Universidade de Lisboa

Faculdade de Farmácia

Evaluation of the anti-inflammatory activity of *Rubus spp* polyphenol metabolites

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Food Quality and Health

2019

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Acknowledgments

I would like to start with a special thank you and express gratitude and acknowledgement to Doctor Regina Menezes Echaniz, my supervisor. I am very grateful for not only all the support and guidance, but also for the patience comprehension for my situation. Also, Doctor Cláudia Nunes dos Santos, who always had a little time to help me when I as in doubt.

My sincere appreciation to Doctor Maria Eduardo Figueira for accepting to be my supervisor at Faculdade de Farmacia, thank you for the support offered during all my work.

I would also like to acknowledge my laboratory colleagues, who all helped me during my time at the lab. Thanks to Andreia Gomes, Inês Fiqueira and Rita Ramos for their hospitality and for always being there to help, explain everything and give advice. To Diogo Carregosa and Maria Inês Bento who were always my company.

I cannot forget to thank my parents, my brother and my boyfriend. Without their support it would have been impossible to keep working while doing my internship. They gave me strength and never let me give up. Also, a heartfelt thank you to Fernanda Albuquerque for always being my listener and my “mother from Lisbon” and Mr. Manfred who was always supportive and comprehensive.

Abstract

Chronic diseases have become a matter of high priority for Europe and several studies point to (non-infectious) inflammatory processes as a determining factor in their origin. It has been reported that high concentrations of inflammatory markers have been detected in these diseases. Several studies confirm that a diet based on fruit and vegetable intake is inversely related to the incidence of chronic diseases.

Berries play an important role amongst fruits due to their high content of phenolic compounds, which studies have pointed out for their anti-inflammatory effects. Over the years, the interest for polyphenols has increased largely, but their mechanism of action it is still far from being fully understood. Several studies, including cell-based in vitro studies, have been used to understand and unknot them.

The nutritional profile of raspberries evaluated in this study was determined by proximal analysis, the total phenolic compounds were quantified using the Folin–Ciocalteu assay method, and the major classes of phenolic compounds profile was determined by high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS). Selected raspberries, based on their chemical profile, were subjected to *in vitro* digestion (IVD) and the evaluation of their anti-inflammatory activity was carried out using yeast reporter assays.

The results showed that phenolic compounds are abundant in the matrix and suggest that their interactions with fiber and protein present in the matrix may affect their bioaccessibility and bioactivity upon *in vitro* digestion. All raspberry fractions exhibited high anti-inflammatory potential, particularly Glen Ericht. Remarkably, a yellow raspberry (2J19) with low amounts of anthocyanins showed 66% of protection, suggesting that other classes of phenolic compounds may be associated with the identified bioactivities. The hydroxybenzoic acids (ellagic acids and ellagitannins) could be potential bioactive molecules as they were shown to be abundant in raspberry extracts and fractions resulting from the *in vitro* digestion.

Keywords: Raspberry, Phenolic compounds, Anthocyannins, Flavonols, Ellagic acids, Bioaccessibility, Bioactivity, Inflammation, NFAT, Yeast, Crz1

Resumo

As doenças crônicas tornaram-se uma questão de alta prioridade na Europa devido ao aumento da sua incidência nos últimos anos. Diversos estudos apontam os processos inflamatórios (não infeciosos) como um fator determinante na sua origem visto que concentrações elevadas de marcadores inflamatórios estão normalmente associadas com este tipo de doenças. A literatura científica indica que uma dieta baseada no consumo de frutas e vegetais está inversamente relacionada com a incidência de doenças crônicas. Neste contexto, os pequenos frutos desempenham um papel importante devido ao seu alto teor de compostos bioativos, nomeadamente os compostos fenólicos, com reconhecidas propriedades anti-inflamatórias.

Neste estudo, o perfil nutricional de framboesas foi determinado por análise proximal, os fenóis totais quantificados pelo método de Folin-Ciocalteu e a determinação das principais classes de compostos fenólicos foi feita por cromatografia líquida de alta eficiência acoplada à espectrometria de massa (HPLC-MS). As framboesas foram sujeitas a um processo de digestão *in vitro* e após a determinação do perfil de compostos fenólicos das frações, a sua atividade anti-inflamatória foi avaliada *in vivo*.

Os resultados indicam que os compostos fenólicos são abundantes nas matrizes avaliadas e sugerem que as interações com fibras e proteínas presentes na matriz podem afetar sua bioacessibilidade e bioatividade após a digestão *in vitro.*

Todas as frações apresentaram um elevado potencial anti-inflamatório, particularmente a framboesa Glen Ericht. Notavelmente, a framboesa amarela (2J19), que apresenta menor quantidade de antocianinas em comparação com as demais framboesas, também revelou altos níveis de proteção (66%). Estes resultados indicam que outros compostos fenólicos, para além das antocianinas que lhes conferem a cor, podem estar associados as bioatividades identificadas. Compostos como os ácidos hidroxibenzóicos (ácidos elágicos e os elagitaninos) são potenciais candidatos para as bioatividades observadas uma vez que os resultados indicam a sua presença abundante tanto nos extratos de framboesa como nas frações resultantes da digestão *in vitro*.

Palavras-chave: Framboesas, compostos fenólicos, Antocianinas, Flavonols, Ácidos elágicos, Bioatividade, Bioacessibilidade, Inflamação, NFAT, Yeast, Crz1

Abbreviations

ABS – Absorbance

CaM – Calmodulin

CaN – Calcineurin

CDRE – Calcineurin-dependent response element

Cnb1 – CaN regulatory subunit B, see CaN

CD - Chronic Diseases

Crz1 – CaN-responsive zinc finger transcription factor, see CaN

DW - Dry weight

IVD - *In vitro* digestion

NFAT – Nuclear Factor of Activated T cells

OD – Optical Density

G – G force

RTG – Retrograde

TFs – Transcription factors

UV – Ultraviolet

WT – Wild-type

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1. Objectives

As the years go by, the idea that a diet based on fruit and vegetable intake is inversely related to the incidence of diseases has been highly increased (Giampieri *et al*., 2015). Berries have been pointed out for their high content of bioactive compounds like polyphenols (Elisabetta, *et al*., 2013, Kula *et al*., 2016).

The health benefits provided by the consumption of these compounds depend on their absorption and metabolism, many physiochemical changes occur to these compounds as soon as they enter the human body, in particularly in the stomach and/or in the small intestine. Which means that a study carried out using crude berry extracts, much probably, will not have these changes in mind, making it an unrealistic study.

With all this in mind, in the present study an *in vitro* digestion method was used, which could mimic the physiochemical changes, and also produce a characteristic set of phytochemicals that could have come in contact with the colonic epithelium in situ (Olsson, M. *et al*., 2004; Ship, P. *et al*., 2005). Five raspberry lines went throw this *in vitro* digestion, performed at in James Hutton Institute, resulting into two different digested fractions, called IN (mimics the serum bioaccessible fraction) and OUT (mimics the colon bioaccessible fraction).

The first two tasks involved the phenolic compounds quantification and evaluation of the bioactivity of the digested raspberry fractions. In the second task the β-galactosidase assay was performed in which a yeast model of Crz1 activation was used to identify fractions with anti-inflammatory potential.

The third task consisted in analyzing the nutritional profile of the raspberries and their quality parameters and phenolic compounds discrimination to later correlate all the results and provide valuable information to identify the raspberries with the highest levels of beneficial phenolic compounds.

2. Introduction

The World Health Organization (WHO) defines chronic diseases (CD) as "diseases of long duration and generally slow progression". Many years have gone since CD were considered to be a problem only related to rich and elderly population (WHO, 2010). Nowadays, it is well known that CD affects people from high-income to poor countries as well as young, middle-aged and elderly population (WHO, 2010; Busse, B. *et al*., 2010). The Food and Agriculture Organization (FAO), back in 2002, reported a joint stating that the burden of CD was rapidly increasing worldwide. According to WHO (2002), 46 % of total population lives with it or has the risk of having these types of diseases in the future, estimating an increase to 57% by 2020. In 2008, a study compared prevailing expositions of population CD trends with epidemiologic models (theoretical and empirical), leading to several assumptions regarding the impact of these types of diseases in their mortality rates (Milbank Q., 2008). The study highlighted four leading diseases, such as, the cancer, diabetes, cardiovascular (CVD) and respiratory diseases, and pointed out that the risk of CD would rise. The study also predicted that in the future more than 31 million people would die from chronic noncommunicable disease, with rates rising faster in developing countries than in developed ones. Economic assumptions indicated serious implications in the expenses in health care in developing countries (Milkbank, Q., 2008). They were further referred in a conference in 2010, here relating CD with the decrease of wages and earnings, labor productivity, as well as increasing early retirement, high job turnover and disability. Overall, the spending on CD care has risen across Europe, taking up increasingly greater proportions of public and private budgets (Busse, B. *et al*., 2010).

Many studies link CD not only to the ageing of the society but also to lifestyle choices, such as smoking, sexual behaviors, diet and exercise, never putting apart the genetic predispositions (WHO, 2010). With so, CD have become a matter of high priority for Europe and several studies point to (non-infectious) inflammatory processes as a determining factor in their origin (Lukens, J.R.*et al*., 2012). High concentrations of inflammatory markers and activated inflammatory cells at the site of the affected tissue and systemic circulation, have been detected in these diseases (Calcer, P.C., *et al*., 2009).

2.1. The inflammation

The immune system is divided in two functional divisions, the innate and the acquired. The innate is the result of the activation of mediators that later are released to prevent the infection and attack the foreign organism/material. These reactions lead to an increase of the blood supply. In other words, when an immunological stimulus happens the initial response triggers an inflammatory process (Lawrence, T. *et al.*, 2002, Calcer, P.C., *et al.*, 2002). The stereotyped inflammatory response involves diverse biochemical, physiological, and immunological changes at the injured site (figure 1). It pledges pathogen killing as well as tissue repairing, restoring homeostasis at the affected/damaged sites (Calcer, P.C., *et al.*, 2009).

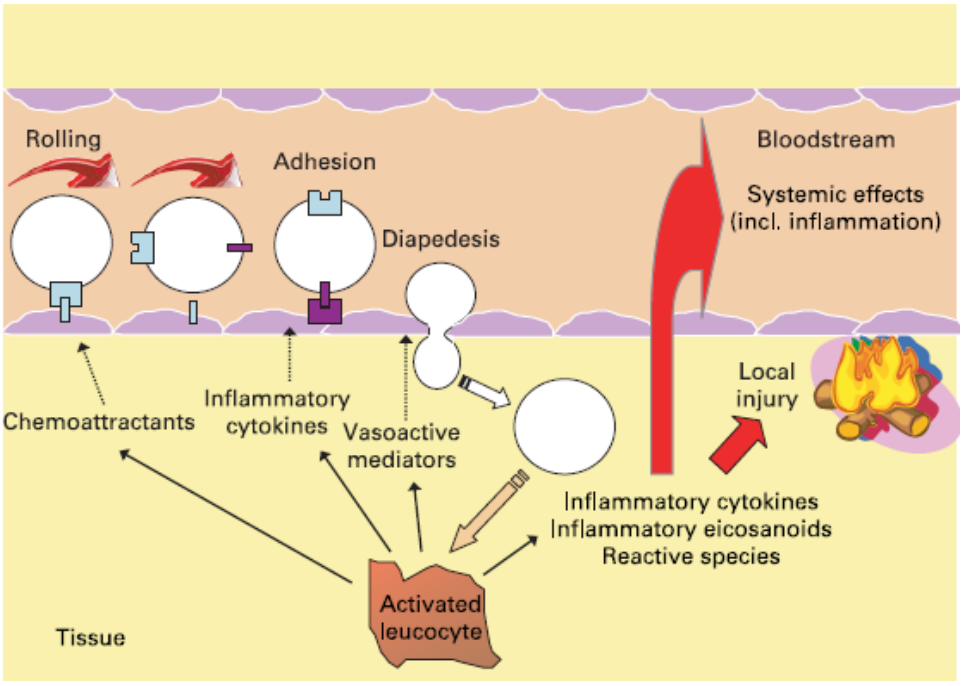


Figure 1 - Generic scheme of inflammation. Leucocyte migration occurs from the capillaries into the surrounding tissue. This is promoted by release of chemo-attractants from the site of inflammation and by the upregulation of adhesion molecules on the endothelium. Once in the tissue the leucocytes move to the site of inflammation. Release of mediators from leucocytes at the site (Calcer, P. *et al.*, 2009).

