Cosmeceutical potential of apple pomace phenolic compounds: development of a natural-based dermal hydrogel as proof of concept

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Abstract

Currently, there is an evident trend towards incorporating natural-based ingredients in dermal formulations. In addition to the growing consumers' acceptance, these natural ingredients are not associated with such a high number of side effects as in the case of most artificial components. Likewise, and following a circular economy approach, there is an overall attempt to find added-value applications among materials that would be otherwise discarded or considered as waste. Apple pomace might be considered as a striking example of these industrial by-products.

In fact, apple pomace remaining from cider and juice pressing industries represents a competitive source of a large number of phenolic compounds. Interestingly, the major part of these bioactive compounds is found in apple peel and remain in apple pomace after the pressing procedure. Phenolic compounds present in apple pomace (*e.g.*, hydroxycinnamic acids, flavonoids and di-hydrochalcones) display health promoting activities, mainly based on their antioxidant, anti-inflammatory and antimicrobial properties. Therefore, they seem to be a promising cosmeceutical aimed to promote the health and beauty of skin. In addition, apple pomace has a high concentration of pectin, which can be used for its jellifying properties.

This research study focused in the evaluation of bioactive and chemical characterization of different apple pomace extracts. Special attention was dedicated to their phenolic profile, as well as their antioxidant and antimicrobial (specifically against different bacteria present on the skin, *e.g.*, *Propionibacterium acnes*, which is linked to acne). The best extract was the mixture of ethanol:water (80:20), which proved to have the highest antimicrobial potential against *P. acnes*. Accordingly, this extract was later incorporated in a novel dermal hydrogel, where pectin, extracted from apple pomace by hot acid extraction, was used as jellifying agent. This hydrogel was further evaluated for its bioactivity, revealing the maintenance of antioxidant and antibacterial activity of the incorporated extract.

Keywords: apple pomace; phenolic compounds; natural cosmeceuticals; *P. acnes*; pectin; bioactivity.

Resumen

Hoy en día existe una tendencia evidente hacia la incorporación de ingredientes de origen natural en formulaciones cosméticas. Además de la aceptación creciente de este tipo de productos por parte de los consumidores, estos ingredientes naturales no están asociados con tantos efectos secundarios como en el caso de la mayoría de componentes artificiales. Al mismo tiempo, teniendo en cuenta el enfoque de la economía circular, existe un interés general por encontrar aplicaciones de valor añadido a materiales que de otra manera serían desechados o considerados como desperdicios. La magaya de manzana podría considerarse como un ejemplo notable de estos subproductos industriales. De hecho, la magaya procedente de las industrias de sidra y zumo representa una fuente competitiva de compuestos fenólicos. Curiosamente, la mayor parte de estos compuestos bioactivos se encuentran en la piel de la manzana y permanecen en la magaya tras el prensado. Los compuestos fenólicos presentes en la magaya (p. ej., ácidos hidroxicinámicos, flavonoides y dihidrochalconas) presentan actividades beneficiosas para la salud, principalmente debido a sus propiedades antioxidantes, antiinflamatorias y antimicrobianas. Por lo tanto, parecen tener un gran potencial como cosmecéuticos destinados a promover la salud y belleza de la piel. Además, la magaya presenta una alta concentración de pectina, susceptible de ser usada por sus propiedades gelificantes.

Este trabajo se centró en la evaluación bioactiva y química de diferentes extractos de magaya. Una atención especial se ha dedicado a su perfil fenólico, así como a su actividad antioxidante y antimicrobiana (en específico contra bacterias presentes en la piel como es el caso de *Propionibacterium acnes*, que está relacionada con la aparición del acné). El mejor extracto fue la mezcla de etanol:agua (80:20), que resultó tener el mayor potencial antimicrobiano frente a *P. acnes*. En consecuencia, este extracto se incorporó posteriormente a un nuevo hidrogel dérmico, donde se utilizó pectina, extraída de la magaya mediante hidrólisis ácida, como agente gelificante. Este hidrogel fue después evaluado por su bioactividad, demostrando la manutención de la actividad antioxidante y antibacteriana del extracto incorporado.

Palabras clave: magaya; compuestos fenólicos; cosmecéuticos naturales; *P. acnes*; pectina, bioactivida

Resumo

Atualmente, há uma tendência evidente para a incorporação de ingredientes de origen natural em formulações dérmicas. Além da crescente aceitação por parte dos consumidores, esses ingredientes naturais não estão associados a um número tão grande de efeitos colaterais como no caso da maioria dos componentes artificiais. Da mesma forma, e seguindo uma abordagem de economia circular, tem havido uma tentativa geral de encontrar aplicações de valor agregado entre materiais que seriam de outra forma seriam rejeitados. O bagaço de maçã pode ser considerado como um exemplo notável desses subprodutos industriais. De fato, o bagaço de maçã proveniente das indústrias produtoras de cidra e sumo representa uma fonte competitiva de compostos fenólicos. Curiosamente, a maior parte desses compostos bioativos é encontrada na casca da maçã e permanece no bagaço de maçã após o procedimento de prensagem. Os compostos fenólicos presentes no bagaço de maçã (p. ex., ácidos hidroxicinâmicos, flavonóides e di-hidrochalconas) exibem atividades benéficas para a saúde, principalmente debido ás suas propriedades antioxidantes, antiinflamatórias e antimicrobianas. Com isto, tal produto parece ser um promissor cosmecêutico destinado a promover a saúde e a beleza da pele. Além disso, o bagaço de maçã contém uma elevada concentração de pectina, a qual se recorre frequentemente devido às suas propriedades gelificantes. Este estudo teve como objetivo avaliar a caracterização bioativa e química de diferentes extratos de bagaço de maçã com ênfase ao seu perfil fenólico, bem como a sua actividade antioxidante e antimicrobiana (especificamente contra diferentes bactérias presentes na pele, por exemplo, *Propionibacterium acnes*, que está ligado à acne). O melhor extrato foi a mistura de etanol: água (80:20), que provou ter o maior potencial antimicrobiano contra *P. acnes*. Assim, este extrato foi posteriormente incorporado em um novo hidrogel dérmico, onde a pectina, extraída do bagaço de maçã por hidrolisis ácida, foi utilizada como agente gelificante. Este hidrogel foi ainda avaliado quanto à sua bioatividade, revelando a manutenção da atividade antioxidante e antibacteriana do extrato incorporado.

Palavras-chave: bagaço de maçã; compostos fenólicos; cosmecêuticos naturais; P. acnes; pectina; bioatividade.

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List of abbreviations

AIJN: European Fruit Juice Association

COXs: Cyclooxygenases

DAD: Diode array detector

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

DPPH: 2,2-diphenyl-1-picrylhydrazyl

FAO: Food and Agriculture Organization of the United Nations

FDA: Food and Drug Administration

EMA: European Medicines Agency

GalA: Galacturonic acid

GSH: Glutathione

HGA: Homogalacturonan

HPLC: High-performance liquid chromatography

HPLC-RI: High-performance liquid chromatography - refractive index detector

iNOS: Inducible nitric oxide synthase

INT: *p*-Iodonitrotetrazolium chloride

LC: Liquid chromatography

LC-DAD-ESI/MS^n: Liquid cromatography with a diode array detector coupled to an electrospray ionization mass detector

LPS: Lipopolysaccharide

MHB: Muller-Hinton broth

MIC: Minimal inhibitory concentration

MRSA: Methicillin Resistant Staphylococcus Aureus

NED: *N*-(1-naphthyl) ethylenediamine dihydrochloride

NO: Nitric oxide

PGs: Prostaglandins

ROS: Reactive oxygen species

rpm: Rotations per minute

TBS: Typtic soy broth

UV-Vis: Ultraviolet-visible

I. Introduction

The plant kingdom has been a source of natural substances with potential application in protecting, healing or keeping the beauty of human skin since ancient times. In fact, herbal and seed extracts, or aromatized waters, were already used as cosmetic ingredients in ancient oriental cultures and also in the Palaeolithic (Bandoni, Ferraro, Martino, & Naidic, 2012). Nowadays, there is a growing association between beauty, health and well-being, which has led to a great increase in the so-called "natural cosmetics", with Europe and the USA leading the way (Bandoni et al., 2012). Although the natural cosmetic market is still relatively small, it is growing much faster than the overall cosmetic industry (Joshi & Pawar, 2015; ProFound, Jones, & Duerbeck, 2008)

The mainstream cosmetics contain a vast number of chemicals, most of which are not under the regulatory purview of the Food and Drug Administration (FDA) or the European Medicines Agency (EMA). Only a few of these chemicals (*e.g.*, parabens, phthalates, polycyclic aromatic hydrocarbons, siloxanes and triclosan) have been assessed for their potential risk to human health (Chow & Mahalingaiah, 2016). Some of these compounds have demonstrated dermal absorption and are likely to work as endocrine disruptors causing cancerous tumours, birth defects, and other developmental disorders (Rattan et al., 2017).

In contrast, herbal and natural products contain several potentially beneficial active compounds that may be useful in the treatment of different skin disorders such as wounds, acne, premature skin aging or eczema (Bandoni et al., 2012; Mukherjee, Maity, Nema, & Sarkar, 2011).

The increased awareness of consumers about health concerns associated with conventional cosmetics, together with the need for a better scientific understanding of the physiology of the skin, led to a growing demand for cosmetic products with innovative natural ingredients intended to promote the health and beauty of skin (Charles Dorni, Amalraj, Gopi, Varma, & Anjana, 2017).

The application of natural ingredients might be even better when using widely available natural matrices, especially if these are possible to be obtained at low cost. Such demand could be effectively fulfilled by the fruit by-products that remain from their industrial processing. Actually this approach could solve an ecological problem, since the growth of the horticultural sector has led to the generation of large amounts of fruit and vegetable wastes (Gullón, Garrote, Alonso, & Parajó, 2007). These bio-residues deserve special attention when originated from products with high dimensionality, such as observed with apple. A considerable part of apple production is processed to obtain juice or cider, originating great volumes of residues, known as apple pomace (Krawitzky et al., 2014). Although being typically considered as waste, this remaining material is rich in valuable compounds, such as polyphenols, vitamins, carotenoids, or fibres (Barbulova, Colucci, & Apone, 2015). Furthermore, the polyphenols in apple are mainly located in the peel (Krawitzky et al., 2014), thereby being highly retained in apple pomace, which raises its interest as a natural low-cost source of bioactive compounds.

For all the above, cosmetic industry can certainly represent a remunerable solution to valorise disposable by-products such as apple pomace, especially because it also fulfils major issues, such as sustainability and human safety, generally associated to cosmetic products (Barbulova et al., 2015).

1.1. Apple production and processing

Apple (*Malus sp., Rosaceae*) is among the most popular fruits in the world, being well-known for its beneficial effects in the prevention of several diseases such as chronic heart and vascular disorders, respiratory and pulmonary dysfunction, diabetes, obesity, and cancer (Tu, Chen, & Ho, 2017). It is cultivated worldwide and its global production exceeded 89 million tons in 2016, according to FAO statistics.

A significant part of harvested apples is processed and converted into juice and cider. In the European Union, for instance, more than three million tons of apples were processed in this way, in 2014 (AIJN/ European Fruit Juice Association, 2014). Apple pomace is a residual product that remains after juice and cider pressing and represents up to 25% of the fresh fruit weight (Bhushan, Kalia, Sharma, Singh, & Ahuja, 2008). The Spanish cider industry, in particular, produced around 20 thousand tons of apple pomace in 2003 (Gullón, Garrote, et al., 2007).

Therefore, this industrial activity generates a large amount of underused by-products, which can be expensive and complex to remove. Accordingly, finding suitable ways of disposal or adding value to those materials might render economic and environmental benefits.

1.1.1. Current uses of apple pomace

Apple pomace (**Figure 1**) consists of apple peels, leftover flesh, core with seeds and stems of a mixture of apple varieties (Waldbauer, McKinnon, & Kopp, 2017).

Its high moisture (66.4-78.2%) and sugars (48.0-62.0% on a dry weight basis) contents (Joshi & Attri, 2006), turns apple pomace in a highly perishable product, hindering its storage (**Table 1**). While refrigeration and freezing can be applied to extend storage time, the energy and space required make them economically unfeasible and unpractical. The other possible solution is drying by reducing the moisture contents to ≈10 %, providing longer storage period and reducing storage space and transportation costs, but it also requires high energy levels (Jung, Cavender, & Zhao, 2015).

Figure 1 *- Fresh apple pomace.*

Table 1 *- Proximate nutritional composition of apple pomace (Joshi & Attri, 2006).*

Constituent	% wet weight basis
Moisture	66.4-78.2
Total carbohydrate	9.5-22.0
Crude fibre	4.3-10.5
Protein	1.0-1.8
Fat (ether extract)	0.8-1.4
Pectin	1.5-2.5
Ash	1.4-1.6

Currently, apple pomace has some applications, but it is far from being used for its full potential. Some of those applications include:

i) **Livestock feed** - for its nutritional value, apple pomace has been traditionally used as cattle food. It has been shown to decrease the feed cost, both in ruminants and non-ruminants, without having harmful effect (Wadhwa & Bakshi, 2013).

ii) **Food products** - once again, due to its nutritional value, apple pomace is susceptible to be used in manufacturing food products. In fact, it contains high levels of fibre and polyphenols, which are known to play an important role in human health (Sudha, Baskaran, & Leelavathi, 2007). Some established examples, include the incorporation of finely ground apple pomace in several bakery products (Rupasinghe, Wang, Huber, & Pitts, 2008; Sudha et al., 2007) and also to brown rice based crackers (Mir, Bosco, Shah, Santhalakshmy, & Mir, 2017).

iii) **Pectin extraction** - apple pomace is one of the main sources of pectin at industrial level. Apple pectin is produced commercially as a white to light brown powder and is used in the nutritional and cosmetic industry as a gelling or thickening agent. The exploitation starts with a hot water-acid extraction, followed by the precipitation in an organic solvent (May, 1990). At industrial scale, ethanol precipitation is one of the most common treatments to purify pectins (Garna et al., 2007). However, acid treatments filtrations and washing steps lead to pectin with lower degrees of polymerization and esterification, producing high amounts of chemical waste. Therefore, alternative, environmentally friendly approaches of pectin extraction with high yields are needed. Hot-compressed water extraction was used to replace the acid extraction. The pectin obtained showed a lower viscosity rate, dry matter and protein content but higher ash and neutral sugar content (Wang & Lu, 2014). Enzymatic pectin extraction seems to be a promising alternative, since it eliminates serious disadvantages of the acidic process, such as requirement for high temperatures, low pH, equipment corrosion and large volumes of sewage that needs neutralization (Wikiera, Mika, & Grabacka, 2015; Wikiera, Mika, Starzyńska-Janiszewska, & Stodolak, 2016).

iv) **Fuel purposes and biotransformation** - the use of biomass for the production of energy has become an important strategy aimed at reducing the harmful environmental impact of today's use of fossil fuels. The most popular method to obtain biofuel is the fermentation of different food waste by anaerobic bacteria (Thi, Lin, & Kumar, 2015). This bacterial fermentation of food waste can also be used to product value added chemicals, such as organic acids or aroma compounds (Ali, Anwar, Irshad, Mukhtar, & Warraich, 2016; Dhillon, Kaur, & Brar, 2013).

