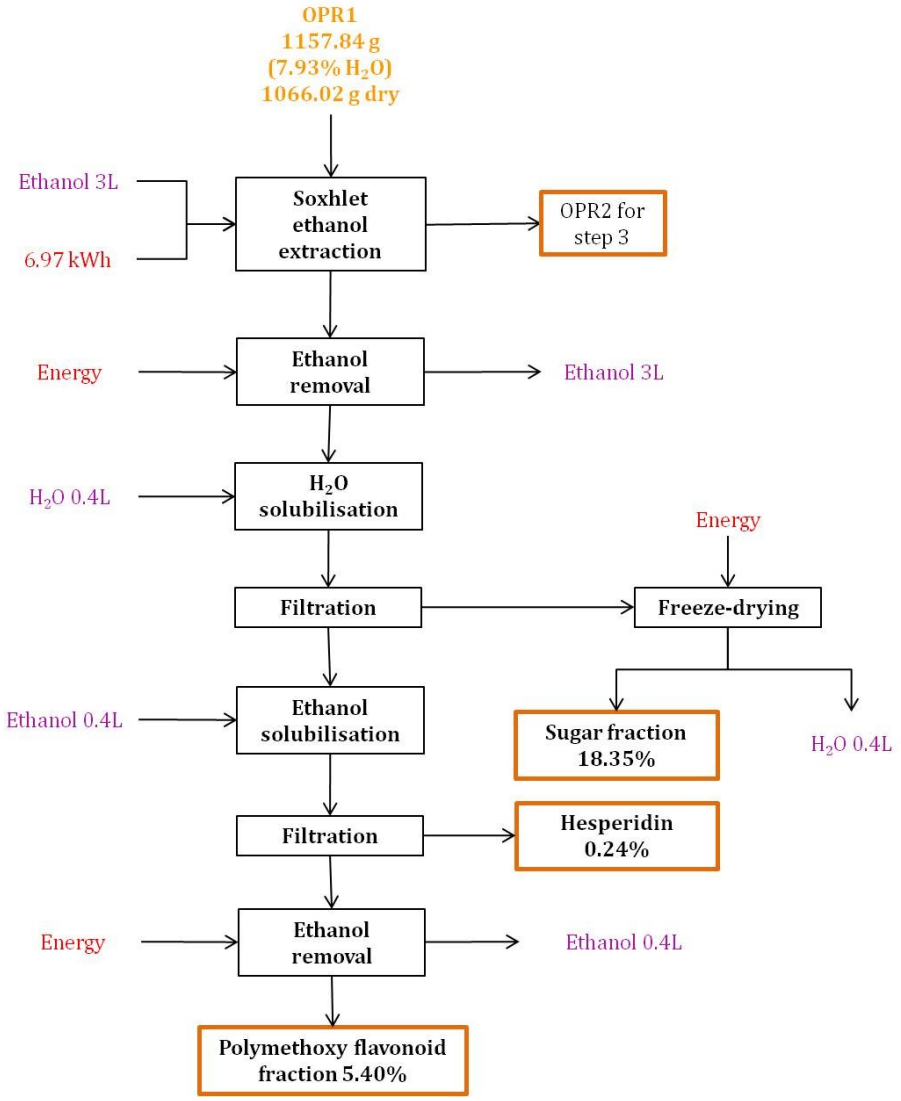


Figure 82: Detailed process flow sheet of step 2
(flavonoids extraction using an ethanol soxhlet, experiment SO3) (originally in colour).

Following the obtention of the ethanol extract, a mass balance was calculated for the ethanol soxhlet extraction, as seen in Table 32). A difference of 10.08 g was observed between the amount of material coming in and the amount of material coming out. This difference corresponds to less than 1% of the total mass of materials entering step 2, which is negligible, especially when working on a 3 L scale.

Table 32: Mass balance for step 2 (flavonoids extraction SO3).

IN		OUT	
Material	**Mass (g)**	**Material**	**Mass (g)**
OPR1	1157.84	Polymethoxy flavonoids	62.58
		Sugar fraction	212.41
		Hesperidin	2.75
		OPR2	870.02
Ethanol	2683	Ethanol	2683
H_2O	400	H_2O	400
TOTAL	4240.84	TOTAL	4230.76

It is interesting to note that out of the different species of flavonoids present in OP, PMFs only are extracted. No non-methoxylated flavonoids have been detected (i.e. naringin, narrutin). In order to understand the influence of the MW extraction of *D*-limonene on the results observed for soxhlet extraction, the soxhlet extraction should be carried out on virgin orange peel. This would have allowed us to see whether hydroxylated flavonoids are degraded during the microwave extraction of *D*-limonene. Hydroxyl groups on the benzo-γ-pyrone ring act as "radical scavengers" due to their hydrogen and electron donating and metal chelating ability.[150, 151] Flavonoids displaying methoxy functions will likely have a higher stability than hydroxylated flavonoids, possibly explaining the sole extraction of polythoxylated flavonoids after microwave treatment.

6.5 Quantification & characterisation of key extracted components

Each fraction isolated was carefully characterised using a minimum two techniques of different nature whenever possible. The polymethoxy flavonoids were characterised by ESI, HPLC-MS and ^{13}C NMR following separation by supercritical carbon dioxide chromatography. Hesperidin was characterised by ATR-IR, ^{13}C NMR and elemental analysis. Sugars have been identified by ^{13}C NMR and quantified using an internal standard.

6.5.1 Polymethoxylated flavonoids

6.5.1.1 Electrospray Ionisation Mass Spectrometry analysis

Electrospray Ionisation (ESI) Mass Spectrometry is a soft ionisation technique used at atmospheric pressure. When developed, the technique was used for protein molecular weight determination. ESI is often used as a detector when coupled with separation techniques such as HPLC (HPLC/ESI-MS).[282] The application of liquid chromatography-electrospray ionisation-mass spectrometry is known on PMFs for example.[254] Ionisation is accomplished by the loss or gain of a proton, generating a molecular ion. No fragmentation of the molecule analysed occurs. This technique relies on the the Coulombic fission: a high voltage (2000-5000 V) is applied to a capillary containing the analyte, causing the formation of charged droplets which will split eventually forming desolved ions in the vapour phase from the molecule initially injected. This is possible as the repulsion forces between the charges formed exceed the surface tension of the solvent. Their separation is then carried-out in vacuum based on their m/z via a mass analyser.[283] Figure 83 highlights the set-up used for the ionisation of the analyte in ESI.

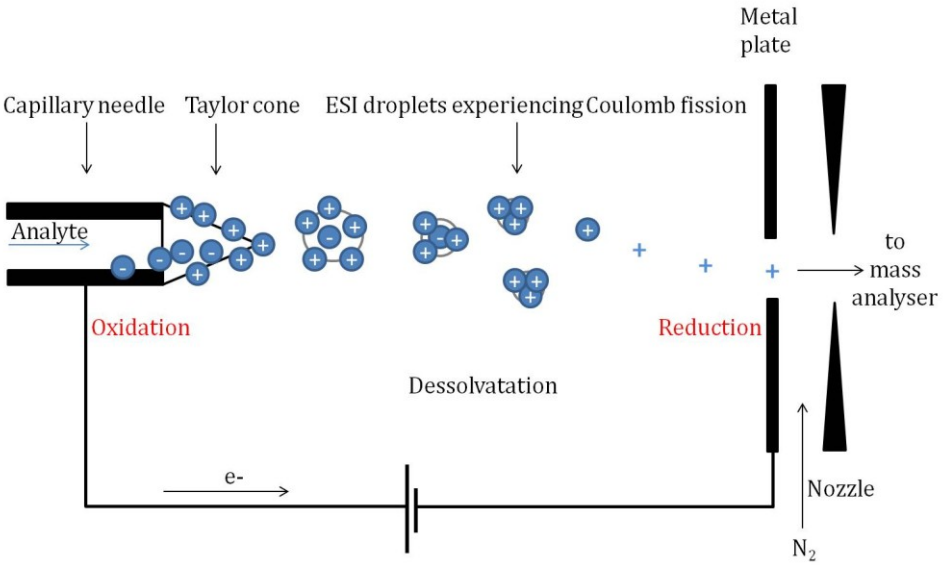


Figure 83: Production of molecular ions by ESI (originally in colour).

ESI analysis is a fast and convenient method requiring only minimum sample preparation. The only prerequisites are the use of a polarisable analyte and the use of an protic solvent such as ethanol, water or acetone to allow the formation of the $[M-H]^+$ ion (in the positive mode).

The polymethoxy flavonoid fraction (labelled SO1daf) was recovered, dried from the extraction solvent and analysed by ESI. The analysis confirmed the presence of the four PMFs previously detected during the purification of *D*-limonene (see Table 33).

Table 33: ESI results for sample SO1daf and SO4daf.

Sample name	Measured m/z (mDa)	Attributed molecular formula	Corresponding m/z (mDa)	Error (mDa)	Identified compound
SO1daf (acetone)	343.1181	$C_{19}H_{19}O_6$	343.1176	-0.4	Tetra-O-methyl scutellarein
	373.1282	$C_{20}H_{21}O_7$	373.1282	-0.3	Tangeritin
	403.1385	$C_{21}H_{23}O_8$	403.1387	0.2	Nobiletin
	433.1494	$C_{22}H_{25}O_9$	433.1493	0.0	Heptamethoxy flavone
SO4daf (ethanol)	343.1177	$C_{19}H_{19}O_6$	343.1176	-0.1	Tetra-O-methyl scutellarein
	373.1280	$C_{20}H_{21}O_7$	373.1282	0.2	Tangeritin
	403.1389	$C_{21}H_{23}O_8$	403.1387	-0.2	Nobiletin
	433.1500	$C_{22}H_{25}O_9$	433.1493	-0.7	Heptamethoxy flavone

Tetra-O-methyl scutellarein, tangeritin, nobiletin and heptamethoxy flavone were all identified using this technique in both extracts, whether acetone or ethanol was used. Each m/z peak for the molecular ion was attributed to the given PMF within ± 0.5 mDa error in both fractions, successfully identifying the above PMFs in the extract obtained following work-up.

To further confirm the ESI mass spectroscopy results, the analysis of both extracts obtained in acetone and ethanol were analysed by High Performance Liquid Chromatography coupled to a Mass Spectrometry detector (HPLC-MS).

6.5.1.2 High Performance Liquid Chromatography - Mass Spectrometry HPLC-MS

High Performance Liquid Chromatography coupled to an Atmospheric Pressure Chemical Ionisation (APCI) ion source is a standard technique used here for the identification of polymethoxylated flavonoids.[282] In comparison to gas chromatography (GC), HPLC is a separation technique appropriate for heat labile non-volatile molecules. Separation is occurs based on the difference of affinity for a given analyte for the stationary and mobile phase. The polarity of the mobile phase

used to elute the analytes one by one can be changed on line. The coupling of liquid chromatography with mass spectrometry allowed the obtention of structural information further allowing the use of chromatography for characterisation of mixture components.[284] The APCI is an atmospheric pressure ionisation technique allowing the formation of non-fragmented molecular ions at atmospheric pressure comparable to ESI. Ionisation is triggered by the formation of $N_2^{\cdot+}$ and $O_2^{\cdot+}$ species which will the transfer their protons to the analytes.[282]

HPLC-MS/APCI analysis was carried out at Centre for Novel Agricultural Products (CNAP). This particular LC-MS method has been previously used by CNAP to detect sesquiterpenes ($C_{15}H_{24}$) from *Artemisia annua* extracts. A C18 reverse phase column was used under gradient conditions with a methanol based mobile phase. It has been proven to successfully separate and detect three PMFs present in this species, the predominant compound being casticin $C_{19}H_{18}O_8$. HPLC-MS/APCI has been previously used to identify oxygen heterocycles in citrus essential oil.[118] This method was applied to the flavonoid mixture obtained under the conditions reported herein in order to confirm the structures of flavonoids detected by ESI and attempt to separate them. The results can be seen in Table 34.

Table 34: Summary of the results obtained by HPLC-MS of sample SO1daf.

Sample name	Compound name	Systematic name	Molecular formula	Molecular mass (g/mol)	Expected molecular mass $(M+H)^+$ ion (g/mol)	HPLC-MS match	Retention time (seconds)
SO1daf	Tetra-O-methyl-scutellarein	4',5,6,7-tetramethoxy flavone	$C_{19}H_{18}O_6$	342.34	343.34	m/z 343.1176 $C_{19}H_{19}O_6$ (error: 0.1613 ppm)	59.77
	Tangeritin	4',5,6,7,8-pentamethoxy flavone	$C_{20}H_{20}O_7$	372.37	373.37	m/z 373.1282 $C_{20}H_{21}O_7$ (error: 0.1613 ppm) m/z 373.1283 $C_{20}H_{21}O_7$ (error: 0.1613 ppm)	53.23 and 66.14
	Nobiletin	3',4',5,6,7,8-hexamethoxy flavone	$C_{21}H_{22}O_8$	402.39	403.39	m/z 403.1385 $C_{21}H_{23}O_8$ (error: -0.5487 ppm) m/z 403.1385 $C_{21}H_{23}O_8$ (error: -0.1613 ppm)	60.24 and 55.70
	Heptamethoxy flavone	2', 3, 3',4',5,7,8-heptamethoxy flavone	$C_{22}H_{24}O_9$	432.42	433.42	m/z 433.1489 $C_{22}H_{25}O_9$ (error: -0.886 ppm)	62.51

Nobiletin, tetra-O-methyl-scutellarein and heptamethoxyflavone have been successfully identified, confirming the results obtained by ESI mass spectroscopy. Furthermore, two new flavonoid components were identified using HPLC-MS in comparison to ESI: two chromatographically resolved candidates for isomers of tangeritin and nobiletin were identified. The use of HPLC-MS allowed the detection of two peaks corresponding to a molecular weight of ~ 373 g/mol and ~ 403 g/mol. Given the difference in retention time, the two peaks must correspond to different compounds with the same molecular formula, or in other words, isomers. For the isomer of tangeritin, a possible match might be sinensetin, or 3',4',5,6,7-pentamethoxyflavone, which only differs by the position of the five methoxy groups on the C_6-C_3-C_6 rings. The structure of sinensetin is given below in Figure 84.

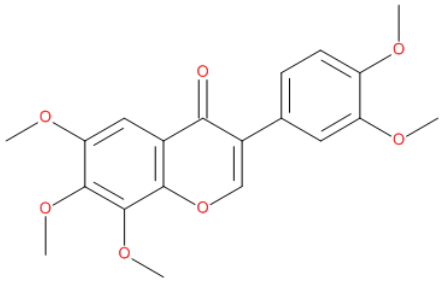


Figure 84: Chemical structure of sinensitin or 3',4',5,6,7-pentamethoxyflavone (originally in colour).

The isomer of nobiletin could be 3,3',4',5,6,7-hexamethoxyflavone. This compound possesses the same number of methoxy groups on the C_6-C_3-C_6 rings and the same molecular formula. Another possible match might be 3',4',5',5,6,7-hexamethoxyflavone. The structures of the possible isomers of nobiletin are given in Figure 85. The isomers for tangeritin and nobiletin could be attributed using MS2 fragmentation data if the -OH and -OCH_3 groups are switched between rings.

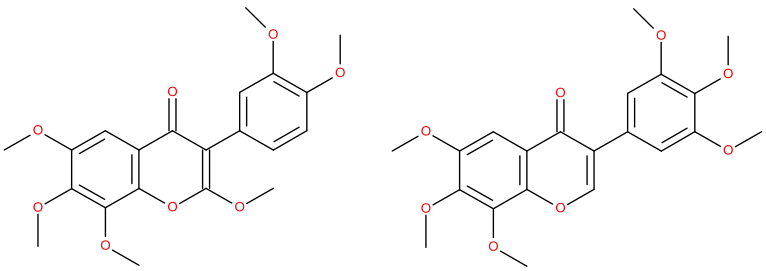


Figure 85: Possible chemical structures of the isomers of nobiletin 3,3',4',5,6,7-hexamethoxyflavone –left; 3',4',5',5,6,7-hexamethoxyflavone –right (originally in colour).

A good approximation of the PMFs relative proportions can be deduced from the HPLC-MS data as relative percentages of the absolute Extracted Ion Chromatogram (XIC) areas obtained for each mass tag. Nobiletin, tetra-O-methyl-scutellarein, heptamethoxyflavone and tangeritin/sinensitin account respectively for 34.13%, 9.10%, 15.19% and 19.80% of the sample analysed. Therefore PMFs represent 78.21% of the sample SO1daf. A typical ion chromatogram is given in the figure below.

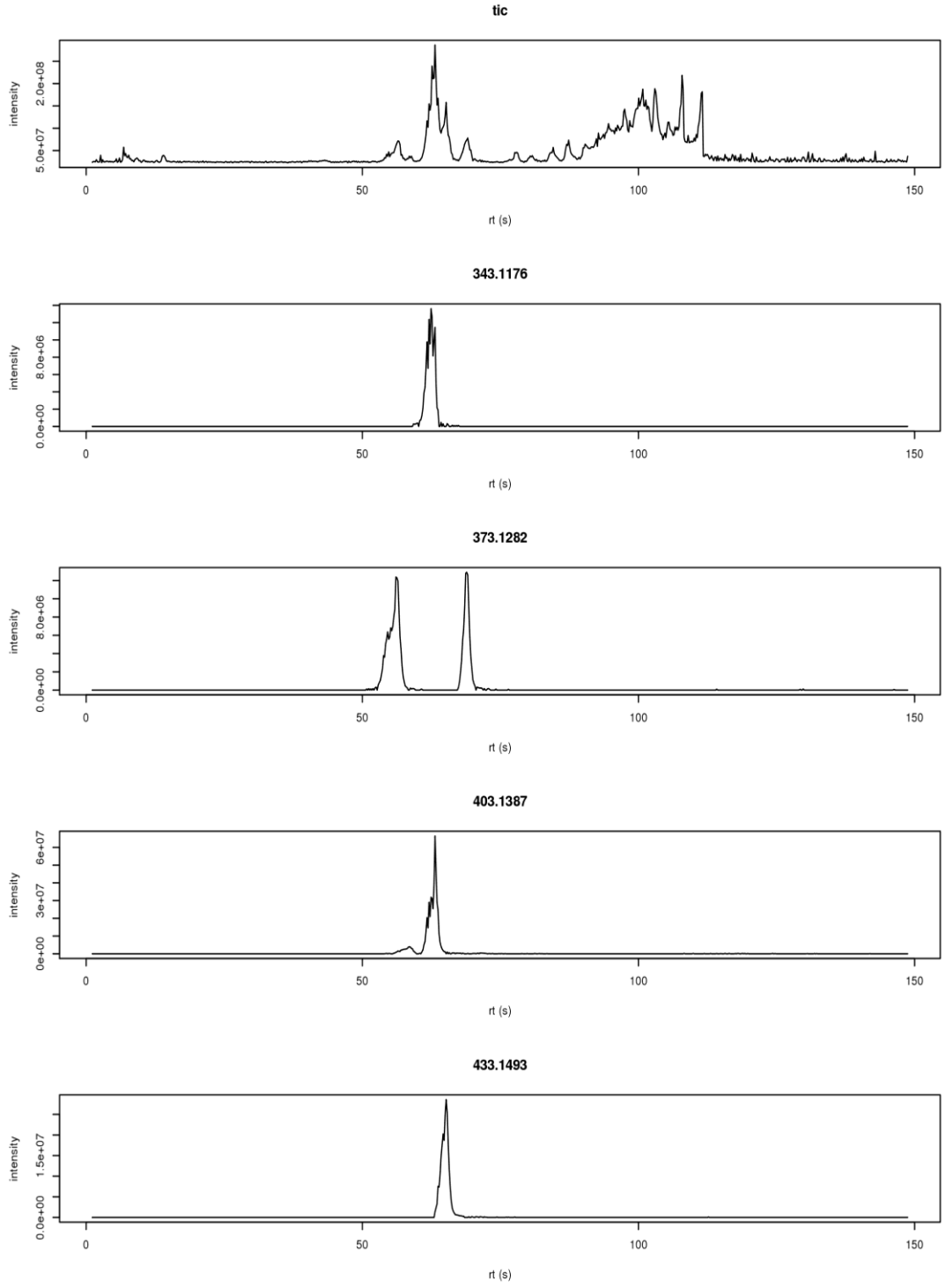


Figure 86: TIC and XIC obtained for the HPLC-MS of sample SO1daf.

The same process was applied to the PMF fraction obtained using an ethanol soxhlet to confirm the results obtained using ESI. The HPLC-MS results obtained for this fraction are summarised in Table 35. The same four compounds were detected in the ethanol fraction, confirming the results obtained by ESI. Relative retention times between the same peaks observed differed by 2.5 and 3.07 seconds further strengthening this conclusion. However, in this case, nobiletin and tetra-O-methyl scutellarein were found to co-elute. A separate peak for tetra-O-methyl scutellarein could not be detected. Instead, the peak previously identified as nobiletin and eluting at 63 seconds contains a masstag for a molecular ion with a m/z of 343 g/mol previously attributed to tetra-O-methyl scutellarein (see Figure 87). This can be explained by the increased age of the column between the analysis of samples SO1daf and SO4daf. The quality of the separation was inferior explaining the inability of the column to separate analytes with a difference in retention time of ~ 1 second. Nonetheless, this is still within the ± 5 ppm limit acceptable for high resolution MS.

Table 35: Summary of the results obtained by HPLC-MS of sample SO4daf.