In order for the organism remaining healthy and maintaining homeostasis the regulation of the inflammatory responses becomes essential and must be controlled properly. Self-regulation involves activation of negative feedback mechanisms such as inhibition of pro-inflammatory signaling cascades, shedding of receptors for inflammatory mediators and activation of regulatory cells. When this type of response lingers chronic inflammation occurs and, over time, it will have a negative impact resulting in irreparable damages to host tissues and disease can happen (Calcer, P.C., *et al.*, 2009).

There are several pathways that can be involved in inflammatory processes, such as the Nuclear Factor of Activated T-cells (NFAT) and the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB). Both of them belong to families of transcription factors (TFs) whose activities are involved in the positive regulation of pro-inflammatory genes (like the TNFα) (Garcia, G.,*et al*., 2016; Oeckinghaus, A., 2009; Sharma, V. *et al*., 2015).

2.2. Yeast models

Even before the systematic sequencing of genomes, studies had reported the similarities between proteins encoded from genes from both yeast and mammals (Botstein, D., et al., 1997). In fact, yeasts are nowadays used in several studies, p.e. in neurodegenerative diseases studies, due to its versatility, one of its main advantages is the fact that its complexity is reduced once compared with the mammalian models. Yeast can also offer the advantage of powerful genetics and proteomics including a completely sequenced genome, comprehensive single gene deletion and overexpression libraries, determination of protein localization in vivo and tandem affinity purification-tagging approaches (Puig, 2001; Giaever, 2003; Suter, 2006).

Regarding NFAT activation, it is known that its control is performed through Ca^{2+}-dependent signaling pathways, and that in its phosphorylated state it remains inactivated in the cytosol (Garcia, G. et al., 2016). As a consequence, when these cells are injured there's an increase of the cytosolic Ca^{2+} levels, resulting in the binding of Ca^{2+} with the high affinity protein Ca^{2+}-calmodulin (CaM), which in turn activates calcineurin (CaN) (Oeckinghaus, A., 2009; Garcia, G. et al., 2016). Upon the rise of intracellular calcium levels, CaN activation leads to NFAT dephosphorylation and migration to the nucleus. Once in there, NFAT proteins bind to their respective recognition sequences in the promoter region of various pro-inflammatory genes (Oeckinghaus, A., 2009; Pan, M.G. et al, 2013; Garcia, G. et al., 2016) (figure 2a).

Some studies point out that the NFAT mechanism reacts only to a sustained elevation of Ca^{2+} levels within the cell to keep its position for as long as it needs, not responding to transient rises of Ca^{2+}, setting it apart from many other regulators of calcium signaling (Graef, I.A., et al., 2001; Wu, H. et al., 2007; Pan, M.G. et al, 2013).

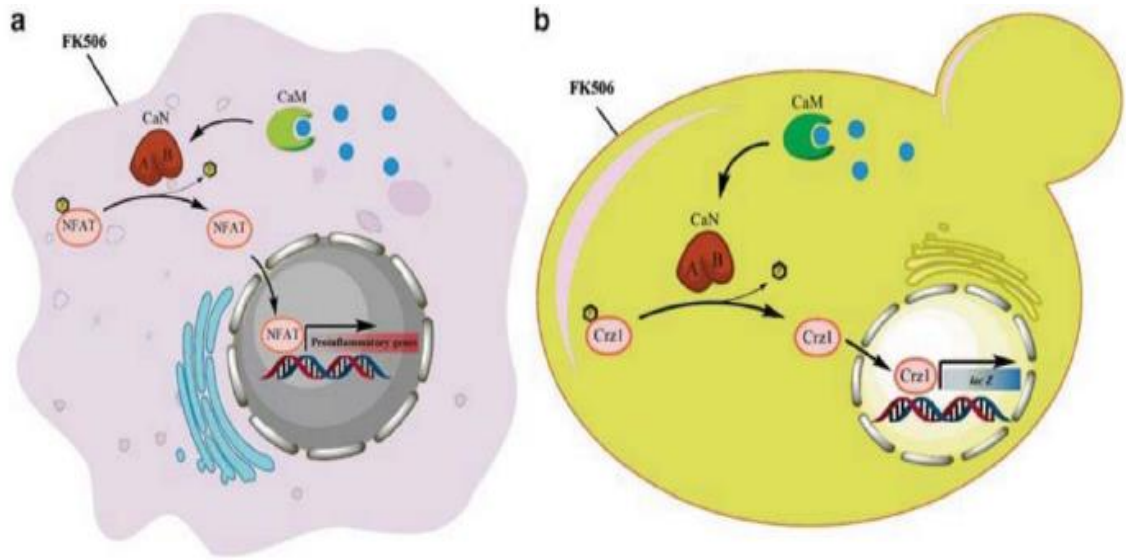


Figure 2 - Illustration of Ca $^{2+}$ dependent signaling pathways: a. Activation of NFAT in mammalian cells; b. Activation of Crz1 in yeast cells. Source: Garcia, G. et al., 2016.

Due to the ability to recapitulate some of the fundamental cellular mechanisms, particularly those associated with the induction of Ca^{2+} signal cascades that lead to the activation of Crz1 (the yeast orthologue of human NFAT gene), the yeast *Saccharomyces cerevisiae* becomes a very valued model organism. In very similar way to the NFAT activation mechanism, Crz1 is also kept inactivated in the cytosol in its phosphorylated form. The increase of Ca^{2+} levels unleash the activation of CaM/CaN that will later on dephosphorylate Crz1, leading to its translocation into the nucleus and activating the expression of target genes via binding to the CaN-Dependent Response Element (CDRE) (Figure 2b). The evolutionary conservation of the mechanism of activation of NFAT and Crz1 allows the use of *S. cerevisiae* as platforms for the screening of compounds that modulate the activation of Crz1 (NFAT) (Garcia, G. et al., 2016; Thewes, S. et al., 2012).

2.3. Evidences of health benefits of fruits and vegetables

Diet and nutrition are important factors throughout human's life course, they promote and help maintaining its good health (WHO, 2002). Several studies indicate that a diet based on fruit and vegetable intake is inversely related to the incidence of CD (Giampieri *et al*., 2015). Berry fruits play an important role amongst fruits due to their high content of bioactive compounds, namely polyphenols. Evidences of their protective role towards inflammation and a variety of human diseases are growing (Elisabetta, *et al*., 2013, Kula *et al*., 2016). Moreover, phenolic compounds are also known for their anti-inflammatory, antimutagenic and antimicrobial effects (Pan *et al*., 2010).

Nowadays the public has become highly aware of the health benefits that a diet based on vegetables and fruits can imply. With so, the interest of the consumers regarding food products rich in polyphenols raised, and with that the consumption of fruits such as berries has been increasing. Especially concerning red raspberries (*Rubus idaeus* L.), scientific data indicates that they contain a large variety of bioactive phytochemicals with beneficial health effects (Kula *et al*., 2016).

2.4. The raspberry (*Rubus spp*)

Raspberries (*Rubus idaeus L.*), the fruits of the raspberry tree, are an aggregation of multiple small drupes formed by the union of several ovaries of the same flower, adhering to a common receptacle (figure 3). Each drupe contains a hard seed surrounded by pulp. The proportion of seeds in the fruit represents between 3 and 10% of the fruit. After the abscission of the ripe fruit, and after harvest, the receptacle remains connected to the plant and, consequently, the fruit becomes hollow with a fragile structure. This fragile structure becomes easily perishable, so its conservation and consumption time is very limited. It is a fruit whose flavor is sweet and smooth, and due to that, can be used for various purposes, such as ice cream, syrups, jellies, liqueurs and sweet. For a satisfactory production, it is necessary that the raspberry tree is subjected to at least 700 hours per year at a temperature below 7 ° C for (Zoratti *et al.*, 2016). According to Câmara-Correia (2016), each 100 g of raspberry (*Rubus idaeus L.*) contains the constituents indicated in Table 1.

Figure 3 - *Rubus idaeus L*. Source: Seseys

Table 1 - General composition of Raspberry, per 100 g of fresh fruit. Source: Câmara – Correia, M., 2016.

Constituent	Quantity
Water	81-84 g
Protein	1.2-1.5 g
Lipids	0.4-0.65 g
Carbohydrates	14-16 g
Crude fiber	<1 g
Anthocyanins	20-65 mg
Vitamin A	130 U.I.
Thiamin B1	25 mg
Riboflavin B2	0.02 a 0.05 mg
Niacin	2.5 a 5 mg
Vitamin C	28 mg
Ca	35 mg
P	30 mg
Na	1-3 mg
K	40 mg
Fe	1.5 mg

The mature fruits of *Rubus spp*. are abundant in polyphenols, plant secondary metabolites that intervene in growth and development, influencing the pigmentation of the fruits and their organoleptic characteristics. Thus, the phenolic profile shows quantitative and qualitative variations throughout fruit development and maturation, as well as between harvests of consecutive years (Mazur, S., *et al*., 2014; Mullen *et al*., 2002). Polyphenols are also responsible for the antioxidant mechanisms of the raspberry, the protection of UV light radiation and the scavenging of free radicals generated during the photosynthetic process. Table 2 shows the total content of phenolic compounds in total flavonoids and total anthocyanins of the raspberry. Literature points ellagitannins and anthocyanins as the majority compounds that integrate this matrix (Prior, R., 2003; Mullen *et al.*, 2002). Carvalho M. (2010) and Mullen *et al.* (2016) refer that the concentration of ellagitannins in these fruits (determined in ellagic equivalents ($C_{14}H_6O_8$) or in gallic acid equivalents) varies between 20 and 40 mg. 100 g^{-1} of dry weight depending of the variety.

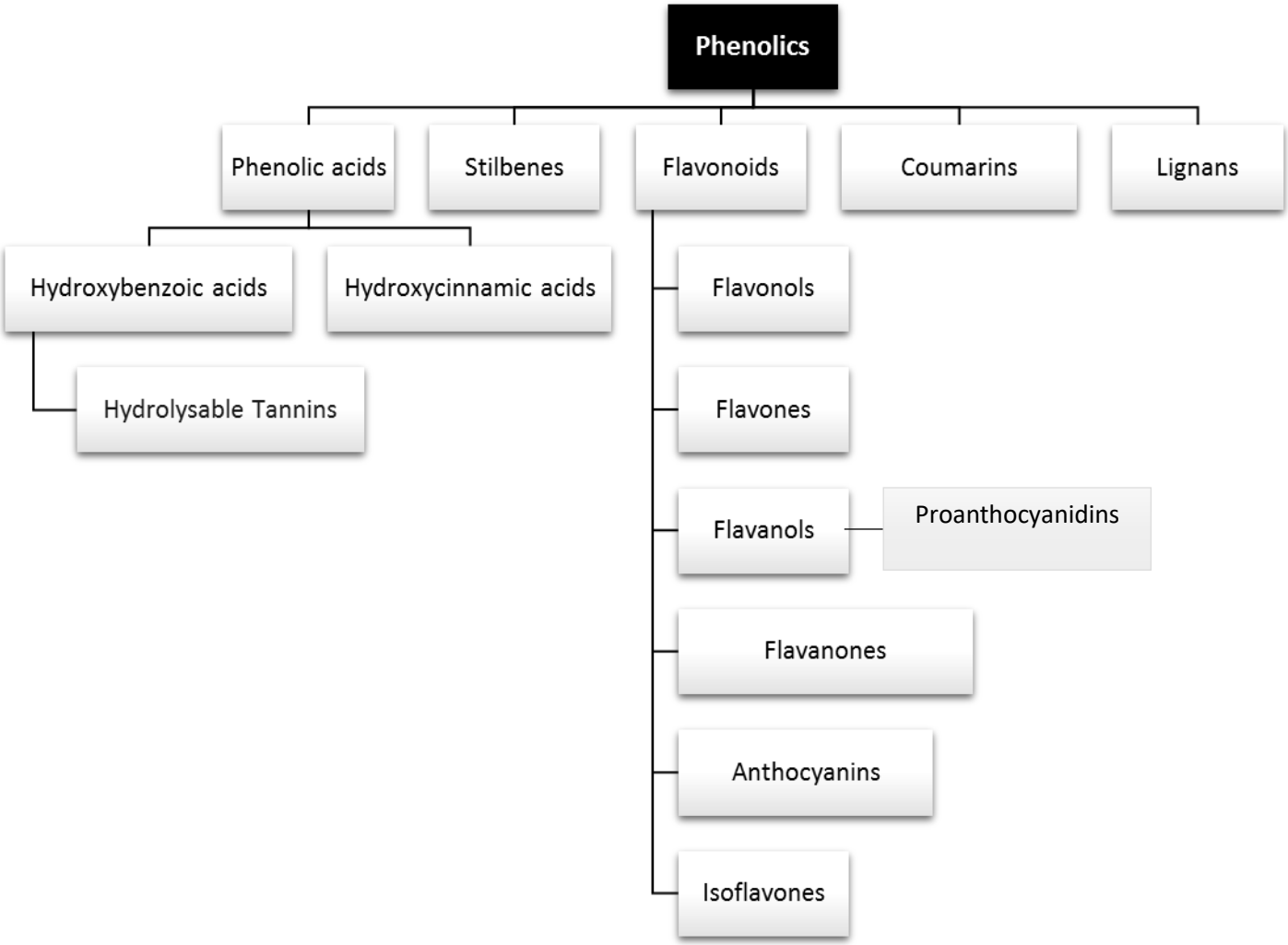
Table 2 - Total contents of flavonoids and anthocyanins in *Rubus idaeus* L. fruits. Source: Câmara-Correia, M., 2016; Remberg, S.F. *et al*.., 2010; Ordidge, M., *et al.,* 2010).