Some efforts have been made to produce bioethanol, hydrogen or methane from apple pomace via anaerobic fermentation (Doi, Matsumoto, Abe, & Morita, 2010; Evcan & Tari, 2015; Thi et al., 2015). Additional work was performed on the generation of a gas product (through the slow pyrolysis of apple pomace) to be further used as a starting material for hydrogen production (Guerrero, Salinas Gutiérrez, Meléndez Zaragoza, López Ortiz, & Collins-Martínez, 2016).

1.1.2. Circular Economy and bio-resources.

It is estimated that the worldwide agricultural sector generates around 140 billion tons of biomass every year (Perlatti, Forim, & Zuin, 2014). The main part of these by-products are regarded as waste, but considering their available volume and practical low cost, this material should be analysed as a potential source of chemical and bioactive components, as well as an alternative source of energy (Lin et al., 2014).

In this context, biorefineries are becoming very important, as analogous to today's petroleum refinery, which produce multiple fuels and products from petroleum. Biorefineries integrate biomass conversion processes and equipment to produce fuels, power, heat, and value-added chemicals from biomass waste (**Figure 2**).

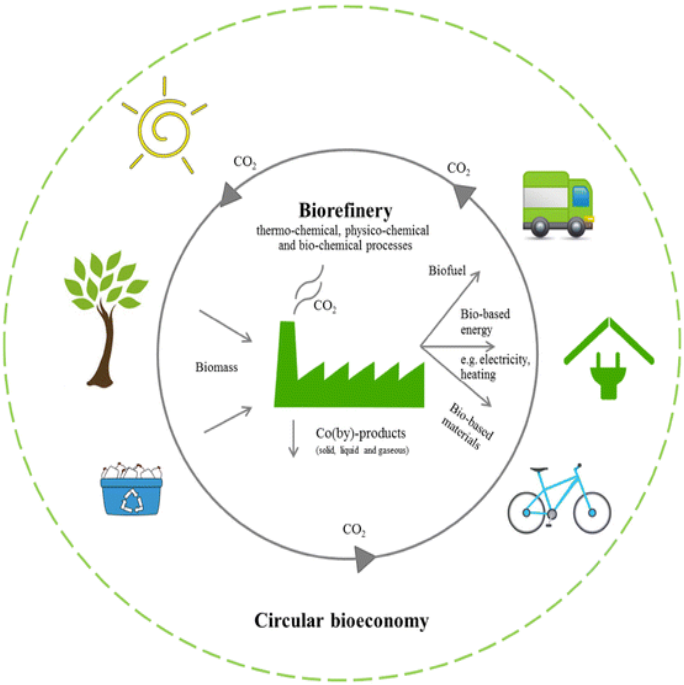


Figure 2 *- Holistic biorefinery model integrating biomass, biofuel, biomaterials and bioenergy cycle, based on green and sustainable technologies in the scope of bioeconomy and circular economy (Zuin & Ramin, 2018).*

From the very beginning, identifying and evaluating all potentially high value-added chemicals that could be removed from available renewable feedstocks requires robust, efficient, selective, reproducible, and benign analytical approaches. Bearing this in mind, green and sustainable separation of natural products from agro-industrial waste is clearly attractive, considering both socio-environmental and economic aspects. Therefore, innovation and research efforts are needed in this field, in order to find the most sustainable and efficient separation of natural products from agro-industrial by-products (Zuin & Ramin, 2018).

In fact, this paradigm is currently connected to other strong concepts, *i.e.*, bioeconomy and circular economy; the latter is described as an industrial system that is restorative by intention and design. This idea replaces the end-of-life concept with regeneration, shifts on the use of renewable energy, eliminates the use of toxic chemicals (which impair reuse), and aims for the elimination of waste through the superior design of materials, products, systems, and, within this, business models (The Ellen MacArthur Foundation, 2012).

1.2. Bioactive compounds in apple pomace

As previously stated, apple consumption is associated to an improvement of human health and protection against chronic diseases. The health benefits of apple are primarily attributed to their antioxidant compounds and dietary fibre (Koutsos, Tuohy, & Lovegrove, 2015; Tu et al., 2017). During apple juice and cider processing, most of the water content is lost, leading to an increase of the polyphenol content in apple pomace in comparison to the fresh fruit. In fact, and although polyphenols are partially transferred to the juice, their predominant location is the apple peel, thereby remaining mostly in apple pomace (Krawitzky et al., 2014).

Essentially, apple pomace represents a low-cost source of phytochemical and active compounds, such as polyphenols, dietary fibre, pectin, triterpenoids and volatile compounds. Nevertheless, this by-product is far from being used at its full potential, particularly considering its potential application in food and pharmaceutical industry (Waldbauer et al., 2017).

In the next sections, polyphenols and pectin, as bioactive and functional compounds present in apple pomace will be described in detail.

1.2.1. Phenolic compounds.

Phenolic compounds, which are synthesized through the pentose phosphate, shikimate and phenylpropanoid pathways, are found to be among the most important groups of plant secondary metabolites (Dewick, 2009; Randhir, Lin, & Shetty, 2004). These compounds play an important role in plant defence (*e.g.*, against microbial pathogens) and development, helping to establish the ecological relationships and participating in their morphological development and physiological processes (Mierziak, Kostyn, & Kulma, 2014). Several studies approved their use, for instance, as antimicrobials in human medicine, raising the possibility of their use against microbial resistance (Fialova, Rendekova, Mucaji, & Slobodnikova, 2017; Musilova, Ridl, Polivkova, Macek, & Uhlik, 2016).

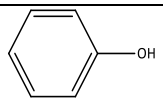
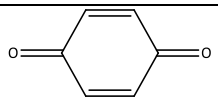
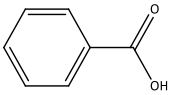
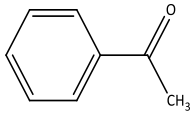
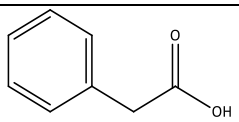
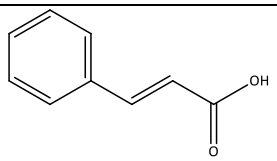
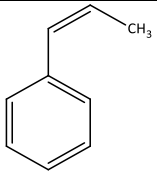
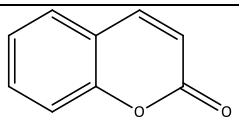
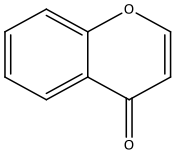
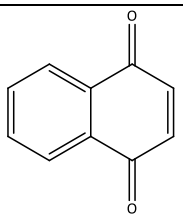
Phenolic compounds are highly active antioxidant compounds, being acknowledged for their broad spectrum of biological properties. Several studies support the perception that a polyphenol-rich diet may directly benefit human health and be associated with reduced chronic diseases risk, thereby promoting optimal aging. Among their biological properties, and in addition to antioxidant activity, is worth mentioning their anti-inflammatory, antimicrobial, or cytotoxic properties (Costa et al., 2017; Działo et al., 2016), especially when used in combined formulations, instead of single molecules (Costa et al., 2017).

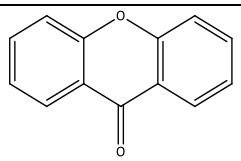
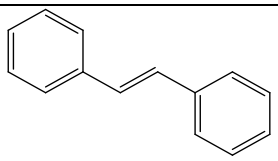
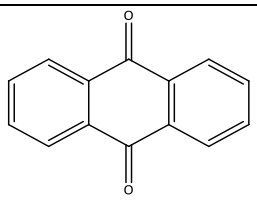
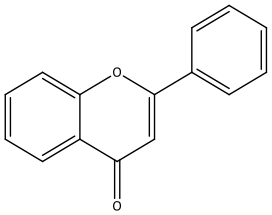
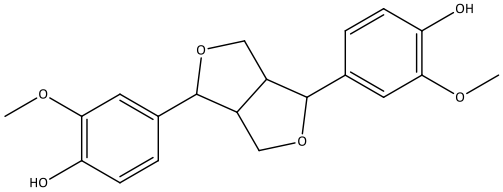
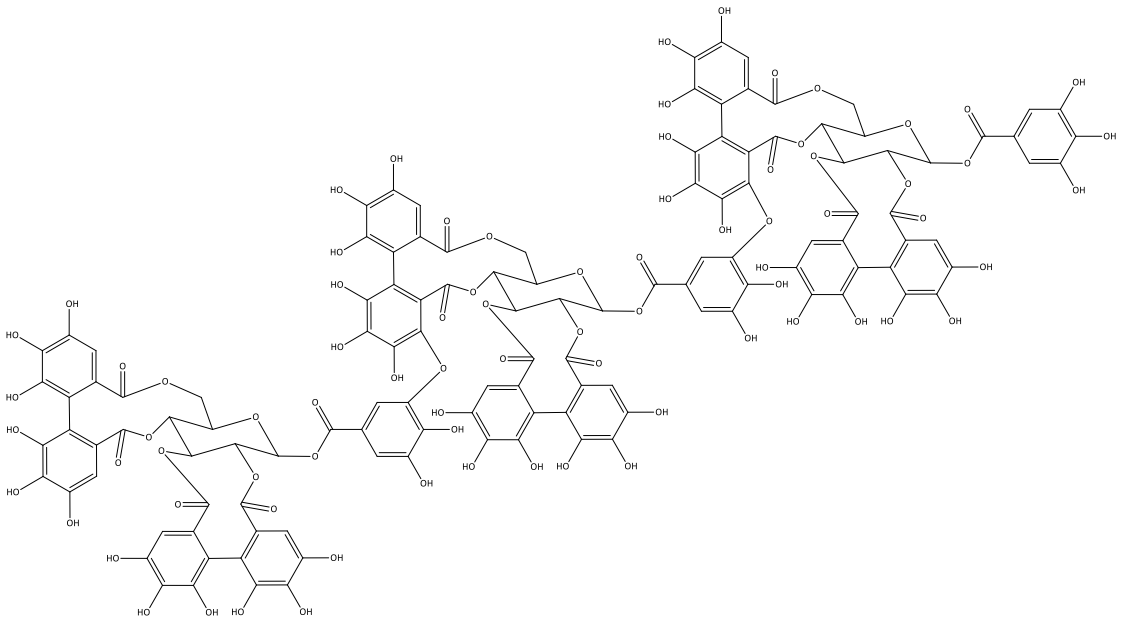
1.2.1.1. Chemical structure and bioactivity of phenolic compounds

Phenolic compounds constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with more than 8000 phenolic structures currently known. Chemically, they comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules, such as phenolic acids, to highly polymerized compounds, such as tannins (**Table 2**). Usually, plant polyphenols contain more than one phenolic ring, and thus they are called polyphenols (Bravo, 2009).

The bioactivity of polyphenols is explained by their chemical properties, such as the ability to neutralize free radicals, stabilizing them by delocalization, due to the interaction of the hydroxyl groups of phenolics with the p-electrons of the benzene ring. The formation of these relatively long-lived radicals is able to modify radical-mediated oxidation processes, an important aspect of their biological role as antioxidants. Therefore phenolics with two ortho-positioned hydroxyl groups are very good antioxidants, but they are relatively instable and may oxidize to quinones during storage or processing (Parr & Bolwell, 2000). Other aspects like the capacity to chelate metal ions are also relevant in their biological action. In this case vicinal hydroxyl groups are required, as we can observe in quercetin or caffeic acid (**Figure 3**). This metal-binding capacity may also be relevant for the antioxidant activity, since free transition metal ions are pro-oxidant that produce free-radicals in the presence of hydrogen peroxide (Croft, 1998; Psotová, Lasovský, & Vicar, 2003).

Table 2 *- Basic structures of some common phenolic compounds.*

Class	**Basic skeleton**	**Basic structure**
Simple phenols	C_6	
Benzoquinones	C_6	
Phenolic acids	C_6–C_1	
Acetophenones	C_6–C_2	
Phenylacetic acids	C_6–C_2	
Hydroxycinnamic acids	C_6–C_3	
Phenylpropenes	C_6–C_3	
Coumarins	C_6–C_3	
Chromones	C_6–C_3	
Naphtoquinones	C_6–C_4	

Xanthones	$C_6–C_1–C_6$	
Stilbenes	$C_6–C_2–C_6$	
Anthraquinones	$C_6–C_2–C_6$	
Flavonoids	$C_6–C_3–C_6$	
Lignans	$(C_6–C_3)_2$	
Ellagitannins	$(C_6–C_3)_n$	

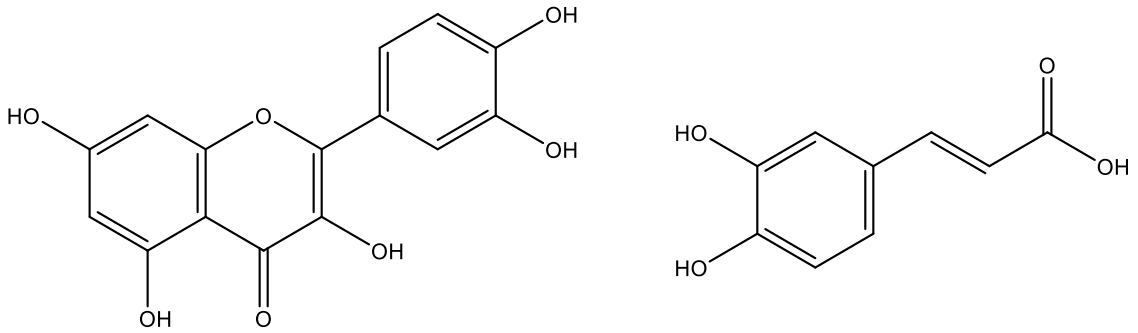


Figure 3 *- Quercetin* (B ring) *and caffeic acid as examples of polyphenols with vicinal hydroxyl groups.*

Other properties of biological relevance also come from the presence of phenolic groups that are readily ionised because of their potential for electronic delocalisation. This has an influence on the reactivity of phenolics and in the formation of hydrogen bonds, where they are good H donors. Some polymeric phenolics carry large numbers of such donor groups, resulting that the complexes formed with other molecules are extremely stable and tend to precipitate. This is the basis for the astringent taste and tanning abilities of the tannins (Parr & Bolwell, 2000).