Sample name	Compound name	Systematic name	Molecular formula	Molecular mass (g/mol)	Expected molecular mass $(M+H)^+$ ion (g/mol)	HPLC-MS match	Retention time (seconds)
SO4daf	Tangeritin	4',5,6,7,8-pentamethoxy flavone	$C_{20}H_{20}O_7$	372.37	373.37	m/z 373.1272 $C_{20}H_{21}O_7$ (error: -2.6973 ppm) m/z 373.1273 $C_{20}H_{21}O_7$ (error: -2.4049 ppm)	56.3 and 68.9
	Nobiletin/ Tetra-O-methyl-scutellarein	3',4',5,6,7,8-hexamethoxy flavone/ 4',5,6,7-tetramethoxy flavone	$C_{21}H_{22}O_8$	402.39	403.39	m/z 403.1375 $C_{21}H_{23}O_8$ (error: -3.6476 ppm) m/z 403.1377 $C_{21}H_{23}O_8$ (error: -3.0922 ppm) (contains Tetra-O-methyl-scutellarein)	58.2 and 63
	Heptamethoxy-flavone	2', 3, 3',4',5,7,8-heptamethoxy flavone	$C_{22}H_{24}O_9$	432.42	433.42	m/z 433.1482 $C_{22}H_{25}O_9$ (error: -2.4425 ppm)	65.2

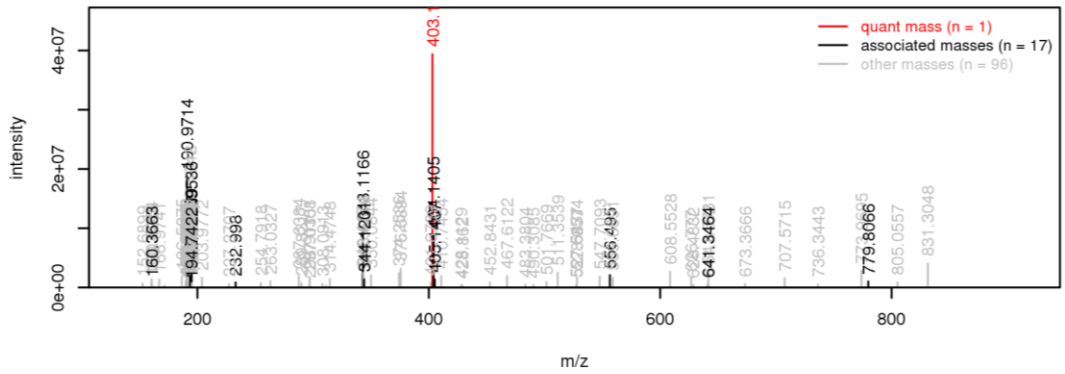


Figure 87: Extraction ion chromatogram (XIC) of the nobiletin peak eluting at 63 seconds for sample SO4daf (originally in colour).

The proportion of the PMFs has been estimated from the HPLC-MS data as relative percentages of the absolute Extracted Ion Chromatogram (XIC) areas for each m/z value. Nobiletin/tetra-O-methyl-scutellarein, heptamethoxyflavone and tangeritin/sinensitin account to give 26.56%, 11.70% and 14.59% respectively. These values should be regarded as relative and should not be used as absolute values. PMFs represent 52.85% of the sample SO4daf. Compared to SO1daf, this represents a decrease of ~ 33% in the content of PMF present in the fraction. Taking into account the change in solvent from acetone to ethanol led to a 2.5 increase in the yield of PMF recovered, it is an interesting result, highlighting the difference in selectivity between the two solvents for this step.

In each PMF fraction obtained, a significant proportion of compounds are not flavonoids. In SO1daf this amounts to 21.79% and in SO4daf it corresponds to 47.15%. ESI analysis of each PMF fraction dissolved in ethanol allowed qualitative analysis of the rest of the components present. In each case, major peaks were present at 203.05 Da and 231.08 Da. They could be allocated to the following molecular formulas respectively: $C_6H_{12}NaO_6$ and $C_8H_{16}NaO_6$. In each case, the error was below 5 mDa. The results obtained for the sodiated molecular ions show the presence of C_6 sugar monosaccharides and ethyl D-glucopyranoside. C_6 sugars are expected to be extracted in ethanol. The latter molecule, ethyl D-glucopyranoside, has been known to form under acidic conditions starting from D-glucose in ethanol at 100 °C over 200 mg Silica-SO_3H.[285]

6.5.1.3 Supercritical Fluid Chromatography (SFC) separation

Experimental observations confirmed by the literature have shown that the extraction of PMFs can be carried out using hexane-based solvent extraction.[277, 286] Hexane is commonly used to mimic the non-polar properties of supercritical carbon dioxide ($scCO_2$) for extraction or separation at atmospheric pressure. This led to the trial of Supercritical Fluid Chromatography (SFC) for the separation of the PMFs extracted with $scCO_2$.[287] Obtaining a pure fraction of one PMF would allow its characterisation by ^{13}C NMR for example, confirming the results obtained by HPLC-MS. On the other hand, demonstrating the possible separation of the compounds could be of interest, generating additional revenue to the biorefinery. The extraction and isolation of PMF is especially valuable since "due to their potential use as a chemopreventive agent based on in vitro studies, a rapid reproducible method for the purification of PMF's is critical."[277] The supply of a large quantity of individual PMFs is particularly desired, notably for *in vivo* studies.[255, 288]

Carbon dioxide SFC is based on the ability of CO_2 to exist in a supercritical state above 31 °C and 73 bars. These specific conditions are called the "supercritical point" where "the densities of both gas and liquid become identical," generating a hybrid state for carbon dioxide.[284, 289] SFC used in conjunction with carbon dioxide functions on the same principle as liquid chromatography except the mobile phase here is $scCO_2$ and a modifier. The separation of non-volatile analytes still occurs based on their affinity for both the mobile and the stationary phase. The density of carbon dioxide is affected by varying the pressure and temperature. Additionally, the amount of polar modifier used can increase the polarity of $scCO_2$, making it a tunable solvent. SFC particularly suits the separation of heat sensitive, non-volatile components.[284, 289] A diagram of the experimental set-up used for SFC separation is given in Figure 88.

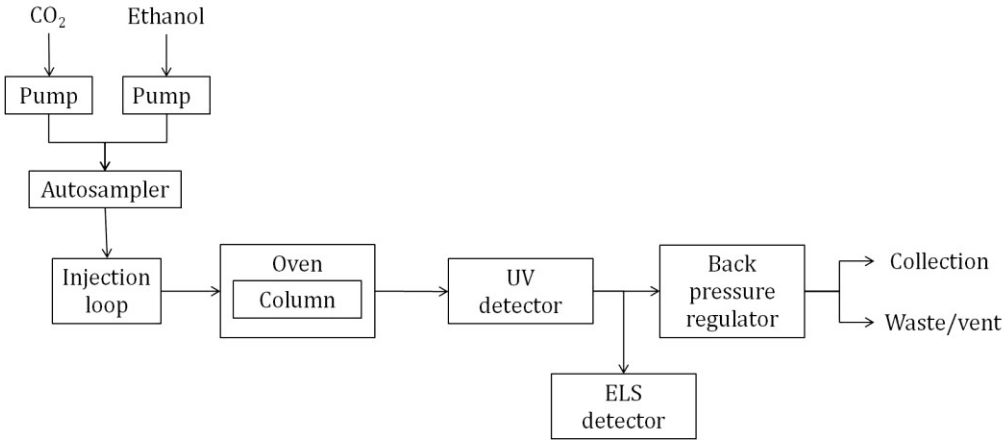


Figure 88: SFC chromatography equipment.

Typically, CO_2 in its supercritical state is regarded as polar as hexane although the polarity of the mobile phase can then be modulated using pressure and temperature. The polarity of $scCO_2$ can also be fine-tuned using co-solvents such as methanol or ethanol.[290] Although energy requirements of $scCO_2$ are high, the technology has been commercially used for hop extraction, decaffeination of coffee and dry cleaning.[291] This technique possesses other practical advantages: the low viscosity of $scCO_2$ insures good diffusivity and allows the use of high flow rates with low back pressures. The depressurisation of $scCO_2$ ensure the mobile phase can be removed quickly and efficiently for the collected analyte with minimal residues.[292] This, together with the fact that carbon dioxide is considered as a cheap, recyclable, renewable, non-flammable food grade solvent (it has obtained a GRAS status and has a toxicity threshold limit value set at 5000 ppm), asserts the privileged position this technique has for the extraction and isolation of plant metabolites for example.[293] Ultimately large volumes of organic solvents typically used for liquid chromatography are not necessary when using SFC technology. This represents an advantage, especially at larger scale.

Separation resolution is affected by the flow rate of the mobile phase, the pressure and the percentage of modifier used. An increase in resolution can be achieved by decreasing the flow rate, lowering the pressure and the amount of modifier used. A variation of temperature affects the density of carbon dioxide primarily: as the temperature increases, the density of CO_2 decreases, and the retention time of a given compound increases.[289]

Based on this knowledge, the pressure, flow rate of the mobile phase together with the nature and percentage of polar modifier were used to design a separation

method for the PMFs obtained following soxhlet extraction with acetone and ethanol. A first set of conditions were identified as promising: using a total flow rate of 3 mL/min modified with 20% ethanol as modifier at 75 bars and 40 °C. Figure 89 displays the chromatogram obtained when using these conditions.

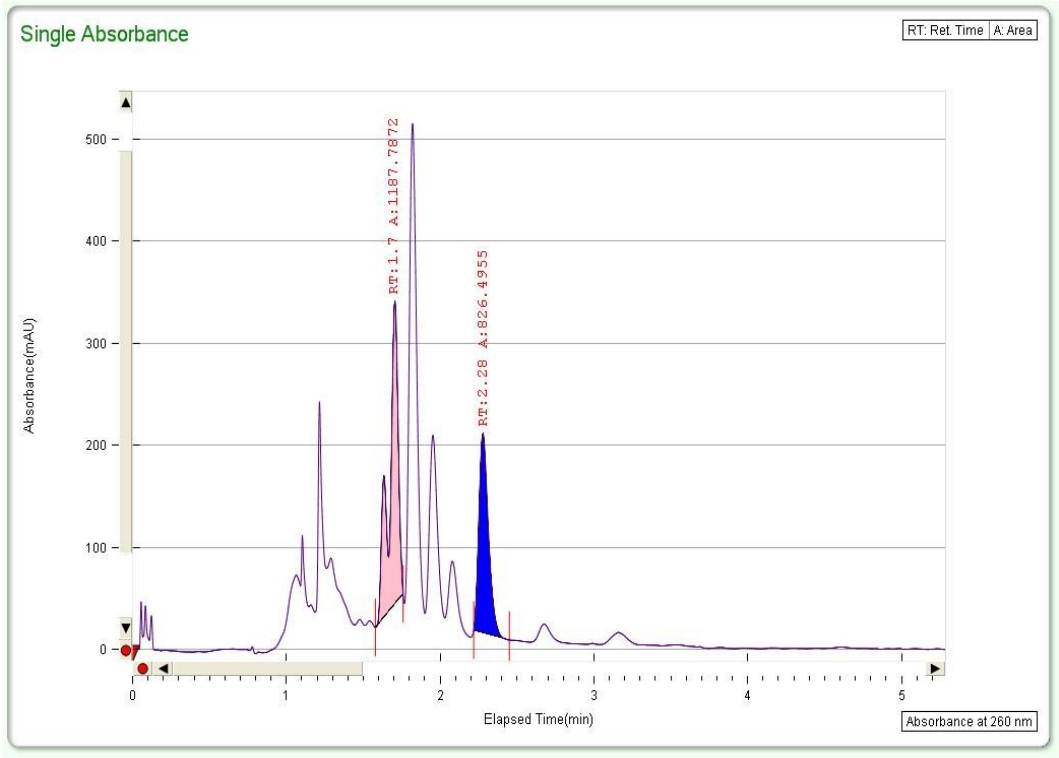


Figure 89: SFC chromatogram of SO1daf (originally in colour).

Following the collection of the fraction separated and NMR analysis it was established that the separation wasn't 100% efficient and that further optimisation work had to be carried-out. A dominant PMF molecule was identified in each fraction corresponding to each peak but cross-contamination still occurred. It was decided to try to lengthen the chromatography method allowing better separation while trying to keep a sharp resolution. This can be achieved by lowering the pressure, the flow rate and/or the percentage of co-modifier.

Further tests were carried out and ultimately an optimised separation method was obtained by lowering the percentage of ethanol to 8%. Samples SO1daf and SO4daf were analysed using this method yielding very similar chromatograms, except for the non-UV active compounds. In this section of the chromatogram, a lower number of peaks were detected for SO4daf which was obtained using an ethanol soxhlet,

yielding a cleaner mixture. Consequently, and given the same retention times were observed for the UV-active peaks of interest, efforts were concentrated on sample SO4daf only. Especially as a higher PMF yield was obtained for this sample, which extracted using ethanol. Figure 90 displays the chromatogram obtained.

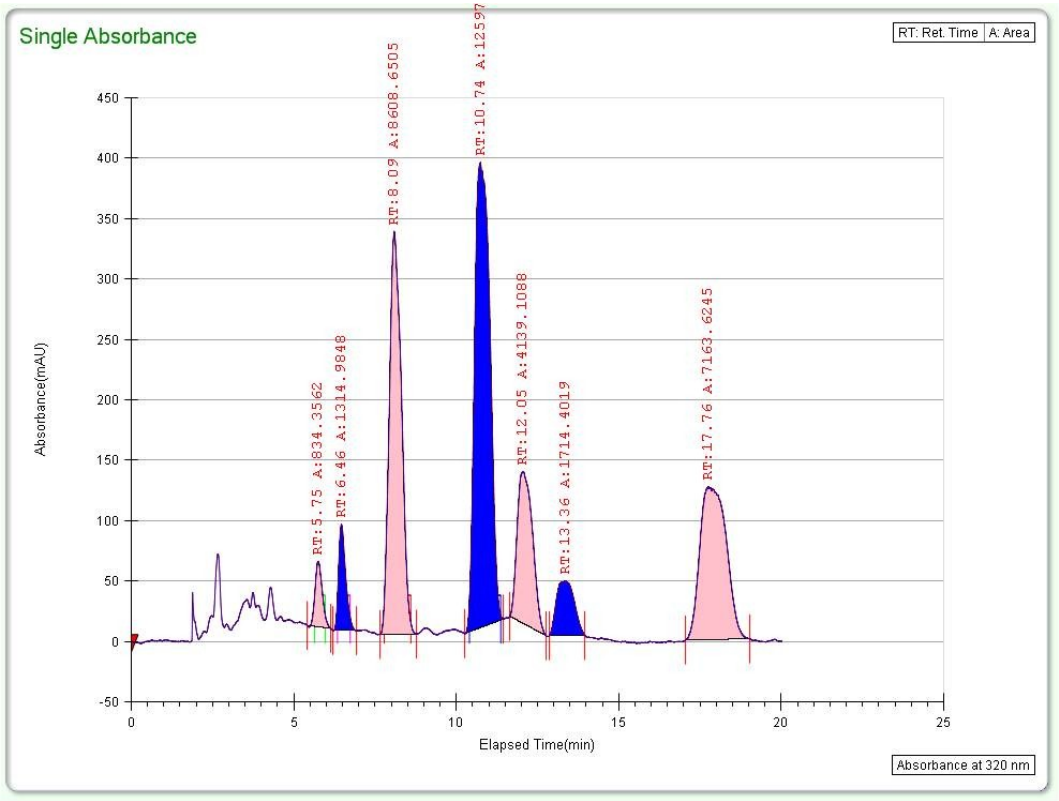


Figure 90: SFC chromatogram of SO4daf following optimisation (originally in colour).

Little work has been reported on the separation of PMFs using SFC. The first paper published in this area was a feasibility study on the separation of PMFs in citrus oil. Using 10% methanol as a polarity modifier, tangeretin, nobiletin, sinensitin and tetramethylisoscutellarein were separated under 8 minutes at 35 °C, 222.92 bars and with a carbon dioxide flow rate of 3 mL/min.[294] SFC has also been applied on a commercial extract following flash chromatography to separate tangeritin from heptamethoxyflavone (50% methanol) and nobiletin from 5, 6, 7, 4'-tetramethoxyflavone (45% methanol) at 30 °C, 100 bars, 70 mL/min.[255] The separation was carried-out in under 8 minutes. However, it can be argued that the conditions used are below the critical point with the modifier being employed at such a high loading. This is especially true as the temperature used is below 35 °C. This separation is useful but does not fall into the category of CO_2 SFC. Another type

of flavonoids were separated by SFC: isorhamnetin, kampcetin, quercetin and fisetin were separated on a phenyl column at 50 °C, 250 bars, with a 1.05 mL/min flow rate of $scCO_2$ containing 9.98% ethanol. The separation was run in 14 minutes.[295] Comparatively, the separation of polyhydroxylflavonoids using HPLC is run in approximately one hour.[271, 296] The full automation of the analysis and the possibility of performing stacked injections with SFC renders the production of several mg of compound feasible in a convenient fashion and a relatively short amount of time.

In conclusion, based on previous published work, a novel method was designed to separate the PMF content of an experimental extract, namely sample SO4daf. Although a complete separation method was developed, ^{13}C NMR data for each PMF present in the mixture could not be obtained due to lack of time on the project. ^{13}C NMR data would however be useful to confirm the results obtained by HPLC-MS and identify the isomers of the nobiletin and tangeritin providing they are separated using sc CO_2.

6.5.2 Hesperidin

A second product stream has been detected during the work-up the polymethoxy flavonoids. Following solubilisation in ethanol of the water insoluble fraction, a white solid was filtered off the PMF containing mixture. The ethanol insoluble solid was dried and subjected to ATR-IR analysis with the aim to identify functional groups which could help determine its identity. Following information found in the literature it was successfully compared against a commercial sample of hesperidin (see section 6.5.2.1). Hesperidin is one of the main flavanone glycosides present in sweet oranges.[153] The hesperidin content of citrus fruits diminishes as the fruit matures.[297] It is known for its anti-cancer activity and its antioxidant properties.[298] The molecule has been identified as one of the factors causing "cloudiness" in fruit juices.[272]

Hesperidin belongs to another class of flavonoids: flavanones. They differ from flavones by the absence of unsaturation on the B ring (see Figure 73). Flavonones exist in two forms; the glycone and the aglycone, of which the glycone corresponds to the flavonoid grafted with a sugar monomer such as glucose or rhamnose. It is the latter form which is mostly present in nature.[272, 299] In the case of hesperidin, the monosaccharide residue is constituted of rhamnose and glucose and is named 6-O-α-L-rhamnosyl-D-glucoside. The common name of the dissacharide residue is

rutinose.[297] The use of an alcohol such as ethanol for the separation of hesperidin from the PMF mixture is important as it can be directly crystallised out of ethanol below 10 °C.[300]

This section will focus on the characterisation of the two experimental samples of hesperidin obtained following soxhlet extraction with acetone and ethanol. In each case, the compound was isolated with a yield of 0.14 and 0.24% respectively. Although the yield of PMF obtained seemed high in comparison to previous values quoted in the literature, the highest yield of hesperidin extracted only represents a small portion of the available compound in peel: hesperidin has been reported to account for ~ 4% of mature blood orange fruits.[298] As a matter of comparison, extraction using a saturated solution of $Ca(OH)_2$ yielded 3.1% hesperidin.[298] Under closed-vessel microwave assisted extraction conditions at 140 °C in a 70% aqueous solution of ethanol, 19.4 ± 4 mg of hesperidin were extracted by gram of peel.[300] The literature results indicate the yield of hesperidin so far reported in this work could be increased by changing the extraction technique implemented. There is an opportunity to use microwave technology for the recovery of hesperidin, increasing the yield obtained for this specific compound and avoid a switch in technology in the middle of the process, as both step 1 and 3 are both microwave based. This would have the advantage of allowing the design of a more integrated process.

In the next sections, ATR-IR and ^{13}C NMR will be used to confirm the identity of the solid obtained following ethanol solubilisation of the water insoluble fraction of the extract.

6.5.2.1 ATR-IR analysis of hesperidin

ATR-IR was primarily used to identify the class of compound the white solid belonged to. Sample preparation being minimal with this technique, ATR-IR permits a fast determination of functional groups such as carbonyls, alkanes or amines, and identification against a standard for example, as most substances show a characteristic spectrum that can be directly recognised. The spectra obtained for samples SO1e and SO4e can be found in Figure 91. SO1e has been obtained following acetone soxhlet extraction and SO4e has been obtained following ethanol soxhlet extraction. The spectrum was compared to a commercial sample of hesperidin (see molecular structure in Figure 92). Good correlation between the four spectra was

observed, particularly below 1500 cm^{-1}, in the fingerprint region, where the position of absorption bands is specific to the stretching vibrations of a defined compound.[301]

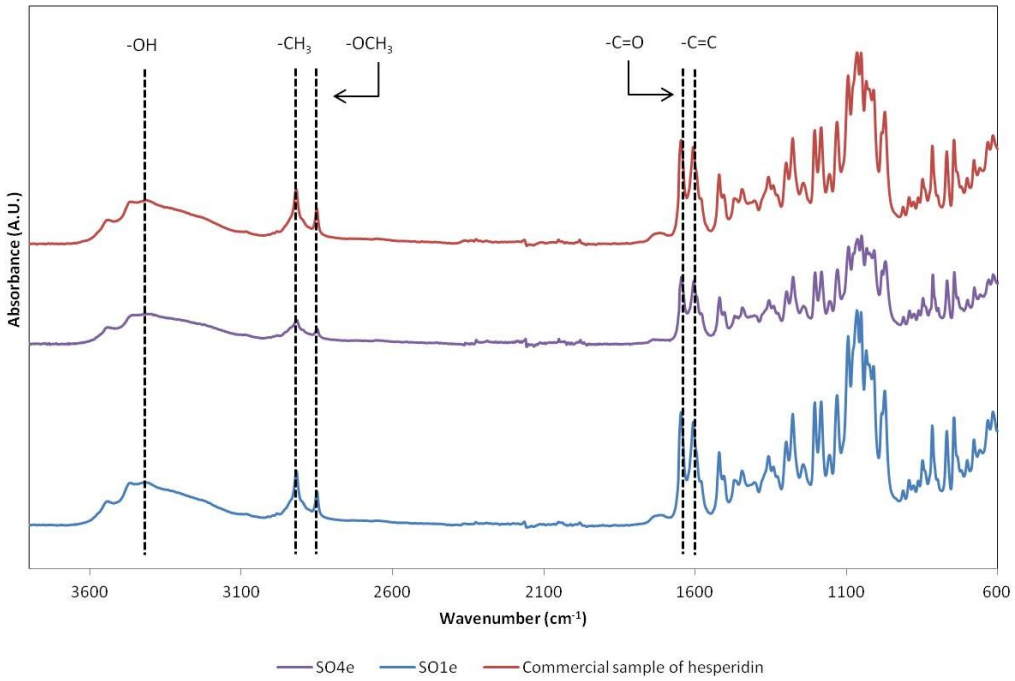


Figure 91: Comparison of the ATR-IR spectra of sample SO1e and SO4e against a commercial sample of hesperidin (Acros Chemicals) (originally in colour).