Parameter	**Content**
Total Phenols (mg GAE. 100 g^{-1} DW)	57.5 – 2062.3
Total Flavonoids (mg EC. 100 g^{-1} DW)	9.6 – 279.3
Total Anthocyanins (mg EC3G. 100 g^{-1} DW)	2.1 – 325.5

GAE -> Gallic acid equivalents; EC -> (+) – catechin equivalents; EC3G -> cyanidin-3-glucoside equivalents.

2.5. Phenolic compounds

Polyphenols have a large variety of structures and naturally occur as mono- and polysaccharide conjugates, usually connected to one or more phenolic groups. They may also appear as functional derivatives, such as esters and methyl esters (Proteggente, A.R., *et al*., 2003; Serrano, J., *et al*., 209). These compounds are known to comprise an aromatic ring, bearing one or more hydroxyl substituents. These structures can range from simple molecules to highly polymerized compounds (Balasundram, N., *et al.,* 2005). Polyphenols can mainly be divided into phenolic acids, stillbenes, flavonoids, coumarins and lignans (scheme 1). This paperwork will only be focusing in the phenolic acids and de flavonoids.



Scheme 1 – Main polyphenol classes. Adapted from Figueira. I., *et al*. 2017

Phenolic acids can then be divided into two subgroups, hydroxybenzoic acids and hydroxycinnamic acids. The first subgroup has in common the C_6-C_1 structure and gallic acid and protocatechuic acid are two examples of this kind of structures (figure 4a). Inside this subgroup of phenolic compounds there is a very important group that needs to be highlighted, the hydrolysable tannins, which have relatively high molecular weight. Often called tannins, they are constituted mainly by esters of gallic acid (gallo- and ellagi-tannins, figure 5) or polymers formed by the condensation of two or more of hydroxyflavan-3-ol monomers (like proanthocyanidins). A third type on tannins can be mentioned, although it is not important in the human diet, complex tannins or phlorotannins consisting entirely of phloroglucinol and have been isolated from several plant material such as genera of brown algae (Balasundram, N. *et al*., 2006; Aguilar, C. N. *et al.*, 2007).

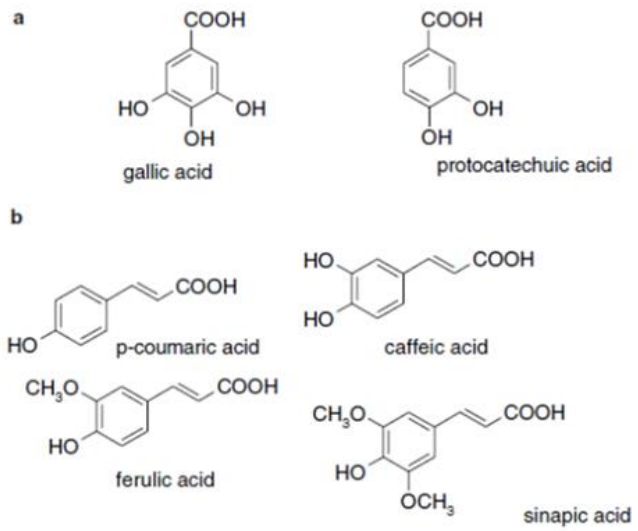


Figure 4 - Hydroxybenzoic (a) and hydroxycinnamic (b) acids. Source: Balasundram, N. *et al*.,2006

As for the second group they show a three-carbon side chain (C_6-C_3), and their most common forms include caffeic, ferulic, *p*-couramic, vanilic and sinapic acids (figure 4b). (Balasundram, N. *et al*., 2006; Kristina B. Martinez, K.B. & McIntosh, M.K, 2017).

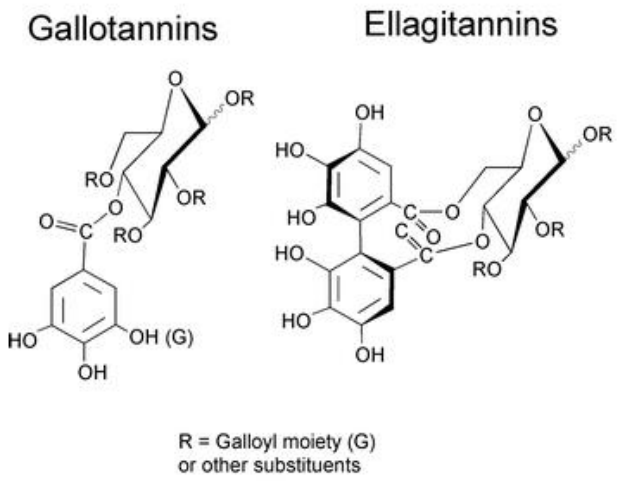


Figure 5 – Hydrolysable tannins, gallotannins and ellagitannins structure. Source: Khanbabaee, K. & Van Reeb, T., 2001

Flavonoids are the largest group of polyphenols in plants having more than 10,000 different structures that have been identified. They are low molecular weight compounds constituted by a diphenyl propane skeleton ($C_6C_3C_6$) (figure 6). This family includes monomeric flavonols, flavones, flavanols, flavanones (or catechins), anthocyanidins and isoflavones (scheme 1 and figure 6). Alongside with the phenylpropanoids or hydroxycinnamic acid derivatives (C_6C_3), flavonols and flavones are found in almost every plant, although the last one in lesser extent.

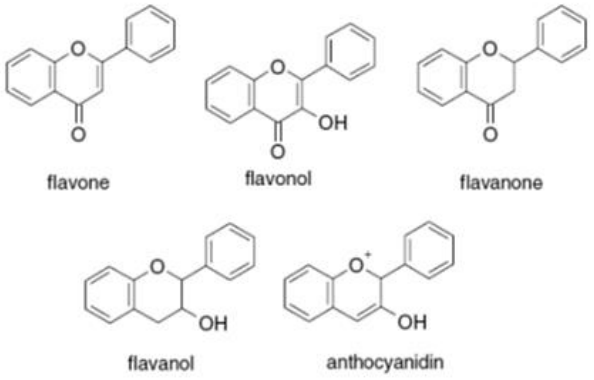


Figure 6 - Major classes of flavonoids and their generic chemical structure. Source Balasundram, N. *et al*., 2006

Changes/substitutions in rings A and B (figure 7) may give rise to different compounds within each class of flavonoids. Giving examples of possible substitutions, it can be included: oxygenation, alkylation, glycosylation, acylation and sulfation (Rice-Evans, A.C., *et al.*, 1995; Balasundram, N. *et al*., 2006). Whereas flavanones and flavones may often be found together

in some fruits (e.g., citrus), connected by specific enzymes, the same does not happen between flavones and flavanols. The same does not happen between flavones and flavanols. Rice-Evans, A.C *et al.* (1995) quoted that "a certain mutual exclusion" between flavones and flavonols existed in many plant families and also stated that anthocyanins were almost absent in flavanone-rich plants.

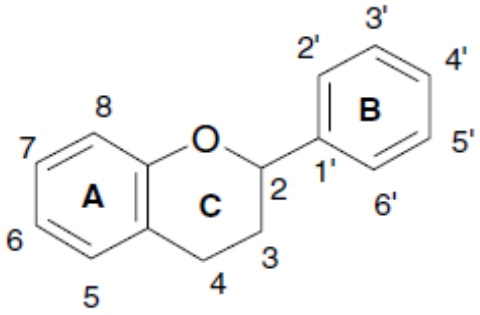
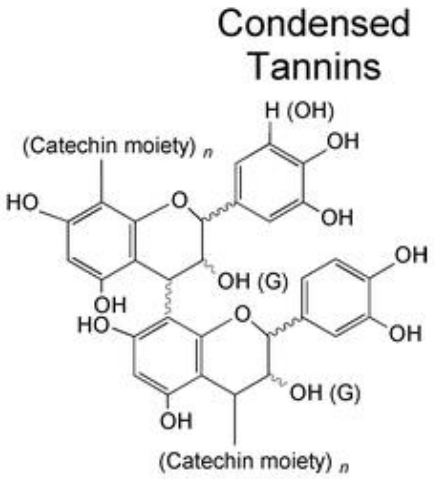


Figure 8 - Flavonoids generic structure molecule. Source: Balasundram, N. *et al.*,2006

The proanthocyanidins appear inside de flavonols and they are also considered tannins, but condensed tannins (figure 8), which are constituted by dimers, oligomers and polymers of catechins (Proteggente, A.R. *et al.*, 2003; Serrano, J. *et al.*, 2009).



All these diferrences in the polyphenol stuctures will influence their biological activity and therefore its potential activity.

2.6. Bioavailability of polyphenols

Polyphenols can be metabolized both in tissues and by the colonic microbiota, and their health effects may depend not only on the amount consumed but also on their bioavailability, i.e., what is truly absorbed from the gut and later enters the blood circulation (McDougall, G. *et al.,* 2005; Coates, E.M., *et al.,* 2007). In 2017, Gary Williamson and Michael N. Clifford published a review article about the "Role of the small intestine, colon and microbiota in determining the metabolic fate of polyphenols", in which they state that "*The route of absorption can be either through the stomach, small intestine or, if not absorbed at those sites, by the colon, after chemical modification by the colonic microbiota*". This statement leads to the idea that most of the *in vitro* studies that are carried out using crude berry extracts, without consideration of the physiochemical changes that can occur in the stomach/ small intestine/ colon, were unrealistic studies.

Also, polyphenol's bioavailability can diverge significantly from one to another, and it doesn't necessary means that the most abundant/consumed polyphenols in our diet are the ones that lead to the highest concentrations of active metabolites in target tissues. Most of polyphenol parent compounds are not well absorbed in the small intestine (Manach, C. *et l.,* 2005) and over 90 – 95 % of the residual polyphenols enter the large intestine lumen where they are catabolized by the colonic microbiota (Ozdal *et al*. 2016).

3. Materials and methods

3.1. Material

Dimethyl sulfoxide (DMSO) was purchased from Duchefa Biochemie, Netherlands. Folin-Ciocalteau (FCR) reagent was purchased from VWR CHEMICALS, USA. Gallic acid monohydrate (GA) was purchased from Sigma–Aldrich®, China. Yeast extract and Agar were purchased from Himedia, India. Bacto™ Peptone BD - Biosciences, France. D-+-glucose was purchased from Sigma–Aldrich®, USA. Yeast nitrogen base (YNB) was purchased from BD Difco, USA. Complete Supplement Mixture (CSM) was purchased from MP Biomedicals, USA. ONPG was purchased from Sigma–Aldrich®, UK. Na_2HPO_4 was purchased from ROTH. $NaH_2PO_4 \bullet H_2O$ was purchased from AppliChem. Potassium chloride (KCl) and Magnesium sulfate heptahydrate ($MgSO_4 \bullet 7H_2O$) was purchased from MERCK. Pepsin was purchased from Sigma (Product number P6887), UK. Y-PER cell lysis reagent was purchased from Pierce® Protein Research, USA.

3.2. Sample Characterization

3.2.1. Raw material

Five raspberries, provided by Dr. Derek Stewart, from the Division of Enhancing Crop Productivity and Utilization, James Hutton Institute (JHI, England), were selected for undergoing IVD (*in vitro* digestion) treatment. They derived from different germplasm lines (quasi-isogenic) and were generated by artificial selection. The resulting raspberry polyphenolic fractions used in this study were: Glen Ericht, 0304F6, 00123A7, Tulameen (industrial standard) and 2J19 (yellow raspberry) (McDougall *et al*. 2005; Garcia, G., 2014).

Table 3 - Quasi-isogenic raspberries used in this study: Glen Ericht, 0304F6, 00123A7, Tulameen (an industrial standard) and 2J19 (yellow raspberry).