1.2.1.2. Classification of phenolic compounds

Phenolic metabolites can be classified into flavonoid and non-flavonoid compounds (**Figure 4**). The chemical structure of **flavonoid** consists of two aromatic rings connected by a three carbons bridge (C_6-C_3-C_6). They are divided in seven subclasses: flavonols, flavones, flavanones, flavan-3-ols, isoflavones, chalcones and anthocyanins. In the physiological state, flavonoids occur usually in association with sugar (glycoside derivatives). The second class of phenolics, **non-flavonoid** metabolites, consists on a very structurally heterogeneous group, composed by the following subgroups: phenolic acids (hydroxybenzoates C_6-C_1, hydroxycinnamates C_6-C_3), lignans $(C_6\text{-}C_3)_2$ and stilbenes C_6-C_2-C_6. Tannins and lignins are other two subsclasses of non-flavonoids compounds that occur mainly as complicated biopolymers (Del Rio et al., 2013). However, tannins are divided in two others subgroups, hydrolysable tannins, that are formed in the pathway of phenolic acids with sugar polymerization and condensed tannins that consist

of a combination of flavonoids, therefore they are usually consider as a subclasse of flavonoids compounds (Ashok & Upadhyaya, 2012)

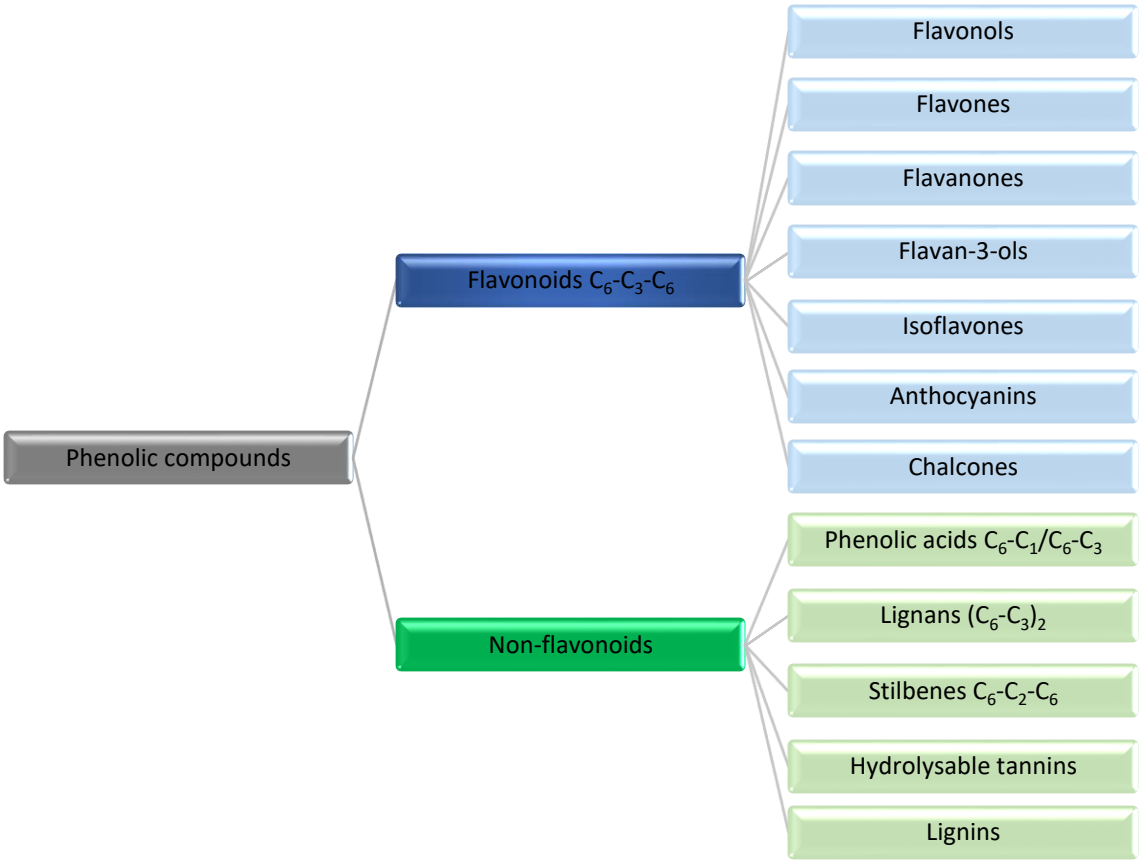


Figure 4 *- Classification of phenolic compounds.*

Flavonoids

Flavonoids are low-molecular-weight phenolics that are widely distributed in Plant kingdom, where these compounds have long been known to be responsible for the colour and aroma of flowers and fruits, intended to attract pollinators and consequently fruit dispersion to help in seed and spore germination (Panche, Diwan, & Chandra, 2016). Flavonoids protect plants from different biotic and abiotic stresses and act as unique UV-filter, as they are able to absorb the harmful UV radiation (Takahashi & Ohnishi, 2004), having also been studied for their photoprotective properties in human (Adhami, Syed, Khan, & Afaq, 2008).

Flavonoids are associated with a broad spectrum of health-promoting effects and seem to become promising active compounds in cosmetics. This is because of their venotropic action that helps to reduce the redness produced by superficial capillaries in the human skin. They also have local anti-inflammatory properties, owing to their ability to inhibit several enzymes, such as xanthine oxidase, cyclooxygenase, lipoxygenase and phosphoinositide 3-kinase (Bandoni et al., 2012; Iio, Moriyama, Matsumoto, Takaki, & Fukumoto, 1985; Walker et al., 2000).

Flavonoids can be subdivided into different subgroups depending on the carbon of the C ring on which the B ring is attached and the degree of unsaturation and oxidation of the C ring (**Figure 5**).

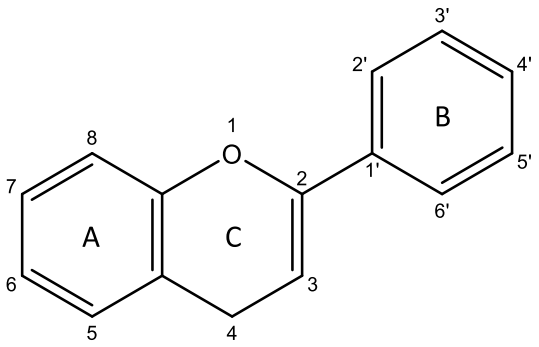
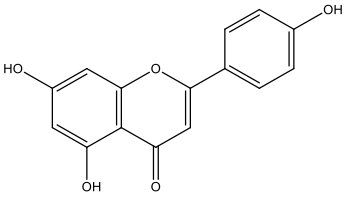
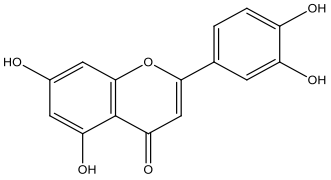
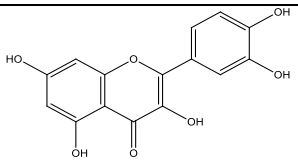
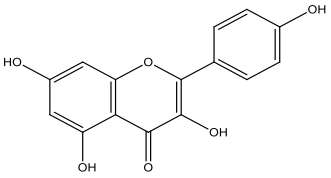
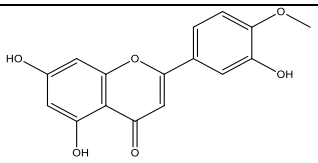
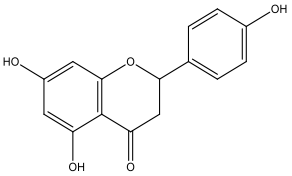
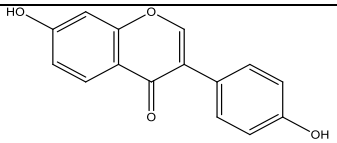
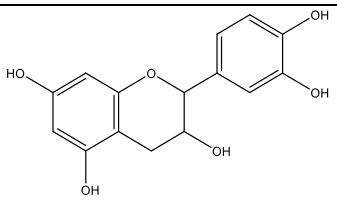
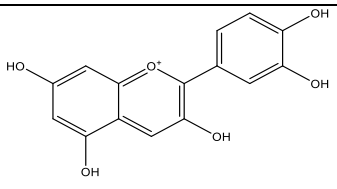
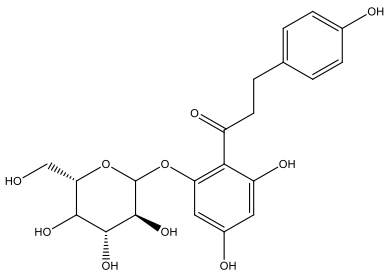


Figure 5 *- Basic structure of a flavonoid.*

Flavonoids in which the B ring is linked in position 3 of the C ring are called isoflavones. Those in which the B ring is linked in position 4 are called neoflavonoids, while those in which the B ring is linked in position 2 can be further subdivided into several subgroups on the basis of the structural features of the C ring (**Table 3**). These subgroups are: flavones, flavonols, flavanones, flavanols or catechins, anthocyanins and chalcones (Panche et al., 2016).

Table 3 - *Classification of flavonoids* (Panche et al., 2016).

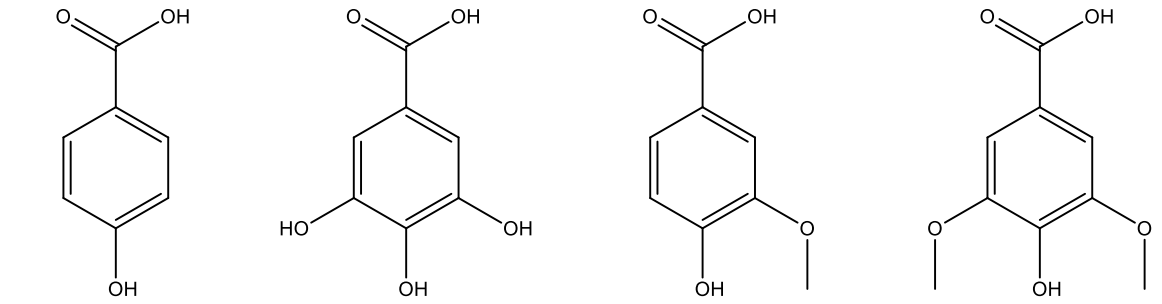
Subgroup	Occurrence	Health benefits	Examples	Chemical structure
Flavones	One of the most important groups. Present in leaves, flowers and fruit, usually as glycosides.	Protection of neurodegenerative and cardiovascular diseases	Apigenin	
			Luteolin	
Flavonols	Occur abundantly in in a variety of fruits and vegetables.	Antioxidant potential. Reduced risk of vascular disease.	Quercetin	
			Kaempferol	
Flavanones	Generally present in all citrus fruits such as oranges, lemons and grapes.	Free radical-scavenging properties. Antioxidant potential.	Hesperitin	
			Naringenin	
Isoflavonoids	Predominantly found in soybeans and other leguminous plants.	Phyto-oestrogens.	Daidzein	
Flavanols, flavan-3-ol or catechins	Abundantly in bananas, apples, blueberries, peaches and pears	Antioxidant potential.	Catechin	
Anthocyanins	Pigments responsible for red, blue and purple colours in plants, flowers and fruits.	Potent antioxidants. Vaso-protective and anti-inflammatory properties.	Cyanidin	

Chalcones	Tomatoes, pears, apples, strawberries and certain wheat products.	Numerous nutrition benefits.	Phlorhizin	

Phenolic acids

Phenolic acids comprise a group of phenolic compounds that is extensively spread throughout the Plant kingdom. They play a key role in the growth and reproduction of plants and their synthesis is conditioned by environmental factors, such as light, chilling or pollution (Kefeli, Kalevitch, & Borsari, 2003). They are also well-known for their antioxidant activity and have been described to have numerous benefits in human health (Kaushik et al., 2015). Phenolic acids are divided in two subgroups: hydroxybenzoic acid derivatives (C_6-C_1) (**Figure 6**) and hydroxycinnamic acid derivatives (C_6-C_3) (**Figure 7**).

Hydroxybenzoic acids are commonly present in the bound form and are typically a component of complex structures like lignins and hydrolysable tannins. They can also be found linked to sugars or organic acids in plants.



p-hydroxybenzoic acid | gallic acid | vanillic acid | syringic acid

Figure 6 *- Chemical structures of exemplifying hydroxybenzoic acids.*

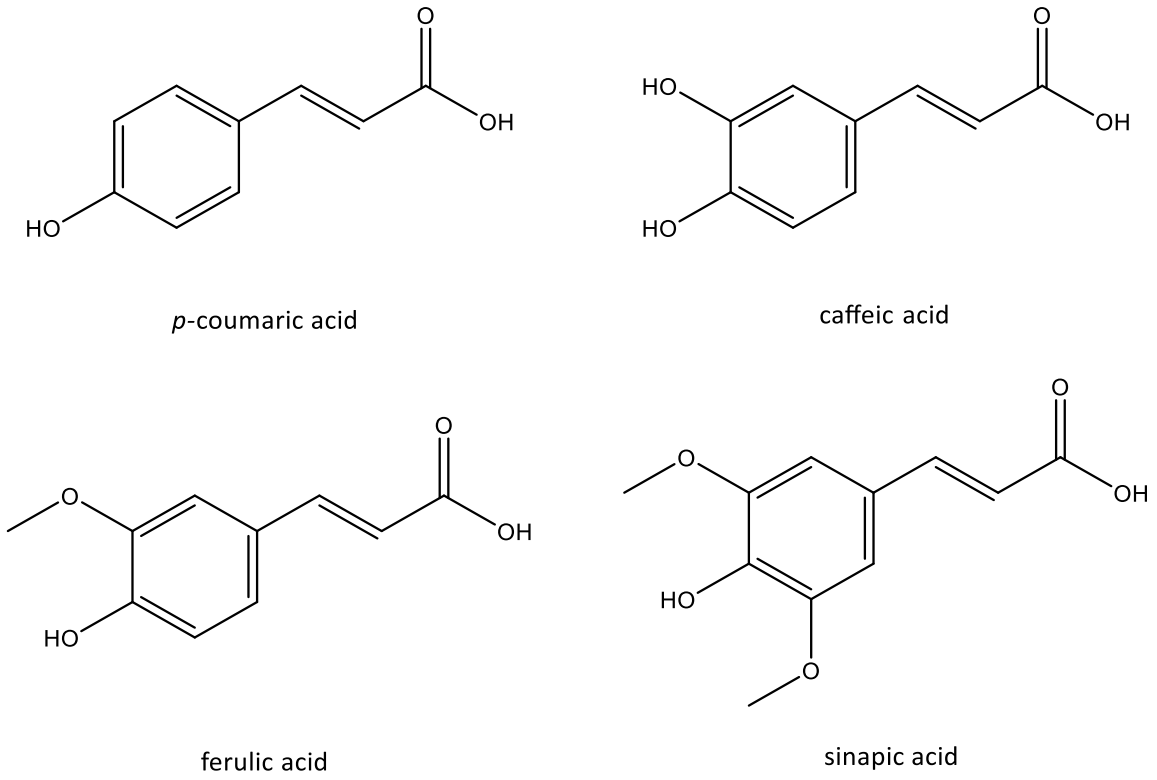


Figure 7 - Chemical structures of exemplifying hydroxycinnamic acids.