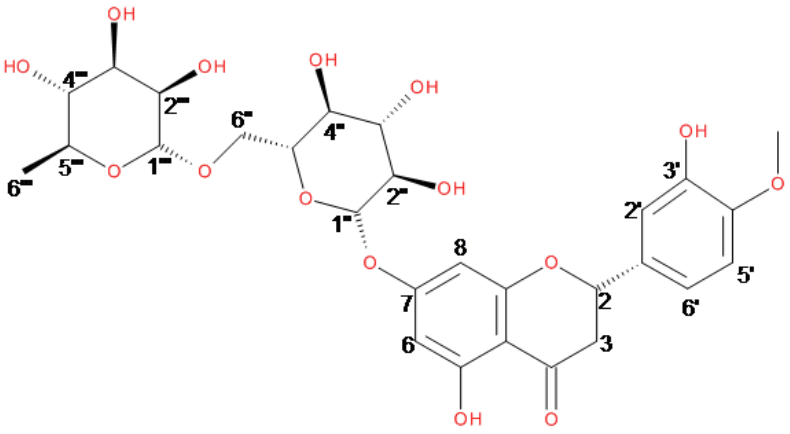


Figure 92: Molecular structure of hesperidin ($C_{28}H_{34}O_{15}$).

Figure 91 shows absorption bands corresponding to –OH groups (stretching vibration, 3420 cm^{-1}), -CH_3 groups (stretching vibration, 2950-2850 cm^{-1}), the -OCH_3 groups (stretching vibration, 2850-2810 cm^{-1}) and the ketone function (1700-1650 cm^{-1}).[302] Peaks associated with Ar-H groups (3040-3010 cm^{-1}) are often hidden. The absorbance observed at 3540 cm^{-1} could correspond to free O-H stretching and

bending bond vibrations as it is sharper than the broad than the band observed for H-bonded O-H. The signal at 1609 cm^{-1} has been previously tentatively attributed to the ν(C=C) on the aromatic ring. The same applies for the δ (COH), ν (C–O–C) appearing at 1285 cm^{-1}.[303] Very little infra red data was available for the analysis of hesperidin. A lack of information was especially observed regarding the wavelength of the functional groups present in the disaccharide unit of the molecule (i.e. $–CH_2$, -CH-OH, -C-O-C-). Therefore the compound isolated was further characterised using NMR.

6.5.2.2 ^{13}C NMR analysis of hesperidin

NMR analysis was used to further confirm the extraction of hesperidin. It was especially important to verify the glycone (hesperidin) of the molecule was isolated, as opposed to the aglycone (hesperetin). A commercial sample of hesperidin was used as a standard. The chemical shifts obtained for all samples have been compared to the ones obtained by Inoue *et. al.* to allocate the peaks obtained for SO1e and SO4e and the standard. All the chemical shifts are given in Table 36.[300, 302]

Table 36: Chemical shifts obtained via ^{13}C NMR for samples SO1e, SO4e and a commercial sample of hesperidin (d-DMSO, 500 MHz, 1024 scans at 60 °C).

Carbon position	Inoue *et. al.* δ(ppm)	Hesperidin Acros Organics δ(ppm)	SO1e δ(ppm)	SO4e δ(ppm)
-O<u>C</u>H$_3$	56.3	55.69	55.69	55.69
2	78.7	78.07	78.10	78.07
3	42.5	41.87	41.89	41.87
4	197.2	196.48	196.54	196.49
5	163.5	162.78	162.82	162.79
6	97.0	96.31	96.32	96.31
7	165.6	164.99	165.01	164.99
8	96.1	95.43	95.44	95.43
9	162.9	162.24	162.28	162.30
10	103.9	103.20	103.22	103.20
1'	131.5	130.90	130.91	130.91
2'	114.6	113.98	114.00	113.99
3'	147.1	146.41	146.43	146.42
4'	148.5	147.81	147.83	147.81
5'	112.9	112.30	112.28	112.30
6'	118.2	117.55	117.60	117.56
1''	100.1	99.52	99.52	99.52
2''	76.1	75.46	75.48	75.47
3''	-	-	-	-
4''	71.3	70.62	70.64	70.63
5''	76.8	76.20	76.21	76.20
6''	66.6	65.94	65.96	65.94
1'''	101.0	100.40	100.42	100.40
2'''	70.2	69.54	69.55	69.54
3'''	70.7	70.08	70.11	70.08
4'''	73.5	72.88	72.89	72.88
5'''	68.7	68.06	68.09	68.06
6'''	18.1	17.47	17.51	17.47

By comparing the chemical shifts between the experimental samples and the commercial sample, one can effectively verify the identity of a molecule. A consistent difference between the experimental and the standard chemical shift across all peaks can be used to confirm the identity of a molecule. When comparing SO1e against the standard, the chemical shifts obtained for SO1e differ by a maximum of 0.03 ppm with the exception of peaks at 196.54, 165.01, 162.28 and 117.60 ppm. A DEPT experiment was done on SO1e and permitted to attribute the peaks at 41.89 and 65.96 ppm to the C3 and the C6'' respectively which are the only two $-CH_2$ groups present in hesperidin, confirming the results reported in the literature. The spectra is given in Figure 93.

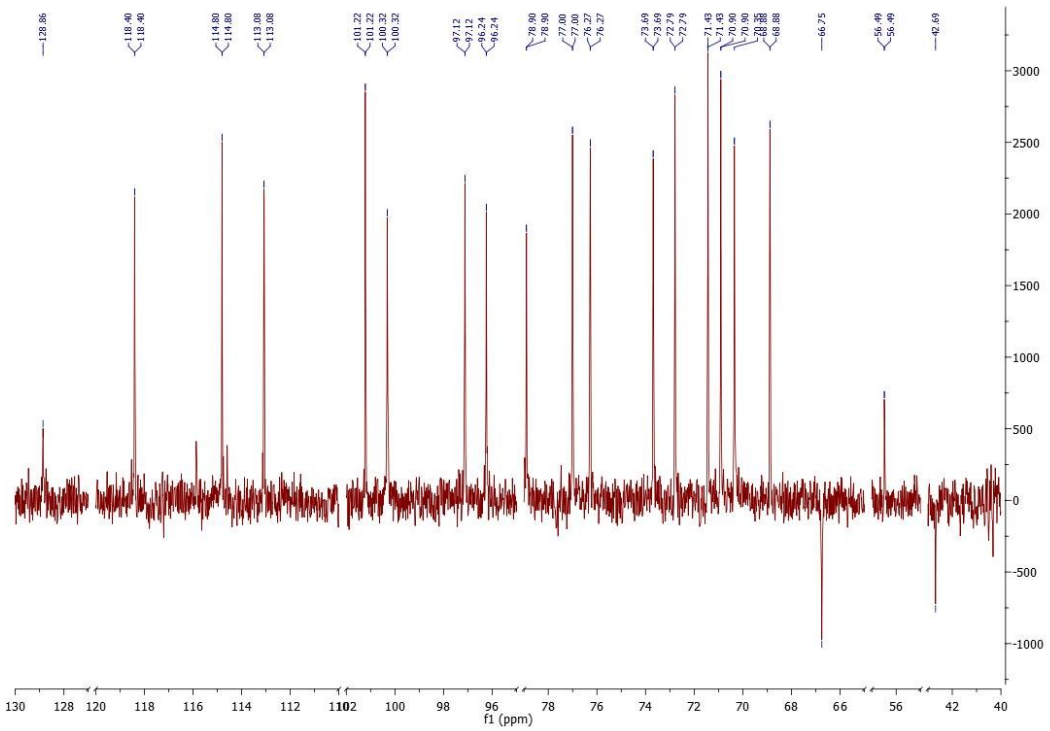


Figure 93: ^{13}C NMR DEPT spectra of hesperidin sample SO1e (d-DMSO, 500 MHz, 1024 scans at 60 °C) (originally in colour).

The match between sample SO4e and then standard is even better. With the exception of the peak at 162.30 ppm, the difference in chemical shift observed does not exceed 0.02 ppm. These results confirm hesperidin has been isolated following soxhlet extraction with acetone (SO1e) and ethanol (SO4e). The final experimental products are shown in Figure 94 alongside a commercial sample of hesperidin. The full ^{13}C NMR spectra for sample SO4e is given in Figure 95.

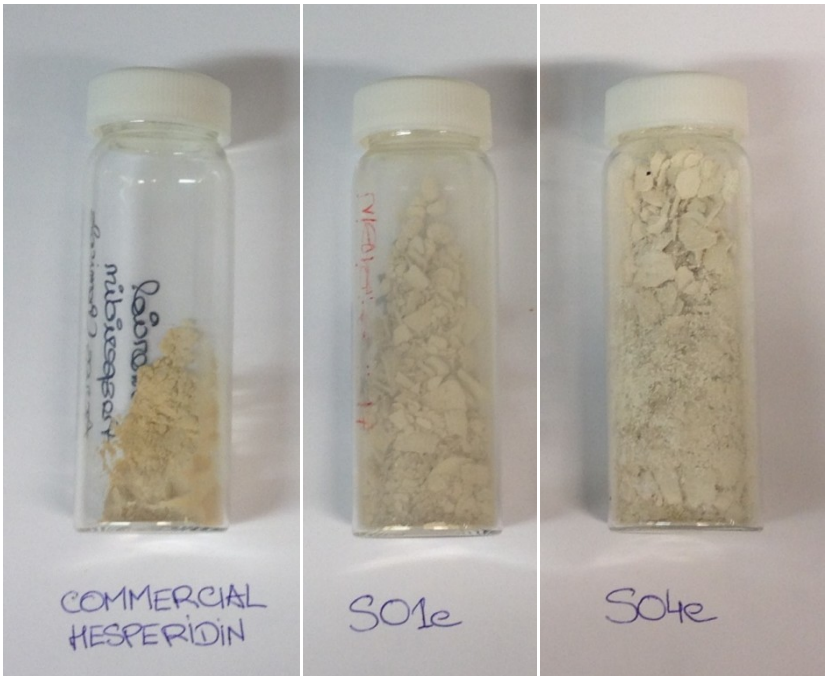


Figure 94: Experimental samples of hesperidin SO1e and SO4e against commercial hesperidin (originally in colour).

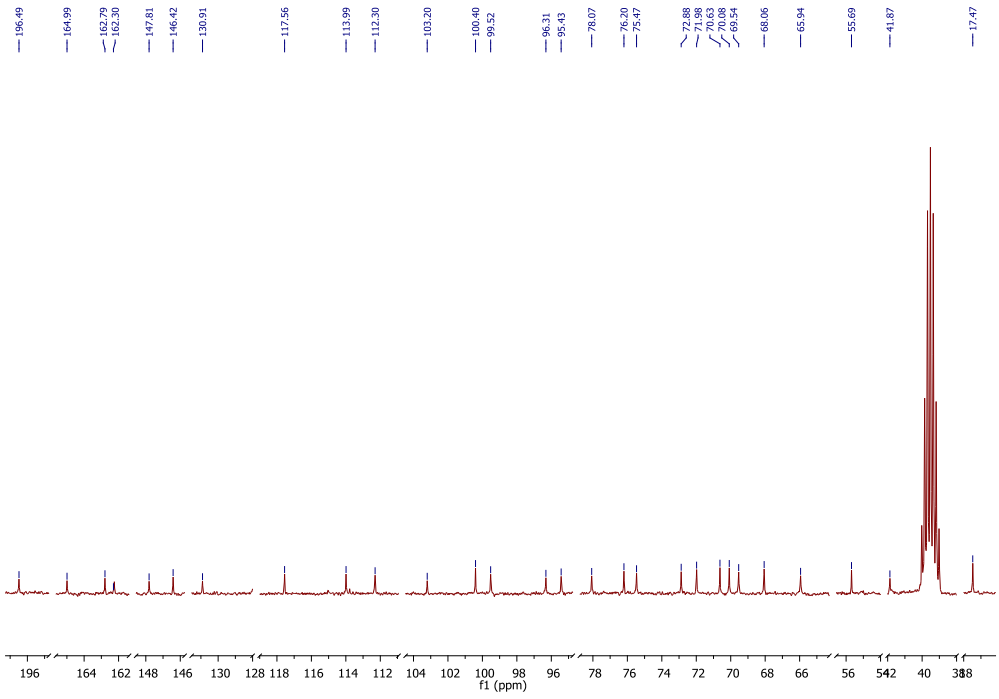


Figure 95: ^{13}C NMR spectra of hesperidin sample SO4e (d-DMSO, 500 MHz, 1024 scans at 60 °C) (originally in colour)

6.5.3 Quantification & characterisation of sugar components

Sugar monosaccharides are the building blocks of the biopolymer content of biomass. Common sugar monosaccharides include glucose, rhamnose, galactose, mannose, xylose and arabinose for example. They all follow the molecular formula $C_nH_{2n}O_n$ where n is equal to 3, 5 or 6. Monosaccharides fall into two sub-categories depending on the chemical nature of the anomeric carbon (C1). If it is an aldehyde, the monosaccharide is classified as an aldose and if it is a ketone, it is classified as a ketose.

The analysis of the crude acetone extracted mixture was subjected to ESI analysis (Figure 96). Alongside the PMFs, the presence of sugar monosaccharides was detected under the form of molecular ions at 203 Da.

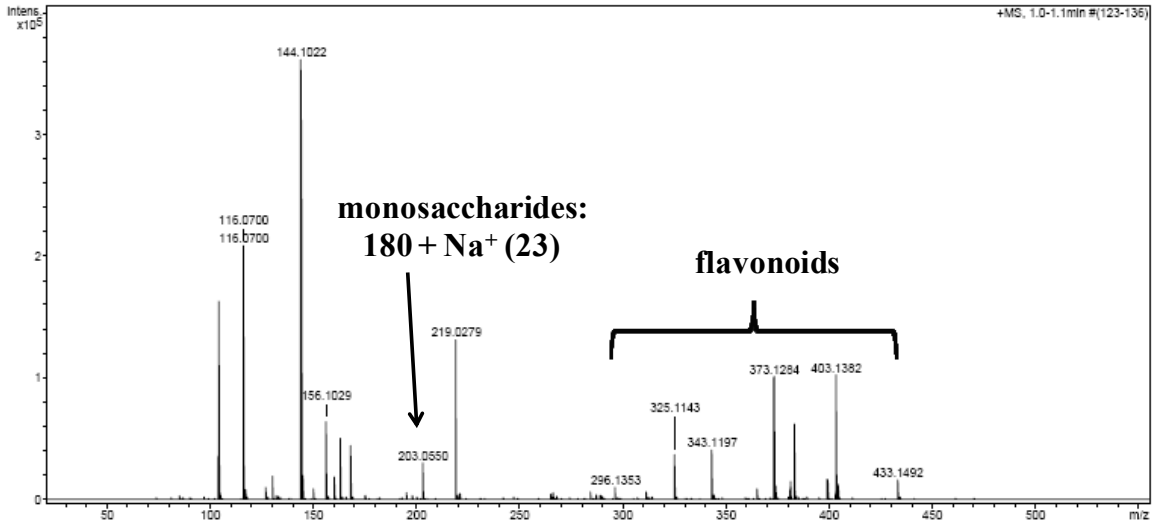


Figure 96: ESI spectrum of the acetone soxhlet extract from OPR1.

Following soxhlet extraction, the isolation of a water soluble monosaccharide rich fraction was obtained after freeze-drying. As seen in sections 6.3 and 6.4, both acetone and ethanol extraction allowed the obtention of a brown solid with a yield of 4.34% and 18.35% on a wet basis. Dry yields corresponded to 4.52% for acetone and 19.93% for ethanol. A sample has been photographed in Figure 97. The switch from acetone to ethanol and from 100 mbars to 500 mbars *D*-limonene extracted peel proved to have an important effect on the yield of the monosaccharide fraction recovered. The change in solvent and starting material is responsible for a four fold

increase in yield for this specific fraction. It represents the greatest increase in yield out of the three fractions recovered following soxhlet extraction. This is beneficial for the development of the OPEC process at larger scale. Sugar monosaccharide are valuable as they can be used as a fermentation feedstock for the production of bio-ethanol or as a substrate for the production of platform chemicals such a diacids (i.e. succinic acid, levulinic acid), representing another marketable product stream.[129]

Figure 97: Water-soluble fraction obtained by ethanol soxhlet extraction (originally in colour).

Given the extraction conditions used, the type of biomass extracted and the solubility of the compounds in acetone and water, the presence of sugars is expected. Following ESI analysis ^{13}C NMR should further help to characterise and quantify this particular fraction to understand how much monosaccharides are present and in which relative proportion. ^{13}C NMR experiments in D_2O were carried out to identify the sugars present. ^{13}C NMR was preferred to ^{1}H NMR as a simplified spectra would be obtained.

Glucose and fructose were identified as the two sole monosaccharides present in the mixture. Their presence was confirmed by the use of glucose and fructose standards against which the experimental samples SO1H2O and SO4H2O respectively extracted in acetone and ethanol, were compared. Figure 98 shows a representative ^{13}C NMR spectra obtained for sample SO4H2O.

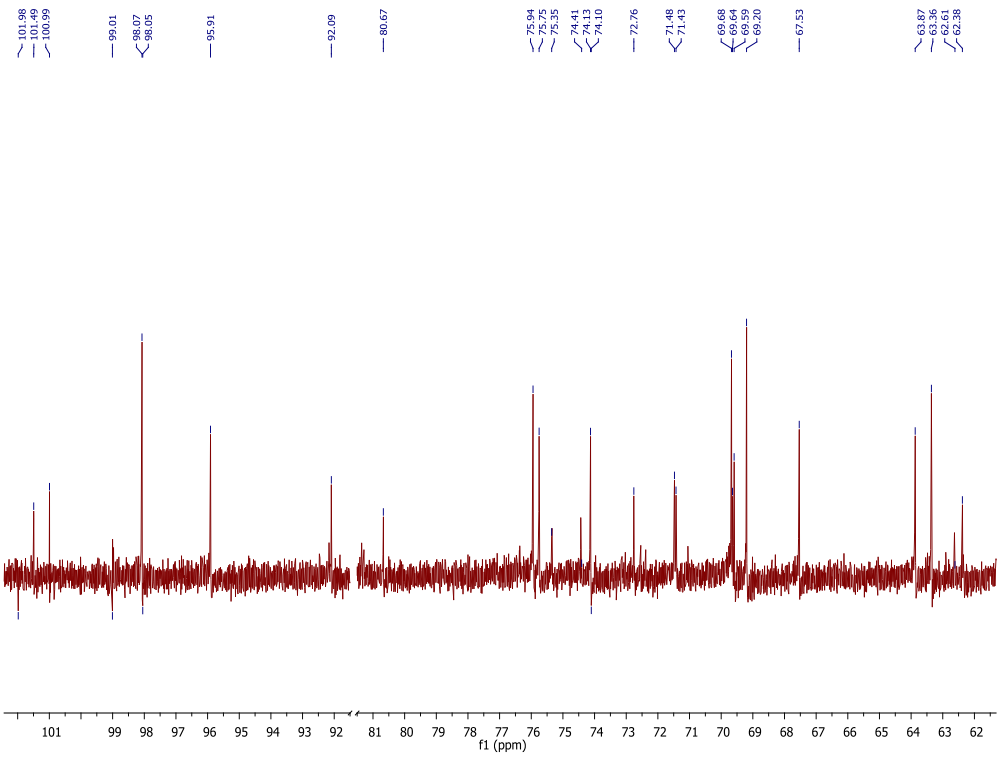


Figure 98: ^{13}C NMR spectra of fraction SO4H2O (quantitative run, 500 MHz, D_2O, 30 seconds delay and 1024 scans) (originally in colour).

Peak attribution was carried-out by comparison of the chemical shifts obtained with standards run in D_2O. The chemicals shifts are given in Table 37 and Table 38. In each case, the majority of the peaks are attributed to glucose and fructose. In each sample, the difference in chemical shift between the values obtained for the experimental samples and the standards are below 0.1 ppm in absolute value. Additionally, the difference in chemical shift between the experimental sample and each standard is relatively constant. These two observations univocally confirm the presence of glucose and fructose in the water soluble fraction of the extract.

Table 37: Chemical shifts observed for fraction SO1H2O following ^{13}C NMR (500 MHz, D_2O, 30 seconds delay and 1024 scans) (originally in colour).

SO1H2O chemical shifts (ppm)	Glucose chemical shifts (ppm)	Difference (ppm)	Fructose chemical shifts (ppm)	Difference (ppm)
101.45			101.4	0.05
99.02				
98.07			98.09	-0.02
97.99	98	-0.01		
95.86	95.91	-0.05		
92.09	92.1	-0.01		
80.64			80.67	-0.03
75.94	75.95	-0.01		
75.75	75.73	0.02		
75.34			75.3	0.04
74.4			74.38	0.02
74.13	74.14	-0.01		
72.74	72.76	-0.02		
71.48	71.48	0		
71.42	71.44	-0.02		
71.13				
69.67			69.6	0.07
69.64	69.65	-0.01		
69.59	69.6	-0.01		
69.2			69.15	0.05
68.33				
67.53			67.49	0.04
63.85			63.86	-0.01
63.35			63.33	0.02
62.63			62.54	0.09
62.37			62.31	0.06

Table 38: Chemical shifts observed for fraction SO4H2O following ^{13}C NMR (500 MHz, D_2O, 30 seconds delay and 1024 scans) (originally in colour).