	Glen Ericht	0304F6	00123A7	Tulameen	2J19 – "Yellow Raspberry"
Raspberries					

3.2.2. *In vitro* digestion and fractions preparation

After being selected, the raspberries were freeze-dried (powder) and went through an *In vitro* treatment (figure 9) described by McDougall *et al.* This model mimics the alterations that phytochemicals undergo throughout both gastric and small intestine digestion. Briefly, the pH of the extracts (2 g in 20 mL of water) was adjusted to 1.7 (using HCl). They were then incubated in the presence 0.56 g of pepsin with agitation at 37 °C for 2h. The samples corresponding to post -gastric digestion were removed and frozen and the remaining material was placed in a glass beaker in interaction with pancreatin and bile salts. In the same beaker, the resulting digested fractions went through a dialysis process for about 2h at 37°C, using a cellulose tube that contains $NaHCO_3$ to neutralize titratable acidity. In the end two different fractions/solutions were collected: one that entered the dialysis tubing ("IN" - bio-accessible fractions of the serum) and one that remained outside the tubing ("OUT" - bio-accessible fractions of the colon). Both fractions were centrifuged and, to remove possible interfering compounds from the *in vitro* digestion model, the resulting soluble materials were next extracted using a C18 solid phase extraction (SPE) column (GIGA tubes, 1000 mg capacity, Phenomenex Ltd.). (McDougall *et al.* 2005; Tavares, L. *et al.*, 2012; Garcia, G., 2014)

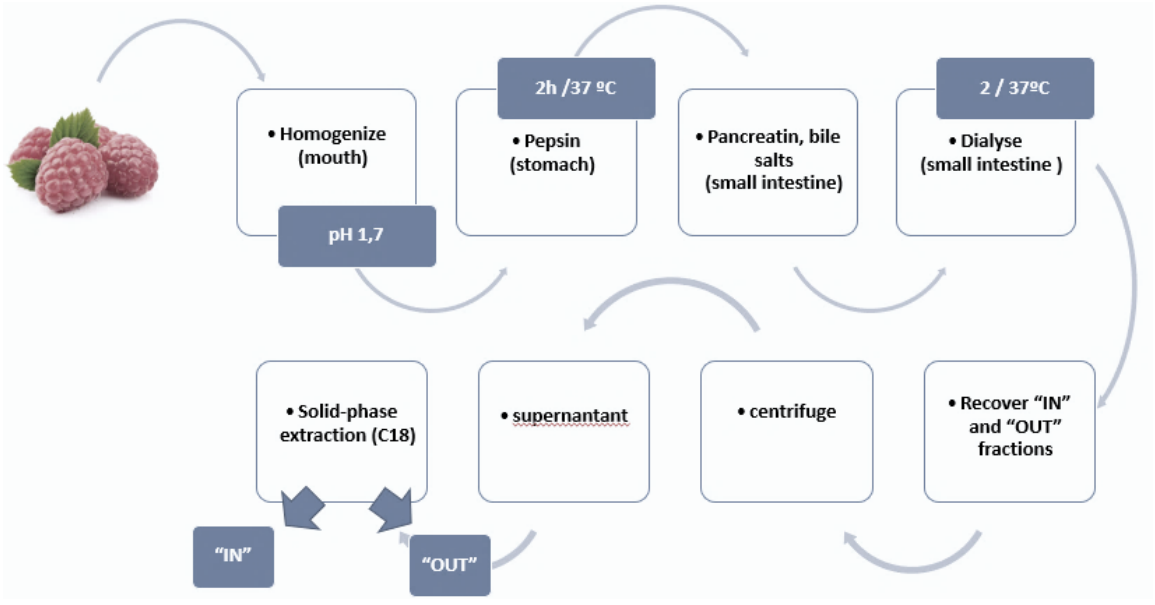


Figure 9 – *In vitro* digestion procedure scheme, mimicking the alterations that phytochemicals undergo throughout both gastric and small intestine digestion. The pH of freeze-dried raspberries extracts was adjusted to 1.7 followed by incubation with pepsin. Post - gastric digestion samples were frozen, and an aliquot was treated with pancreatin and bile salts. The resulting digested fractions were submitted to a dialysis process. The resulting fractions were collected: IN" - bioaccessible fractions of the serum and "OUT" - bioaccessible fractions of the colon. Fractions were centrifuged and the resulting soluble materials were extracted using a C18 solid phase extraction (SPE) column. Adapted from: McDougall *et al.*, 2005

3.2.3. Nutritional profile

The original matrix went through proximal analysis, following the AOAC Official Methods^{SM}. Most specific, the total protein was measured by the Kjeldhal method (AOAC 990.03) and total fiber content by the Weende method (AOAC BA 6A-05) (Washington DC/USA).

3.2.4. Raspberry quality parameters and phenolic compounds discrimination

Total anthocyanins determination (pre and post IVD treatment) was made by HPLC, followed by quantification via photodiode array (HPLC-PDA, UV). Flavonols and relative amounts of ellagic acids and ellagitannins were determined by HPLC and quantification HPLC-MS.

3.3. Total phenols quantification

Total phenolic compounds (TPC) present in samples were quantified using the Folin–Ciocalteu (F-C) assay, a colorimetric method based on an oxidation/reduction reaction between the F-C reagent and the polyphenolic compounds, forming a blue chromophore complex (constituted by phosphotungs-ticphosphomolybdenum) that can be quantified by UV-Vis spectrophotometry, (Blainski, A. *et al.*, 2013; Schofield, P., *et al.*, 2001).

Two different sets of IN and OUT samples were resuspended, one using purified water (500 µL – **1**) and the other DMSO (60 µL – **2**), with agitation. The samples in **1** were first diluted in a volume of 350 µL of water, for one of the extracts (Tulameen) was necessary to add 150 µL of ethanol for total resuspension of the fraction, the remaining were added more water to a final volume of 500 µL. After the material in samples **2** were diluted, they were subjected to sequential dilutions, according to figure 10 (final dilution factor, F.D., 1:40). For the preparation of the calibration curve it was used a stock solution of gallic acid monohydrate (GA) (1 $g.L^{-1}$) and purified water. The solutions for the curve had concentrations of 0, 50, 200, 400, 550 and 700 $mg.L^{-1}$ of GA. In a 96 well plate it was added (by order) 235 µL of H_2O, 5 µL of each standard or sample, 15 µL of Folin-Ciocalteau reagent, and finally 45 µL of Na_2CO_3 (106 $g.L^{-1}$, Saturated solution, previously warmed up until 40 °C, with agitation) (Farmacopeia Brasileira, 2010). The plates were for 30 min at 40°C in a microplate reader (UV-Vis

spectrophotometer, Biotek powerwave XS), and optical density at 765 nm (OD_{765}) was recorded using the Gen5Software.

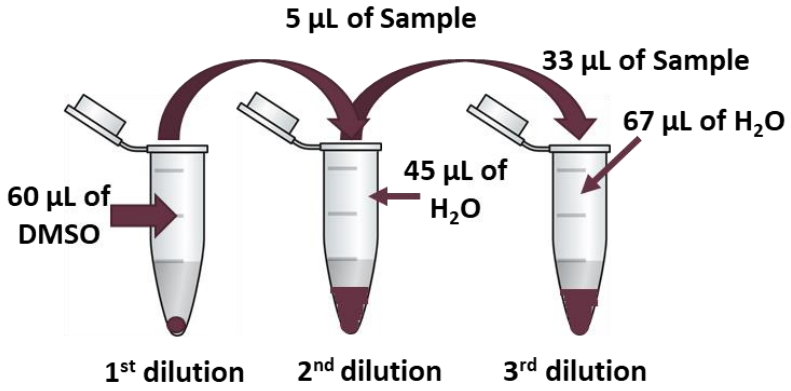


Figure 10 - Dilution scheme of the extracts resuspended with DMSO. To the samples were added 60 µL of DMSO and mixed until total resuspension of the material (1st dilution). Then, 5 µL of the previous solution were added to 45 µL of purified H_2O (2nd dilution). Finally, 33 µL of the 2nd dilution samples were diluted again in 67 µL of H_2O, making a final F.D of 1:40.

3.4. *Saccharomyces cerevisiae* strains and growth conditions

S. cerevisiae strains used in this study are listed in table 4, YAA5 is a recombinant reporter strain encoding an integrated copy of the bacterial *lacZ* gene under the control of four in tandem repeats of CaN-Dependent Response Element (CDRE) (the *Crz1* recognition elements). YAA6 is isogenic to YAA5 lacking the *CRZ1* gene, acting as negative control.

Table 4 –*Saccharomyces cerevisiae* strains used in the study

Strain	Code	Genotypic information	Source
BY4742_*CDRE-lacZ*	YAA5	*MATa his3 leu2 lys2 ura3 aur1::AUR1-C-4xCDRE-lacZ*	Araki, Y. *et al.*, (2009)
BY4742_*CDRE-lacZ_crz1*	YAA6	*MATa his3 leu2 lys2 ura3 YNL027W::HIS3MX4 aur1::AUR1-C-4xCDRE-lacZ*	Araki, Y. *et al.*, (2009)

All strains were maintained in YPD solid medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2 % (w/v) agar, pH 6.5]. For the pre-cultures, one single colony of each strain was inoculated in 2 mL of Synthetic Complete medium (SC) [0.79 % (w/v) CSM, 0.67 % (w/v) YNB, and 2 % (w/v) glucose], overnight at 30 °C with orbital agitation at 5.814 G. OD_{600} was measured and the pre-cultures were diluted (volumes calculated by equation 1) to obtain a

final OD_{600} = 0.5 in 5 mL of SC after 16 h incubation at 30 °C with orbital agitation at 5.814 G. The OD_{600} was measured and cell were diluted in fresh SC to a final OD_{600} = 0.1 (figure 11).

$$Seeding\ Volume\ (ml) = \frac{OD_{600} of\ the\ culture\ \times Culture\ Volume\ (ml)}{OD_{600\ of\ the\ pre-cultures}} \quad \textbf{(1)}$$

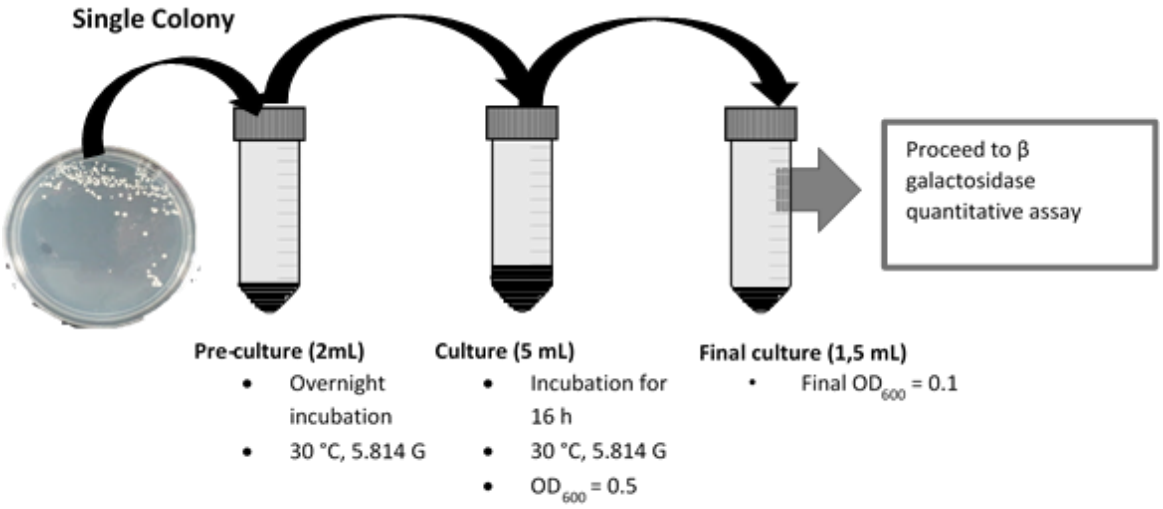


Figure 11 - Short scheme of growth conditions. Strains YAA5 and YAA6 were inoculated in 2 mL fresh SC medium, overnight at 30 °C. Pre-cultures were diluted in fresh SC medium to OD_{600} = 0.5 in 5 mL. and incubated for 16 h at 30 °C. OD_{600} was measured and cell were diluted in fresh SC to a final OD_{600} = 0.1.