Hydroxycinnamic acids appear mainly in the bound form, linked to cell-wall structural components, such as cellulose, lignin, and proteins through ester bonds (Barros, Dueñas, Ferreira, Baptista, & Santos-Buelga, 2009).

One of the most widespread hydroxycinnamic acid is caffeic acid, which occurs in several species such as coffee, apples, potatoes, spinach lettuce, cabbage, olive oil, wine and tobacco leaves (Mattila & Hellström, 2007).

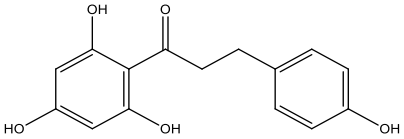
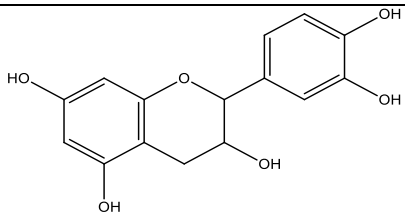
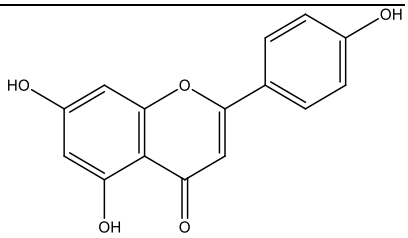
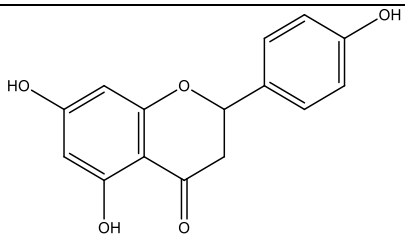
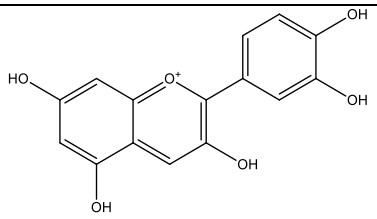
1.2.1.3. Phenolic compounds in apple pomace

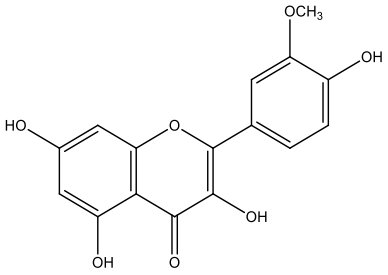
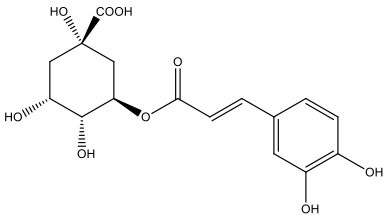
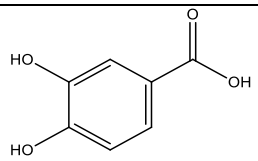
There is a wide range of polyphenols in apple, depending on the cultivar. Flavanols (catechin and proanthocyanidins) are the major classes of apple polyphenols (71-90%), followed by hydroxycinnamates (4-18%), flavonols (1-11%), di-hydrochalcones (2-6%), and (in red cultivars) anthocyanins (1-3%) (Vrhovsek, Rigo, Tonon, & Mattivi, 2004). In a study carried out in France, apple varieties used for cider proved to have a higher polyphenolic content than the typical dessert cultivars, such as Golden Delicious (Sanoner, Guyot, Marnet, Molle, & Drilleau, 1999). Therefore, apple pomace coming from cider industry can be of especial interest for phenolic extraction.

The concentration of apple polyphenols in pomace seems also to be influenced by the method of juice extraction. A comparative study of different apple cultivars showed a higher concentration in the pomace obtained from clear juice production (Kołodziejczyk et al., 2009).

Extensive studies on the identification of apple pomace polyphenols have been performed. The main polyphenols founded were di-hydrochalcones, flavanols, flavones, flavanones, flavonols, hydroxycinnamic acids and anthocyanins (in red cultivars). All of them are well-known for their antioxidant properties, suggesting this raw material as an important source of antioxidants (Ramirez-Ambrosi et al., 2013; Sánchez-Rabaneda et al., 2004). In fresh apple pomace, the polyphenolic pattern showed high contents of chlorogenic acid, caffeic acid, (+)-catechin, (−)-epicatechin, rutin, and quercetin glycosides (Ćetković et al., 2008). In contrast, phloridzin was the most prominent polyphenol in apple pomace after freeze-drying (Andreas Schieber, Petra Hilt , Petra Streker , Hans-Ulrich Endreß, Christine Rentschlerb, Carle, 2003). In fact, di-hydrochalcones, in which phloridzin is included, are the main polyphenols founded in apple seeds and stems, whereas the flesh part consists mainly of chlorogenic acid and flavonol glycosides (Górnaś et al., 2015). These compounds present properties that go beyond conventional and indirect antioxidant activities or chemo-preventive effects, which are common to most phenolic compounds (Stevenson & Hurst, 2007). Some uses of phlorizin include the adjuvant treatment of type 2 diabetes, weight loss agent for obesity, or acute management of hyperglycaemia (Ehrenkranz, Lewis, Kahn, & Roth, 2005).

Table 4 *- Polyphenols identified in apple pomace (Ramirez-Ambrosi et al., 2013; Sánchez-Rabaneda et al., 2004).*

Compound class	Compound name	Exemplifying structure
Di-hydrochalcones	Phloretin, phloretin-2-*O*-glucoside, phloretin-2-*O*-xylosyl-glucoside, phloretin-pentosylhexoside, phloretin-hexosyl-hexoside, 3-hydroxyphloretin-2'-*O*-xylosyl-glucoside, 3-hydroxyphloretin-2'-*O*-glucoside.	 Phloretin
Flavanols	(+)-Catechin, (−)-epicatechin, procyanidin B1, procyanidin B2, procyanidin B3, procyanidin B5, procyanidin C1, (epi)catechin trimer, (epi)catechin tetramer.	 Catechin
Flavones	Apigenin, chrysoeriol, luteolin, luteolin-7-*O*-galactoside, luteolin-7-*O*-glucoside.	 Apigenin
Flavanones	Hesperidin-*O*-pentoside, eriodictyol, eriodictyol-hexoside, naringenin-7-*O*-glucoside, naringenin-7-*O*-neohesperidoside, naringenin-7-*O*-rutinoside, naringenin-*O*-glucuronide.	 Naringenin
Anthocyanins	Cyanidin-3-*O*-galactoside Cyanidin-3-*O*-hexoside	 Cyanidin

Flavonols	Isorhamnetin-3-*O*-galactoside, isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, isorhamnetin-3-*O*-rhamnoside, kaempferol-*O*-glucoside, quercetin-3-*O*-diglucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-xylanoside, quercetin-*O*-xylosyl-pentoside, rhamnetin-3-O-glucoside.	 Isorhamnetin
Hydroxycinnamic acids	3-*O*-Caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, caffeic acid-*O*-hexoside, ferulic acid-*O*-hexoside, *p*-coumaric acid-*O*-hexoside, sinapic acid-*O*-glucoside, 4-*O*-*p*-coumaroylquinic acid, 5-*O*-*p*-coumaroylquinic acid	 3-*O*-Caffeoylquinic acid
Other	Protocatechuic acid, salicylic acid	 Protocatechuic acid

Other bioactivities detected among apple polyphenols include anti-inflammatory, antiproliferative, anti-tumour, antimicrobial and cardio-protective properties. Therefore, they are seen as promising nutraceuticals for the management of chronic diseases (Friedman, Henika, & Levin, 2013; Rana & Bhushan, 2016).

Nevertheless, after apple pressing, there is a decrease in phenolic content, mainly due to the presence of enzymes, such as polyphenol oxidases and peroxidases, which are liberated from cell vacuoles or cell wall compartments and start the oxidation of phenolic compounds (Yoruk & Marshall, 2003). In consequence, the most important step for polyphenol preservation is the immediate and effective drying process after pressing. Mild drying methods such as lyophilisation seem to be one of the best alternatives, since low temperatures prevent thermal degradation (Schieber, Keller, & Carle, 2001), but the whole phenolic extraction process still need to be optimised.

1.2.2. Pectin

Pectin (derived from the Greek term “pektos”, meaning firm and hard) is a structural heteropolysaccharide present in the primary cell walls of terrestrial plants (Srivastava & Malviya, 2011). Pectins are synthesized in the Golgi stacks of plant cells and released into the wall by exocytosis, aimed to attribute strength and flexibility to the wall, separate microfibrils and link adjacent cells in the middle lamellae (Braidwood, Breuer, & Sugimoto, 2014).

Pectins constitute an extremely diverse group of complex high molecular weight polysaccharides that are soluble in aqueous and acidic conditions, being considered as part of the soluble dietary fibre. Their main industrial sources are apple pomace and citrus peel (Willats, Knox, & Mikkelsen, 2006), being mostly used as a gelling agent with application in foods, pharmaceuticals or cosmetics. This is due to their ability to form gels in the presence of water and to provide charged surfaces that regulate pH (Thakur, Singh, & Handa, 1997). It is also reported that pectin has several biological and physiological functions, such as reduction of serum cholesterol (Gunness & Gidley, 2010), immuno-modulation (Inngjerdingen et al., 2007) and inducing apoptosis of colon cancer cells (Olano-Martin, Rimbach, Gibson, & Rastall, 2003). All these functional properties lead to consider pectin as a promising nutraceutical, a possible prebiotic, or a delivery vehicle for probiotics (Naqash, Masoodi, Ahmad Rather, Wani, & Gani, 2017). Pectin is also a promising polymer in drug delivery due to the lack of toxicity and the low production cost (Klemetsrud, Jonassen, Hiorth, Kjøniksen, & Smistad, 2013).

1.2.2.1. Chemistry and structure of pectin

Pectin structure is difficult to determine, since it can change during isolation from plant, storage and processing of plant material. Pectin consists of a complex mixture of polysaccharides that makes up about one third of the cell wall dry substance of higher plants (Sriamornsak, 2003). Pectin is mainly composed by three polysaccharide domains: homogalacturonan (HGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II).

Homogalacturonan is the major component of pectin polysaccharides, and consists of galacturonic acids (GalA) joined in chains by means of a (1,4) glycosidic linkage (Munarin, Tanzi, & Petrini, 2012; Sriamornsak, 2003).

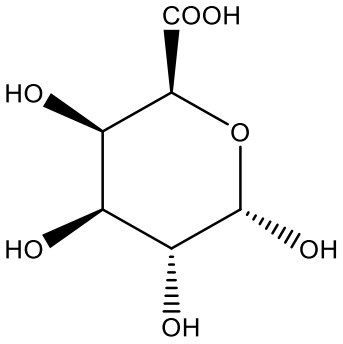


Figure 8 *- Galacturonic acid. The main monomer of pectin.*

The carboxyl groups of the uronic acids can be methyl-esterified or remain free (Munarin et al., 2012).

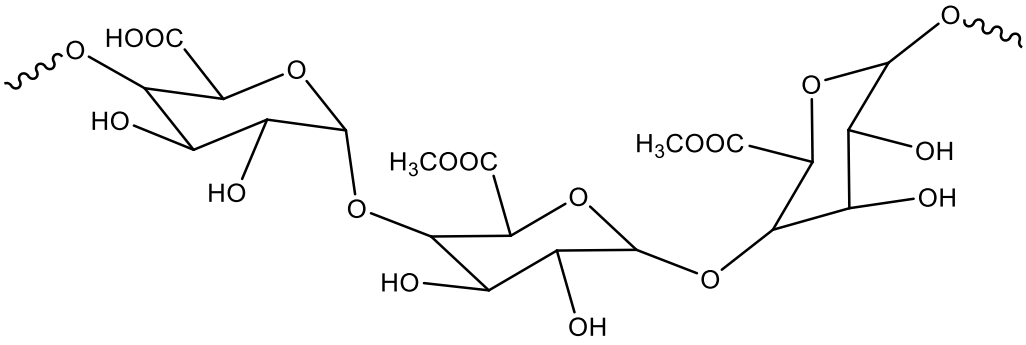


Figure 9 *- Homogalacturonan (HGA). Galacturonic acid joined by (1,4) glycosidic linkage.*

The most important use of pectin is based on its ability to form gels when dissolved in water. Gel formation occurs when portions of HGA are cross-linked forming a three dimensional crystalline network in which water and solutes are trapped. This ability depends on various factor, such as temperature, molecular weight, degree of esterification, pH, sugar and other solutes, and calcium. In high-ester pectins, the junction zones are formed by the cross-linking of HGA by hydrogen bridges and hydrophobic forces between methoxyl groups, both promoted by high sugar concentration and low pH. In turn, in low-ester pectins junction zones are formed by calcium cross-linking between free carboxyl groups (Sriamornsak, 2003; Willats et al., 2006).

1.3. Potential applications of apple pomace in cosmetic industry

Considering the information presented in the former sections, the bioactive compounds in apple pomace are likely to be used as cosmeceuticals, which are characterized as holding potential ability for health promotion, aside from their cosmetic application. The cosmeceutical concept was first defined in four complementary points: (1) “A cosmeceutical is a scientifically designed product intended for external application to the human body”; (2) “A cosmeceutical produces a useful, desired result”; (3) “A cosmeceutical has desirable aesthetic properties”; (4) “A cosmeceutical meets rigid chemical, physical and medical standards” (E. Reed, 1964).

Skin is the largest organ of the body in vertebrates (Metcalfe & Ferguson, 2007) and presents many functions in humans, especially in the defence against physical, chemical or biological factors. It also acts in the regulation of water and electrolyte homeostasis and plays a secretory role, besides being involved in perception and immunological response of the organism (Brohem et al., 2011). Therefore, the search for cosmetic products that not only seek an improvement in the appearance of skin, but also seek to promote its health, is one of the challenges facing the cosmetic industry today.

The potential application of phenolic compounds and pectin, as cosmeceuticals, will be analysed in this section in detail.

1.3.1. Phenolic compounds

The health promoting activity of phenolic compounds is based on their antioxidant, anti-inflammatory and antimicrobial properties. For these bioactivities, they deserve recognition in natural medicine and may be highly effective in treatment of various skin problems. These properties constitute the underlying mechanisms of action against various skin disorders (Działo et al., 2016).

1.3.1.1. Antioxidant properties

As it was already explained, the antioxidant activity of phenolic metabolites is associated with their chemical structure that allows them to inhibit reactive oxygen species (ROS), interrupting the cascade of free radical reactions in lipid peroxidation and protecting other compounds from oxidant effect.