SO4H2O chemical shifts (ppm)	Glucose chemical shifts (ppm)	Difference (ppm)	Fructose chemical shifts (ppm)	Difference (ppm)
101.49			101.4	0.09
99.01				
98.07			98.09	-0.02
98.05	98	0.05		
95.91	95.91	0		
92.09	92.1	-0.01		
80.67			80.67	0
75.94	75.95	-0.2		
75.75	75.73	-0.39		
75.34			75.35	-0.01
74.4			74.41	-0.01
74.13	74.14	-0.01		
74.1				
72.76	72.76	0		
71.48	71.48	0		
71.43	71.44	-0.01		
69.68				
69.64	69.65	-0.01		
69.59	69.6	-0.01		
69.2			69.15	0.05
67.53			67.49	0.04
63.87			63.86	0.01
63.35			63.33	0.02
62.63			62.54	0.09
62.37			62.31	0.06

An internal standard was selected to carry-out the quantification of glucose and fructose. A known amount of *N,N*-dimethyl formamide (DMF) was used to compare the integration obtained for the equivalent methyl groups belonging to DMF with the integration obtained for the α- and ß- anomeric carbons of glucose and fructose. Figure 99 gives the chemical structure of the standard used. This method has been previously used for the quantification of glucose produced during the microwave assisted hydrolysis of cellulose. In this case, the solvent, deuterated DMSO, was used as an internal standard and a reference.[304]

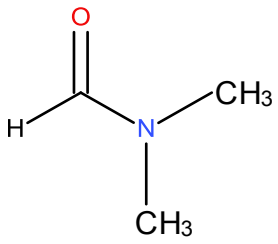


Figure 99: Chemical structure of *N,N*-dimethyl formamide (originally in colour).

The ratio of the sum of the integration of each monosaccharide anomeric carbon over the integration of the methyl groups belonging to DMF allowed correlation between the number of moles of DMF and the number of moles of each monosaccharide identified. Using this method, the percentage of glucose and fructose present in fraction SO1H2O was of 19.10% and 25.99%. The experimental data is given Table 39. The total percentage of monosaccharides in the sample was therefore equal to 45.08%. When using ethanol as an extraction solvent, those values were notably increased: for sample SO4H2O, the percentage of glucose and fructose present was of 74.21% and 25.35%. The experimental data is given in Table 40. The total percentage of monosaccharides in the sample was therefore equal to 99.55%. This is interesting as the high content in sugar monosaccharide extracted when using ethanol could potentially be used without any further separation and/or purification as a fermentation substrate.

Table 39: Experimental data used for the monosaccharide quantification by ^{13}C NMR for sample SO1H2O (originally in colour).

	Glucose standard chemical shift (ppm)	Integration	Difference (ppm)	Fructose standard chemical shift (ppm)	Integration	Difference (ppm)	*N,N*-DMF chemical shift (ppm)	Integration	Difference (ppm)	Sum of integration for each compound
101.23				101.4	1.85	-0.17				6.75
97.8				98.09	4.9	-0.29				
95.66	95.91	2.95	-0.25							4.96
91.83	92.1	2.01	-0.27							
31.13							31.4	39.13	-0.27	-

Table 40: Experimental data used for the monosaccharide quantification by ^{13}C NMR for sample SO4H2O (originally in colour).

	Glucose standard chemical shift (ppm)	Integration	Difference (ppm)	Fructose standard chemical shift (ppm)	Integration	Difference (ppm)	*N,N*-DMF chemical shift (ppm)	Integration	Difference (ppm)	Sum of integration for each compound
101.21				101.4	3.11	-0.19				8.16
97.76				98.09	5.05	-0.33				
95.61	95.91	9.83	-0.3							23.89
91.79	92.1	14.06	-0.31							
31.16							31.4	45	-0.24	-

6.6 Conclusion and future work

In this chapter, the extraction and isolation of four PMFs (tetra-O-methylscutellarein, tangeritin, nobiletin and heptamethoxyflavone) which can all be separated using SFC, hesperidin which is a molecule with known antioxidant properties and a mixture of glucose and fructose (> 97% monosaccharides) was demonstrated using food grade solvents only. Continuous liquid-solid extraction was carried-out using a soxhlet equipment on a 3 L scale. The following yields were respectively obtained on a wet basis for the mixture of four PMFs, hesperidin and a mixture of sugars: 5.87%, 0.24% and 18.35%.

These results represent an important achievement compared to the molasses usually recovered following treatment of the peel with inorganic salts. This step change has the potential to allow for the efficient extraction and separation of compounds usually obtained together, using renewable food grade solvents instead of polluting inorganic salts.

The PMFs were characterised by ESI and HPLC-MS. The latter technique allowed for the separation of the compounds and the identification of tangeritin and nobiletin. Supercritical CO_2 chromatography was used for separation of the compounds with the aim of further allowing the characterisation of the separated PMFs by ^{13}C solution NMR. This technique was also used in combination to ATR-IR for the characterisation of hesperidin. Furthermore, glucose and fructose have been quantified by ^{1}H quantitative NMR while using DMF as an internal standard. This technique showed glucose and fructose respectively accounted for 74.21% and 25.35% of the sample weight, making possible the production of a high purity fermentation feedstock alongside the extraction of valuable flavonoids. It was concluded that the soxhlet extraction of OPR1 was a good addition to the overall process for the valorisation of WOP. The use of ethanol in the work-up to isolate the mixture of PMFs indicated it would also be suitable extraction solvent. This would have the advantage of simplifying solvent recovery at large scale as acetone would no longer be used and second solvent recovery system wouldn't be necessary.

In terms of future work, additional experiments should be carried-out to evaluate the suitability of an alternative solid-liquid extraction technique based on microwave technology. This will enable further integration of the process and should be the next step towards the scale-up. Continuous microwave extraction is known and has been reported as an efficient alternative to soxhlet extraction. In

addition, the use of microwave technology to prevent "the binding of polyphenols to the plant matrix" is also known.[51] Microwave-assisted extraction of hesperidin has been reported as a faster and more efficient process for this particular compound under "hot and pressurised conditions (< 180 °C)".[300] Furthermore, reproducibility of the results should be assessed and all extractions should be re-run at least twice, to obtain standard deviation data, especially for the extraction experiment carried-out in ethanol with *D*-limonene peel extracted at 500 mbars.

Chapter 7

Materials and Methods

Materials and methods

Materials & reagents

Biomass used

Experiments were conducted using waste orange peels (*Citrus sinensis* or sweet oranges) of mixed varieties obtained from a London-based juicer, Sundance. Upon deliverance to our facilities, they were frozen within 12 hours following juicing at -18 °C. Before use, WOPs were defrosted at 4 °C and milled using a Retsch™ Knife Mill Grindomix GM300 food processor (<5 mm particles). Whenever stated, fresh WOP has also been used for specific experiments. This relates to WOP which was used fresh as opposed to following defrosting.

Cleaning procedure

Dedicated glassware was used for the extraction of *D*-limonene, the flavonoids or pectin whenever possible to avoid contamination with any residual acid. Large pieces of glassware which could not solely be used for the purpose of this project (e.g. 1 L pyrex microwave vessel, 3 L soxhlet) were thoroughly washed with hot water and soap to avoid any bacterial contamination from degrading waste orange peel between batches. Lastly, an ethanol rince was used to remove traces of organic components. This method was also applied to wash glassware between experiments. NMR tubes were washed with acetone to remove d-DMSO whenever used. Otherwise acetone was used on them. 5 mm 7" Select Series NMR tubes used were purchased from Norell (*S-5-400-7).*

Commercial samples and standards

All the chemicals were used as received. All purity percentages are given as a weight percentage and have been obtained from the chemical retailer. De-ionised water was used as supplied by chemistry stores.

D-limonene extraction

D-limonene (97% purity) was purchased from Sigma Aldrich and used as a standard for ^{1}H NMR, GC-FID and GC-MS identification and quantification. Ethyl benzene (>97% purity) which was used as an internal standard was also purchased from Sigma Aldrich. Cyclopentyl methyl ether (99.90**%** purity minimum) used for the liquid-liquid extraction was obtained from ZEON Corporation.

Standards of α-myrcene , α- and ß- pinene, *p*-cymene, α-terpineol and linalool were used for characterisation purposes for GC-TOF analysis of the *D*-limonene fractions obtained by steam distillation and microwave assisted solvent-less extraction. They were all obtained from Sigma-Aldrich (>98% purity).

Flavonoids extraction

A sample of hesperidin (>97% purity) was used for comparison purposes and purchased from Acros Organics Chemicals.

Sugar monosaccharides extraction

Glucose, fructose, mannose, arabinose, galactose, xylose and galacturonic acid standards were all purchased from Sigma-Aldrich (>99% purity) and used as references for ^{1}H NMR analysis and GC-MS analysis. *N,N*-dimethyl formamide (>99% purity) was purchased for Sigma Aldrich and used as an internal reference for the quantification of glucose and fructose by quantitative ^{1}H solution NMR.

Pectin extraction

Seven different commercial pectin samples were used for comparison purposes. Citrus derived commercial pectin P3935(>74% galA) was purchased from Sigma Aldrich. Pectin sample CU201 (DE 72%; galA content of 83%), CU301 (DE 65%; galA content of 84%), CU401 (DE 63%; galA content of 87%), CU501 (DE 56%) and

CU701 (DE 38%; galA content of 85%) were provided by Herbstreight & Fox. Non-standardised high methoxy commercial pectin was obtained from Kelco (DE 77.2%; galA content of 82.6%). All samples were accompanied by a specification sheet from the pectin manufacturer.

Deuterated solvents

Deuterated water (99.9% atom D) and dDMSO (≥99.9%) were used as such for NMR analysis. Both were purchased from Sigma Aldrich.

Buffers and other solvents:

Buffers were used for gel formation tests. They were all produced by Fluka and purchased from Sigma-Aldrich, alongside tartaric acid. The list of the three buffers tested can be found below:

- Buffer concentrate pH 2.00 for 500 mL buffer solution, citric acid / hydrochloric acid / sodium chloride.
- Buffer concentrate pH 3.00 for 500 mL buffer solution, citric acid / sodium hydroxide / sodium chloride solution.
- Buffer concentrate pH 4.00 for 500 mL buffer solution, citric acid / sodium hydroxide / sodium chloride solution.

Acetone and ethanol (99.9% purity) were purchased from VWR Company.

Equipment used

CEM Discovery SP microwave, 300 W, 2.45 GHz:

CEM Discover SP-D closed vessel system with attached auto-sampler (CEM EXPLORER 48, 72, & 96). 35 mL Pyrex® vials were used with silicon caps as reaction vessels. All equipment can be seen in Figure 100. It possessed an IR temperature probe for temperature measurement. All experiments performed in this microwave were done with a maximum power mode switched off (a setting which would

maintain steady microwave power, with cooling achieved using compressed air) at several holding temperatures. Before each experiment, the IR temperature probe was calibrated at 120 °C using glycerol.

Figure 100: CEM Discovery SP microwave equipment
- left and CEM Discovery 35 mL Pyrex® vial with corresponding silicon cap- right (originally in colour).

CEM MARS 6 microwave, 1800 W, 2.45 GHz:

Pectin extraction was carried-out in a CEM MARS 6 with One Touch™ Technology microwave, using EasyPrep™ Plus Easy Prep Teflon 100 mL closed vessels. This equipment was used in power dynamic mode. This microwave was fitted with a dual IR probe (within the microwave cavity) and fibre optic probe (positioned in a glass insert on the control vessel as seen in Figure 101) for accurate temperature measurements following automatic calibration. Pressure feedback was available for safely control.

Figure 101: CEM MARS 6 equipment
–left, and control vessel & support –right (originally in colour).

Sairem Mini Flow 200SS, 200 W, 2.45 GHz:

This device was used to gain accurate information on the energy efficiency of microwave-assisted hydrothermal experiments. This equipment was used in power dynamic mode. It possessed a built-in optic fibre temperature probe. Two experiments were carried out in pyrex glass closed vessels (20 mL). The temperature was also measured externally using a Calex® PyroUSB 151 IR sensor. The IR sensor had an emissivity of 0.1 to 1.0, adjustable via software. Two samples were analysed: a blank one (de-ionised water) and an aqueous pectin solution (1 g in 10 mL). Each vessel was irradiated at 100 W (fixed forward power) until a determined temperature was reached, at which point the reflected power was measured on the Sairem equipment.

Milestone ROTO SYNTH, 1200 W, 2.45 GHz:

The *D*-limonene extraction was carried out in a *Milestone RotoSYNTH* microwave reactor, a 45° rotative solid-phase microwave reactor. The Milestone microwave was used in conjunction with a 2 L Pyrex® vessel (see Figure 102). The pump used to apply vacuum to the system was a CVC2 mode vacuum pump made by Vacuubrand®. The process vacuum was monitored at all times. Liquid fractions were collected in a series of round bottom flasks fitted within the vacuum line. This equipment was only used in a fixed power mode.

Figure 102: Milestone ROTOSYNTH equipment
containing WOP for the purpose of the *D*-limonene microwave extraction
–left and 1 L Pyrex® glass reactor –right (originally in colour).

NMR spectrometry equipment:

Different NMR spectrometer were used for the recording of NMR spectra. They are listed below:

- Jeol ECS 400 MHz console, with a narrowbore 9.4T Oxford AS400 shielded superconducting magnet a TH5 (Jeol) 5mm NMR probe with pulsed-field gradients along Z and running Delta 4.3.6.
- 400 MHz Bruker Avance III HD spectrometer equipped with a Bruker 4 mm H(F)/X/Y triple-resonance probe and 9.4T Ascend® superconducting magnet.
- Bruker Avance 500 MHz console, with a narrowbore 11.7T superconducting magnet and a 5mm BBI probe with pulsed-field gradients along Z and running the Topspin 2.1.
- Bruker Avance II 700 MHz console, with a narrowbore 16.4T shielded superconducting magnet and a 5mm BBO probe with pulsed-field gradients along Z and running Topspin 3.0.

Balance:

Two balances were used according to the scale at which the work was carried out. *A Kern Analytical Balance ACJ 220-4M with an internal calibration system was used for weights between 0.01 and 220 g (± 0.0001 g). For masses between 0.5 and 610 g, a Mettler Toledo PB602-S balance was used (± 0.01 g).*

Freeze-drier:

A VirTis SP Scientific Sentry 2.0 freeze-drier was used to dry the pectin obtained in step 3 (chapter 4) as well as the glucose and fructose mixture obtained in step 2 (chapter 6). All drying experiments were done at -103.3 °C and at a pressure of 29 mTorr. All samples were freeze-dried from water for a duration of 48 hours in 500 mL or 250 mL Pyrex round-bottom flasks. Prior to freeze-drying, all samples were frozen in liquid nitrogen

Rotary evaporator:

Solvent removal was carried out using a Heidolph™ Hei-Vap™ rotary evaporator. The vacuum was achieved an ILMVAC pump.

Centrifuge:

Liquid-solid separation carried out for the work-up of pectin was done using a Thermo Scientific Heraeus Megafuge 40R centrifuge. It was set at 3600 rpm for 20 minutes at room temperature. The acceleration and deceleration rates were set at 9 and 6 rpm, respectively.

Food processor:

A Retsch™ Knife Mill Grindomix GM300 was used for the size reduction (<5 mm particles) of WOP prior to *D*-limonene extraction (Figure 103).

Figure 103: Macerated WOP prior to *D*-limonene extraction (originally in colour).

Condensing chiller

A Thermo Scientific HAAKE DC10-K10 3-liter Digital Refrigerated Circulating Water Bath was connected to the condenser to cool the liquid phase obtained during the extraction of *D*-limonene when using the ROTO SYNTH microwave equipment.

Powermeter:

Energy consumption measurements were carried out using a plug-in Power and Energy Monitor Prodigit 2000MU. It was used for all three steps under the best conditions determined following optimisation. All results were reported in kWh with an accuracy of 30 ppm, as stated by the manufacturer (Prodigit).

Oven:

The moisture content of peel residues WOP, OPR1, OPR2 and OPR3 was all determined following oven drying in a PF60 (200) oven manufactured by Carbolite. Samples were dried at a maximum temperature of 70 °C until constant weight was reached. Samples were allowed to reach room temperature inside a desiccator before weighting.

Laboratory & Analysis techniques

Microwave assisted *D*-limonene extraction and quantification

D-limonene extraction using microwave technology with hexane

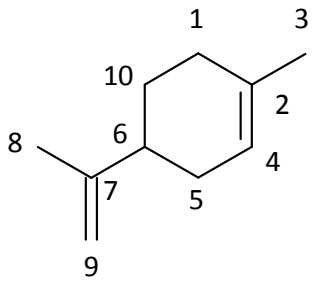
D-limonene was extracted using an average of 3.5 kg of WOP per experiment in the presence of 1.5 L of hexane. The CEM MARS microwave was used in an open vessel configuration. For each extraction a microwave power of 1800 W was used for 5 to 9 minutes, depending on how fast the solvent used started to condense. All

experiments were done under a blanket of nitrogen. After two runs, hexane was separated from the peel by filtration and the extracted orange oil was recovered after hexane evaporation. This oil was then subjected to steam distillation to isolate *D*-limonene for 4 hours. The yield of oil recovered following purification was determined by mass in relation to the wet mass of WOP peel used.

$$Yield\ of\ oil\ (\%) = \frac{mass\ of\ oil\ recovered\ (g)}{mass\ of\ wet\ OPR\ (g)} \times 100$$

The latter was characterised using ^{13}C NMR. The obtained spectrum was recorded on a 400 MHz Jeol spectrometer at 100 MHz using the central resonance of $CDCl_3$ as a reference (δC = 77.16 ppm). 256 scans and a relaxation time of 10 seconds were used. The chemical shifts are given in the table below (Table 41).

Table 41: Chemical shifts obtained for the characterisation of *D*-limonene obtained by microwave assisted extraction in hexane ($CDCl_3$, 100 MHz).

Compound	carbon number	^{13}C shift (ppm)
	C1	30.75
	C2	133.89
	C3	23.62
	C4	120.80
	C5	30.96
	C6	41.24
	C7	150.42
	C8	20.96
	C9	108.50
	C10	28.07

D-limonene extraction using microwave technology in presence of water

The MASD was carried out using a *Milestone RotoSYNTH* microwave reactor and WOP. For each run, between 800 and 900 g of WOP were weighted into a microwave Pyrex® vessel. The later was then connected to two condensers placed in line outside the microwave cavity. Between them, a separating funnel was used as collection flask. The second condenser was then fixed to a trap and connected to a cooler (-1 °C; HAAKE DC10-K10). To carry out the extraction, WOP was first irradiated with microwaves at 1200 W for 6 minutes. The power was then decreased to 800 W for the desired

amount of time, following the collection of the first drop of distillate in the separation funnel. The liquid phase produced was directly collected into a separating funnel. The glassware was allowed to stand for a few hours to allow the *D*-limonene containing oil phase to separate from the water phase. Yields of *D*-limonene containing oil were calculated based on wet WOP according to the equation below.

$$Yield\ of\ oil\ (\%) = \frac{mass\ of\ oil\ (g)}{mass\ of\ wet\ OPR\ (g)} \times 100$$

Typically, four microwave runs were separately carried out on fresh batches of WOP and their extraction product were combined. The influence of the WOP:H_2O ratio was studied with ratios of 1:1, 1:0.5 and 1:0. Furthermore, in order to find the optimal extraction time, the length of the MWSD was studied (10, 15, 20, 25 and 30 minutes) to find the optimal conditions for the steam distillation. This was done using a specific WOP:H_2O ratio 1:1. Characterisation was done by GC-FID.

D-limonene extraction using microwave technology under solvent-less conditions

The extraction was carried out at in a *Milestone RotoSYNTH* microwave reactor. The same set-up was used as in section 0. The set-up only differed by its connection to the vacuum. Four microwave runs were carried-out on fresh batches of WOP separately and their extraction product were combined. 850 ± 50 g of WOP were used per run. The microwave vessel was subjected to vacuum (100 or 500 mbars) and then irradiated with microwaves at 1200 W for 6 minutes. The power was decreased to 800 W for 19 minutes, following optimisation experiments.

After the irradiation process was finished, microwave heating was switched off and the vacuum was stopped. The whole system was left to reach atmospheric pressure before opening the system to avoid the loss of any volatiles. Following microwave treatment, the liquid fraction was mixed with cyclopentyl methyl ether in order to extract the oil phase. The solvent-extract mixture obtained was all added to 500 mL volumetric flasks prior to GC analysis. The purified *D*-limonene fraction was stored

at 5 °C and further characterised using GC-FID and GC-TOF. Each experiment was carried out twice at 100 mbars and three times at 500 mbars. Four to six runs were carried-out per experiment. The orange peel residue obtained after *D*-limonene extraction (OPR1) was subsequently used for flavonoid extraction. Energy consumption of the system composed of the microwave, chiller and pump was measured using the powermeter described in section 0.

The orange peel residue obtained after *D*-limonene and flavonoid extraction (OPR2) was subsequently used for pectin extraction.

Conventional D-limonene extraction by steam distillation

100 g of defrosted and milled WOP were extracted by steam distillation using 1 L of water. Prior to the steam distillation, the moisture content of the WOP was measured. The extraction was carried-out for 240 minutes at 100 °C until no more oil was obtained. The purified *D*-limonene fraction was stored at 5 °C and further characterised using GC-FID and GC-TOF following liquid-liquid extraction with cyclopentyl methyl ether. This experiment was repeated three times. Energy consumption of the system composed of the microwave, chiller and pump was measured using the power meter described in section 0.

Flavonoid and sugar extraction and work-up:

The flavonoids were extracted using an acetone or an ethanol soxhlet on a 3 L scale. All yields were determined based on a wet weight of OPR1. For each soxhlet carried out, between 450 and 475 g of *D*-limonene extracted WOP (OPR1) were used. Every soxhlet extraction was left to complete over four extraction cycles (the sample containing soxhlet body was allowed to fill/empty 4 times). After acetone soxhlet extraction, acetone was removed at 40 °C and 556 mbars using a rotary evaporator. The extract was then worked-up to recover two types of flavonoids as well as sugars. In the case of ethanol soxhlet extraction, ethanol was removed at 60 °C and 175 mbars in a rotary evaporator.