3.5. β-galactosidase assay

Cell cultures obtained as indicated above were incubated for 90 min in the presence of 100 µg. mL^{-1} of IN and OUT raspberry digested fractions in a final volume of 1500 µL. FK506 (dissolved in DMSO) at a final concentration of 10 µg. mL^{-1} was used as positive control. Crz1 was induced with 3 mM of $MnCl_2$ for 90 min. After incubation, 10 µL of cell suspensions were transferred onto a 96 well plate containing 20 µL of Y-PER cell lysis reagent. After incubation, 10 µL of each cell-treated suspension microtube (thoroughly resuspended) were transferred onto a 96 well plate already containing, per well, 20 µL of Y-PER cell lysis reagent. The samples were incubated for 20 min at 30 °C without agitation and 240 µL of a solution containing 2 µg. mL^{-1} of O-nitrophenyl-β-D-galactopyranoside (ONPG) dissolved in LacZ buffer [8.5 $g.L^{-1}$ Na_2HPO_4; 5.5 $g.L^{-1}$ $NaH_2PO_4 \bullet H_2O$; 0.75 $g.L^{-1}$ KCl; 0.246 $g.L^{-1}$ $MgSO_4 \bullet 7H_2O$] were added to each well. The plates were incubated in the Biotek Powerwave XS spectrophotometer for 2 h at 30

°C and OD_{420} and OD_{550} reading were recorder every ten minutes. Before Cell lysis, a 100 µL aliquot of cells suspensions were kept for OD_{600} measurements (Garcia, G. *et al.,* 2016).

Miller units were calculated according to equation 2 (Miller, 1972; Garcia, G., *et al.*, 2016), which were necessary to the calculation of the percentages of induction and protection showed in equation 3 and 4.

$$Miller\ Unit = 1000 \times \left(\frac{OD_{420} - 1.75 \times OD_{550}}{t \times V \times OD_{600}}\right) \quad \textbf{(2)}$$

Where:

t = reaction time (in minutes);

V = volume of culture assayed (used for lysis, in mL).

$$\%\ Induction = 100 \times \left(\frac{100 \times [\overline{DMSO}]_{H2O}}{[\overline{DMSO}]_{MnCl2} - [\overline{DMSO}]_{H2O}} + \frac{100 \times [\overline{FK506}]_{MnCl2}}{[\overline{DMSO}]_{MnCl2} - [\overline{DMSO}]_{H2O}}\right) \quad \textbf{(3)}$$

Where:

$[\overline{DMSO}]_{H2O}$ = control of cell suspensions incubated in DMSO with o induction (average, in miller units);

$[\overline{DMSO}]_{MnCl2}$ = control of cell suspensions incubated in DMSO inducted with $MnCl_2$ (average, in miller units);

$[\overline{FK}]_{MnCl2}$ = control of cell suspensions incubated in FK506 inducted with $MnCl_2$ (average, in miller units).

$$\%\ Protection = 1 - \%\ Induction \quad \textbf{(4)}$$

3.6. Statistical analysis

Data are presented as mean values ± standard errors (SE) or standard deviations (SD). Statistical differences were tested using unpaired one-way ANOVA with multiple comparisons tests, only considering significant when $p < 0.05$.

4. Results

4.1. HPLC and proximal analysis

Starting with the proximal analyses, several parameters were analyzed but, in this study, the focus will be on fiber and protein since it is well established that polyphenols stability and their physical/chemical interactions that modulate their release during digestion are related with these two parameters (Palafox-Carlos, H. *et al*. 2011).

For fiber, results show that Tulameen, 00123A7 show similar contents, ranging from 31 to approximately 32 g.100g $^{-1}$ DW. Glen Ericht follows with values near the 28 g.100g $^{-1}$ DW, 2J19 shows small content of fiber (around 21 g.100g $^{-1}$ DW). The extract 0304F6 shows the lowest score for this parameter, having contents below 20 g.100g $^{-1}$.

In general, protein showed small contents in all five raspberries, with values only ranging from 4 to 9 g.100g $^{-1}$ DW. The extracts that showed the highest content of protein were 2J19 and Glen Ericht, ranging from 8 to 9 g.100g^{-1} DW. Extracts 00123A7 and Tulameen revealed the same content, ≈ 6.3 g.100g $^{-1}$ DW and the lowest was again attributed to 0304F6 (4.4 g.100g $^{-1}$ DW).

The results in figure 12 show that the raspberry extract with the highest content of polyphenols was 00123A7 (9602 mg.100g $^{-1}$ DW), followed by Tulameen and 2J19, (5851 and 5270 mg.100g $^{-1}$ DW). 0304F6 and Glen Ericht were the extracts with the lowest values of polyphenols (4412 and 3392 mg.100 g^{-1} DW, respectively).

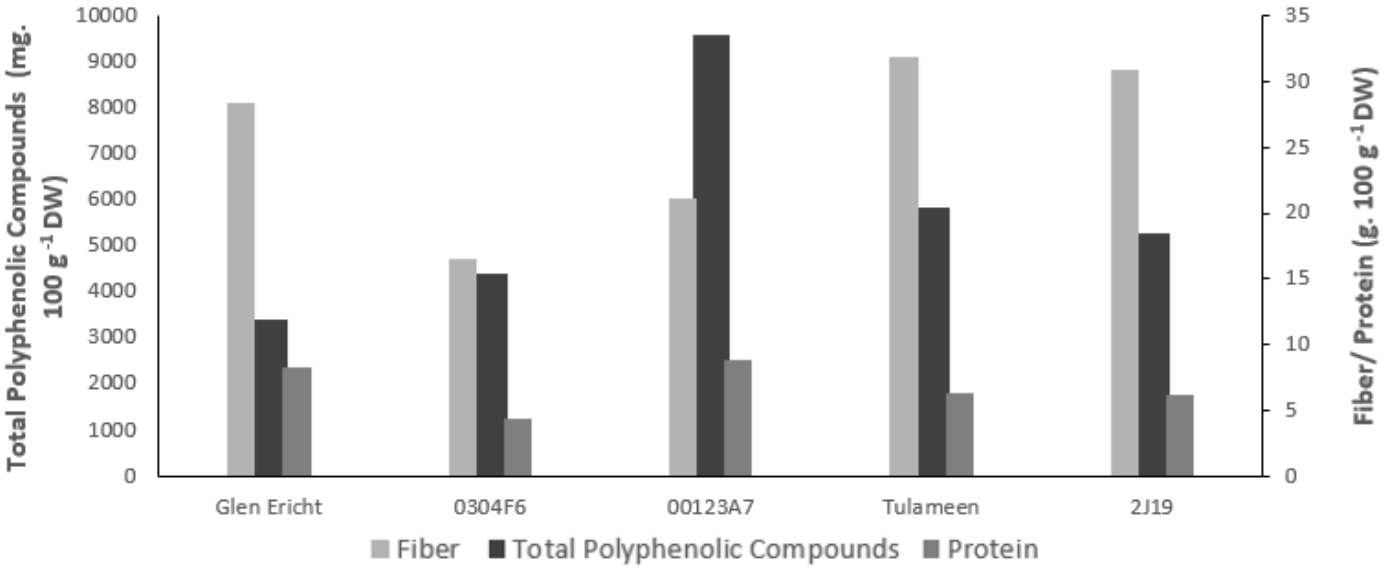


Figure 12 – Fiber, total phenolic compounds, and protein content of the indicated raspberries. Fiber (solid light grey bar) was determined by the Weende method, total phenolic compounds (solid light grey bar) by HPLC and protein (solid grey bar) by the Kjeldhal method.

Figure 13 represents a comparative analysis of the total phenolic compounds and two of the main groups inside this category, anthocyanins and flavonols. Concerning the anthocyanins, their concentration in the matrix varies between 3 and 681 mg.100 g $^{-1}$ DW, being that the highest value belongs to Tulameen and the extremely low value in shown by 2J19. Glen Ericht also shows a significant content of anthocyanins followed by 00123A7 and 0304F6.

Overall, flavonols are present in very low concentrations in all extracts and the values do not surpass the 28 mg.100 g^{-1} DW, which belongs to 00123A7. Tulameen follows 00123A7 with a very close value of 21 mg.100 g^{-1} DW, Glen Ericht showed a medium value of 13 mg.100 g^{-1} DW and the lowest values belong to 0304F6 (5 mg.100 g^{-1} DW) and 2J19 (8 mg.100 g^{-1} DW).

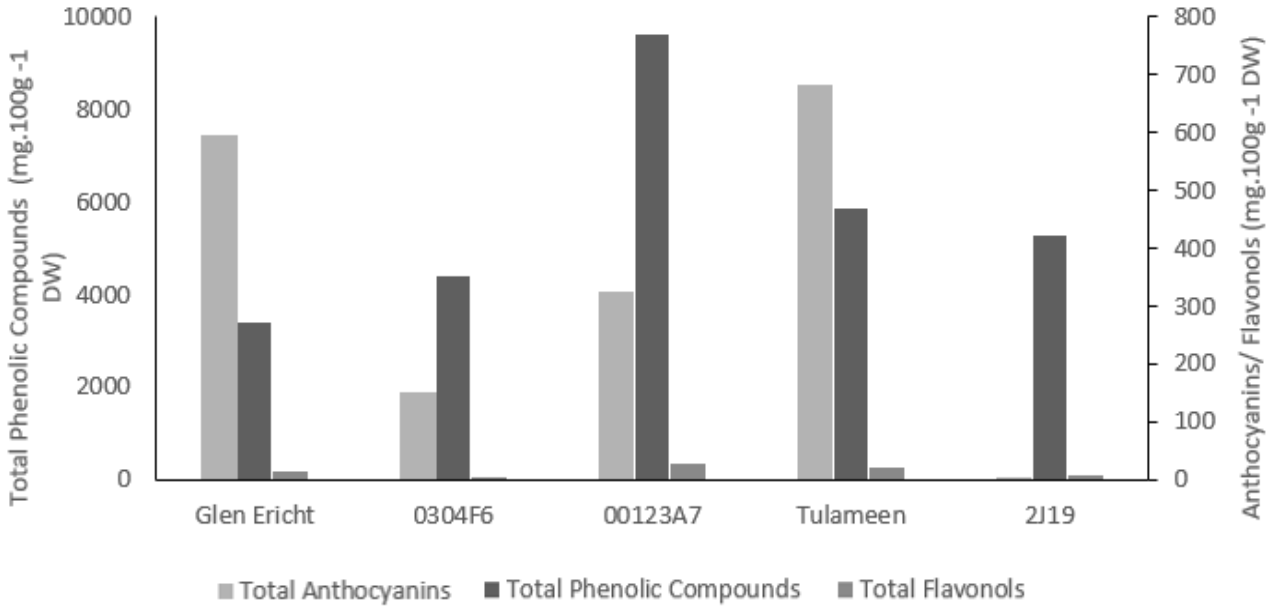


Figure 13 –Total anthocyanins, total phenolic compounds and total flavonols content. Total anthocyanins (solid light grey bar) by HPLC-PDA (UV). Total phenolic compounds (solid dark grey bar) were determined by HPLC-PDA-MS. Flavonols (solid grey bars) by HPLC-MS (MS peak).

The values for total ellagic acids and ellagitannins are referred as relative amounts calculated by the MS peak.

Total ellagic acids (conjugated) show very similar values in all samples, varying from 12 to 20. Both Glen Ericht and Tulameen have the highest content followed by 00123A7, 20 and 18 respectively. The lowest score was attributed to 2J1912 with the value of 12.

Overall, total ellagitannins was the parameter with the lowest contents with barely detectable values varying from 1 to 2.4. Once again, all extracts show similar values, being Glen Ericht the one with the highest value and 0304F6 the one with the smallest (2.4 and 1, respectively).

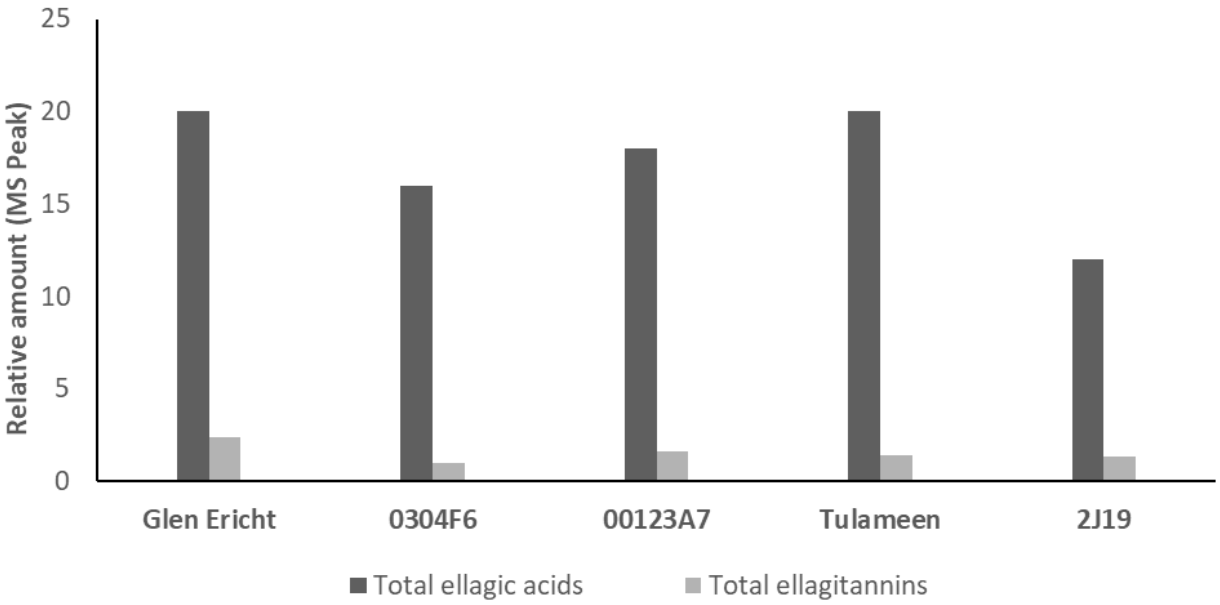


Figure 14 - Total ellagic acids and total ellagitannins. Total ellagic acids (conjugated, solid dark grey bar) and total ellagitannins (solid light grey bar) were determined by HPLC-MS (MS peak).