Nowadays, the use of antioxidants in cosmetic products is becoming increasingly popular (Kusumawati & Indrayanto, 2013). Skin is well equipped with a network of protective enzymatic antioxidants such as glutathione peroxidase, superoxide dismutase and catalase, and non-enzymatic low-molecular-weight antioxidants such as vitamin E, vitamin C, glutathione (GSH), uric acid, and ubiquinol, which help maintaining its homeostasis by reducing the number of ROS (Shindo, Witt, & Packer, 1993). However, this balance can be disturbed by several factors such as ultraviolet radiation, pollution or lifestyle associated issues like smoking or inadequate diet (Kandola, Bowman, & Birch-Machin, 2015). This imbalance is called oxidative stress and may lead to certain skin disorders, such as erythema, acne, psoriasis or even cancer (Black, 2004; Briganti & Picardo, 2003). Accordingly, the use of natural antioxidants, such as polyphenols, can be of great help in the prevention of certain skin diseases and the maintenance of a healthy skin.

1.3.1.2. Anti-inflammatory properties

Every day, our organism is exposed to external agents, which may cause various types of damage, irritation or allergies. The body's defensive reaction against the negative effects of these factors is inflammation, a complex process in which an excess of free radicals is produced (Medzhitov, 2008). Polyphenols are able to inhibit several pro-inflammatory mediators, such as nitric oxide (NO), ROS, cytokines and prostaglandins (PGs), as well as enzymes, *e.g.*, inducible nitric oxide synthase (iNOS) and cyclooxygenases (COXs), reducing the inflammatory response (Bandoni et al., 2012; Pastore et al., 2009).

1.3.1.3. Antimicrobial activity

One of the functions of polyphenols in the plant is the defence against microbial pathogens, such as fungi, bacteria and viruses, raising the possibility of their use in human medicine (Fialova et al., 2017). In the case of skin infection or disease, one of the most common treatments is the use of broad-spectrum antibiotics. However, this may lead to the negative influence of antibiotics on natural microflora of the skin, boosting the resistance of many bacterial strains (Dryden, 2009).

Therefore, the search for new drugs and new sources of antibacterial agents is of utmost importance. The use of polyphenols seem to be a promising tool to overcome the problem, since their activity has a special significance in the case of strain resistant to antibiotics (Anani et al., 2015; Pinho et al., 2014; Zuk et al., 2014). The antibacterial properties of phenolics result mainly from their mechanism of action on cell membranes (Wu et al., 2013).

1.3.1.4. Apple pomace polyphenols as cosmeceuticals

Among the polyphenols found in apple pomace, phloretin and phloridzin seem to be the ones with highest potential to be used as natural alternatives to synthetic antioxidants and antimicrobials (Zhang et al., 2016). Apple polyphenols seem to have potential as a therapeutic agent against skin premature aging caused by UV irradiation (Park et al., 2014).

In addition, a recent *in vitro* study showed the inhibitory capacity of a polyphenolic apple extract over the fat production of sebaceous cells. This suggests that apple phenolic compounds may be useful in the regulation of sebum production, alleviating skin diseases, such as acne, in which fat production is altered (Lee, Youm, Lee, Kang, & Kim, 2017).

Therefore, the next section will focus on analysing how apple polyphenols may inhibit inflammation and *Propionibacterium acnes* activity in acne vulgaris.

1.3.1.5. Polyphenols in the treatment of skin disorders: Acne vulgaris

Numerous studies *in vitro* and *in vivo* have shown the potential of phenolic compounds in several skin disorders, such as premature skin aging, psoriasis, rosacea, acne vulgaris, skin allergies, atopic dermatitis, wounds, burns and even potentially life-threatening diseases, such as cancer (Działo et al., 2016). For all this, phenolic compounds seem to be a promising cosmeceutical.

Herein, special attention will be given to the potential use of phenolic compounds in the treatment of acne vulgaris, since apple polyphenols seem to decrease the sebum production of the skin, which may help to treat this disease (Shoaib Khan et al., 2011).

Acne vulgaris is a very common skin disorder that causes many physical and psychological problems. It commonly occurs during adolescence but sometimes persists throughout life and leave permanent scarring on the face. The causes for acne development are complex, but the principal factors involved are stimulation of sebaceous gland secretion by androgens, hyperkeratinization (which leads to the obstruction of sebaceous follicles), microbial colonization of pilosebaceous units by *Propionibacterium acnes* and inflammation. The clinical classification of acne can range from a mild form to severe inflammatory cystic acne of the face, chest and back (Toyoda & Morohashi, 2001).

Typical treatment is based on topical therapies, systemic antibiotics, oral retinoid and hormonal agents, which are moderately effective but may be associated with several side effects. For example, oral isotretinoin, one of the most effective treatments, has potentially serious side effects including teratogenicity and dyslipidaemia. Topical retinoids, as well as topical and systemic antibiotics, may cause a burning sensation and antibiotic resistance (Zaenglein et al., 2016).

Therefore, the use of medicinal plants and natural products is becoming increasingly popular in the treatment of acne as they have several advantages, such as better patient tolerance, long history of use, fewer side-effects and being relatively less expensive (Nasri et al., 2015; Rafieian-kopaei, 2012). As we can see, the main properties valued in the treatment of acne are: anti-bacterial, anti-inflammatory, antioxidant, and anti-androgenic activity (Działo et al., 2016; Toyoda & Morohashi, 2001).

There are a few examples of natural extracts that were characterized as having activity against *P. acnes*. The extracts from bitter melon leaf, for example showed good activity levels, most likely due to the presence of gallic, chlorogenic, caffeic, ferulic, and cinnamic acids, as well as myricetin, quercetin, luteolin, apigenin, and thymol (Huang et al., 2015). Likewise, the effect of mixtures of phenolic compounds and other drugs on *P. acnes* was also studied. Mixed formulations (kaempferol or quercetin and either erythromycin or clindamycin) due to their synergic action, caused the inhibition of antibiotic resistant *P. acnes* growth. In general, the combination of clindamycin with kaempferol or quercetin showed greater effect than other formulations (Lim, Kim, & Seo, 2007).

Polyphenols have also showed to be able to reduce dermal inflammation probably due to their antioxidant properties. Specifically, Jumihaidokuto, a traditional Japanese medicine, that contains a broad variety of polyphenols, suppresses *P. acnes*-induced dermatitis in rats (Matsumoto et al., 2015).

1.3.2. Pectin

Despite its ability to form gels, which makes it interesting in the formulation of cosmetics gels, pectin also presents potential in wound healing, since the properties of pectin impart several advantages, such as hydrophilicity, retention of an acid environment (which may act as a barrier against bacteria) and the ability to bind active molecules, such as drugs, or growth factors to heal the wounds. Pectin also serves as a binding agent, protecting cell growth factors from degradation (Munarin et al., 2012).

1.3.3. Similar experiences: grape pomace in cosmetics

Grape pomace is already used in several cosmetic formulations in two different forms, as grape seed oil, or antioxidant directly extracted from pomace (Dwyer, Hosseinian, & Rod, 2014). Grape seeds, coming from the wine industry, are used to product grape seed oil that is known to have several health promoting properties, such as antioxidant, anti-inflammatory, antimicrobial and also controlling the cell life-cycle. These effects have been related to their constituents, mainly tocopherol, linolenic acid, resveratrol, quercetin, procyanidins, carotenoids, and phytosterols (Garavaglia, Markoski, Oliveira, & Marcadenti, 2016). The polyphenols extracted from grape pomace are known for their strong antioxidant properties and therefore are commonly used in anti-aging cosmetics (**Figure 8**) (Wittenauer, Schweiggert-Weisz, & Carle, 2016).

Figure 10 *- Anti-aging cream from Uvas Frescas (a Spanish company).*

1.4. Objectives

The main objective is the exploitation of an industrial by-product, specifically apple pomace, as an innovative source of cosmeceuticals, and further developing a novel natural-based formulation, in that way adding value to this industrial bio-waste.

Besides the thorough chemical characterization of apple pomace, it was intended to test different extraction conditions to maximize the yield in phenolic compounds. The best extracts were also evaluated for their antioxidant and antimicrobial activities. The microorganism tested in the antimicrobial assay were bacteria that are typically found in human skin, including *Propionibacterium acnes*, that is linked to the skin condition of acne vulgaris.

Bioactivity results, with particular emphasis in antimicrobial activity, were then used to define the appropriate amount of extract to incorporate in the cosmetic formulation. Instead of using typical commercial gelling agents (such as carbopol 940), it was intended to isolate the pectin from apple pomace by hot acidic extraction, in this way using this by-product considering two distinct aims. Pectin was also selected for being a product of natural origin, free of toxicity and with some potential in the treatment of skin lesions. The antioxidant and antimicrobial activities of the hydrogel obtained were also determined and compared to the activity of a blank gel formulation (without being added with apple pomace phenolic extract).

II. Material and Methods

2.1. Standards and reagents

The standards used in the antioxidant activity, gallic acid and catechin, were purchased from Sigma (St. Louis, MO, USA); 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene were obtained from Alfa Aesar (Ward Hill, MA, USA).

For the characterization of phenolic compounds, HPLC-grade acetonitrile was obtained from Fisher Scientific (Lisbon, Portugal). The phenolic compound standards were from Extrasynthese (Genay, France).

In the antimicrobial assays, the culture media Muller Hinton broth (MHB) and Tryptic Soy Broth (TSB) were obtained from Biomerieux (Marcyl'Etoile, France), as well as the blood agar with 7% sheep blood and MacConkey agar plates. The dye *p*-iodonitrotetrazolium chloride (INT) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was used as microbial growth indicator.

For the cosmetic gel preparation propanediol, citric acid and triethanolamine were purchase from Sigma (St. Louis, MO, USA).

Ethanol and methanol for the phenolic extraction were of analytical grade purity purchased from Fisher Scientific (Lisbon, Portugal).

All other chemicals and solvents were of analytical grade and acquired from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Sample collection and preparation

Apple pomace samples were kindly supplied by Corporación Hijos de Rivera S.L. (Spain), and Tuinsappen Lombarts Calville (Belgium). The pomaces was obtained from a mixture of apple varieties harvested during 2017 season. Sample from Spain consisted mainly of a mixture of Rabosa, Pero, Príncipe, Verdeña, Perezosa, and De la Riega varieties.

Pomace was immediately frozen after the pressing step in the beginning of November. This sample was collected on 4th of December 2017 in Chantana (Lugo, Spain) and delivered to CIMO, Bragança (Portugal) were it was kept at -80 °C , until it was lyophilized. After lyophilization, pomace was grinded to obtain a fine powder. This material was stored protected from light and humidity, until further analysis.

Samples obtained from the Belgian company consisted of approximately 25 different regional "old variaties", such as Jacques Lebel, Keuleman, Lombarts Calville, Reinette de Flandre, Quastresdouble, Collapuis, President van Dievoet. Pomace samples were dried at 50 °C immediately after the pressing step, in December 2017. Two different pomace types were provided: one from cider production and the other from juice production, where the varieties were sweeter. They were delivered to CIMO, Bragança, in January 2018. After reception, the material was reduced to a fine dried powder, mixed to obtain a homogenate sample and stored protected from light and humidity, until further analysis.

Apple pomace from cider (Spain) | Apple pomace from cider (Belgium) | Apple pomace from juice (Belgium)

Figure 11 *- Apple pomace samples. The Belgian samples were dried at 50 °C at the processing company, while the Spanish samples were lyophilized at our laboratory facilities.*

2.3. Proximate composition

Although our ultimate goal was to develop a dermocosmetic formulation, we decided to determine the proximate composition to have an idea about the quality and properties of our samples. Additionally, as we already mentioned, apple pomace is susceptible to be use in Food products for its nutritional value. The proximate composition was determined according to AOAC procedures (AOAC, 2016). Samples from the three apple pomace types were characterized for moisture (only in Spanish samples, since Belgian samples were obtained from the processing company already dehydrated), ash, fat and carbohydrate contents. The soluble sugar were also determined by HPLC-RI.

2.3.1. Moisture content determination

Moisture was determined comparing the weight before and after freeze drying. The moisture content was calculated using the following equation:

$$\%moisture = \frac{(m_i - m_f)}{m_i} \times 100$$

Where m_i represents the initial mass, before the sample is lyophilized, and m_f represents the final mass, after the elimination of water by freeze drying.

2.3.2. Ash content determination

For the determination of mineral content, 300 mg of dried sample were added to calcined and previously weighed crucibles. Immediately after, the crucibles were introduced in the muffle (Lenton Thermal designs; ECF12/22), where they were incinerated at 550±15 °C, until a white ash was formed (approximately 20 h). The crucibles were cooled in a desiccator and the weigh was determined.

The results were expressed in ash percent using the following equation:

$$\%ash = \frac{m_f}{m_i} \times 100$$

Where m_i represent the initial mass introduced in the crucible and m_f represent the final mass after the incineration in the muffle.

Figure 12 *- Muffle used in ash determination.*

2.3.3. Fat content determination

To determinate the crude fat the powdered samples (5 g) were extracted with petroleum ether using a Soxhlet apparatus. After 8 hours, the extraction was interrupted and the flask, containing the fat solubilized in ether, was removed. Subsequently the solvent was evaporated and the fat content was determined using the following expression:

$$\%fat = \frac{m_f}{m_i} \times 100$$

Where m_i represents the initial mass of apple pomace and m_f represent the mass of fat obtained in the flask after the evaporation of the solvent.

Figure 13 *- Soxhlet equipment used in the determination of fat content.*

2.3.4. Protein content determination by Kjeldahl method

Kjeldahl method allows to quantify the crude protein content based in the nitrogen content of the sample. In this analysis, 500 mg of previously grinded samples were introduced in the digestion tubes with 15 ml of sulfuric acid and two selenium tablets to catalyze the reaction. The tubes were introduce in the digester (FossTM Digestor) during approximately 2 h at 410 °C. After cooling, the digestion tubes were placed in the Kjedahl equipment, where a distillation and a titration were automatically performed. First, the sample was diluted and neutralized using water and a solution of sodium hydroxide. Subsequently, the ammonia was distillated and dragged to another solution that contained boric acid, and methylene blue and methyl red, where the ammonia was collected. With the addition of ammonia to the solution, boron ion was formed in the same amount as nitrogen. The boron ion was then titrated with a standard solution of chloric acid. The protein content was calculated by multiplying the obtained value by a specific conversion factor that can vary from 5.18 to 6.38 depending on the matrix, in this case the factor selected was 6.25.