Following the removal of the extraction solvent, the crude extract was dissolved in de-ionised water to allow the precipitation of flavonoids present in the crude extract

from the soluble sugars, which will be water soluble. The flavonoid containing mixture was then filtered off and rinsed with water repeatedly to remove all sugars. The water-soluble fraction was freeze-dried to obtain a mixture of glucose and fructose.

The water insoluble fraction was then solubilised in ethanol, forming two phases isolated by filtration. The ethanol soluble fraction would yield the mixture of PMFs whereas the ethanol insoluble phase will contain hesperidin. Hesperidin was filtered off the PMF containing ethanol solution using a Buchner funnel over vacuum. QL100 Fisher Scientific filter papers were used (product code 11566873). It was repeatedly washed with two portions of ethanol (50 mL). Following their separation, ethanol was removed by evaporation from the PMF mix at 60 °C and 175 mbars in a rotary evaporator. All products were kept below 5 °C prior to their characterisation. Energy consumption of the soxhlet heater was measured using the power meter described in section 0.

Pectin extraction and work-up

Microwave assisted pectin extraction under acid-free conditions

D-limonene and flavonoids extracted peel (OPR2) or PWOP and fresh WOP were used for pectin extraction under acid-free microwave conditions. PWOP corresponded to *D*-limonene and flavonoids extracted peel (OPR2). Pectin extraction was carried out using two different types of microwave equipment: the CEM Discovery microwave, which was used on a 35 mL scale, and the CEM MARS, which was used on a 100 mL scale. In both cases a combination of different operational parameters were trialled: the variation of the peel:water ratio (w./v.), microwave irradiation temperature, microwave temperature holding time and MMPD were investigated. At a 100 mL scale, a 1:10 peel:water ratio was always used. In this case, 7 g of fresh WOP were used together with 70 mL of de-ionised water. For PWOP, taking into account the reduced moisture content, 1.4 g of PWOP in 70 mL of de-ionised water were used. The different microwave methods used can be found in Table 42.

Table 42: Microwave methods used at 120 °C for acid-free pectin extraction for both the CEM Discovery and the CEM MARS microwave equipment.

	CEM Discovery microwave	CEM MARS microwave
Power mode	Dynamic	Dynamic
Stirring	high	high
Hold temperature (°C)	120	120
Temperature hold time (minutes)	10	15
Number of vessels per run	1	6
Average number of runs per experiment	20	6

Upon completion of microwave heating, samples were cooled using compressed air. When room temperature was achieved, the liquid phase was then recovered by solid-liquid filtration. Pectin containing extracts were worked-up following a modified version of the method used by Fishman *et. al.*[148] Pectin was precipitated with 96% ethanol, using twice the volume of the pectin containing aqueous phase. On a 35 mL and a 100 mL sample scale, this amounted respectively to 2 and 5 litres of ethanol. The precipitation mixture was left to stand overnight. Following precipitation, the ethanol was removed using a centrifuge (Thermo Scientific Heraeus Megafuge 40R) set at 3500 rpm for 20 minutes. The recovered pectin was washed twice using an excess of acetone (1 litre in each case) followed by hot ethanol filtration (0.75 L in each case) to remove neutral sugars monomers, organic acids, low molecular weight peptides and amino acids amongst others.[186, 305-307] Care was taken never to leave pectin more than 10 minutes in refluxing ethanol (78 °C). Between each step, the washing solvent was removed using centrifugation (same settings as above). Following hot filtration, the pectin sample was dissolved in a 100 mL of water. Finally, pectin was dried using a freeze-drier. Pectin yields were calculated on a wet basis according to the equation below.

$$Pectin\ yield\ (\%) = \frac{mass\ of\ dried\ pectin\ recovered\ (g)}{mass\ of\ wet\ OPR\ (g)} \times 100$$

For samples P0002 and P0003, an ethyl acetate extraction step was included between the ethanol precipitation and the acetone washing steps. It was eliminated for samples P0004 and onwards as it was time consuming and did not make a difference on the pectin end result. In all cases, a sample of the peel residue (OPR3) post pectin extraction was kept and frozen (~10 g).

Study of the reflected power on a system composed of WOP in water

The absorption of microwave power by fresh orange peel was studied on a Sairem Miniflow 200SS microwave equipment. 15 mL Pyrex closed-vessels were used. The equipment uses a fiber optic probe to measure the temperature via a Calex® PyroUSB 151 infrared sensor. The experiment was done on a reference sample containing 10 mL of water only and another one containing 1 g of fresh WOP in 10 mL of water. The microwave power was set to 100 W. The reflected power was recorded at 115 °C and 130 °C based on previous work done on acid-free pectin extraction on the CME Discovery microwave equipment.

Conventional pectin extraction via acid hydrolysis:

Both dried WOP and PWOP were used for conventional acid hydrolysis extraction using the adapted protocol reported by Kratchanova *et. al.*[146] Briefly, 25 g of dried WOP were added to 250 mL of de-ionised water. The pH was lowered to 1.5 using 0.5 M HCl. The pH was verified using pH paper. The mixture was then heated to 81°C for an hour, after which solid-liquid filtration was used to collect the pectin containing liquid phase. The same protocol used than in section 0 was used for the isolation and work-up of pectin.

After cooling, the filtrate was coagulated using twice the volume of ethanol and left to stand overnight. The coagulated pectin was separated by centrifugation, washed again with ethanol and then twice with acetone. Between each step, the solvent was removed using centrifugation. Finally a hot ethanol filtration was performed to remove neutral sugars.[186, 307] Pectin was then dried using a freeze-drier. Two ethanol washes were used to make sure no acid remained in the pectin, making sure the stainless steel tank of the freeze-drier would not be corroded by the acid. The same procedure was used for PWOP except 20 g of peel were used in 100 mL of distilled water for the extraction. A sample of the peel residue post pectin extraction (OPR3) was kept in all cases and frozen at –18 °C (~10 g).

Biomass moisture content:

The moisture content of WOP, PWOP, OPR1, OPR2 and OPR3 was determined following air drying in a 70 °C oven until constant weight was reached. Following cooling in a closed vial, the equation below was used for the calculation of the moisture content:

$$Moisture\ content\ (\%) = \frac{Sample\ mass\ post\ drying\ (g)}{Sample\ mass\ pre\ drying\ (g)} \times 100$$

The average of two duplicate drying experiments is reported in Table 43 for moisture content of the peel samples studied.

Table 43: Average moisture content of the extracted peel residues over two samples.

Peel residue used for extraction	Moisture content (%)
Fresh WOP	77.36
Defrosted WOP	17.68
OPR1 (following *D*-limonene extraction)	4.16
OPR2 (following *D*-limonene & flavonoid extraction)	0.11

The figure below illustrates the different peel residues extracted for this project.

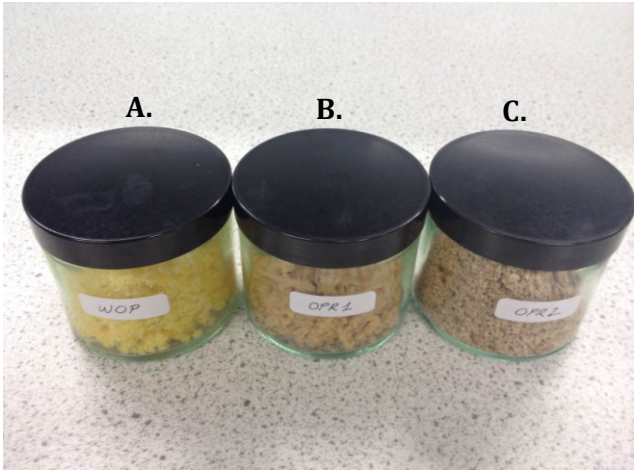


Figure 104: WOP examples used for the different extraction steps A) WOP for D-limonene extraction, B) OPR1 for flavonoids and sugars extraction and C) OPR2 for pectin extraction (originally in colour).

Analysis technique

GC-FID analysis for the quantification of *D*-limonene content:

GC-FID was performed using an Agilent Technologies 6890N Network gas chromatograph system equipped with the 7638B series Agilent injector. In the case of solvent-less microwave extraction and steam distillation extraction of *D*-limonene, GC-FID was used for the determination of the *D*-limonene content. Following the recovery of the *D*-limonene containing organic fraction of cyclopentyl methyl ether in a 500 mL volumetric flask together with 2.51g of the internal standard ethyl benzene (IS), a 1 mL sample of the later mixture was then analysed by GC-FID to determine quantitatively the amount of *D*-limonene extracted and calculate a yield of *D*-limonene extracted.

The chromatography conditions are described as follows: a 1 µL injection volume was used (splitless injection) with He as a carrier gas at 300 °C, 23.27 PSI and a flow of 114 mL.min^{-1}. A ZB-5HT (30 x 0.25 X 0.25 m) column was used with an initial flow of 2 mL.min^{-1}. The oven temperature was initially set at 60 °C (5 minute holding time) and increased to 300° C (5 minute holding time) using a 15 °C.min^{-1} ramp (total running time of 26 minutes)

The mass of *D*-limonene extracted was obtained following the equation below where k corresponds to the response factor:

$$\frac{Area\,(D-limonene)}{mass\,(D-limonene)} = k\,\frac{mass\,(IS)}{Area\,(IS)}$$

The response factor was determined by doing a calibration curve using *D*-limonene and ethyl benzene as an IS using varying known concentrations. The chromatograms obtained are shown in Figure 105.

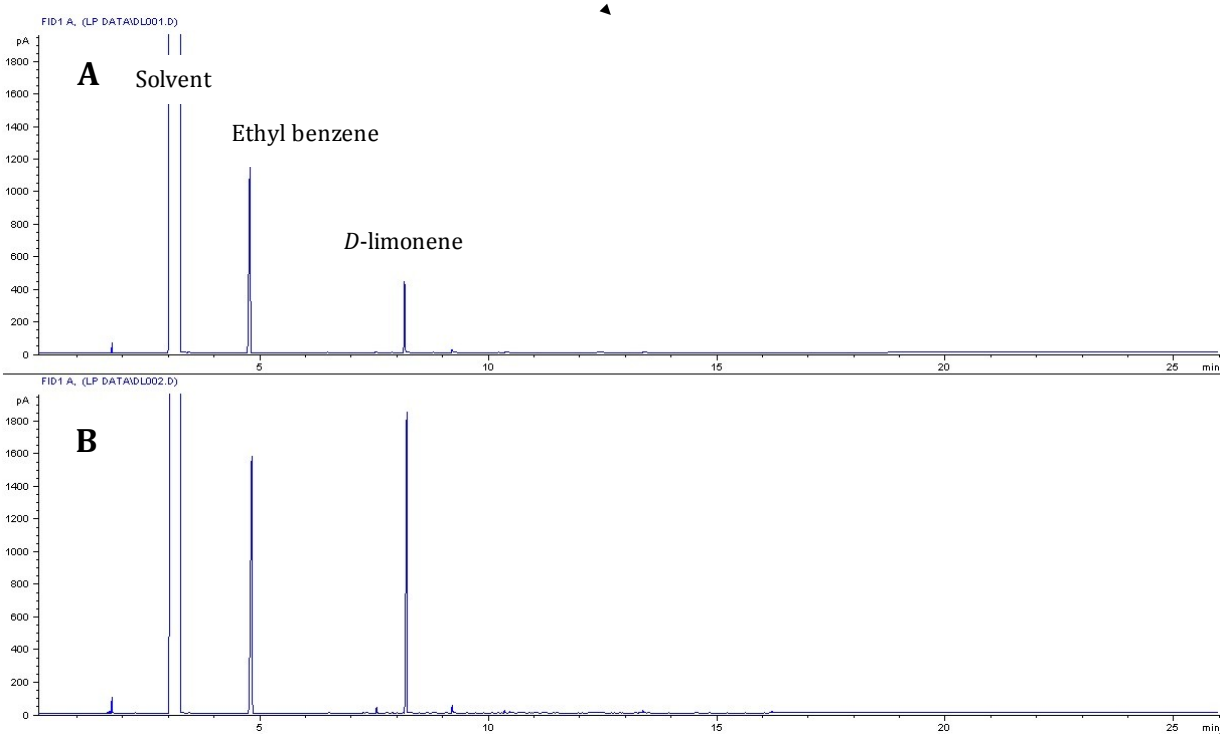


Figure 105: GC-FID chromatograms obtained for samples DL001 (chromatogram A) and DL002 (chromatogram B) (originally in colour).

The exact calculations used for the determination of the *D*-limonene content are highlighted for sample DL001 obtained at 100 mbars (Table 44). 1.001 g of *D*-limonene were extracted per litre of liquid phase obtained. 2,81 g of liquid phase have been collected following the extraction, meaning 2.81 g of *D*-limonene had been extracted in total. The wet yield therefore amounts to 0.16% (0.22 % on a dry basis) based on the extraction of 1734.56 g of wet WOP (17.13 %, 1437.43 g WOP on a dry basis).

Table 44: GC-FID data for the calculation of the *D*-limonene concentration extracted with a response factor of k = 0.8482 (sample DL001) at 100 mbars.

	Boiling point (° C)	Retention time (minutes)	Peak area	Concentration (mol/L)
Ethyl benzene (IS) $C_6H_5CH_2CH_3$	136	4.80	2728.9	0.061
***D*-limonene** $C_{10}H_{16}$	176	8.18	561	1.001

GC-TOF analysis of *D*-limonene fractions

GC-TOF analysis was carried-out on an Agilent 6890 GC coupled to a Pegaus IV TOF mass spectrometer (Leco). The GC was fitted with a 30 m x 0.25 mm Rxi-5Sil MS column (Thames Restek). The film thickness was of 0.25 µm. The carrier gas was He. It was used in constant flow mode at 1 $mL.min^{-1}$. A 1 µL sample was injected at a 100:1 split ratio. The oven program was isothermal at 40 °C for 2 minutes then ramped at 5°$C.min^{-1}$ to 300 °C and held for 2 minutes. Electron impact mass spectra were generated at 230 °C and 70 eV. Data was collected at unit mass resolution over the range 20 - 450 m/z and 20 $scan.s^{-1}$. Data was analysed using the ChromaTof 4.5 software (Leco). Compounds were identified by reference to authentic standards or by spectral matches to the NIST 05 and Wiley 7 libraries.

ESI analysis of polymethoxy flavonoids

Analysis was carried out at the Center of Excellence for Mass Spectroscopy of the University of York by Karl Heaton. ESI analysis was carried-out on a Brucker microTOF mass spectrometer (orthogonal Time of Flight) coupled to an Agilent series 1200 liquid chromatograph with a 108 position autosampler and a photodiode array with a wavelength range from 190 nm to 640 nm. The mass spectrometer included a nominal mass range of 3,000 and a 10,000 FWHM. The flow rate regulated by the injector pump ranged typically from > 1 $\mu L.min^{-1}$ to 100 $\mu L.min^{-1}$. Samples were typically diluted in ethanol for identity confirmation.

HPLC-MS of polymethoxy flavonoids:

Analysis was carried out independently by Dr. Tony Larson at the CNAP laboratory from the Biology Department of the University of York. Ethanol was used to dissolve the samples: typically, 10.5 mg of flavonoid mixture were dissolved in 1 mL of solvent. HPLC-MS was performed on a Waters Acquity Ultra Performance LC coupled with a Thermo LTQ-Orbitrap detector with APCI ionization source operating in full scan positive mode, scanning 150-900 Da. The resolution of the detector was set to 7500 FWHM at m/z 400 with a 4 Hz scan rate. $[M+H]^+$ ions were obtained with a mass accuracy inferior to 5 ppm , allowing to calculate an empirical formula for the eluted compound. 2 µL samples were injected in full loop mode at 5 °C and separated on a 50 x 2.0 mm Phenomex Luna HST 2.5 µm C18 column set at a temperature of 60 °C (reverse phase). The gradients used for the combination of mobile phase A (5% methanol and 0.1 % formic acid in water (v./v.)) and mobile phase B (0.99 % methanol and 0.1 % formic acid (v./v.)) are given in Table 45.

Table 45: Mobile phase linear gradient used for the HPLC-MS analysis of the polymethoxylated flavonoids extracted.

Time (minutes)	Mobile phase A (%)	Mobile phase B (%)	Column flow rate ($mL.min^{-1}$)
0	60	40	0.6
1.3	0	100	0.6
1.8	0	100	0.6
2	60	40	0.6

Separation of the polymethoxy flavonoids by supercritical CO_2 chromatography

All supercritical fluid chromatography was carried out on a Thar SuperPure Discovery Series SFC with a Water 2995 photodiode array detector (ultraviolet/visible light spectrophotometer) and a Water 2424 Evaporative Light Scattering detector. Optimisation work was carried out using a Phenomex Luna Silica (100 Å, 3 µm) column (50 mm x 4.6 mm) was used. For optimised work, the separations were performed on a Waters SunFire Silica Semi prep column (250 mm

x 10 mm) packed with 5 μm silica (100 Å). All were normal phase separations. An injection loop of 100 μL was used and the sample injection volume was of 20 μL.

The mobile phase used was carbon dioxide (99.8% purity from BOC). Methanol and ethanol were both tested as co-solvents. A Thar SuperPure Automated Back Pressure regulator (Discovery serie) was used for fractionation of the targeted compounds.

Typically a sample containing 65 mg of the polymethoxy flavonoids mixture obtained following soxhlet extraction was dissolved in 1 mL of ethanol. The pressure, flow rate and modifier percentage were optimised until a satisfactory separation was achieved. All analysis was done at a standard temperature of 40 °C. All data was obtained three times to check for reproducibility issues. Following satisfactory reproduction of the optimised separation method, collection of the separated analytes was performed on the Waters SunFire Silica Semi prep column, using a flow rate of 9 $mL.min^{-1}$, an injection volume of 100 μL, an ethanol percentage of 8% at 75 bars and 40 °C.

Fourier-Transform Infrared Spectroscopy

DRIFT-IR analysis of samples was carried out on a Bruker Vertex 70 instrument equipped with "Specac" Golden Gate Single Reflection Diamond ATR probe. Sample spectra were collected with a resolution of 4 cm^{-1} between 400 and 4000 cm^{-1}. Spectrums of commercial pectin galacturonic acid, galacturonic acid sodium salt, polygalacturonic acid and polygalacturonic acid sodium salt were also obtained to determine the peaks of the functional groups in pectin samples. This technique was also used to identify hesperidin against a commercial standard. All finely milled samples were used as received.

Solution NMR

Polymethoxy flavonoids

^{1}H NMR was used on the mixture of polymethoxy flavonoids to identify functional groups and establish the class of compounds extracted. The NMR was run in $CDCl_3$

on a 400 MHz Jeol spectrometer at room temperature and using 1,024 scans and a relaxation time of 4 seconds.

Hesperidin:

^{13}C NMR spectra of experimental hesperidin samples were obtained in dDMSO at 60 °C using 1,024 scans (Brucker 500 MHz). A standard relaxation time of 2 seconds was used. The number of scans was increased to 1,024 in order to increase the S/N ratio as hesperidin was notably difficult to dissolve, even in deuterated DMSO. The analysis was done at 60 °C to avoid hesperidin precipitating out of the dDMSO over the duration of the experiment. The ^{13}C spectra of commercial hesperidin was obtained at room temperature using 256 scans and a relaxation time of 2 seconds on a 400 MHz spectrometer (Jeol 400 MHz) in dDMSO.

Quantification of sugar monosaccharides

The protocol used was adapted from a method initially used by Fan *et. al.*[304] The integration area of a peak can be correlated to the mass of a compound present in the mixture when compared to the integration area of a standard of known mass. In this case, *N,N*-dimethylformamide was used as an internal standard. The signals considered can be found in Table 46.

Table 46: Chemical shifts of glucose, fructose and DMF together with the integration range used for each peak considered, as used for their respective quantification.

Reference compound	Chemical shift reference (ppm)	Integration range used (ppm)
α-fructose	101.25	101.22-101.28
ß-fructose	97.80	97.79-97.85
ß-glucose	95.66	95.62-95.72
α-glucose	91.83	91.80-91.88
DMF	31.13	31.00-31.21

The composition in mass percentage of glucose and fructose was determined by using the average integration ratio of the signal obtained for anomeric carbon of each sugar (α- and ß-) over the average integration ratio obtained for the $-CH_3$ group

of the tertiary amine of DMF. The equation below describes the calculation used for glucose as an example:

$$Mass_{glucose}(g) = \frac{Average\ integration\ C1_{glucose}}{Average\ integration\ CH_{3_{DMF}}} \times n_{DMF}(mol) \times M_{glucose}\ (\frac{g}{mol})$$

The mass percentage of glucose present in the extract was then determined as follow:

$$Glucose\ (\%) = \frac{Mass_{\ glucose}\ (g)}{Mass_{\ sample}\ (g)} \times 100$$

The protocol was first tested on 4 samples containing known varying amounts of glucose and fructose together with a known DMF. Following application of an integration method, 1.79% for glucose and 15.34% for fructose was determined.
Regarding the preparation of the experimental sample, typically, a 70 mg sample of extract was prepared in D_2O. 40 mg of DMF were added for the sample to be run. The quantitative ^{13}C NMR spectra was recorded on a 500 MHz spectrometer (Jeol) at 125 MHz using a relaxation delay of 30 seconds and 1,024 scans. All spectra were acquired in D_2O and samples were processed using the same integration range (including the standards).

Pectin

GPC analysis for small scale pectin extraction:

The analysis was carried out independently at RAPRA by Dr. Steve Holden. The column set-up consisted of an Agilent PLaquagel-OH Guard plus column and 2 Plaquagel-OH Mixed-H columns (30 cm, 8µm). The eluent was a x/y mixture of 0.2 M $NaNO_3$ and 0.01 M NaH_2PO_4 aqueous solutions adjusted to pH 7. The nominal flow rate was 1.0 mL.min^{-1} and the temperature was set at 30 °C. Triple detection was used consisting of a Malvern/Viscotek Triple Detector Array TDA301 , a refractive index detector and right angle light scattering. The system was calibrated using Malvern/Viscotek Pullulan standard for triple detection and a series of individual Agilent PL Pullulan standards,[223] with 10 individual standards having molecular weights ranging between 180 and 708,000. Sample solutions were prepared to accurate concentrations by dissolving ~20 mg of pectin in 10 mL of eluent. Samples

were stirred overnight assuring maximal dissolution of the pectin. Following, the sample was filtered through a 0.45 µm PVDF membrane Data was collected and analysed using the "OminSec" software provided by Malvern/Viscotek. The GPC results (molecular weight distribution) were based on back-calculated concentrations: a calibration curve (log Mw=f(retention volume)) was determined using the light scattering detector and the absolute Mw was determined for each size-exclusion chromatography elution volume increment. The calibration curve was then used to determine the average molecular weights and their distribution.