For the HPLC-PDA-MS analysis of all compounds in the matrix, pre and after IVD, the results were not only analyzed just focusing on graphic images but also by calculating recovery percentages (RP, equation 5). RP were not calculated for the ellagic acid in ellagic acids conjugates and for the ellagitannins due to possible breakdown products related with these compounds.

$$RP = \frac{[Compound]_{pre-IVD}}{[Compound]_{IN/OUT}} \times 100 \quad \textbf{(5)}$$

HPLC analysis of pre IVD treatment and afterwards IN and OUT reveals a great loss of anthocyanins. Both fractions showed varying recovery, ranging from 4-34 % for IN, and from 0-54 % for OUT. Despite the lower levels of anthocyanins in 0304F6 (compared with Tulameen, for example), this was the extract with the highest RP, ranging from 4-34% in IN and 0-54% in OUT. The 0123A7 follows 0304F6, with RP varying from 0-26 % for IN, and from 0-41 % for OUT. Although Tulameen had the highest values for anthocyanins, it was not the one with the higher percentage of recovery on both fractions (1-26% in IN and 0-37% In OUT). Glen Ericht comes in the fourth place (0-16% in IN and 0-34% In OUT) and once more, the lack of anthocyanins in the "yellow – raspberry" is showed.

It is possible to highlight certain compounds, like que cyanidin diglucoside, which for almost all extracts revealed the bests RP although it was not the most abundant compound in most of them. Also, the recovery of cyanidin glucoside was higher in the 2J19 IN fraction (25%).

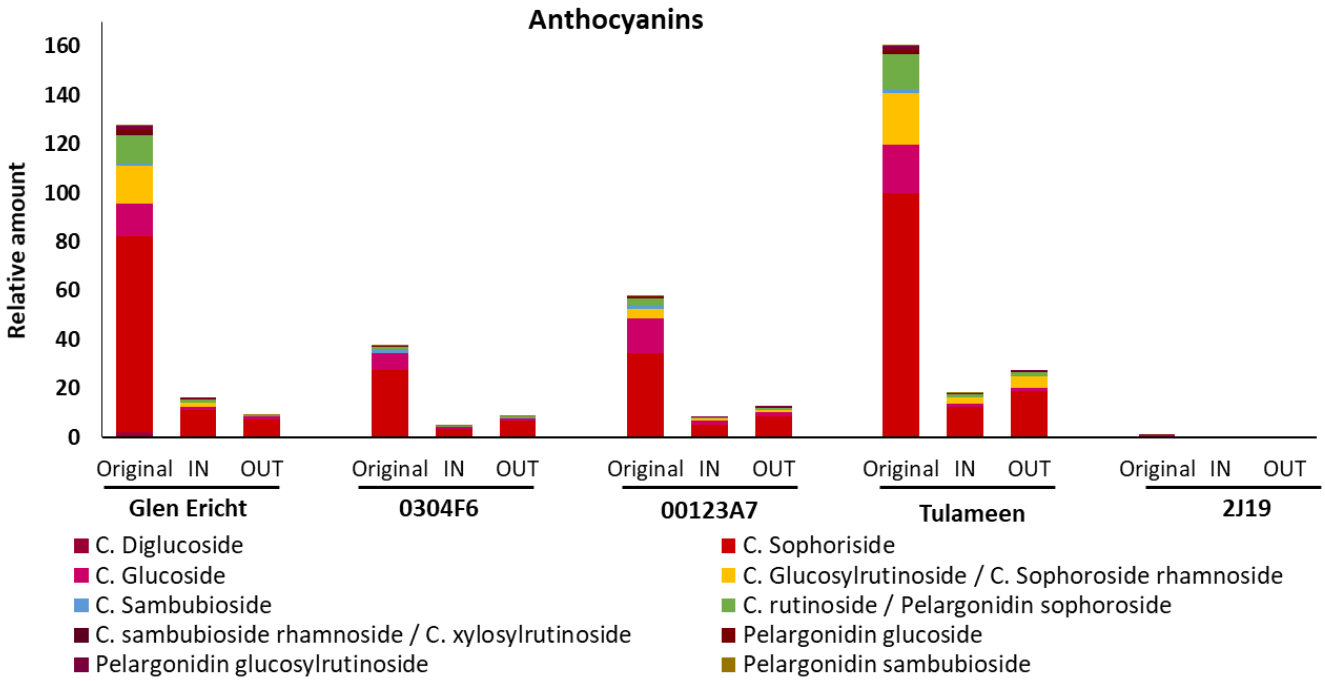


Figure 15 – Relative amounts of anthocyanins present in raspberries before and after IVD treatment as determined by HPLC. C - cyanidin.

Like the anthocyanins, flavonols also show varying RP post treatment, higher than the previous ones, with the IN ranging from 0-47 % and the OUTs 0-79 %.

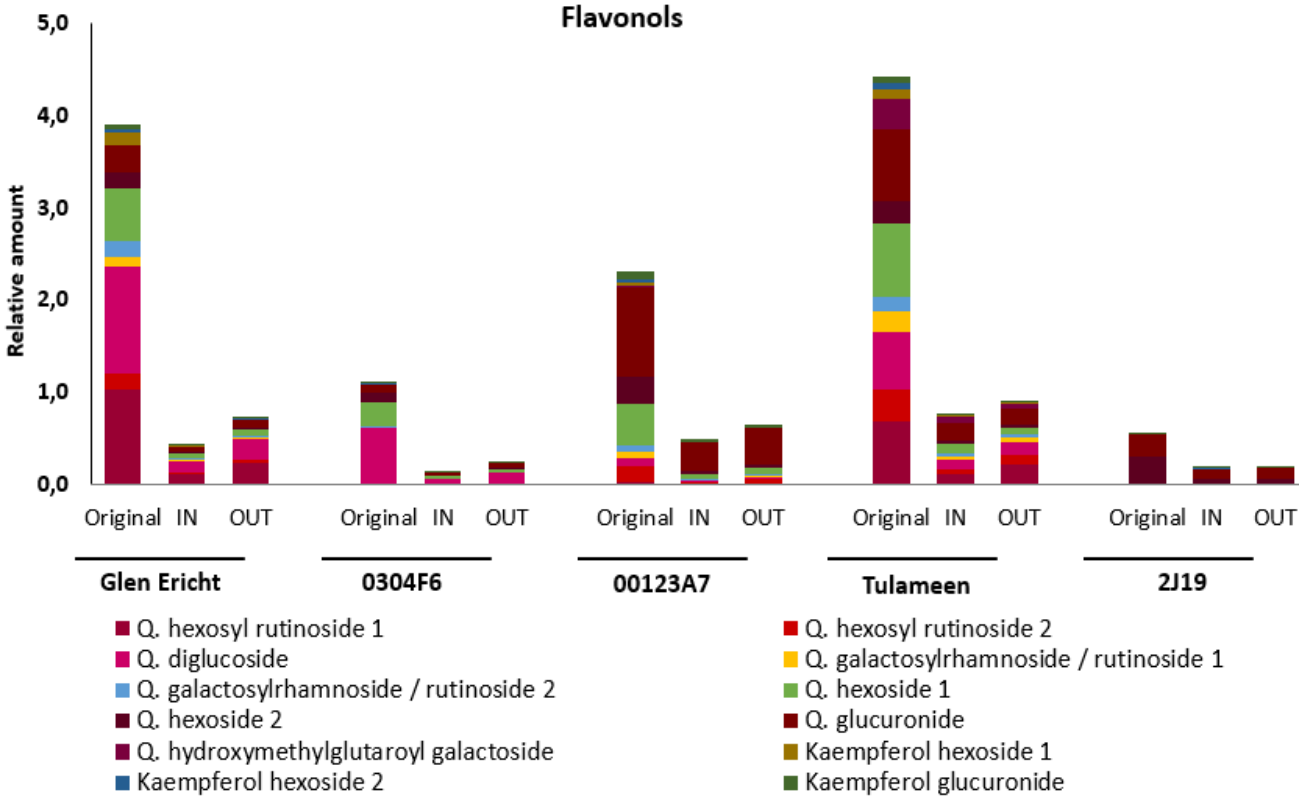


Figure 16 - Relative amounts of flavonols present in raspberries before and after IVD treatment as determined by HPLC. Q - quercetin.

The 0304F6 showed varying RP from 8 to 30% in IN and from 9 to 69% in OUT and although this is the highest RP (in OUT), in the overall this was not the extract with the best RP for all compound. 00127A7 and Tulameen revealed better capacity of recovery and comparing the two of them had similar RP values in most of the compounds (varying from 7 to 31% in IN and 13 to 41% in OUT for 00127A7 and from 13 to 25% in IN and 8 to 33% in OUT for Tulameen). As expected, flavonols are more abundant in the yellow raspberry than anthocyanins. Compounds like quercetin glucuronide showed RP values of 47% for the IN fraction and 54% for the OUT fraction). 2J19 also revealed 79% of recovery of quercetin galactosylrhamnoside / rutinoside 2 in the OUT fraction and a higher concentration of this compound in the IN fraction than in the matrix.

Ellagic acids conjugates were present in lower levels in the matrix (figure 17), it was possible to see that the recovery of these components had the highest levels so far (figure 18). In this parameter the RP calculations showed percentages above 100 % in one specific compound for the OUT extracts. The ellagic acids in Glen Ericht, 0304F6 and 00123A7, presented higher concentration in the OUT extract than it had before the IVD treatment. This suggests that these compounds may suffer breakdown during IVD treatment, being that their structure may be altered. For this reason, RP will not be calculated for these compounds.

Unlike in the other classes, the extract that showed the best RP for several compounds was 2J19 (from 28 to 54% for IN and 31 to 75% for OUT). All the other 4 extracts showed similar RP for most of the compounds.

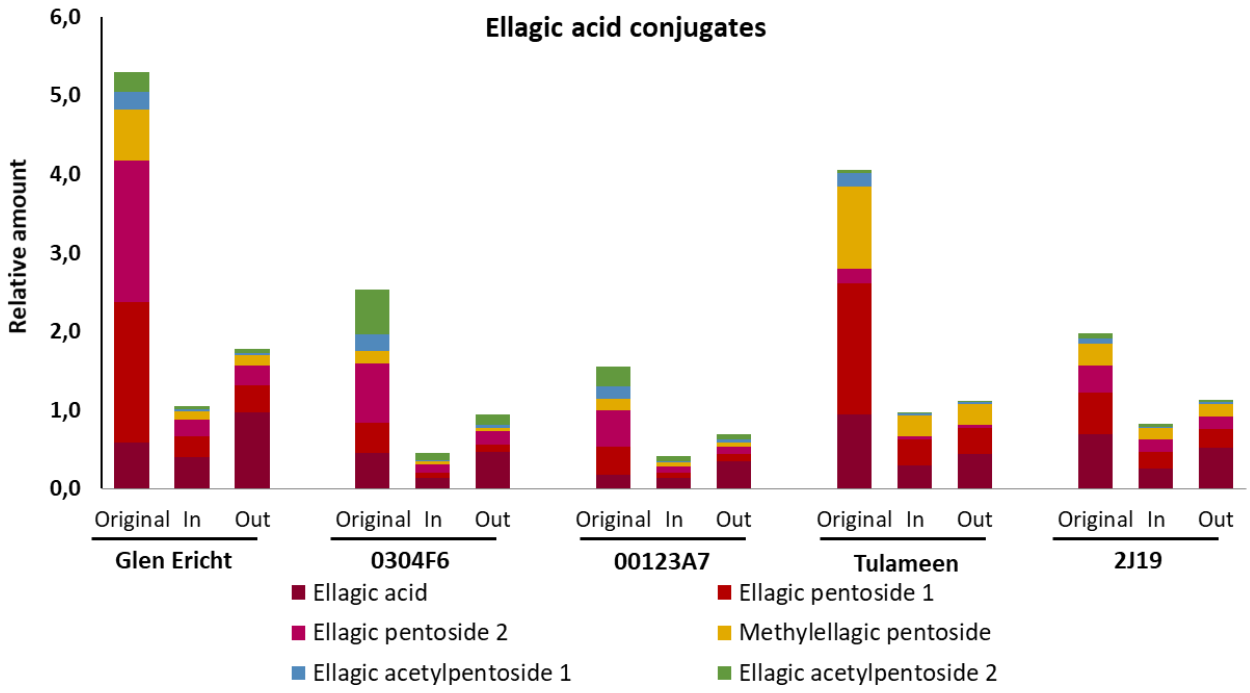


Figure 17 - Relative amounts of ellagic acids conjugates present in raspberries before and after IVD treatment as determined by HPLC.