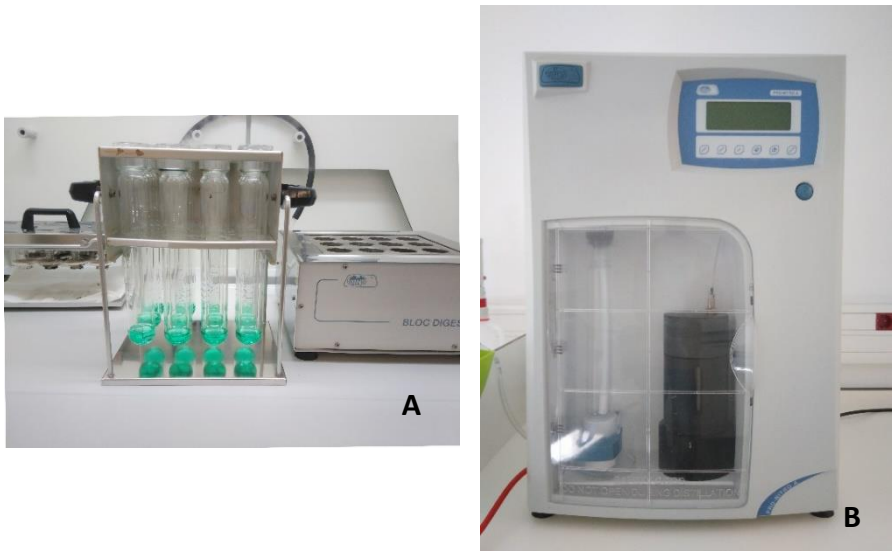


Figure 14 *- Equipment used in the determination of protein content. A - digestor. B - Kjeldahl equipment.*

2.3.5. Determination of soluble sugars

Soluble sugars were also determined in the powdered sample that remained after the crude fat extraction, as fat could interfere in sugar determination. The determination was carried out by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany), as already described (Barros et al., 2013). The defatted sample (1.0 g) was spiked with melezitose as internal standard (IS, 5 mg/ml), and was extracted with 40 ml of 80% aqueous ethanol at 80 °C for 1.5 h. The supernatant was concentrated at 60 °C under reduced pressure and the solid residues were dissolved in water to a final volume of 5 ml and filtered through 0.2 µm nylon filters from Whatman 9. Separation was achieved using a Eurospher 100-5 NH2 column (5 µm, 4.6 × 250 mm, Knauer) and quantification was performed using the internal standard method. The results were expressed in g per 100 g of pomace dry weight.

Figure 15 *- Equipment (HPLC-RI) used in soluble sugars identification.*

2.4. Polyphenol extracts preparation

For the extraction of the polyphenols, two different procedures were used. Each extraction procedure was conducted twice.

2.4.1. Decoction

In this procedure, 100 ml of deionized water were heated to its boiling point; then, 1 g of each powered sample was added and extracted during 5 min, followed by filtration through a Whatman filter paper No. 4. The extract was frozen and lyophilized. Every extraction was performed twice.

2.4.2. Maceration

Three different solvents were used in this extraction:

- Methanol: water 80:20
- Ethanol:water 80:20
- Ethanol

The extracts were prepared by mixing 1 g of the powered dried apple pomace sample with 30 ml of solvent, at 25 °C during 1 h under continuous stirring, followed by filtration through a Whatman filter paper No. 4. The remain residue was re-extracted with an additional portion of 30 ml under the same conditions, and the combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and stored at −20 °C for further analysis.

To eliminate the remaining water, the corresponding extracts were frozen and lyophilized.

2.5. Phenolic compounds analysis

The phenolic profile was determined after dissolving each dried extract in 20% aqueous ethanol at 10 mg/ml (decoctions and hydromethanolic maceration) or 20 mg/ml (hydroethanolic and ethanolic macerations) and filtration through a 0.22-µm disposable LC filter disk.

Chromatographic analysis was performed in a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system equipped with a diode array detector coupled to a electrospray ionization mass detector (LC-DAD-ESI/MS^n), a quaternary pump, an auto-sampler (kept at 5 °C), a degasser and an automated thermostatted column compartment, as previously described by Dias et al., (2016) and Pires et al., (2018). Chromatographic separation was achieved using a Waters Spherisorb S3 ODS-2 C18 (3 µm, 4.6 × 150 mm) column thermostatted at 35 °C. For the double online detection, a DAD (280, 330 and 370 nm as preferred wavelengths) and a mass spectrometer performed in negative mode (Linear Ion Trap LTQ XL) equipped with an ESI source, ThermoFinnigan, San Jose, CA, USA) were used and connected to the HPLC system.

The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% B (5 min), 15% B to 20% B (5 min), 20-25% B (10 min), 25-35% B (10 min), 35-50% B (10 min), and re-equilibration of the column, using a flow rate of 0.5 ml/min.

The identification was performed using standard compounds, when available, by comparing their retention times, UV–vis and mass spectra. If no standard compound was available, phenolic compounds were identified by comparing the obtained information with available data reported in the literature.

For quantitative analysis, a calibration curve for each available phenolic standard was created, when no commercial standard was available a similar compound from the same phenolic group was used as a standard. Quantification was made from the areas of the peaks recorded at 280 nm or 370 nm, depending on the compound, by comparison with the calibration curves obtained from standards. The results were expressed in mg per g of lyophilized extract.

***Table 5**- Standard used in the quantification of phenolic compounds*

Standard	Identified compound
Acido cafeico	Caffeic acid hexoside
Ac. Clorogenico	4-*O*-Caffeoylquinic acid
Ac. Clorogenico	5-*O*-Caffeoylquinic acid
Catequina	B-type (epi)catechin dimer
Epicatechin	Epicatechin
Catequina	B-type (epi)catechin trimer
Catequina	B-type (epi)catechin tetramer
Isoliquiritigenin	3-Hydroxyphloretin-2'-*O*-xylosyl-glucoside
Quercetina-3-O-rutinósido	Quercetin-3-*O*-rutinoside
Quercetina-3-O-rutinósido	Quercetin-3-*O*-glucoside
Quercetina-3-O-rutinósido	Quercetin-*O*-hexoside
Isoliquiritigenin	Phloretin-2-*O*-xyloglucoside
Quercetina-3-O-rutinósido	Quercetin-*O*-pentoside
Quercetina-3-O-rutinósido	Isorhamnetin-3-*O*-rutinoside
Isoliquiritigenin	Phlorizin (phloretin-2-*O*-glucoside)

***Figure 16** - Equipment (HPLC-MS) used in the determination of the phenolic compounds.*

2.6. Antioxidant activity

The antioxidant activity was evaluated after dissolving each extracts in its respective solvent. Successive dilutions were then prepared (10 to 0.3125 mg/ml).

2.6.1. DPPH radical-scavenging activity

This assay was performed using an ELX800 microplate reader (Bio-Tek Instruments, Inc.). As already described (Barreira, Rodrigues, Carvalho, & Ferreira, 2013), the reaction mixture in every microplate consisted of 30 µl of the diluted extract and 270 µl of a methanolic solution of DPPH radicals (6×10^{-5} mol L^{-1}). A control was also prepared adding 30 µl of the solvent without extract. The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation:

$$\%RSA = \frac{A_{DPPH} - A_S}{A_{DPPH}} \times 100$$

Where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against sample concentration.

Figure 17 *- Microplate reader for absorbance determination.*

2.6.2. Reducing power

This assay was performed using the same microplate reader described above. The methodology was previously described by (Barreira et al., 2013). Sodium phosphate buffer (200 mmol/l, pH 6.6, 0.5 ml) and potassium ferricyanide (1%, w/w, 0.5 ml) were added to each extract solution (0.5 ml). After 20 minutes of incubation at 50 °C,

trichloroacetic acid (10%, w/v, 0.5 ml) was incorporated. The mixture (0.8 ml) was poured in the 48-wells, as also deionized water (0.8 ml) and ferric chloride (0.1%, w/v, 0.16 ml).

Additionally, a control with the solvent not containing the extract was prepared for later comparison.

The absorbance was measured at 690 nm and the concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance *vs*. sample concentration.

2.6.3. Inhibition of β-carotene bleaching

A solution of β-carotene was prepared by dissolving it (2 mg) in chloroform (10 ml). Two milliliters of this solution were pipetted into a round-bottomed flask. The chloroform was removed at 40 °C in a rotary evaporator. Linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 ml) were added to the 0.2 mg of β-carotene that remained in the flask. Immediately after, the flask was vigorously shaken to obtain an emulsion.

Aliquots (4.8 ml) of this emulsion were transferred into test tubes containing the extracts at different concentrations (0.2 ml). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm (Analytik Jena 200-2004 spectrophotometer). Absorbance was also measured after 2 h of incubation. β-Carotene bleaching inhibition was calculated using the following equation:

$$\%Inhibition = \left({}^{A_{470(t_{120})}}\Big/ {}_{A_{470(t_0)}} \right) \times 100$$

Where $A_{470(t120)}$ is the absorbance after 2h of incubation, and $A_{470(t0)}$ is the absorbance at the zero time. The concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of β-carotene bleaching inhibition percentage against sample concentration.

2.7. Antibacterial activity of the extracts

2.7.1. Bacterial strains

Bacterial strains were clinical isolates from patients hospitalized in the Local Health Unit of Bragança or Central Hospital of Trás-os-Montes and Alto-Douron (Vila Real):

Staphylococcus aureus (Gram positive) isolated from wound exudate, *Proteus mirabilis* (Gram negative) isolated from urine, *Pseudomonas aeruginosa* (Gram negative) isolated from expectoration; or purchased from ATCC (11827, Liofilchem, Italy): *Propionibacterium acnes* (Gram negative). These bacterial strains were used to screen the antimicrobial activity of the developed extracts because they are typically found in human skin with special focus on *P. acnes* for being linked to the skin disorder *acne vulgaris*.

2.7.2. Determination of the minimal inhibitory concentrations (MIC)

MICs were determined by the microdilution method together with the rapid colorimetric assay using *p*-iodonitrotetrazolium chloride (INT). Briefly, a stock solution was prepared from the hydroalcoholic and ethanolic extracts, by dissolving 100 mg in 5 ml of TSB media with 5% DMSO. After, 200 µl of this solution was pipetted to the firs well of a 96 microplate and 100 µl were transferred to the wells below containing 100 µl of culture media, and so on making successive dilutions (20-0.15625 mg/ml).

Afterwards, 10 µl of inoculum (1.5×10^8 CFU/ml) were added to all wells containing the tested concentrations. Three negative controls were prepared (one TSB media, another one with the extract, and the third one with medium and inoculum). Ampicillin, methicillin and streptomycin (for *P. acnes*) were used as positive controls.

The plates were then incubated in an oven (Jouan, Berlin, Germany) at 37 °C for 24 h, except for *P. acnes*, that due its relatively slow-growth was incubated during a week. MICs were determined after addition of INT (0.2 mg/ml, 40 µl) and after incubation at 37 °C for 30 min. The viable microorganisms reduced the yellow dye to pink. MICs were defined as the lowest extract concentration that prevented this change and exhibited the complete inhibition of bacterial growth. The assays were carried out in duplicate.

2.8. Pectin extraction

Pectin was obtained by hot acid extraction, following a previously established procedure (Miceli-Garcia, 2014), with minor modification. The extraction was performed at two different pHs, for later comparison of the yield. For each extraction, 50 g of frozen apple pomace were blended with 200 ml of distilled water, and this slurry was transferred to a 600 ml glass beaker in which 200 ml of distilled water were added and heated under continuous stirring up to 20 °C. Hydrochloric acid was added to the dispersion to set the pH to 1.5 or 3; pH was monitored using and pH meter equipped with a temperature compensation probe (Hanna Instruments, Microprocessor pH meter 211, UK). Dispersions were heated at 90 °C under continuous stirring (350 rpm) using a laboratory heating plate and a magnetic stirrer.

The slurry was cooled to room temperature, in about 30 minutes, using an ice bath. To separate the liquid portion, the slurry was centrifuged at 6000 rpm for 30 minutes at 15 °C in a centrifuge (Centurion K24OR refrigerated centrifuge, West Sussex, UK). The supernatants were filtered through a Buchner funnel with a Whatman No.4 filter paper connected to a vacuum filtration system. The remaining solids were centrifuged and filtered under the same conditions given above. The filtrated supernatants were combined and the pH was adjusted to 3.5 with 1 M sodium hydroxide.

Then, the extract was mixed with an equal volume of ethanol, and stirred for 10 min at room temperature. The precipitate was separated by centrifugation, under the same

conditions given above. Afterwards, the precipitate was dispersed in 500 ml of 70% ethanol and stirred for 10 min at 250 rpm at room temperature, and the alcohol was removed by centrifugation as explained above. The extracted pectin was freeze dried under -50 °C and 0.22 mbar by a bench top freeze drying system (Labconco FreeZone 4.5, Kansas City, MO, USA) for approximately 60 h. The yield was gravimetrically determined, dividing the weight of pectin extracted by the total mass of used apple pomace:

$$\%pectin = \frac{m_{pectin}}{m_{app}} \times 100$$

Where m_{pectin} is the mass of pectin obtained and m_{app} is the total mass of frozen apple pomace used in the extraction, in our case, 50 g.

2.9. Dermocosmetic gel formulation

Pectin was dissolved in water by the direct addition method (Featherstone, 2015). Citric acid (300 mg) was dissolved in distilled water (30 ml) to create an acidic environment and facilitate the pectin dissolution. Immediately afterwards, pectin (500 mg) was added and heated (60-100 °C) under vigorous agitation during 20 minutes until complete dissolution (pectin solutions are not recommended to be maintained above 71 °C for more than 1 h, since pectin can break down).

Then, 175 mg of the phenolic extract were dissolved in 500 µl of distilled water and 1 g of propylene glycol, used as a humectant agent. The dissolved extract was added to the pectin gel and stirred at room temperature to obtain a homogeneous distribution of the phenolic extract. The pH was adjusted adding 500 mg of triethanolamine, obtaining a final pH of 5.69. The final concentration of the phenolic extract in the gel was 5 mg/ml, since the final volume was 35 ml. Other gels were prepared using 350 mg of phenolic extract, corresponding to a final concentrations of 10 mg/ml. In this case, citric acid was not added, since pectin is acid enough by itself, and in this way, the amount of triethanolamine, added to adjust the pH, was reduced.

A blank gel formulation (without phenolic extract) was also prepared for posterior comparison of bioactivities.

2.10. Bioactivity of the cosmetic gel

The antioxidant and antimicrobial assays were performed for different hydrogel formulations as previously described for the extracts (sections 2.6. and 2.7.).

In the DPPH antioxidant assay, the reaction was performed in a vial and not directly in the wells, as normally done, since pectin precipitated at the bottom of the well, hindering the passage of light through the well and impeding the determination of absorbance. In this case, 90 µl of gel and 810 µl of DPPH (6×10^{-5} M) solution were added to the vials and left to stand in the dark for 1 h. Afterwards, 300 µl of the supernatant were pipetted and placed in the wells to determine the absorbance at 515 nm.