GPC analysis for large scale pectin extraction:

The analysis was carried out independently by the Centre for Water Soluble Polymers at Glyndwr University (Prof. Peter Williams). The GPC system consisted of a Suprema column (dimensions 300 mm x 8 mm; Polymer Standards Service GmbH) with 10 micron beads with a 300 10^{-10} m pore size, protected by a Guard column (Polymer Standards Service GmbH: 10 microns). The eluent used was 0.1M NaCl containing 0.005% sodium azide and was filtered with a GSWP 0.22 µm filter/ Millipore filter and degassed before use (Vacuum degasser CS 1615/Cambridge Scientific Instrument, Ltd). 1% solutions of control and experimental pectin samples were prepared in a 0.22 µm filtered aqueous solution of 0.1M NaCl and by tumbling overnight at 25 ˚C. If the dissolved pectin solutions contained insolubles (i.e. aggregates) solutions were centrifuged for one hour using a Heraeus Centrifuge Biofuge 28RS (speed at 4800 rpm) to remove the insoluble material. The eluent flow rate was set at 0.5 $mL.min^{-1}$ using a Waters Corporation 515 HPLC pump and the loop volume was 200 µL (Rheodyne model: 7125). *Dawn*® DSP *Laser Photometer* and OPTILAB DSP Interferometric Refractometer (Wyatt Technology Corporation) detectors were used in conjunction with the Agilent 1100 series UV detector (280 nm) (Agilent Technologies). Samples were passed through a 0.22 µm pore size nylon syringe filter before being injected onto the column. Measurements were performed in duplicate. The molecular weight was determined using Astra for Windows 4.90.08 QELSS 2.XX. The Debye model was used for all evaluation analyses. A value of 0.146 was used for the refractive index increment (dn/dc).[169]

Pectin titration:

The protocol was adapted from one used at the Centre for Water Soluble Polymers at Glyndwr University (Prof. Peter Williams). A mixture of 5 mL of 2.7M HCl and 100 mL of 60% ethanol in water was added to 2 g of sample in a 250 mL glass bottle and agitated for 10 minutes. The solution was transferred to a fritted-glass filter tube (30 to 60 mL capacity), washed with six 15 mL portions of the same HCl-60% ethanol mixture, followed by 60% ethanol until the filtrate was free of chloride and finally washed with 20 mL of ethanol. 2 g of material (initial weight) were dried at 105 °C for 2.5 hours and the percentage of final material recovered was determined. 0.5 g of the residue were transferred to a 250 ml conical flask and was moistened with 2 mL of ethanol. 100 mL of recently boiled and cooled water were added to the residue and swirled until a complete solution was formed. 5 drops of 1% of phenolphthalein in ethanol were added to the sample solution and titrated with 0.1 M of sodium hydroxide. The result was recorded as the initial titre (v_1). 20 mL of 0.5 M sodium hydroxide was added and the sample solution was shaken vigorously in a stoppered flask and left to stand for overnight. 20 mL of 0.5 M HCl were added to the sample solution which was shaken until colour change was observed. The sample solution was then titrated with 0.1 M of sodium hydroxide to a faint pink colour that persisted after vigorous mixing with high speed magnetic stirrer. The result was recorded as the saponification titre (v_2). Measurements were performed in duplicate. The DE was then calculated following the equation below.

$$DE\ (\%) = 100 \times \left[\frac{v_2}{v_1 + v_2}\right]$$

GC-EI-MS monosaccharide analysis of pectin samples:

The quantification of both neutral and acidic sugars present in pectin is provided by GC-EI-MS analysis of the trimethylsilyl glycoside derivatives of the glycosyl residues originating from the pectin's monosaccharides obtained by methanolysis. The conversion of the sugars into their corresponding alditol acetate and trimethylsilyl (TMS) ether has been a widely used method for gas chromatography analysis of sugar monosaccahrides.[308] The analysis consisted of the following steps:[309]

- pectin depolymerisation using anhydrous methanol containing HCl producing the corresponding methyl glycosides residue (methanolysis), followed by

- conversion of the methyl glycosylated derivatives of the monosaccharides obtained to their trimethylsilyl methyl glycosides derivatives.

The analysis was carried out independently by Dr. Jerry Thomas at the Biotechnology Facility of the Biology Department at the University of York. The protocol used was reported by Doco *et. al.*[191] Methanol containing 0.5 M HCl was prepared by adding acetyl chloride (140 µL) to anhydrous methanol (1 mL). Monosaccharides and polysaccharides (100–500 µg) were suspended in methanol/HCl (0.5 mL) and kept for 16 hours at 80 °C. The cooled solutions were concentrated to dryness at 40 °C under a stream of nitrogen gas. An excess of TriSil® reagent (0.3 ml) was added and the solutions kept for 20 minutes at 80 °C. The reagents were removed at 40 °C with a stream of nitrogen gas. The residue was then extracted with hexane (1 mL). The extract was concentrated to 50 µL and 2 µL were used for GC-EI-MS analysis. All analyses were performed in triplicate. Pectin samples were hard to depolymerise using anhydrous methanol containing HCl. Instead the pectin samples were treated with a 2% molar aqueous solution of TFA at 121 °C for one hour and then subjected to methanolysis yielding the alditol acetate derivative of the glycoside. A similar procedure was used by Georgiev *et. al.*[189]

GC-MS was performed using an Agilent 7890A gas chromatograph together with a 5975C Mass Selective Detector (MSD). The detector was used in full scan mode, targeting a mass range of 50-500 Da. Extracted ion chromatograms were used for quantification: ions at m/z 204 and 217 (sugars) were compared against ions at m/z 305 from inositol, which was used as an internal standard. Two DB-1 fused-silica capillary columns (30 m×0.25 µm i.d., 0.25 µm film thickness, J&W Scientific) were coupled to a single injector inlet. A hydrogen flow (2 $ml.min^{-1}$, average velocity 58 $cm.s^{-1}$) was used as a carrier gas. Samples were injected in the pulsed split mode with a split ratio of 20:1. The injector and the FID were operated at 250 °C. The transfer line to the MSD was set at 280 °C. The GC heating ramp was set to 120–145 °C at 1 $°C.min^{-1}$, 145–180°C at 0.9 $°C.min^{-1}$, and 180–230 °C at 50 $°C.min^{-1}$. EI mass spectra were obtained from *m/z* 50–650 every 2.48 s in the total ion-monitoring mode using a source temperature of 230 °C, a quadrupole temperature of 106 °C, a filament emission current of 34.6 µA and an ionization voltage of 70 eV. All the w./w. percentages reported the different monosaccharide contents were calculated on a moisture free basis.

Solution ^{1}H and ^{13}C NMR:

Both ^{1}H and ^{13}C NMR spectra were recorded. Commercial citrus pectin sample P140-10 was run on a Bruker 700 MHz spectrometer (including the DEPT135). All spectra were acquired in D_2O at 298K. To ensure quantitative data was recorded for ^{13}C solution NMR, spectra were recorded with a relaxation time of 10 seconds using 1,024 scans. Samples P0004 and P0018 were run on a 500 MHz spectrometer (Brucker) at 300K with a relaxation time of 2 seconds and 1,024 scans.

Solid state ^{13}C NMR:

All MAS spectra were acquired using a 400 MHz Bruker spectrometer. CP dynamics were investigated for all samples for contact times ranging from 50-3050 μs. $^{13}C^{203}$ CPMAS spectra with linearly-ramped contact pulses of 1 and 2 ms duration were used for quantification. They were acquired at spinning rates of 10000 ± 2 Hz, recycle delays of 5-8 seconds, spinal-64 heteronuclear decoupling at ν_{rf} = 85 kHz and are a sum of 512 co-added transients. The calibration curve obtained using ^{13}C CPMAS for the determination of the DE was done using five commercial pectin samples (see section 0 of this chapter).

Pectin gel formation:

The USA-SAG protocol:

The protocol used was adapted from Cox *et. al.*[230, 231] Pectin powder was left to dissolve in de-ionised water for 1 hour under stirring. The solution formed was rapidly heated to 100 °C before a defined amount of sucrose was added. A pre-determined amount of water was evaporated from the mixture using a Dean-Stark apparatus, to reach a SS% of 60%. The jelly mixture was then poured in a clean glass beaker containing a 48.8% tartaric acid solution and the gel was left to set at 25 °C for 24 hours.

The method reported was adapted according to the amount of pectin available for gel formation testing. The exact quantities of pectin, sucrose, water and tartaric acid

solutions are reported in Table 47. It should be noted that the setting temperature was of 26 °C instead of 25 °C as the samples were left to set in the instrument room.

Table 47: Gel formation conditions tested using the USA-SAG protocol.

	Commercial pectin CU201	Commercial pectin CU301	P0004	P0020
Gel test #	**PGT1**	**PGT2**	**PGT3**	**PGT4**
Scale-down factor	2	2	4	10
Pectin (g)	1.375	1.375	0.6861	0.274
H_2O (g)	179	179	107.5	43
Sucrose (g)	323.625	323.625	161.8	62.72
SS%	70	70	65	65
48.8% tartaric acid solution (mL)	3.5	3.5	1.75	0.7
Amount of water collected (mL)	36	36	18	8.8
Setting temperature/ room temperature (°C)	26.5	26.5	26.5	26.5
Observations after 24h at 26 °C	Already started to gel 10 min. after the heating was stopped. Formed a stronger gel than CU301.	Weak gel formed	Weaker gel formed than CU301 and P0020	Weak gel formed

The MacDougall protocol:

The method used was adapted from the protocol published by MacDougall *et. al.*[233, 236] 300-600 mg of a 0.2% (w./w.) pectin solution were sampled using a 1 mL plastic syringe. The needle hole was blocked using parafilm to allow the removal of the plunger. 30 µL of a 1 $mol.L^{-1}$ $CaCl_2$ solution was added to the pectin solution and the plunger was replaced on the syringe to avoid spilling. The syringe tubes were stored upright at 4 °C for 24-48 hours. The graduation on the syringe was used as an arbitrary way of determining how much pectin solution was sampled within the 300-600 mg range. The exact quantities of pectin and $CaCl_2$ solutions are reported in

Table 48 for each test carried-out using this protocol. A picture illustrating the experimental set-up is displayed in Figure 106.

Table 48: Gel formation conditions tested using the MacDougall protocol.

Gel test #	0.2% pectin solution (mg)	$CaCl_2$ solution (µL)
PGT5	364.2	30
PGT6	507.7	30
PGT7	650.8	30
PGT8	321.7	15
PGT9	625.4	30
PGT10	358.7	15
PGT11	654.3	30

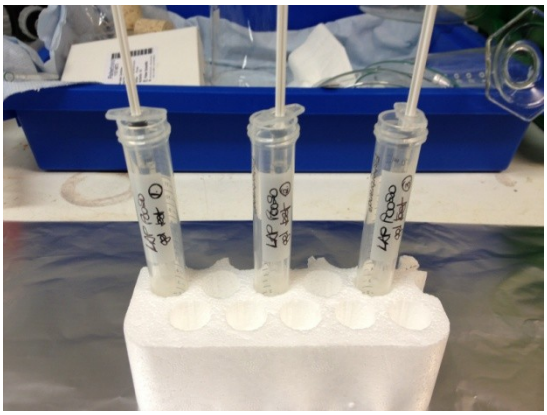


Figure 106: Experimental set-up for the gel formation test using the MacDougall protocol (originally in colour).

The Lofgren protocol:

The protocol used was adapted from a publication by Lofgren *et. al.*[234] A 0.75% (w./v.) pectin solution in a citrate buffer (pH 2, 3 or 4) was made. This solution was left to stir for two hours to ensure total dissolution of the pectin. Afterwards the solution was heated to boiling point using a condenser to avoid any water evaporation, keeping the SS% constant during heating. A defined amount of sucrose was added to reach a 60 or 70 SS%. Whenever necessary, $CaCl_2$ was added to the solution. The solution was left to reach 100 °C again, at the point which the mixture

was poured in a clean glass beaker and left to set at room temperature for 24 hours. All the gel formation conditions tested are summarised in Table 49.

Table 49: Gel formation conditions tested using the Lofgren protocol.

Gel test #	Pectin sample used	Pectin (%)	Pectin (g)	Sucrose (%)	Sucrose (g)	pH	Buffer (g)	Addition of Ca^{2+}
PGT15	P0020	0.75	0.1861	60	15	3	9.81	1.2 ml 0.15% $CaCl_2$
PGT16	P0023	0.75	0.186	60	15	3	9.81	-
PGT17	P0023	0.75	0.186	70	17.5	3	7.31	1.2 ml 0.15% $CaCl_2$
PGT18	P0023	0.75	0.1867	70	17.5	3	7.31	-
PGT19	P0023	0.75	0.1863	60	15	2	9.81	1.2 ml 0.15% $CaCl_2$
PGT20	P0023	0.75	0.1864	60	15	2	9.81	-
PGT21	P0023	0.75	0.1867	70	17.5	2	7.31	1.2 ml 0.15% $CaCl_2$
PGT22	P0023	0.75	0.1861	70	17.5	2	7.31	-
PGT23 (repeat of PGT17)	P0023	0.75	0.187	70	17.5	3	7.31	1.2 ml 0.2% $CaCl_2$
PGT24 (repeat of PGT18)	P0023	0.75	0.1868	70	17.5	3	7.31	-
PGT25	CU201 H&F	0.75	0.1858	70	17.5	3	7.31	-
PGT26	CU201 H&F	0.75	0.1869	70	17.5	2	7.31	-
PGT27	P0023	0.75	0.125	60	10.08	4	6.60	0.81 ml 0.2% $CaCl_2$
PGT28	P0023	0.75	0.125	60	10.08	4	6.60	-
PGT29	P0023	0.75	0.125	70	11.76	4	4.92	0.81 ml 0.2% $CaCl_2$
PGT30	P0023	0.75	0.125	70	11.76	4	4.92	-

List of abbreviations

Table 50: List of abbreviations used.

APCI	-	Atmospheric Pressure Chemical Ionisation
Ara	-	Arabinose
ATR-IR	-	Attenuated Total Reflectance Infra Red
BOD	-	Biological Oxygen Demand
CNAP	-	Centre for Novel Agricultural Products
COD	-	Chemical Oxygen Demand
CPW	-	Citrus Peel Waste
DE	-	Degree of Esterification
DMF	-	Dimethyl formamide
DMSO	-	Dimethyl Sulfoxide
EPA	-	Environmental Protection Agency
ESI	-	ElectroSpray Ionisation
E.U.-27	-	European Union
FT-IR	-	Fourier Transform Infra Red
FSCW	-	Food Supply Chain Waste
Gal	-	Galactose
galA	-	Galacturonic acid
GC	-	Gas Chromatography
GC-MS	-	Gas Chromatography- Mass Spectrometry
GC-TOF	-	Gas Chromatography Time of Flight
GPC	-	Gel Permeation Chromatography
HPAEC	-	High Performance Anion Exchange Chromatography
HPLC-MS	-	High Performance Liquid Chromatography - Mass Spectrometry
IPPC	-	Integrated Pollution Prevention and Control
IR	-	Infra-red
IS	-	Internal Standard
MASD	-	Microwave Assisted Steam Distillation
min.	-	Minute
Mn	-	Number Average Molecular Weight
Mw	-	Weight Average Molecular Weight

MW	-	Microwave
MMPD	-	Microwave Maximum Power Density
MSA	-	Monosaccharide Analysis
n.e.c.	-	not elsewhere classified
NMR	-	Nuclear Magnetic Resonance
NSIs	-	National Statistical Institutes
OPEC	-	Orange Peel Exploitation Company
OPR1, 2 & 3	-	Orange Peel Residue 1, 2 & 3
PAD	-	Pulsed Amperometric Detector
PI	-	Polydispersity Index
PMF	-	Polymethoxy flavonoid
PMFs	-	Polymethoxy flavonoids
polygalA	-	Poly α-1,4- D-galacturonic acid
PWOP	-	Pre-treated Waste Orange Peel
Rha	-	Rhamnose
RI	-	Refractive Index
SEC	-	Size Exclusion Chromatography
SEM	-	Scanning Electron Microscopy
SFC	-	Supercritical Fluid Chromatography
SS%	-	Soluble Solids (%)
TGA	-	Thermo Gravimetric Analysis
TG-IR	-	Thermogravimetric Infra Red Spectroscopy
TIC	-	Total Ion Chromatogram
U.K.	-	United Kingdom
U.S.A.	-	United States of America
WOP	-	Waste Orange Peel
XIC	-	eXtracted Ion Chromatogram

References:

1. C. Rolin, B. U. Nielsen and P.-E. Glahn, in *Polysaccharides: structural diversity and functional versatility*, ed. S. Dumitriu, CRC Press, 1998, ch. 12, pp. 377-432.
2. J. B. Binder and R. T. Raines, *Journal of the American Chemical Society*, 2009, **31**, 1979–1985.
3. A. J. Ragauskas, C. K. Williams, B. H. Davison, G. Britovsek, J. Cairney, C. A. Eckert, W. J. J. Frederick, J. P. Hallett, D. J. Leak, C. L. Liotta, M. J. R., M. R., T. R. and T. Tschaplinski, *Science*, 2006, **311**, 484-489.
4. S. K. C. Lin, L. A. Pfaltzgraff, L. Herrero-Davila, E. B. Mubofu, A. Solhy, J. H. Clark, A. Koutinas, N. Kopsahelis, K. Stamatelatou, F. Dickson, S. Thankappan, M. Zahouily, R. Brocklesby and R. Luque, *Energy & Environmental Science*, 2012, **6**, 426-464.
5. P. Anastas and J. Warner, *Green Chemistry: Theory and Practice*, OUP Oxford, 2000.
6. Basics of Green Chemistry, http://www2.epa.gov/green-chemistry/basics-green-chemistry, Accessed 08.09.2014, 2014.
7. P. T. Anastas and J. C. Warner, *Green Chemistry: Therory and Practice*, Oxford University Press, 1998.
8. J. H. Clark and D. Macquarri, *Handbook of Green Chemistry and Technology*, Wiley-Blackwell Publishing, 2002.
9. P. T. Anastas and M. M. Kirchoff, *Accounts of Chemical Research*, 2002, **35**, 686–694.
10. International Chemical Secretariat, SIN List, http://www.chemsec.org/what-we-do/sin-list, Accessed 13.03.2012, 2012.
11. J. R. Dodson, A. J. Hunt, H. L. Parker, Y. Yang and J. H. Clark, *Chemical Engineering and Processing*, 2012, **51**, 69–78.
12. M. Simon and C. Li, *Chemical Society Reviews*, 2012, **41**, 1415-1427.
13. J. H. Clark, V. Budarin, F. E. I. Deswarte, J. J. E. Hardy, F. M. Kerton, A. J. Hunt, R. Luque, D. J. Macquarrie, K. Milkowski, A. Rodriguez, O. Samuel, S. T. Tavener, R. J. White and A. J. Wilson, *Green Chemistry*, 2006, **8**, 853–860.
14. P. T. Anastas and R. L. Lankey, *Green Chemistry*, 2000, **2**, 289–295.
15. P. H. Nielsen, K. M. Oxenbøll and H. Wenzel, *The International Journal of Life Cycle Assessment*, 2006, **12**, 432-438.
16. U.S. Energy Information Administration, http://www.eia.gov/finance/markets/spot_prices.cfm, Accessed 18.03.2012, 2012.
17. M. Lancaster, *Green Chemistry, An Introductory Cast*, The Royal Society of Chemistry, Cambridge, 2010.
18. What is biomass?, http://www.biomassenergycentre.org.uk/portal/page?_pageid=76,15049&_dad=portal, Accessed 14.03.2012, 2012.
19. Taking Bio-based from promise to market, http://ec.europa.eu/enterprise/sectors/biotechnology/files/docs/bio_based_from_promise_to_market_en.pdf, Accessed 08.09.2014, 2014.
20. B. Vijayendran, *Bio-based chemicals: technology, economics and markets (White Paper)*, 2010.
21. M. Carus, D. Carrez, H. Kaeb, J. Ravenstijn and J. Venus, Level Playing Field for Bio-based Chemistry and Materials file:///C:/Users/Lucie%20Pfaltzgraff/Downloads/nova_Institut_policypaper_bio_economy_18042011_.pdf, Accessed 08.09.2014, 2014.