In general, the values for ellagitannins were similar to thet of the flavonols and the previoust ones. Of all the raspberries, Glen Ericht revealed an extremely larger amount of ellagitannins and possile breakdowns (arround 15) when compared with the others, which showed similar amounts amongt them (between 3 and 6).

Compounds like the sanguiin H6 and H10A seem to exist in all raspberries, with higher content in Glen Ericht and with varying percantages of recory from 12 to 50 %. Sanguiin H10B also appears in all raspberries but show higher content then on the original matrix, in the

extracts Glen Ericht, 0304F6 and 00123A7 . The possibility of breakdown products is showed in this parameter, compounds such as sanguiinH6 without 2 x ellagic A and sanguiinH6 without 2 x ellagic D demonstrate that in some of the raspberries (Glen Ericht, 0304F6 and 00123A7) they were not present in their original form, but were in both extracts.

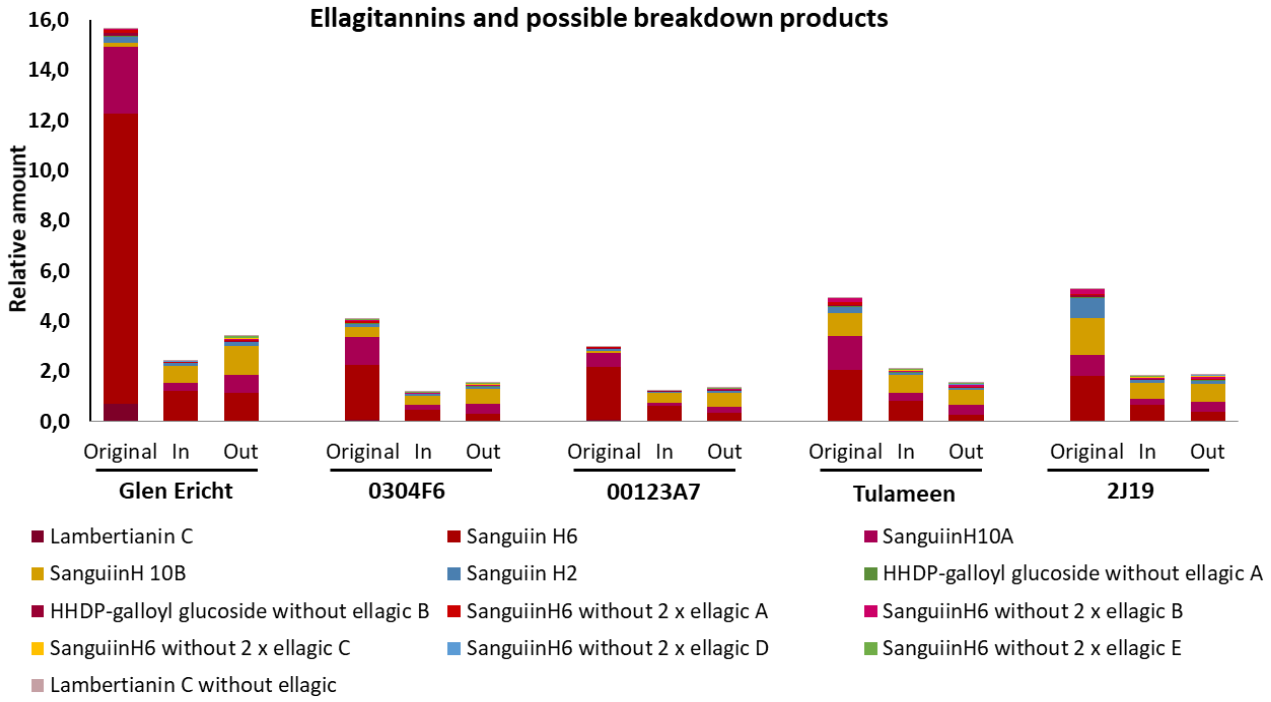


Figure 18 - Relative amounts of ellagitannins and their possible breakdown products in all raspberries before and after IVD treatment as determined by HPLC.

4.2. Total phenolic compound quantification for the bioactivity assays

4.2.1. OUT Fractions resuspended in water

Polyphenol quantification was made with OUT fractions being resuspended in purified water (plus ethanol for Tulameen (table 5). Glen Ericht originated the OUT fraction with the uppermost concentration of polyphenols, with values varying from 0.890 to 1.008 mg GAE. mL^{-1}. The second raspberry showing high concentration of polyphenols in the OUT fraction was 0304F6, with values between 0.610 and 0.650 mg GAE. mL^{-1}. 00123A7 and 2J19 revealed similar values, both very close to 0.540 mg GAE. mL^{-1}. The OUT fraction with the lowest score was Tulameen ranging from 0.355 to 0.375 mg GAE. mL^{-1}. In general, the standard deviation (SD) between all 3 biological replicates was very low, revealing almost no discrepancy between assays.

4.2.2. OUT and IN Fractions resuspended in DMSO

The resuspension method was optimized by using DMSO as solvent. Table 5 showed much more concentrated samples then the ones resuspended in water indicating that DMSO is much more efficient in the solubilization of the polyphenols. Here, Glen Ericht was again the raspberry with the uppermost concentration of polyphenols, but with values varying from 9.647 to 11.183 mg GAE.mL^{-1}. Tulameen followed with values ranging from 6.824 to 7.320. mg GAE. mL^{-1}. The raspberry 0304F6 showed concentrations around 6.271 and 6.855 mg GAE. mL^{-1} and 00123A7 and 2J19 continued having similar values, both varying from 5.250 to 6.001 mg GAE. mL^{-1}.

The fractions IN were only resuspended in DMSO. For the IN fractions, Glen Ericht is still the variety that originates the IN fraction with higher content in polyphenols, but in lower levels then in OUT fractions (values varying from 6.071 to 7.141 mg GAE. mL^{-1}). Tulameen showed levels of polyphenols, ranging from 4.221 to 4.561 mg GAE. mL^{-1} and 00127A7 levels of 3.270 to 4.506 mg GAE. mL^{-1}. 2J19 and 0304F6 showed similar concentrations, with values surrounding 3.707 to 3.779 mg GAE. mL^{-1} in the first one and from 3.275 to 3.545 mg GAE. mL^{-1}in the second. These were the fractions with the lowest levels of polyphenols.

4.2.3. Polyphenol quantification over the years

The first assays of polyphenol quantification were made in 2013 with fractions resuspended in 250 µL of ethanol. Looking at table 5 and comparing these results with the ones made in 2017/2018, it is possible to see that the results of the 2018 quantification in DMSO was higher than the assays done in 2013. This shows, once again, that DMSO is the most efficient solvent in polyphenol solubilization.

In the 2013 results Glen Ericht was also the raspberry with the uppermost concentration of polyphenols, but with values varying from 2.018 to 2.124 mg GAE.mL^{-1}. Tulameen and 0304F6 showed similar values, ranging from 1.746 to 1.776 mg GAE. mL^{-1} for the first and concentrations of 1.660 and 1.770 mg GAE. mL^{-1} for the second one. 2J19 with showed values varying from 1.399 to 1.519 mg GAE. mL^{-1} and finally 00123A7 with values from 1.270 to 1.334 mg GAE. mL^{-1}.

Table 5 – Comparison of total phenolic compounds quantification of OUT fractions between different assays. The first assay was made in 2013 and resuspension was made using ethanol, the second in 2017 with a water, plus ethanol mixture for Tulameen, and the last one in 2018 using DMSO.

	2013 (Ethanol resuspension)	**2017** (Water/ethanol for Tulameen resuspension)	**2018** (DMSO resuspension)
Raspberry	mg GAE.mL^{-1}	mg GAE.mL^{-1}	mg GAE.mL^{-1}
Glen Ericht	2.071 ± 0.053	0.949 ± 0.059	10.415 ± 0.768
0304F6	1.715 ± 0.055	0.630 ± 0.020	6.713 ± 0.142
00127A7	1.302 ± 0.032	0.538 ± 0.016	5.658 ± 0.343
Tulameen	1.761 ± 0.015	0.365 ± 0.010	7.075 ± 0.251
2J19	1.459 ± 0.060	0.541 ± 0.027	5.455 ± 0.205

For the IN fractions polyphenol quantification made in 2013 fractions were also resuspended in ethanol. Comparing these results with the ones made in 2018, for this fractions DMSO was also the best solvent to fully resuspend de extracts.

Glen Ericht showed the highest content in polyphenols with values varying from 1.519 to 1.573 mg GAE. mL^{-1}. Tulameen showed levels of polyphenols ranging from 1.253 to 1.355 mg GAE. mL^{-1} and 2J19 0.938 to 1.024 mg GAE. mL^{-1}. 0304F6 and 00127A7 showed close concentrations, with values of 0.681 to 0.755 mg GAE. mL^{-1} in the first one and from 0.608 to

0.684 mg GAE. mL^{-1} in the second. These were the fractions with the lowest levels of polyphenols.

Table 6 - Comparison of total phenolic compounds quantification of IN fractions between different assays. The first assay was made in 2013 and resuspension was made using ethanol, the second in 2018 using DMSO.

	2013 **(Ethanol resuspension)**	**2018** **(DMSO resuspension)**
Raspberry	mg GAE.mL^{-1}	mg GAE.mL^{-1}
Glen Ericht	1.546 ± 0.027	6.606 ± 0.535
0304F6	0.718 ± 0.037	3.410 ± 0.135
00127A7	0.646 ± 0.038	3.888 ± 0.618
Tulameen	1.304 ± 0.051	4.391 ± 0.170
2J19	0.981 ± 0.043	3.743 ± 0.036

Finally, it is important to highlight some differences on the polyphenol quantification of OUT and IN fractions. For the OUT fractions the sequence of polyphenol concentration (from the highest to the lowest) is: Glen Ericht > Tulameen > 0304F6 > 00127A7 > 2J19; and for the IN fractions: Glen Ericht > Tulameen > 001272A7 > 2J19 > 0304F6.

4.3. Anti-inflammatory potential

Preliminary assays were performed to monitor the proper functioning of the model. Two strains were used, the reporter strain YAA5 and YAA6 as negative control. Figure 20 shows Crz1 activation in cells induced with $MnCl_2$ and the reduction of Crz1 activity in YAA5 cells pre-treated with FK506, a widely used immunosuppressant agent.