2.11. Statistical analysis

For all the experiments conducted in apple pomace extracts, two independent samples of each pomace type were selected; each of these sample was extracted thrice, and each extract was also analyzed three times. The results are expressed as mean values±standard deviation (SD), except for antimicrobial assays.

An analysis of variance (ANOVA), followed by Tukey's test (homoscedastic distributions) or Tamhane's T2 test (heteroscedastic distributions) was used to classify the statistical differences among the mean scores obtained for each apple pomace type or extraction solvent. The fulfillment of the one-way ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Shapiro Wilk's and the Levene's tests, respectively. In cases with less than three groups of results, a *t*-Student test was performed to compare the results. In all cases, a 5% significance level was considered. All statistical analysis were performed using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., USA).

III. Results and discussion

Despite the main purpose of characterizing the phenolic composition of apple pomace to evaluate its potential use as a natural ingredient of a novel dermocosmetic formulation (prepared also using apple pomace pectin as a natural gelling agent), a preliminary characterization of its proximate composition was also performed in order to assess potential food applications of this industrial waste.

3.1. Proximate composition

Samples provided by Tuinsappen Lombarts Calville (Belgium) were already dried by the company itself, so it was not possible to measure water content. Samples provided by Corporación Hijos de Rivera S.L. (Spain) were frozen immediately after juice extraction, allowing to determine their water content. As listed in **Table 5**, apple pomace still retains a high water content (75.3 g/100 g), but the quantified value is in agreement with previous analytical work (Gullón, Falqué, Alonso, & Parajó, 2007).

In dry weight basis, carbohydrates are clearly the major compound (above 90% in all cases). According to the results obtained for soluble sugars, these carbohydrates were mainly present as individual sugars, particularly fructose and glucose, in addition to small sucrose percentages. The Belgian apple cultivars used in juice extraction presented the highest fructose content (55 g/100 g dried pomace), which in turn was detected in the lowest percentage in Belgian apples used in cider production (42 g/100 g dried pomace). Glucose and sucrose were both quantified in the highest percentages in Spanish apple cultivars (26 and 7.4 g/100 g dried pomace, respectively). In either case, the sugar percentages were similar to those detected in previous studies (Gullón, Falqué, et al., 2007).

Owing to the similarity among carbohydrates content and the differences in soluble sugars, it might be hypothesized that the Belgian cider cultivars present the highest percentages of fiber.

The studied samples also presented significant differences (p<0.050) concerning protein, fat and ash contents (as indicated by different letters). Nevertheless, the proximate profiles were highly similar to those characterized in industrial apple pomace from a different company (Rodríguez Madrera, Pando Bedriñana, & Suárez Valles, 2017). Considering the major differences in soluble sugars, the energy values were also significantly different, reaching the maximum values in Spanish cultivars (351 kcal g/100 g dried pomace).

In general, apple pomace revealed an interesting nutritional potential, which might be used in food applications, thereby offering an alternative added value beyond the dermocosmetic application detailed in the next sections.

Table 6 *- Proximate composition (values presented in g/100 g dried pomace, except for water: g/100 g fresh pomace and energy: kcal/100 g dried pomace) of different apple pomace samples.*

	water	**fat**	**proteins**	**carbohydrates**	**fructose**	**glucose**	**sucrose**	**ash**	**energy[3]**
Spanish	75.3±0.4	1.9±0.1 a	3.3±0.1 b	93.0±0.2 b	46±1 b	26±2 a	7.4±0.2 a	1.7±0.2 b	351±12 a
Belgian (cider)	*	1.6±0.1 b	2.8±0.1 c	94.2±0.2 a	42±1 c	23±1 b	1.7±0.1 b	1.4±0.1 c	291±4 c
Belgian (juice)	*	1.0±0.1 c	4.0±0.2 a	93.1±0.3 b	55±1 a	20±1 c	1.8±0.1 b	1.9±0.2 a	329±7 b
Homoscedasticity[1] (p-value) (n = 54)	-	0.136	0.036	0.444	0.008	<0.001	<0.001	0.256	0.007
ANOVA[2] (p-value) (n = 54)	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]p-values <0.050 indicate heteroscedastic distributions, thereby multiple comparison was performed by Tamhane's T2 test; p-values >0.050 indicate homoscedastic distributions, thereby multiple comparison was performed by Tukey HSD test. [2]p-values <0.050 indicate that the corresponding parameter presented significant differences (identified with different letters) for at least one apple pomace type. [3]Energy values were calculated as: $m_{fat} \times 9 + m_{(proteins + fructose + glucose + sucrose)} \times 4$ (where m values were included in grams).
*Samples were provided dried.

3.2. Phenolic compounds

Four different extracts were prepared in order to evaluate the effect of the extraction step in phenolic compounds yield, as well as in bioactivity levels. One of the extracts was prepared by decoction in water, and the other three extracts were prepared by maceration, using the same solid to liquid ratio (1:30), but different solvents: ethanol, ethanol:water (80:20) and methanol:water (80:20) The extraction yields obtained for each apple pomace type varied with the used solvent (capitalized letters in **Table 6**), which might be, in part, explained by the simultaneous extraction of soluble sugars (*e.g.*, apple pomace remaining from juice production from the Belgian company, which contained the highest soluble sugars percentages, reached the maximum yield when extracted with water). Likewise, each apple pomace type allowed different extraction yields according to the selected solvent (small case letters). This result might, once again, be related to the soluble sugars content, since Belgian apple pomace remaining from juice production reached the maximum yields in all cases where water was used (pure or in mixture) as the extraction solvent.

Table 7 *- Phenolic compounds extraction yields according to apple pomace type or solvent composition (values are presented in percentage).*

	EtOH	**H_2O**	**EtOH:H_2O (80:20)**	**MeOH:H_2O (80:20)**	Homoscedasticity[1] (*p*-value) (n = 72)	ANOVA[4] (*p*-value) (n = 54)
Spanish	52±2 aA	44±1 bC	47±3 bB	47±1 bB	<0.001	<0.001
Belgian (cider)	28±1 cB	41±1 bA	41±3 cA	40±1 cA	<0.001	<0.001
Belgian (juice)	37±2 bC	65±8 aA	52±2 aB	50±1 aB	<0.001	<0.001
Homoscedasticity[1] (*p*-value) (n = 54)	<0.001	<0.001	0.008	0.140	-	-
ANOVA[2] (*p*-value) (n = 54)	<0.001	<0.001	<0.001	<0.001	-	-

[1]*p*-values <0.050 indicate heteroscedastic distributions, thereby multiple comparison was performed by Tamhane's T2 test; *p*-values >0.050 indicate homoscedastic distributions, thereby multiple comparison was performed by Tukey HSD test. [2]*p*-values <0.050 indicate that the corresponding parameter presented significant differences (identified with different lower case letters) for at least one apple pomace type. [3]*p*-values <0.050 indicate that the corresponding parameter presented significant differences (identified with different upper case letters) for at least one solvent.

The extraction yield was calculated from the following equation:

$$Extraction\ yield = \frac{m_{ext}}{m_{samp}} \times 100$$

Where m_{samp} represents the apple pomace mass used for the extraction, and m_{extr} represent the mass of extract obtained after complete evaporation of the solvent.

However, besides the extraction yield, it was important to verify the phenolic composition of each prepared extract. Data concerning the retention time, λ_{max}, pseudomolecular ion, main fragment ions in MS^2, tentative identification and concentration of phenolic compounds are presented in **Tables 7** and **8**.

In the case of apple pomace from the Spanish company (**Table 7**), fifteen different phenolic compounds were identified, three phenolic acids (compounds 1 to 3), four flavan-3-ols (compounds 4 to 7), five flavonoids (isorhamnetin and quercetin, compounds 9 to 11, 13 and 14) and three di-hydrochalcones (compounds 8, 12 and 15).

Compounds 3, 5, 9, 10 and 13 were positively identified as 5-*O*-caffeoylquinic acid, epicatechin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside and isorhamnetin-3-*O*-rutinoside, respectively, by comparison with authentic standards, as also by their MS fragmentation patterns, retention times and UV-vis characteristics.

Compounds 1 and 3 were identified as phenolic acids. The pseudomolecular ion of peak 1 ($[M-H]^-$ at *m/z* 341) matched a caffeic acid hexoside, while compound 2 was easily distinguished by its base peak at *m/z* 173 [quinic acid-H-H_2O]$^-$, which allowed identifying it as 4-*O*-caffeoylquinic acid according to the previously described fragmentation pattern (Clifford, Knight, & Kuhnert, 2005).

Compounds 4, 6 and 7 corresponded to proanthocyanidin oligomers of the procyanidin class (*i.e.*, consisting of catechin and/or epicatechin units). Peak 4 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 577 and MS^2 fragmentation patterns coherent with B-type (epi)catechin dimers (*i.e.*, (epi)catechin units with C4–C8 or C4–C6 interflavan linkages). Characteristic product ions were observed at *m/z* 451 (-126 mu), 425 (-152 mu) and 407 (-152-18 mu), attributable to the HRF, RDA and further loss of water from an (epi)catechin unit, and at *m/z* 289, that is associated to the fragment corresponding

to the (epi)catechin unit. Similarly, compound 8 (pseudomolecular ions $[M-H]^-$ at *m/z* 865) and compound 7 (pseudomolecular ions $[M-H]^-$ at *m/z* 1153) can be assigned as B-type (epi)catechin trimers and tetramers, respectively. In all cases, fragmentation patterns are coherent with those expected for these types of compounds, *i.e.*, similar to those observed for procyanidin dimers but with additional fragments from the alternative cleavages of different interflavan bonds.

Peaks 11 ($[M-H]^-$ at *m/z* 463) and 13 ($[M-H]^-$ at *m/z* 433) were assigned to quercetin derivatives. Both presented MS^2 fragments corresponding to distinct losses of hexosyl (-162 mu) and pentosyl (-132 mu) moieties, and an elution order coherent with the type of substituent sugars, according to their expected polarity, although the position and nature of the sugar moieties could not be identified, because their retention times did not correspond to any of the standards available. Therefore, they were identified as quercetin-*O*-hexoside and quercetin-*O*-pentoside, respectively.

Compounds 8 ($[M-H]^-$ at *m/z* 583) 12 ($[M-H]^-$ at *m/z* 567) and 15 ($[M-H]^-$ at *m/z* 435) were respectively identified as hydroxyphloretin-2'-*O*-xylosyl-glucoside, phloretin-2'-*O*-xyloglucoside, phlorizin (phloretin-2'-*O*-glucoside), chalcones that are also commonly present in apple. This identification was performed taking into previous works describing the phenolic profile of apple pomace extracts (Waldbauer et al., 2017).

In the case of Belgian apple pomace remaining after cider production from the Belgian company (**Table 8**), the same phenolic compounds could only be detected in the case of aqueous extracts. Compounds 4 to 6 and 8 to 15 were absent from the ethanolic extracts, while MeOH:H_2O extracts lack compounds 10, 11, 13 and 14 and EtOH:H_2O extracts did not present compounds 5, 7, 8, 10, 14 and 15. In the case of apple pomace remaining from juice extraction, the results were even worse, since the chromatograms obtained from the corresponding extracts did not reveal any peak.

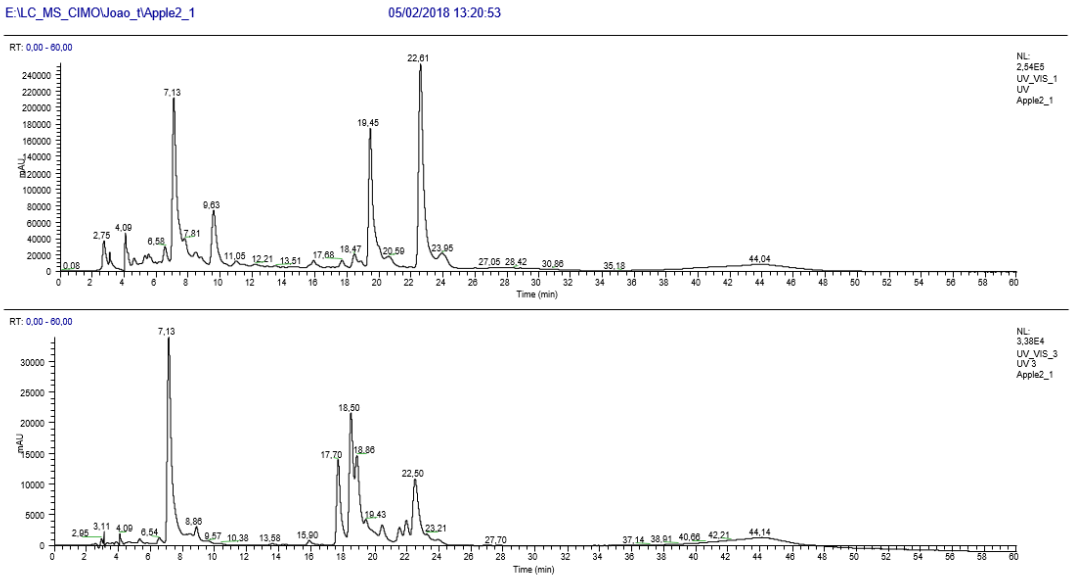


***Figure 18**- Phenolic compounds chromatogram. The upper chromatogram was obtained at a wavelength of 280 nm and the lower chromatogram at 370 nm.*

Besides their higher diversity, the extracts obtained from apple pomace recovered from the Spanish company also presented higher concentrations, as it was particularly noticeable for EtOH, MeOH:H_2O and EtOH:H_2O extracts.

Taking these results in consideration, apple pomace from the Spanish company, which should be reminded that were dried by lyophilisation, instead of thermal drying (as applied in Belgian apple pomace), became our top choice as an alternative source of bioactive compounds to be included in new dermocosmetical formulations.