22. Accelerating the development of the market for Bio-Based products in Europe, http://ec.europa.eu/enterprise/policies/innovation/files/lead-market-initiative/prep_bio_en.pdf, Accessed 08.09.2014, 2014.
23. F. Fava, G. Totaro, L. Diels, M. Reis, J. Duarte, O. Beserra Carioca, H. M. Poggi-Varaldo and B. Sommer Ferreira, *New Biotechnology*, 2013, DOI: 10.1016/j.nbt.2013.1011.1003.
24. IEA, *IEA Bioenergy Annual Report 2009*, 2009.
25. F. Cherubini, *Energy Conversion and Management*, 2010, **51**, 1412-1421.
26. J. H. Clark, F. E. I. Deswarte and T. J. Farmer, *Biofuels, Bioproducts and Biorefining*, 2009, **3**, 72–90.
27. N. Brings Jacobsen, *Journal of Industrial Ecology*, 2006, **10**.
28. F. Chemat, M. Abert Vian and G. Cravotto, *International Journal of Molecular Sciences*, 2012, **13**, 8615–8627.
29. Crodarom, production facilities, http://www.crodarom.com/home.aspx?s=110&r=124&p=896, Accessed 03.03.12, 2012.
30. F. E. I. Deswarte, J. H. Clark, J. J. E. Hardy and P. M. Rose, *Green Chemistry*, 2006, **8**, 39–42.
31. T. Eggeman and R. T. Elander, *Bioresource Technology*, 2005, **96**, 2019–2025.
32. L. Tao, A. Aden, R. T. Elander, V. Ramesh Pallapolu, Y. Y. Lee, R. J. Garlock, V. Balan, B. E. Dale, Y. Kim, N. S. Mosier, M. R. Ladisch, M. Falls, M. T. Holtzapple, R. Sierra, J. Shi, M. A. Ebrik, T. Redmond, B. Yang, C. E. Wyman, B. Hames, S. Thomas and R. E. Warner, *Bioresource Technology*, 2011, **102**, 11105–11114.
33. Y. Fernández, A. Arenillas and J. A. Menéndez, in *Advances in Induction and Microwave Heating of Mineral and Organic Materials*, ed. S. Grundas, InTech, 2011, ch. 31, pp. 723-752.
34. V. L. Budarin, J. H. Clark, B. A. Lanigan, P. Shuttleworth, S. W. Breeden, A. J. Wilson, D. J. Macquarrie, K. Milkowski, J. Jones, T. Bridgeman and A. Ross, *Biorsource Technology*, 2009, **100**, 6064-6068.
35. OECD, Biofuel policies in OECD countries costly and ineffective, http://www.oecd.org/document/28/0,3343,en_2649_37401_41013916_1_1_1_1,00.html, Accessed 06.03.2012, 2012.
36. DEFRA- Sustainable businesses and resource efficiency, http://www.defra.gov.uk/environment/economy/business-efficiency/, Accessed 26.02.2012, 2012.
37. WRAP, 2009 Gate Fee report, http://www.wrap.org.uk/downloads/W504GateFeesWEB.c640bae1.7613.pdf, Accessed 23.03.12, 2012.
38. WRAP, 2011 Gate Fee Report, http://www.wrap.org.uk/recycling_industry/publications/wrap_gate_fees.html, Accessed 23.03.12, 2012.
39. EfW, landfill, RDF, http://www.letsrecycle.com/prices/efw-and-landfill, Accessed 30.06.2014, 2014.
40. S. Giljum, *Overconsumption? Our use of natural resources*, Friends of the Earth, 2009.
41. G. Laufenberg, B. Kunz and M. Nystroem, *Bioresource Technology*, 2003, **87**, 167-198.
42. Europe 2020, http://ec.europa.eu/europe2020/index_en.htm, Accessed 02.02.2012, 2012.
43. L. A. Pfaltzgraff, M. De bruyn, E. C. Cooper, V. Budarin and J. H. Clark, *Green Chemistry*, 2013, **15**, 307-314.
44. J. H. Litchfield, *Food Biotechnology*, 1987, **1**, 29-57.
45. B. Krieger-Brockett, *Research on Chemical Intermediates*, 1994, **20**, 39-49.
46. R. Luque, J. A. Menéndez, A. Arenillas and J. Cot, *Energy & Environmental Science*, 2012, **5**, 5481-5488.

47. D. Stuerga, in *Microwaves in Organic Synthesis*, eds. A. De La Hoz and A. Loupy, Wiley VCH, 2012, vol. 1, ch. 1, pp. 3-56.
48. in *Practical Microwave Synthesis for Organic Chemists*, eds. C. O. Kappe, D. Dallinger and S. S. Murphee, Wiley VCH, 2009.
49. Y. Coquerel, E. Colacino, J. Rodriguez, J. Martinez and F. Lamaty, in *Stereoselective Synthesis of Drugs and Natural Products*, eds. V. Andrushko and N. Andrushko, John Wiley & Sons, 2013, vol. 1, pp. 145-166.
50. C. Leonelli, P. Veronesi and G. Cravotto, in *Microwave-assisted Extraction for Bioactive Compounds: Theory and Practice*, eds. F. Chemat and G. Cravotto, Springer, 2012, ch. 1, pp. 1-14.
51. K. Hayat, X. Zhang, H. Chen, S. Xia, S. Jia and F. Zhong, *Separation and Purification Technology*, 2010, **73**, 371-376.
52. M. Nuchter, B. Ondruschka, W. Bonrath and A. Gum, *Green Chemistry*, 2004, **6**, 128-141.
53. C. O. Kappe, *Chemical Society Reviews*, 2008, **37**, 1127-1139.
54. A. A. Clifford, Change of water properties with temperature, http://www.criticalprocesses.com/Use%20of%20enthalpies%20to%20calculate%20energy%20needed.htm, Accessed 26.06.2014, 2014.
55. P. Lidstrom, J. Tierney, B. Wathey and J. Westman, *Tetrahedron*, 2001, **57**, 9225-9283.
56. J. D. Moseley and C. O. Kappe, *Green Chemistry*, 2011, **13**, 794-806.
57. R. Morschhauser, M. Krull, C. Kayser, C. Boberski, R. Bierbaum, P. A. Pushner, T. N. Glasnov and C. O. Kappe, *Green Processing and Synthesis*, 2012, **1**, 281-290.
58. D. Obermayer and C. O. Kappe, *Organic and Biomolecular Chemistry*, 2010, **8**, 114-121.
59. C. R. Strauss, in *Handbook of Green Chemistry and Technology*, eds. J. H. Clark and D. Macquarrie, Blackwell Science Ltd, Oxford, 2002, ch. 17, pp. 397-415.
60. A. De la Hoz, A. Diaz-Ortiz and A. Moreno, *Chemical Society Review*, 2005, **34**, 164-178.
61. M. J. Gronnow, R. J. White, J. H. Clark and D. J. Macquarrie, *Organic Process Research & Development*, 2005, **9**, 516–518.
62. T. Razzaq and K. C. Oliver, *ChemSusChem*, 2008, **1**, 123-132.
63. S. Perino-Issartier, J.-F. Maingonnat and F. Chemat, in *Alternatives to conventional Food Processing*, ed. A. Proctor, RSC, Cambridge, 2011, ch. 11, pp. 415-458.
64. M. Ferhat, B. Meklati, J. Smadja and F. Chemat, *Journal of Chromatography A*, 2006, **1112**, 121-126.
65. R. Meredith, *Engineers Handbook of Industrial Microwave Heating*, The Institution of Electrical Engineers, 1998.
66. B. A. Roberts and C. R. Straus, *Accounts of Chemical Research*, 2005, **38**, 653–661.
67. M. Pourbafrani, F. Talebnia, C. Niklasson and M. J. Taherzadeh, *Internatinal Journal of Molecular Science*, 2007, **8**, 777-787.
68. D. J. Macquarrie, J. H. Clark and E. Fitzpatrick, *Biofuels, Bioproducts & Biorefining*, 2012, **6**, 549–560.
69. J. Parfitt, M. Barthel and S. Macnaughton, *Philosophical transactions of the Royal Society of Biological Sciences*, 2010, **365**, 3065-3081.
70. J. Gustavsson, C. Cederberg, U. Sonesson, R. van Otterdijk and A. Meybeck, *Global food losses and food waste. Extend, causes and prevention.*, Food and Agriculture Organization of the United Nations, 2011.
71. A. Segrè and S. Gaiani, *Transforming Food Waste into a Resource*, RSC Publishing, Cambridge, 2012.
72. D. Gunders, *Wasted: How America is Losing Up to 40 Percent of Its Food from Farm to Fork to Landfill*, NRDC, 2012.

73. European Commission, Preparatory study on food waste across E.U.-27 for the European Commission, ec.europa.eu/environment/eussd/pdf/bio_foodwaste_report.pdf, Accessed 24.09.2014, 2014.
74. C. M. Galanakis, *Trends in Food Science & Technology*, 2013, **26**, 68-87.
75. A. Schieber, F. C. Stintzing and R. Carle, *Trends in Food Science & Technology*, 2001, **12**, 401-413.
76. C. O. Tuck, E. Pérez, I. T. Horváth, R. A. Sheldon and M. Poliakoff, *Science*, 2012, **337**, 695-699.
77. T. Fox and C. Fimeche, *Global Food. Waste Not, Want Not.*, Institution of Mechanical Engineers, 2013.
78. G. Barilla, S. Blanco, B. Buchner, A. Segré, J. Bloom, R. Wang, R. Cavallo, T. Stuart, M. Cedroni and D. Nierenberg, *Combating Waste- Defeating the Paradox of Food Waste*, Barilla CFN, 2013.
79. *Food Wastage Footprint- Impacts on Natural Resources*, Food and Agriculture Organization of the United Nations, 2013.
80. J. C. Buzby, J. Hyman, H. Stewart and H. F. Wells, *The Journal of Consumer Affairs*, 2011, **45**, 492–515.
81. *United Kingdom Food & Drink Processing Mass Balance*, C-Tech Innovation, 2004.
82. W. Skibar, G. Grogan, J. McDonald, M. Pitts, A. Higson, M. Moustras, E. Milsom, S. Turney and J. Whittall, *UK Expertise for Exploitation of Biomass-Based Platform Chemicals* Royal Society of Chemistry, 2009.
83. C. S. K. Lin, A. A. Koutinas, K. Stamatelatou, E. B. Mubofu, A. S. Matharu, N. Kopsahelis, L. A. Pfaltzgraff, J. H. Clark, S. Papanikolaou, T. H. Kwan and R. Luque, *Biofuels, Bioproducts and Biorefining*, 2014, DOI: 10.1002/bbb.1506.
84. J. K. Hughes, in *Renewable Raw Materials*, eds. R. Ulber, D. Sell and T. Hirth, Wiley-VCH, Weinheim, 1st edn., 2011, ch. 3, pp. 33-47.
85. Food Industry- Processed agricultural products, http://ec.europa.eu/enterprise/sectors/food/eu-market/processed-products/index_en.htm, Accessed 27.10.2012, 2012.
86. Recycling of catering and food waste, http://sciencesearch.defra.gov.uk/Default.aspx?Menu=Menu&Module=More&Location=None&Completed=0&ProjectID=17580, Accessed 18.07.2014, 2014.
87. *Data & Trends of the European Food and Drink Industry 2012*, Food Drink Europe, 2012.
88. *Facts & figures on the European Chemical Industry*, The European Chemical Industry Council, 2011.
89. R. Rathmann, A. Szklo and R. Schaeffer, *Renewable Energy*, 2010, **35**, 14–22.
90. G. T. Kroyer, *Lebensmittel-Wissenschaft und Technologie- Food Science and Technology*, 1995, **28**, 547–552.
91. B. Mahro and M. Timm, *Engineering in Life Science*, 2007, **7**, 457-468.
92. C. A. Zaror, *Food Control*, 1992, **3**, 190–199.
93. S. Djilas, J. Canadanovic-Brunet and G. Cetkovic, *Chemical Industry & Chemical Engineering Quaterly*, 2009, **15**, 191-202.
94. V. Ferreira-Leitão, L. M. Fortes Gottschalk, M. A. Ferrara, A. Lima Nepomuceno, H. B. Correa Molinari and E. P. S. Bon, *Waste and Biomass Valorization*, 2010, **1**, 65-76.
95. G. C. Matteson and B. M. Jenkins, *Bioresource Technology*, 2007, **98**, 3098–3105.
96. I. O. Okonko, T. A. Ogunnusi, F. E. Aloysius, O. D. Adejoye and O. A. Adewale, *Electronic Journal of Environmental, Agricultural and Food Chemistry*, 2009, **8**, 263-286.
97. C. Bartlett, *Mapping Waste in the Food and Drink Industry*, Oakdene Hollins Research & Consulting, 2010.

98. H. Møller, O. J. Hanssen, J. Gustavsson and K. Östergren, *Report on review of (food) waste reporting methodology and practice* Fusion, 2014.
99. O. J. Hanssen, Å. Stenmarck, P. Dekhtyar, C. O'Connor and K. Östergren *Review of EUROSTATs reporting method and statistics*, FUSION, 2013.
100. Statistics on the production of manufactured goods (prom), http://epp.eurostat.ec.europa.eu/portal/page/portal/prodcom/data/tables_excel, Accessed 02.06.2014, 2013.
101. PRODCOM survey quality and methods, file:///C:/Users/Lucie%20Pfaltzgraff/Downloads/qmiprodcomdec13finalforpub_tcm77-346329.pdf, Accessed 21.07.2014, 2014.
102. Quality report on Prodcom - 2012 annual data, http://epp.eurostat.ec.europa.eu/cache/ITY_SDDS/Annexes/prom_esms_an1.pdf, Accessed 09.09.2014, 2014.
103. Z. B. Maroulis and G. D. Saravacos, in *Food Plant Economics*, CRC Press, 1 edn., 2007, ch. 7, pp. 175-269.
104. Integrated Pollution Prevention and Control Reference Document on Best Available Techniques in the Food, Drink and Milk Industries., http://eippcb.jrc.ec.europa.eu/reference/BREF/fdm_bref_0806.pdf, Accessed 03.06.2014, 2014.
105. K. Hill and R. Höfer, in *Sustainable Solutions for Modern Economies*, ed. R. Höfer, Royal Society of Chemistry, 2009, ch. 9.1, pp. 167-237.
106. W. Russ and M. Schnappinger, in *Utilization of By-Products and Treatment of Waste in the Food Industry*, eds. V. Oreopoulou and W. Russ, 2007, ch. 1, pp. 1-13.
107. M. Chandrasekaran, ed., *Valorization of Food Processing By-Products*, CRC Press, 2012.
108. *Handbook for the Prevention and Minimisation of Waste and Valorisation of By-Products in European Agro-Food Industries*, AWARENET: Agro-Food Wastes Minimisation and Reduction Network, 2004.
109. Orange juice, raw density data, http://www.aqua-calc.com/page/density-table/substance/orange-blank-juice-coma-and-blank-raw, Accessed 18.07.2014, 2014.
110. A. M. Ramos and A. Ibarz, *Journal of Food Engineering*, 1998, **35**, 57-63.
111. Food Balance Sheets, http://www.fao.org/economic/the-statistics-division-ess/publications-studies/publications/food-balance-sheets/ar/, Accessed 17.07.2014, 2014.
112. *Citrus: World Markets and Trade for January 2014*, United States Department of Agriculture 2014.
113. J. A. Siles Lopez, Q. Li and I. P. Thompson, *Critical Reviews in Biotechnology*, 2010, **30**, 63-69.
114. P. Singh nee'Nigam and A. Pandey, eds., *Biotechnology for Agro-Industrial Residues Utilisation*, Springer, 2009.
115. R. P. Bates, J. R. Morris and P. G. Crandall, in *Principles and practices of small and medium scale fruit juice processing*, Food & Agriculture Organization, 2001, vol. 2014, ch. 11.
116. D. A. Kimball, *Citrus Processing, A Complete Guide*, Aspen Publication, 1999.
117. R. J. Braddock, *Handbook of Citrus By-Products and Processing Technology*, Wiley-Interscience, 1999.
118. P. Dugo, L. Mondello, L. Dugo, R. Rosanna Stancanelli and G. Dugo, *Journal of Pharmaceutical and Biomedical Analysis*, 2000, **24**, 147-154.
119. G. Dugo and A. D. Giacomo, eds., *Citrus- The genus Citrus*, Taylor & Francis, London, 2002.
120. *Private communication*.
121. E. W. Sample, *US20110305808 A1*, 2013.
122. K. Bevill, Freshly squeezed ethanol, Accessed 23.09.11, 2011.

123. N. Sahraoui, M. Abert Vian, M. El Maataoui, C. Boutekedjiret and F. Chemat, *Innovative Food Science and Emerging Technologies*, 2011, **12**, 163-170.
124. C. Talens, M. Castro-Giraldez, C. Bald and P. J. Fito, presented in part at the InsideFood Symposium, Leuven, 2013.
125. M.-C. Ralet, E. Bonnin and J.-F. Thibault, in *Polysaccharides And Polyamides in the Food Industry: Properties, Production, And Patents*, eds. A. Steinbüchel and S. Ki Rhee, Wiley-VCH, 2005, vol. 1.
126. F. R. Marin, C. Soler-Rivas, O. Benavente-Garcia, J. Castillo and J. A. Perez-Alvarez, *Food chemistry*, 2007, **100**, 736-741.
127. M. Pourbafrani, G. Forgacs, I. S. Horvath, C. Niklasson and M. Taherzadeh, *Bioresource Technology*, 2010, **101**, 4246-4250.
128. V. A. Bampidis and P. H. Robinson, *Animal Feed Science and Technology*, 2006, **128**, 175-217.
129. P. Gallezot, in *Catalysis for Renewables: From Feedstock to Energy Production*, eds. G. Centi and R. A. van Santen, Wiley-VCH, 2008, ch. 3, pp. 53-74.
130. ICIS, *D*-limonene impacted by events in Brazil and Asia, Accessed 23.09.11, 2011.
131. F. Kerton, in *Alternative Solvents for Green Chemistry*, ed. F. Kerton, Royal Society of Chemistry, 2009, ch. 5, pp. 97-115.
132. D. Hudac, Why is the D-limonene price so volatile?, http://www.fbcchem.com/prices/why-is-the-d-limonene-price-so-volatile-2/, Accessed 09.06.2014, 2014.
133. *Ullmann Encyclopedia of Industrial Chemistry*, **A11**.
134. What is limonene?, http://www.emulso.com/store.asp?pid=11250, Accessed 01.08.2011, 2011.
135. Y. Liu, J. Shi and T. A. G. Langrish, *Chemical Engineering Journal*, 2006, **120**, 203-209.
136. A. Fletcher, An economic alternative to pure pectin?, http://www.foodnavigator.com/Science-Nutrition/An-economic-alternative-to-pure-pectin, Accessed 24.07.2014, 2014.
137. J. Holck, A. T. J. Hotchkiss, A. S. Meyer, J. D. Mikkelsen and R. A. Rastall, in *Food Oligosaccharides: Production, Analysis and Bioactivity*, eds. F. J. Moreno and M. Luz Sanz, John Wiley & Sons, 2014, ch. 5, pp. 76-86.
138. S. Daniells, Low-grade apples may give high-grade pectin for industry, http://www.foodnavigator.com/Science-Nutrition/Low-grade-apples-may-give-high-grade-pectin-for-industry, Accessed 03.09.2013, 2013.
139. C. D. May, in *Thickening and Gelling Agents for Food*, ed. A. P. Imeson, Springer, 1997, ch. 10, pp. 230-261.
140. B. R. Thakur, R. K. Singh and A. K. Handa, *Critical Reviews In Food Science and Nutrition*, 1997, **37**, 47-73.
141. C. D. May, *Carbohydrate Polymer*, 1990, **12**, 79-99.
142. M. M. Bomgardner, *Chemical and Engineering News*, 2013, **91**, 20.
143. M. Hennessy, CP Kelco expands production into Brazil, http://www.foodnavigator-usa.com/Suppliers2/CP-Kelco-expands-production-in-Brazil, Accessed 28.07.2014, 2014.
144. R. B. Johnston, Citrus Pectin Peel Preparation, http://www.vincentcorp.com/content/citrus-pectin-peel-preparation, Accessed 24.07.2014, 2014.
145. R. J. Braddock and P. G. Crandall, *Journal of Food Science*, 1978, **43**, 1678-1679.
146. M. Kratchanova, E. Pavlova and I. Panchev, *Carbohydrate polymers*, 2004, **56**, 181-185.
147. M. Kratchanova, I. Panchev, E. Pavlova and L. Shtereva, *Carbohydrate polymers*, 1994, **25**, 141-144.
148. M. L. Fishman, H. K. Chau, P. Hoagland and K. Ayyad, *Carbohydrate research*, 2000, **323**, 126-138.

149. J. A. Manthey and K. Grohmann, *Journal of Agricultural and Food Chemistry*, 1996, **44**, 811-814.
150. E. Tripoli, M. La Guardia, S. Giammanco, D. Di Majo and M. Giammanco, *Food chemistry*, 2007, **104**, 466-479.
151. N. Balasundram, K. Sundram and S. Samman, *Food chemistry*, 2006, **99**, 191-203.
152. H. U. Endress, in *Renewable Resources for Functional Polymers and Biomaterials*, ed. P. A. Williams, RSC, 2011, ch. 8, pp. 210-260.
153. J. Londono-Londono, V. Rodrigues de Lima, O. Lara, A. Gil, T. B. Crecsynski Pasa, G. J. Arango and J. R. Ramirez Pineda, *Food Chemistry*, 2010, **119**, 81-87.
154. W. W. Widmer and A. M. Montanari, *Proceedings of the Florida State Horticultural Society*, 1994, **107**, 284-288.
155. S. A. El-Nawawi, *Carbohydrate Polymers*, 1995, **27**, 1-4.
156. R. H. Higby, *US 2421061 A*, 1947.
157. J. A. Manthey and K. Grohmann, *Journal of Agriculture and Food Chemistry*, 2001, **49**, 3268-3273.
158. Citrus Molasses, http://www.vincentcorp.com/content/citrus-molasses, Accessed 25.07.2014, 2014.
159. G. Aravantinos-Zafiris, V. Oreopoulou, C. Tzia and C. D. Thomopoulos, *Lebensmittel-Wissenschat & Technologie*, 1994, **27**, 468-471.
160. P. Ozmen and S. Aslanzadeh, Master of Science, University of Boras, 2009.
161. M. Pourbafrani, Doctor of Philosophy, Chalmers University of Technology, 2010.
162. K. Grohman, R. Cameron, Y. Kim, W. Widmer and G. Luzio, *Journal of Chemical Technology and Biotechnology*, 2012.
163. C. Scott-Thomas, C P Kelco invests a futher 8 million euros to expand pectin production in Denmark, http://www.foodnavigator.com/Business/CP-Kelco-invests-a-further-8m-to-expand-pectin-production-in-Denmark, Accessed 06.08.2014, 2014.
164. R. H. Walter, ed., *The Chemistry and Technology of Pectin*, Academic Press, Inc., 1991.
165. M. Pinelo, C. Ferrer, A. S. Meyer and G. Jonsson, *Separation and Purification Technology*, 2012, **85** 54-60.
166. J. Harholt, A. Suttangkakul and H. V. Scheller, *Plant Physiology*, 2010, 384-395.
167. A. C. J. Voragen, C. Rolin, B. U. Marr, I. Challen, A. Riad, R. Lebbar and S. H. Knutsen, *Ullmann's Encyclopedia of Industrial Chemistry*, 2003.
168. *Polysaccharides in food*, Butterworths, 1979.
169. B. B. Koubala, L. I. Mbome, G. Kansci, F. Tchouanguep Mbiapo, M.-J. Crepeau, J.-F. Thibault and M.-C. Ralet, *Food Chemistry*, 2008, **106**, 1202–1207.
170. L. D. Melton and B. G. Smith, *Current Protocols in Food Analytical Chemistry*, 2001.
171. M. L. Fishman and H. K. Chau, *US 6,143,337*, 2000.
172. J. A. Donaghy and A. M. McKay, *Bioresource technology*, 1994, **47**, 25-28.
173. M. L. Fishman, H. K. Chau, P. D. Hoagland and A. T. Hotchkiss, *Food Hydrocolloids*, 2006, **20**, 1170-1177.
174. M. Manabe, J. Naohara, T. Sato and J. Okada, *Nippon Shokuhin Kogyo Gakkaishi*, 1988, **35**, 497-501.
175. M. Kratchanova, E. Pavlova, I. Panchev and C. Kratchanov, in *Pectins and Pectinases*, eds. J. Visser and A. G. J. Voragen, Elsevier Science, 1996, vol. 14, ch. 941, pp. 941-946.
176. F. Bagherian, F. K. Ashtiani, A. Fouladitajar and M. Mohtashamy, *Chemical Engineering and Processing*, 2011, **50**, 1237-1243.
177. X. Guo, D. Han, H. Xi, L. Rao, X. Liao, X. Hu and J. Wu, *Carbohydrate Polymers*, 2012, **88**, 441-448.