Table 8 *- Retention time (Rt), waveleghts of maximum absorption in visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in apple pomace from Corporación Hijos de la Rivera S.L. (Spain).*

Peak	Rt (min)	λ_{max} (nm)	Molecular ion (*m/z*)[a]	MS^2 (*m/z*)	Tentative identification	Quantification (mg/100 g dried pomace)				Homoscedasticity[1]	ANOVA[2]
						EtOH	H_2O	MEOH:H_2O	EtOH:H_2O	(*p*-value) (n = 72)	(*p*-value) (n = 72)
1	4.87	364	341	179(100)	Caffeic acid hexoside	tr	tr	tr	tr	-	-
2	6.67	322	353	191(12), 179(1), 173(100), 161(1), 135(2)	4-*O*-Caffeoylquinic acid	14±2 c	20±1 a	18±2 b	11±2 d	<0.001	<0.001
3	7.24	327	353	191(100), 179(6), 173(2), 161(1), 135(1)	5-*O*-Caffeoylquinic acid	114±20 a	69±6 b	108±16 a	66±1 b	<0.001	<0.001
4	8.02	281	577	451(24), 425(100), 407(21), 289(12)	B-type (epi)catechin dimer	18±4 b	16±1 b	29±6 a	19±4 b	<0.001	<0.001
5	9.82	281	289	245(100), 203(5), 187(1), 161(2), 137(2)	Epicatechin	31±1	tr	12±1	tr	<0.001	<0.001*
6	11.39	280	865	739(74), 713(44), 695(100), 577(64), 575(37), 425(10), 407(9), 289(8), 287(7)	B-type (epi)catechin trimer	10±2 b	10±1 b	17±1 a	7±2 c	<0.001	<0.001
7	12.54	280	1153	865(19), 863(18), 577(6), 575(11), 289(3), 287(4)	B-type (epi)catechin tetramer	2.4±0.2 d	4.0±0.1 c	15±2 a	7±2 b	<0.001	<0.001
8	16.13		583	289(100)	3-Hydroxyphloretin-2'-*O*-xylosyl-glucoside	4.5±0.5 b	1.9±0.4 d	3.6±0.4 c	5.6±0.5 a	<0.001	<0.001
9	17.95	353	609	301(100)	Quercetin-3-*O*-rutinoside	13±1 c	21±1 b	24±2 a	12±1 c	<0.001	<0.001
10	18.79	354	463	301(100)	Quercetin-3-*O*-glucoside	13±1 b	nd	24±3 a	13±1 b	<0.001	<0.001
11	19.14	353	463	301(100)	Quercetin-*O*-hexoside	13±1 b	nd	24±1 a	12±1 b	<0.001	<0.001
12	19.70	285	567	273(100)	Phloretin-2-*O*-xyloglucoside	20±4 a	tr	15±1 b	11±1 c	<0.001	<0.001
13	21.84	356	433	301(100)	Quercetin-*O*-pentoside	12±1 c	20±1 b	22±1 a	11±1 d	<0.001	<0.001
14	22.27	350	623	315(100)	Isorhamnetin-3-*O*-rutinoside	12±1 c	20±1 b	24±2 a	12±1 c	<0.001	<0.001
15	22.89	285	435	273(100)	Phlorizin (phloretin-2-*O*-glucoside)	24±1 b	tr	39±1 a	9±1 c	<0.001	<0.001
					Phenolic compounds	**301±32 b**	**182±7 c**	**375±42 a**	**197±5 c**	**<0.001**	**<0.001**

[1]*p*-values <0.050 indicate heteroscedastic distributions, thereby multiple comparison was performed by Tamhane's T2 test. [2]*p*-values <0.050 indicate that the corresponding parameter presented significant differences (identified with different letters) for at least one solvent.
*In this case, differences were evaluated by the *t*-Student test, as only two groups of results were available.

Table 9 *- Retention time (Rt), waveleghts of maximum absorption in visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in apple pomace from Tuinsappen Lombarts Calville (Belgium).*

Peak	Rt (min)	λ_{max} (nm)	Molecular ion (*m/z*)[a]	MS^2 (*m/z*)	Tentative identification	Quantification (mg/100 g dried pomace)				Homoscedasticity[1]	ANOVA[2]
						EtOH	H_2O	MEOH:H_2O	EtOH:H_2O	(*p*-value) (n = 72)	(*p*-value) (n = 72)
1	4.87	324	341	179(100)	Caffeic acid hexoside	0.64±0.02	tr	tr	tr	-	-
2	6.67	322	353	191(12), 179(1), 173(100), 161(1), 135(2)	4-*O*-Caffeoylquinic acid	4.1±0.3 c	15±4 a	10±1 b	9±2 b	<0.001	<0.001
3	7.24	327	353	191(100), 179(6), 173(2), 161(1), 135(1)	5-*O*-Caffeoylquinic acid	2.7±0.3 c	12±3 a	9±1 b	7±1 b	<0.001	<0.001
4	8.02	281	577	451(24), 425(100), 407(21), 289(12)	B-type (epi)catechin dimer	nd	nd	3.3±0.4	5.1±0.5	<0.001	<0.001*
5	9.82	281	289	245(100), 203(5), 187(1), 161(2), 137(2)	Epicatechin	nd	tr	tr	nd	-	-
6	11.39	280	865	739(74), 713(44), 695(100), 577(64), 575(37), 425(10), 407(9), 289(8), 287(7)	B-type (epi)catechin trimer	nd	8±2 a	3.2±0.3 c	6±2 b	<0.001	<0.001
7	12.54	280	1153	865(19), 863(18), 577(6), 575(11), 289(3), 287(4)	B-type (epi)catechin tetramer	2.6±0.2 b	4.4±0.5 a	3.2±0.2 b	nd	<0.001	<0.001
8	16.13	282	583	289(100)	3-Hydroxyphloretin-2'-*O*-xylosyl-glucoside	nd	tr	tr	nd	-	-
9	17.95	353	609	301(100)	Quercetin-3-*O*-rutinoside	nd	18±1 a	18±1 a	15±2 b	<0.001	<0.001
10	18.79	354	463	301(100)	Quercetin-3-*O*-glucoside	nd	18±1	nd	nd	-	-
11	19.14	353	463	301(100)	Quercetin-*O*-hexoside	nd	19±1	nd	15±3	<0.001	<0.001*
12	19.70	285	567	273(100)	Phloretin-2-*O*-xyloglucoside	nd	tr	tr	tr	-	-
13	21.84	356	433	301(100)	Quercetin-*O*-pentoside	nd	19±1	nd	15±2	<0.001	<0.001*
14	22.27	350	623	315(100)	Isorhamnetin-3-*O*-rutinoside	nd	18±1	nd	nd	-	-
15	22.89	285	435	273(100)	Phlorizin (phloretin-2-*O*-glucoside)	nd	tr	tr	nd	-	-
					Phenolic compounds	**10.0±0.4 d**	**132±12 a**	**47±3 c**	**71±6 b**	**<0.001**	**<0.001**

[1]*p*-values <0.050 indicate heteroscedastic distributions, thereby multiple comparisons were performed by Tamhane's T2 test. [2]*p*-values <0.050 indicate that the corresponding parameter presented significant differences (identified with different letters) for at least one solvent.
*In this case, differences were evaluated by the *t*-Student test, as only two groups of results were available.

Comparing the results from each solvent, the best results (higher phenolic compounds concentration) were obtained with ethanol (301 mg of phenolic compounds per 100 g of dried pomace) and MeOH:H_2O (375 mg of phenolic compounds per 100 g of dried pomace). The main compound in these extracts was 5-*O*-caffeoylquinic acid, with concentrations above 100 mg per 100 g of dried pomace, but the di-hydrochalcones (3-hydroxyphloretin-2'-*O*-xylosyl-glucoside, phloretin-2-*O*-xyloglucoside and phloretin-2-*O*-glucoside), owing to their validated cosmetic advantages, should also be highlighted considering the main purpose of this work.

3.3. Antioxidant activity

As it could be somehow anticipated from the phenolic profiles obtained with each extract, the highest antioxidant activity (lowest EC_{50} values) was achieved in the case of apple pomace from the Spanish company, either in the case of DPPH scavenging activity, reducing power, or β-carotene bleaching inhibition (**Table 9**).

When comparing different solvents, it is also evident that the antioxidant activity measured in Spanish apple pomace extracts was provided by other compounds besides phenolics, as there is no direct correlation among the concentration in phenolic compounds (**Table 7**) and the EC_{50} values obtained in each assay (**Table 9**).

In addition, the best extracts in DPPH scavenging activity and reducing power were those obtained with MeOH:H_2O (80:20) and EtOH:H_2O (80:20), while the most active extract against β-carotene bleaching was that prepared with ethanol.

Considering the lipid composition of human skin, the ethanolic extracts could be considered as the best choice, so these were selected to perform the antimicrobial assays described in the next section. Owing to their innocuous nature, the hydroethanolic extracts were also tested.

Table 10 *- Phenolic compounds extraction yields according to apple pomace type or solvent composition (values are presented in percentage).*

DPPH scavenging activity				
	EtOH	**H_2O**	**MeOH:H_2O (80:20)**	**EtOH:H_2O (80:20)**
Spanish	4.1±0.4	1.7±0.2	0.6±0.1	0.7±0.1 c
Belgian (cider)	>10	9±1	>10	10±1 a
Belgian (juice)	>10	>10	>10	9±1 b
Homoscedasticity[1] (*p*-value) (n = 54)	-	<0.001	-	<0.001
ANOVA or *t*-Student[2] (*p*-value) (n = 54)	-	<0.001	-	<0.001
Reducing power				
	EtOH	**H_2O**	**MeOH:H_2O (80:20)**	**EtOH:H_2O (80:20)**
Spanish	1.0±0.1	1.0±0.1	0.35±0.02	0.62±0.02 c
Belgian (cider)	>10	4.2±0.5	>10	5.2±0.3 a
Belgian (juice)	>10	>10	>10	2.5±0.1 b
Homoscedasticity[1] (*p*-value) (n = 54)	-	<0.001	-	0.140
ANOVA or *t*-Student[2] (*p*-value) (n = 54)	-	<0.001	-	<0.001
β-carotene bleaching inhibition				
	EtOH	**H_2O**	**MeOH:H_2O (80:20)**	**EtOH:H_2O (80:20)**
Spanish	0.8±0.1	3.4±0.3	1.4±0.2	1.2±0.3
Belgian (cider)	>10	>10	>10	>10
Belgian (juice)	>10	>10	>10	>10
Homoscedasticity[1] (*p*-value) (n = 54)	-	-	-	-
ANOVA[2] (*p*-value) (n = 54)	-	-	-	-

[1]*p*-values <0.050 indicate heteroscedastic distributions, thereby multiple comparison was performed by Tamhane's T2 test. [2]*p*-values <0.050 indicate that the corresponding parameter presented significant differences (identified with different lower case letters) for at least one apple pomace type (in cases with three different values ANOVA was applied; in cases with only two values, a *t*-Student test was applied).

3.4. Antimicrobial activity

The antimicrobial activity was measured against *P. acnes*, owing to their relevance in acne process, methicillin-resistant *Staphylococcus aureus* (MRSA), *Proteus mirabilis* and *Pseudomonas aeruginosa*. The minimal inhibitory concentration values were not exceptionally low, but in the specific case of *P. acnes* the result might be considered as satisfactory. Ampicillin was used as control in the MIC determination.

Table 11 *- Antimicrobial activity of selected extract from apple pomace of Corporación Hijos de la Rivera S.L. (Spain).*

	EtOH extract	EtOH:H_2O extract
Bacteria	MIC (mg/ml)	MIC (mg/ml)
Propionibacterium acnes	5	2.5
MRSA	5	2.5
Proteus mirabilis	20	10
Pseudomonas aeruginosa	>20	>20

3.5. Dermocosmetic gel formulation and characterization

In order to obtain a formulation free, as much as possible, of artificial ingredients, the pectin present in apple pomace was chosen as the jellifying agent.

This methodology was conducted in our lab for the first time, being especially optimized for the current work. Different pH conditions were assayed, allowing to achieve extraction yields varying from 1.69 % (pH = 1.5) up to 1.87% (pH = 3).

Figure 19*-Pectin extracted from apple pomace.*

Pectin was further incorporated in the formulated hydrogels until the intended consistency was achieved (500 mg in a total volume of 35 mL).

Considering the antimicrobial results obtained with the assayed apple pomace extracts, the hydroethanolic extract was chosen to be incorporated in the hydrogel. The selected concentration was 5 mg/mL.

***Figure 20**- gel and blank gel prepared.*

This gel was afterwards evaluated for its antioxidant and antimicrobial activity. In the first case, the tested assays were DPPH scavenging activity and reducing power. The gel achieved scavenging effect of DPPH over 85%, while the absorbance of the reaction endpoint was 1.858, much higher than the 0.500 value that is considered as the EC_{50} value in this assay.

In the case of antimicrobial activity, the prepared gel was tested only against *P. acnes*, since this species was the most relevant considering the purpose of this work. However, the bacterial growth was not inhibited, probably due to the 2× dilution effect induced by the solubilization of the gel in culture medium.

Therefore, a second gel was prepared with 10 mg/mL of hydroethanolic extract. In this second attempt, the growth of *P. acnes* was effectively inhibited.

IV. Conclusion

Although the ultimate goal of this study was the determination of its potential in cosmetic application, apple pomace also revealed an interesting nutritional value, thereby offering alternative applications to this industrial by-product. Among nutritional parameters, soluble sugars were the most abundant compounds, showing significant differences among apple pomace types. The three different studied samples also presented significant differences concerning protein, fat and ash contents, which lead us to consider the different nutritional value of the pomace considering its origin and the cultivars there present.

Regarding phenolic compounds composition the Spanish apple pomace proved to be the best source of these bioactive compounds, which should be a consequence of the drying process. As the Spanish samples were immediately frozen after the pressing and subsequently lyophilized, instead of thermally dried, the oxidation of phenolic compounds must have been avoided. Fifteen different phenolic compounds were identified in these samples, three phenolic acids, four flavan-3-ol, five flavonoids and three di-hydrochalcones. Taking these results in consideration, apple pomace from the Spanish became our top choice as an alternative source of bioactive compounds to be included in the new dermal formulations. Within Spanish samples, the extracts that presented the highest phenolic concentration were EtOH, MeOH:H_2O and EtOH:H_2O extracts.

Pertaining antioxidant assays, ethanol extract proved to be the most active extract against β-carotene bleaching. Considering the lipid composition of human skin, the ethanol extract was chosen to be added to the hydrogel, as it is also much less toxic than methanol.

For all the above, the extracts tested against the different bacteria were the ethanolic and the hydroethanolic. The hydroethanolic showed to have a better antibacterial activity against *P. acnes*, MRSA, *Proteus mirabilis* and *Pseudomonas aeruginosa*. The antimicrobial activity against *P. acnes* was especially important, since this bacteria is linked to acne and apple phenolics already showed inhibitory capacity of fat production in sebaceous cells.

For the dermal hydrogel formulation, pectin was obtained by hot-acid extraction; however, this method, at industrial scale, leads to pectin with lower degree of polymerization and produces high amounts of chemical waste. Therefore, the study of possible alternative methods, as enzymatic extraction, would be of interest for future research in this field. The resulting hydrogel showed to have antioxidant and antimicrobial activity against *P. acnes* at a concentration of 5 mg/ml, proving its validity in this application.

In general, apple pomace proved to be a valuable source of active and functional compounds, especially polyphenols and pectin, which could be applied in dermal formulations. Apple pomace phenolics not only proved its antioxidant activity, but also its anti-microbial potential against certain skin bacteria, where *P. acnes* is worth to be mentioned. However, further research would be needed on this field to confirm the anti-acne potential of apple pomace polyphenols.

Finally, using a circular economy approach, industrial by-products, which are typically discarded as waste, could have interesting applications to obtain value-added compounds that are likely to be used in different industrial sectors, such as food, cosmetic and pharmaceutical.

V. References

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