178. L. Zhongdong, W. Guohua, G. Yunchang and J. F. Kennedy, *Carbohydrate Polymer*, 2006, **64**, 548-552.
179. *Food Chemicals Codex Monographs*, National Academic Press, Washington, 1981.
180. U. Kalapathy and A. Proctor, *Food Chemistry*, 2001, **73**, 393-396.
181. A. Fellah, P. Anjukandi, M. R. Waterland and M. A. K. Williams, *Carbohydrate Polymers*, 2009, **78**, 847-853.
182. M. A. Monsoor, U. Kalapathy and A. Proctor, *Food Chemistry*, 2001, **74**, 233-238.
183. E. Westerlund, P. Aman, R. E. Andersson and R. Andersson, *Carbohydrate Polymers*, 1991, **14**, 179-187.
184. Z. Kostalova, Z. Hromadkova, A. Ebringerova, M. Polovka, T. E. Michaelsen and B. S. Paulsen, *Industrial Crops and Products*, 2013, **41**, 127-133.
185. D. A. T. Southgate, *Determination of Food Carbohydrates*, Elsevier, 1991.
186. Y. Brummer and S. W. Cui, in *Food Carbohydrates: Chemistry, Physical Properties and Applications*, ed. S. W. Cui, Taylor & Francis, 2005, ch. 2, pp. 67-104.
187. M. Corredig, W. Kerr and L. Wicker, *Food Hydrocolloids*, 2000, **14**, 41-47.
188. H. G. Barth, *Journal of liquid chromatography*, 1980, **3**, 1481-1496.
189. Y. Georgiev, M. Ognyanov, I. Yanakieva, V. Kussovski and M. Kratchanova, *Journal of Bioscience and Biotechnology*, 2012, **1**, 223-233.
190. P. Sriamornsak, *Silpakorn University International Journal*, 2003, **3**, 206-228.
191. T. Doco, M. A. O'Neill and P. Pellerin, *Carbohydrate Polymers*, 2001, **46**, 249-259.
192. J. Burana-osot, N. Soonthornchareonnon, A. Chaidedgumjorn, S. Hosoyama and T. Toida, *Carbohydrate Polymer*, 2010, **81**, 461-465.
193. L. Catoire, R. Goldberg, M. Pierron, C. Morvan and C. Herve du Penhoat, *European Biophysics Journal*, 1998, **27**, 127-136.
194. Q. Wang and S. W. Cui, in *Food Carbohydrates: Chemistry, Physical Properties and Applications*, ed. S. W. Cui, Francis & Taylor, 2005, ch. 4.
195. J. Maynard, in *Methods in Food Analysis: Applied to Plant Products*, Elsevier, 2012, ch. 4, pp. 87-114.
196. R. Gnanasambandam and A. Proctor, *Food Chemistry*, 2000, **68**, 327-332.
197. A. K. Chatjgakis, C. Pappas, N. Proxenia, O. Kalantzi and M. Polissiou, *Carbohydrate Polymers*, 1998, **37**, 395-408.
198. G. D. Manrique and F. M. Lajolo, *Postharvest Biology and Technology*, 2002, **25**, 99-107.
199. A. Kumar and G. S. Chauhan, *Carbohydrate Polymers*, 2010, **82**, 454-459.
200. R. Gnanasambandam and A. Proctor, *Food Chemistry*, 1999, **65**, 461-467.
201. Y. Tamaki, T. Konishi, M. Fukuta and M. Tako, *Food Chemistry*, 2008, **107**, 352-361.
202. S. Nunn, Advanced ATR correction algorithm, http://www.thermo.com/eThermo/CMA/PDFs/Product/productPDF_57540.PDF, Accessed 25.10.2013, 2013.
203. M. V. Marcon, P. I. B. Carneiro and W. E. Beleski-Carneiro, *Annals of Magnetic Resonance*, 2005, **4**, 56-63.
204. M. Iacomini, R. V. Serato, G. L. Sassaki, L. Lopes, D. F. Buchi and P. A. J. Gorin, 2005, **76**, 676-683.
205. J. Li, L. Ji, L. Suna, N. Weia, J. Zhua, X. Chenb, G. Taia and Y. Zhou, *Carbohydrate polymers*, 2012, **87**, 551–556.
206. A. Sinitsya and J. Copiková, *Journal of Carbohydrate Chemistry*, 1998, **17**, 279-292.
207. M.-A. Bornik and L. W. Kroh, *Journal of Agricultural and Food Chemistry*, 2013, **61**, 3494–3500.
208. J. Baeza and J. Freer, in *Wood and Cellulosic Chemistry*, eds. D. N.-S. Hon and N. Shiraishi, CRC Press, 2 edn., 2000, pp. 275-384.

209. C. A. Marsh, in *Glucuronic acid Free and Combined: Chemistry, Biochemistry, Pharmacology, and Medicine*, ed. G. Dutton, Elsevier, 2012, ch. 1, pp. 4-136.
210. H. Garna, N. Mabon, K. Nott, B. Wathelet and M. Paquot, *Food Chemistry*, 2006, **96**, 477-484.
211. H. Garna, N. Mabon, B. Wathelet and M. Paquot, *Journal of Agricultural and Food Chemistry*, 2004, **52**, 4652–4659.
212. B. L. Rheus, in *Food Analysis*, ed. S. S. Nielsen, Springer Science & Business Media, 2010, ch. 4, pp. 445-455.
213. P. Conte, R. Spaccini and A. Piccolo, *Progress in Nuclear Magnetic Resonance Spectroscopy*, 2004, **44**, 215-223.
214. D. D. Laws, H.-M. L. Bitter and A. Jerschow, *Angewandte Chemie International Edition*, 2002, **41**, 3096–3129.
215. A. Synytsya, J. Čopikova and J. Brus, *Czech Journal of Food Sciences*, 2003, **21**, 1-12.
216. S. Nurdjanah, J. Hook, J. Paton and J. Paterson, *European Journal of Food Research & Review*, 2013, **3**, 16-37.
217. R. M. B. O. Duarte and A. C. Duarte, in *NMR Spectroscopy: A Versatile Tool for Environmental Research*, John Williams & Sons, 2014, pp. 193-206.
218. R. Fernandez-Prini, in *High-Temperature Aqueous Solutions: Thermodynamic Properties*, CRC Press, 1991, ch. 1, pp. 1-25.
219. *Release on the Ionization Constant of H_2O* International Association for the Properties of Water and Steam Lucerne, Switzerland 2007.
220. S. Yeoh, J. Shi and T. A. G. Langrish, *Desalination*, 2008, **218**, 229-237.
221. M. Izydorczyk, in *Food Carbohydrates: Chemistry, Physical Properties, and Applications*, ed. S. W. Cui, CRC Press, 2005, ch. 1.
222. J. Guan, Q. Cao, X. Guo and X. Mu, *Computational and Theoretical Chemistry*, 2011, **963**, 453-462.
223. M. L. Fishman, D. T. Gillespie, S. M. Sondey and R. A. Barford, *Journal of Agricultural and Food Chemistry*, 1989, **37**, 584-591.
224. M. L. Fishman, H. K. Chau, D. R. Coffin and A. T. J. Hotchkiss, in *Advances in Pectin and Pectinase Research*, ed. F. Voragen, Kluwer Academic Publishers, 2003, pp. 107-122.
225. H. Kastner, U. Einhorn-Stoll and B. Senge, in *Gums and Stabilisers for the Food Industry*, eds. P. A. Williams and G. O. Phillip, RSC, 2012, vol. 16, pp. 191-197.
226. V. Vaclavik, V. A. Vaclavik and E. W. Christian, eds., *Essentials of Food Science*, Springer, 2007.
227. V. A. Vaclavik and E. W. Christian, in *Essentials of Food Science*, Springer, 3rd edn., 2008, ch. 5, pp. 69-80.
228. A. G. J. Voragen, F. Voragen, H. Schols and R. Visser, *Advances in Pectin and Pectinase Research*, Kluwer Academic, 2003.
229. J. F. Thibault and M. C. Ralet, in *Advances in Pectin and Pectinase Research*, eds. A. G. J. Voragen, F. Voragen, H. Schols and R. Visser, Kluwer, 2003, ch. 7, pp. 91-106.
230. *Pectin standardization- Final Report of the IFT Committee*, Institute of Food Technologists, Chicago, 1944.
231. R. E. Cox and R. H. Higby, *Food Industries*, 1944, **16**.
232. *Gelling Properties of High Methylester Classic Apple Pectin and Classic Citrus Pectins: Comparison in Dependence from Degree of Esterification and Setting Time.*, Herbstreith & Fox, 2005.
233. A. J. MacDougall, P. W. Needs, N. M. Rigby and S. G. Ring, *Carbohydrate Research*, 1996, **923**, 235-249.
234. C. Lofgren, S. Guillotin, H. Evenbratt, H. Schols and A.-M. Hermansson, *Biomacromolecules*, 2005, **6**, 646-652.
235. Y. Yang, G. Zhang, Y. Hong, Z. Gu and F. Fang, *Food Hydrocolloids*, 2013, **32**, 228-234.

236. C. W. Tibbits, A. J. MacDougall and S. G. Ring, *Carbohydrate Research*, 1998, **310**, 101-107.
237. W. G. T. Willats, C. Orfila, G. Limberg, H. C. Buchholt, G.-J. W. M. van Alebeek, A. G. J. Voragen, S. E. Marcus, T. M. I. E. Christensens, J. D. Mikkelsen, B. S. Murray and J. P. Knox, *The Journal of Biological Chemistry*, 2001, **276**, 19404-19413.
238. R. Kohn and P. Kovàc, *Chemicke Zvesti*, 1978, **32**, 478—485.
239. A. Slavov, C. Garnier, M.-J. Crépeau, S. Durand, J.-F. Thibault and E. Bonnin, *Carbohydrate Polymers*, 2009, **77**, 876-884.
240. *The Specialists for Pectin*, Herbstreith & Fox, 2009.
241. S. L. Birla, S. Wang, J. Tang and G. Tiwari, *Journal of Food Engineering*, 2008, **89**, 390-398.
242. I. Fraeye, T. Duvetter, E. Doungla, A. Van Loey and M. Hendrickx, *Trends in Food Science*, 2010, **21**, 219-228.
243. Z. Zhang, N. M. Khan, K. M. Nunez, E. K. Chess and C. M. Szabo, *Analytical Chemistry*, 2012, **84**, 4104-4110.
244. H. Bagherian, F. Z. Ashtiani, A. Fouladitajar and M. Mohtashamy, *Chemical Engineering and Processing*, 2011, **50**, 1237-1243.
245. B. M. Yapo, P. Lerouge, J.-F. Thibault and M.-C. Ralet, *Carbohydrate Polymers*, 2007, **69**, 426-435.
246. B. Min, J. Lim, K.-G. Lee, S. H. Lee and S. Lee, *Bioresource Technology*, 2011, **102**, 3855-3860.
247. J. A. Lopes da Silva and M. P. Goncalves, *Carbohydrate polymers*, 1994, **24**, 235-245.
248. S. Burt, *International Journal of Food Microbiology*, 2004, **94**, 223 – 253.
249. F. Bakkali, S. Averbeck, D. Averbeck and M. Idaomar, *Food and Chemical Toxicology*, 2008, **46**, 446–475.
250. P. E. Shaw, *Journal of Agricultural and Food Chemistry*, 1979, **27**, 246-257.
251. D. Bégin and M. Gérin, *La substitution des solvants par le D-limonène - IRSST*, IRSST, Montréal, 2000.
252. P. K. Mamidipally and S. X. Liu, *European Journal of Lipid Science and Technology*, 2004, **106**, 122–125.
253. C. Hoque, *Chemical and Engineering News*, 2013, **91**, 19-20.
254. S. Li, C.-Y. Lo and C.-T. Ho, *Journal of Agricultural and Food Chemistry*, 2006, **54**, 4176–4185.
255. S. Li, T. Lambros, Z. Wang, R. Goodnow and C.-T. Ho, *Journal of Chromatography B*, 2007, **846**, 291-297.
256. K. Alfonsi, J. Colberg, P. J. Dunn, T. Fevig, S. Jennings, T. A. Johnson, H. P. Kleine, C. Knight, M. A. Nagy, D. A. Perry and M. Stefaniak, *Green Chemistry*, 2008, **10**, 31-36.
257. P. G. Jessop, *Green Chemistry*, 2011, **13**, 1391-1398.
258. M. A. Ferhat, B. Y. Meklati and F. Chemat, *Flavour and Fragrance Journal*, 2007, **22**, 494-504.
259. A. Ferhat, C. Ginies, M. Romdhane and F. Chemat, *Journal of Chromatography A*, 2009, **1216**, 5077-5085.
260. N. Tigrine-Kordjani, B. Y. Meklati and F. Chemat, *International Journal of Aromatherapy*, 2006, **16**, 141-147.
261. J. R. Schmick and N. E. Leadbeater, in *Microwave heating as a tool for Sustainable Chemistry*, ed. N. E. Leadbeater, CRC Press, 2011, ch. 1, pp. 1-24.
262. C. E. Odio, D-limonene recovery, Lakeland, Florida, U.S.A. , 1996.
263. P. Mengal and B. Mompon, *EP0698076B1*, 1998.
264. R. A. Jones, *US7060313 B2*, 2006.
265. M. S. Ladaniya, *Citrus Fruit Biology, Technology and Evaluation*, Elsevier, 2008.
266. A. Farhat, A. S. Fabiano-Tixier, M. El Maataoui, J. F. Maingonnat, M. Romdhane and F. Chemat, *Food Chemistry*, 2011, **125**, 255-261.

267. T. M. Attard, B. Watterson, V. L. Budarin, J. H. Clark and A. J. Hunt, *New Journal of Chemistry*, 2014, **38**, 2278-2283.
268. Inside Florida Chemicals, http://www.floridachemical.com/whatisd-limonene.htm, Accessed 14.02.2014, 2014.
269. Food grade vs. high purity, http://www.greenterpene.com/Food_Grade_vs_High_Purity_d_Limonene_s/50.htm, Accessed 13.02.2014, 2014.
270. N. Bousbia, M. A. Vian, M. A. Ferhat, B. Y. Meklati and F. Chemat, *Journal of Food Engineering*, 2009, **90**, 409-413.
271. C. O. Green, A. O. Wheatley, A. U. Osagie, E. Y. S. A. Morrison and H. N. Asemota, *Biomedical Chromatography*, 2007, **21**, 48-54.
272. G. Gattuso, D. Barreca, C. Gargiulli, U. Leuzzi and C. Caristi, *Molecules*, 2007, **12**, 1641-1673.
273. D. B. Haytowitz, S. Bhagwat and J. M. Holden, *Procedia Food Science*, 2013, **2**, 46-51.
274. Y.-H. Lee, A. L. Charles, H.-F. Kung, C.-T. Ho and T.-C. Huang, *Industrial Crops and Products*, 2010, **31**, 59–64.
275. S. Li, M.-H. Pan, C.-Y. Lo, D. Tan, Y. Wang, F. Shahidi and C.-T. Ho, *Journal of Functional Foods*, 2009, **1**, 2-12.
276. M. Stobiecki, *Phytochemistry*, 2000, **54**, 237-256.
277. R. M. Uckoo, G. K. Jayaprakasha and B. S. Patil, *Separation and Purification Technology*, 2011, **81**, 151–158.
278. Y. Choi, Y. Kim, H. Ham, Y. Park, H.-S. Jeong and J. Lee, *Journal of Agricultural and Food Chemistry*, 2011, **59**, 12843–12849.
279. M. S. Kim, H. J. Hur, D.-Y. Kwon and J.-T. Hwang, *Molecular and Cellular Endocrinology*, 2012, **358**, 127-134.
280. R. M. Uckoo, G. K. Jayaprakasha and B. S. Patil, in *Emerging Trends in Dietary Components for Preventing and Combating Disease*, ACS, 2012, ch. 1, pp. 3-15.
281. M. D. Luque de Castro and F. Priego-Capote, *Journal of Chromatography A*, 2010, **1217**, 2383-2389.
282. E. de Hoffmann and V. Stroobant, in *Mass Spectrometry Principles and Applications*, Wiley, 3rd edn., 2007, ch. 1, pp. 15-83.
283. N. B. Cech and C. G. Enke, *Mass Spectrometry Reviews*, 2001, **20**, 362– 387.
284. D. C. Harris, in *Quantitative Chemical Analysis*, W. H. Freeman and Company, 4 edn., 1995, ch. 23, pp. 655-698.
285. B. Liu and Z. Zhang, *RSC Advances*, 2013, **3**, 12313-12319.
286. G. Raman, G. K. Jayaprakasha, M. Cho, J. Brodbelt and B. S. Patil, *Separation and Purification Technology*, 2005, **45**, 147-152.
287. B. Subramaniam, R. A. Rajewski and K. Snavely, *Journal of Pharmaceutical Sciences*, 1997, **86**, 885-890.
288. X. Wang, F. Li, H. Zhang, Y. Geng, J. Yuan and T. Jiang, *Journal of Chromatography A*, 2005, **1090**, 188-192.
289. M. Saito, *Journal of Bioscience and Bioengineering*, 2013, **115**, 590-599.
290. F. Sahena, I. S. M. Zaidul, S. Jinap, A. A. Karim, K. A. Abbas, N. A. N. Norulaini and A. K. M. Omar, *Journal of Food Engineering*, 2009, **95**, 240–253.
291. M. Arshadi, A. J. Hunt and J. H. Clark, *RSC Advances*, 2012, **2**, 1806-1809.
292. L. Dolak, in *Today's chemist at work*, American Chemical Society, 2004, vol. 47-48.
293. A. B. Osuna, A. Serbanovic and M. N. da Ponte, in *Green Separation Processes*, eds. C. A. M. Afonso and J. G. Crespo, Wiley-VCH, 2005, pp. 207-218.
294. P. Morin, A. Gallois, H. Richard and E. Gaydou, *Journal of Chromatography*, 1991, **586**, 171-176.
295. Z. Liu, S. Zhao, R. Wang and G. Yang, *Journal of Chromatographic Science*, 1999, **37**, 155-158.
296. Y. Nogata, K. Sakamoto, H. Shiratsuchi, T. Ishii, M. Yano and H. Ohta, *Bioscience, Biotechnology, and Biochemistry* 2006, **70**, 178-192.

297. A. Garg, S. Garg, L. J. D. Zaneveld and A. K. Singla, *Phytotherapy research*, 2001, **15**, 655-669.
298. A. Di Mauro, B. Fallico, A. Passerini, P. Rapisarda and E. Maccarone, *Journal of Agricultural and Food Chemistry*, 1999, **47**, 4391-4397.
299. I. Erlund, *Nutrition Research*, 2004, **24**, 851-874.
300. T. Inoue, S. Tsubaki, K. Ogawa, K. Onishi and J.-I. Azuma, *Food Chemistry*, 2010, **123**, 542-547.
301. D. H. Williams and I. Fleming, *Spectroscopic Methods in Organic Chemistry*, McGraw-Hill Higher Education, New York, 2008.
302. N. Aghel, Z. Ramezani and S. Beiranvand, *Pakistan Journal of Biological Sciences*, 2008, **11**, 2451-2453.
303. S. B. Etcheverry, E. G. Ferrer, L. Naso, J. Rivadeneira, V. Salinas and P. A. M. Williams, *Journal of Biological Inorganic Chemistry*, 2008, **13**, 435-447.
304. J. Fan, M. De bruyn, V. L. Budarin, M. J. Gronnow, P. S. Shuttleworth, S. Breeden, D. J. Macquarrie and J. H. Clark, *Journal of the American Chemical Society*, 2013, **135**, 11728–11731.
305. H. H. Sun, J. B. Wooten, W. S. J. Ryan and G. H. Bokelman, *Carbohydrate Polymer*, 1987, **7**, 143-158.
306. A. J. Gordon, G. J. A. Ryle, D. F. Mitchell and C. E. Powell, *Journal of Experimental Botany*, 1982, **33**, 241-250.
307. J. N. BeMiller, in *Food Analysis Laboratory Manual*, ed. S. S. Nielsen, Springer, 2 edn., 2010, ch. 10, pp. 147-178.
308. Mono and disaccharides, by GC and GC-MS, https://sites.google.com/site/masonaco/Home/applications/services-offered-2/gc-sugars, Accessed 09.09.2013, 2013.
309. T. Doco, M. A. O'Neill and P. Pellerin, *Carbohydrate Polymers*, 2001, **46**, 249-259.