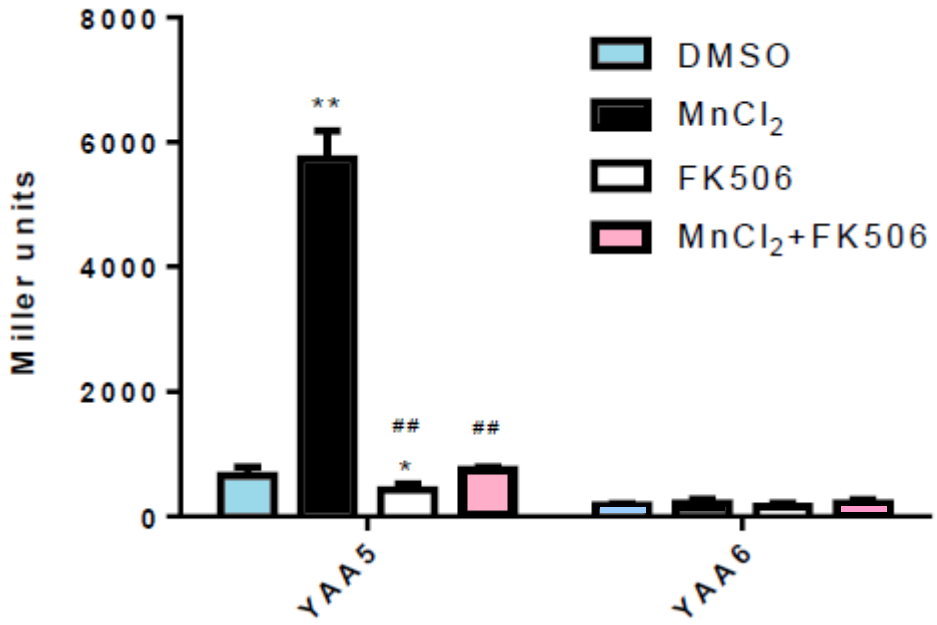


Figure 19 – Yeast reporter assay to monitor Crz1 activation. YAA5 and YAA6 cells were treated or not with 3 mM $MnCl_2$ to induce Crz1 activation. The immunosuppressant FK506 (10 µg/mL) was used as a positive control. Basal (blue bars) Crz1 activation. Activation of Crz1 with $MnCl_2$. Inhibition of Crz1 activity by FK506 (pink bars). YAA6 was used as negative control. Data were obtained from 3 biological replicates. Values are presented in mean ± SD. Statistical differences are denoted as ***p < 0.001, **p < 0.01 relative to control and as ###p < 0.001 relative to $MnCl_2$ condition.

Also, by analyzing non-induced cells (DMSO-black bars), a basal β-galactosidase activity is clearly showed. The negative control (YAA6) worked as it was supposed to, showing no activation in all the conditions tested.

4.3.1. Bioactivity of raspberry digested OUT fractions

The β-galactosidase assay demonstrated remarkable inhibition Crz1 activation for cells pre-treated with all OUT fractions and induced with 3 mM of $MnCl_2$. Overall, all the OUT fractions showed significant differences when compared with the cells induced with $MnCl_2$. Glen Ericht OUT fraction exhibited the highest capacity of attenuation of Crz1 activity followed by Tulameen, and 0304F6 showed the lowest score. When comparing the extract treatment with the immunosuppressant (FK506), it is showed again that Glen Ericht and Tulameen had nearest levels of protection to it and 0304F6 the farthest. Although 2J19 is in fact the yellow raspberry, it shows more protection than 0304F6 (one of the red raspberries), corroborating the fact that anthocyanins may not be the only compounds responsible for the protection

effects. A possible sequence (of protection, from the lowest to the highest) would be 0304F6 < 00123A7 < 2J19 < Tulameen < Glen Ericht.

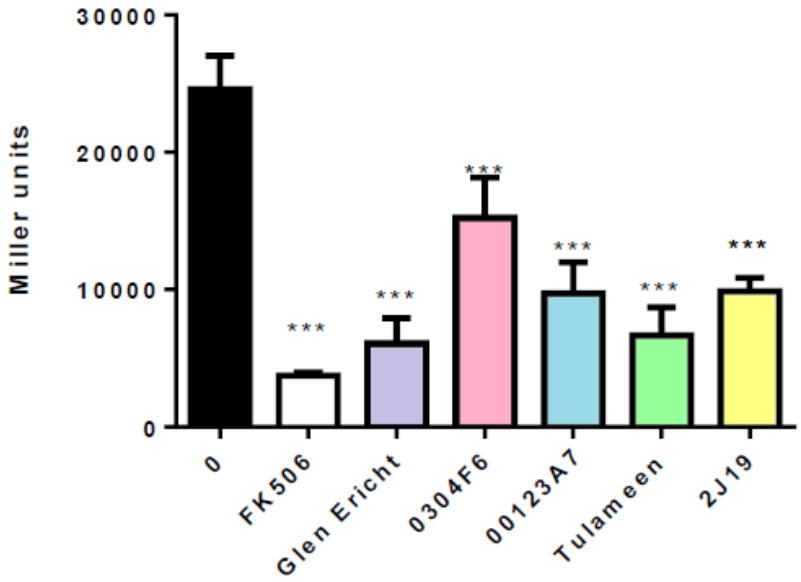


Figure 20 – Anti-inflammatory potential of OUT raspberry fractions. YAA5 cells were pre-treated with 100 µg GAE.mL^{-1} of raspberry OUT fractions and treated or not with 3 mM of $MnCl_2$ to induce Crz1 activation. The immunosuppressant FK506 (10 µg.mL^{-1}) was used as a positive control. Data were obtained from 3 biological replicates. Values are presented in mean ± SD. Statistical differences are denoted as ***p < 0.001 relative to $MnCl_2$ condition.

The percentage of protection (% Protection) was calculated as a parameter to standardize and facilitate the inference of protection levels of each sample (table 7).

Table 7 - Standard values of protection (%) for OUT fractions

Raspberry	Protection (%)
Glen Ericht	86
0304F6	43
00123A7	69
Tulameen	83
2J19	68

4.3.2. Bioactivity of raspberry digested IN fractions

It was also possible to verify a great inhibition of Crz1 activation in cells pre-treated with IN fractions and then induced with 3 mM of $MnCl_2$. All the IN fractions showed significant differences when compared with the cells only induced with $MnCl_2$. Glen Ericht IN extract was

again the one with the highest capacity to attenuate the Crz1 activity. The lowest score was attributed to 00123A7, and when comparing the fractions treatment with FK506, Glen Ericht was still on top of the protection levels followed closely by Tulameen and 0304F6 the farthest.

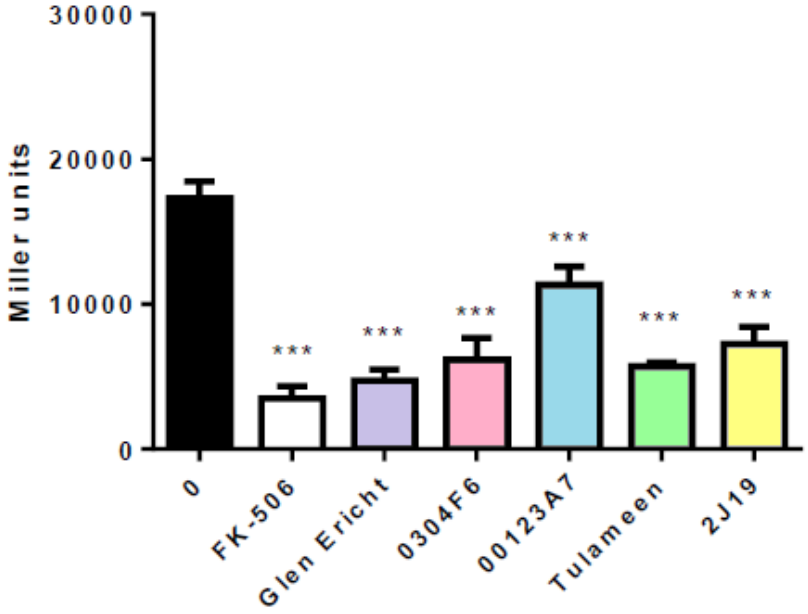


Figure 21 - Anti-inflammatory potential of IN raspberry fractions. YAA5 cells were pre-treated with 100 µg GAE.mL^{-1} of raspberry IN fractions and treated or not with 3 mM of $MnCl_2$ to induce Crz1 activation. The immunosuppressant FK506 (10 µg.mL^{-1}) was used as a positive control. Data were obtained from 3 biological replicates. Data were obtained from 3 biological replicates. Values are presented in mean ± SD. Statistical differences are denoted as ***$p < 0.001$ relative to $MnCl_2$ conditions.

As done for the OUT fractions, the standard values of protection (%) for IN fractions were calculated and are indicated in table 8.

Table 8 - Standard values of protection (%) for IN fractions

Raspberry	Protection (%)
Glen Ericht	86
0304F6	76
00123A7	41
Tulameen	79
2J19	69

5. Discussion

5.1. HPLC and proximal analysis

The composition of the matrix and its variation between the different raspberries was made with the hope that it could help provide valuable information to identify not only the ones with the highest levels of beneficial phenolic compounds, but also to understand which polyphenols could be responsible for that. Proximal analyses and total amount of polyphenols was first analyzed, more specifically fiber and protein were compared with total polyphenol amount. The results indicate a high content in polyphenols. This was expected, since it is already known the composition of the berries is very rich in polyphenols. The elevated polyphenol content showed for the yellow raspberry, 2J19, indicates that these fruits are not only rich in anthocyanins and will later help to build some conclusions. The raspberry extract with the highest content of polyphenols was 00123A7 (9602 mg.100g $^{-1}$ DW), followed by Tulameen and 2J19, (5851 and 5270 mg.100g $^{-1}$ DW). 0304F6 and Glen Ericht were the extracts with the lowest values of polyphenols (4412 and 3392 mg.100 g^{-1} DW, respectively).

There are evidences that relate polyphenol stability and their physical/chemical interactions with fiber and protein, being able to modulate their release during digestion (Palafox-Carlos, H. *et al.* 2011). Starting with fiber, the term dietary fiber is related to the indigestible part of the cell wall (or pant material) and many studies indicate that a wide range of polyphenols can be associated with it. In general, dietary fiber can act in the small intestine or appear as fiber-polyphenol complexes in 3 different forms, as soluble polymer chains in solution, as insoluble macromolecular assemblages and as hydrated (sponge-like) systems (Renard, C., *et al.*, 2017; Palafox-Carlos, H. *et al.*, 2011). Overall, dietary fibers are known for retarding the absorption of some nutrients, and some have the ability of entrapping polyphenols in the lumen of the gut. Although the real effect of fiber on polyphenol absorption in humans or their interaction mechanism is limited many studies indicate that fiber-polyphenol complexes (or polyphenol entrapped in the lumen of the gut) could reduce polyphenol absorption in the small intestine, leading to an increase of their bioavailability for bacterial catabolism (Renard, C., *et al.*, 2017). Tulameen, 00123A7 show similar and the highest contents of fiber, ranging from 31 to approximately 32 g.100g $^{-1}$ DW. Glen Ericht revealed values near the 28 g.100g $^{-1}$, 2J19 showed small content of fiber (around 21 g.100g $^{-1}$ DW) The extract 0304F6 shows the lowest score for this parameter, having contents below 20 g.100g $^{-1}$ DW. As for protein, it is described that dietary polyphenols can bind to the protein chains, displaying many important roles in several physiological activities (such as digestion) as

well as in polyphenol bioavailability (Bandyopadhyay, P. *et al.*, 2012, Raghavendra, M.P., *et al.*, 2007). Meaning that the interactions between polyphenols and proteins will also affect their activity. Several studies describe polyphenol-proteins interactions happening through hydrophobic or hydrophilic interactions, formatting soluble polyphenol-protein aggregates. Then, soluble complexes self-associate, producing large aggregates which subsequently precipitate from the solution. They also depend on their structure, protein/polyphenol ratio, medium pH, etc., and may affect the bioavailability and protective properties of both the individual components (Bandyopadhyay, P. *et al.*, 2012, Raghavendra, M.P., *et al.*, 2007). So, polyphenol-protein complex formation can either reduce or enhance the antioxidant activity of polyphenols and affect the digestion ability of several digestive enzymes present in our body. In studied carried out by Naz *et al.* a polyphenol-protein complex was compared to a pure polyphenol, both were submitted to an *in vitro* duodenal digestion and the results showed that the activity of digestive enzyme was less aggressive in the polyphenol-protein complex. Thus, the polyphenol-protein complexes showed to be more stable than the pure polyphenol, also revealing an increase in their bioavailability. In general, protein showed small contents in all five raspberries. The extracts that showed the highest content of protein were 2J19 and Glen Ericht, ranging from 8 to 9 $g.100g^{-1}$. Extracts 00123A7 and Tulameen revealed the same content, ≈ 6.3 $g.100g^{-1}$ and the lowest was again attributed to 0304F6 (4.4 $g.100g^{-1}$ DW).

This study also compared the levels of anthocyanins and flavonols in each extract, the first ones showed values varying from 3 to 681 $mg.100\ g^{-1}$ DW and the second ones did not go over 28 $mg.100\ g^{-1}$ DW. So overall, it is possible to conclude that other polyphenol the extracts revealed much higher content of anthocyanins than flavonols, except for the yellow raspberry that, as it was expected did not show any content of anthocyanins. The highest content of anthocyanins belonged to Tulameen. Glen Ericht also showed a significant content of anthocyanins followed by 00123A7 and 0304F6. For flavonols 00123A7 was the raspberry with the highest level, closely followed by Tulameen follows 00123A7. Glen Ericht showed a medium value and the lowest values belong to 0304F6 and 2J19.

The HPLC-PDA-MS analysis of the matrix before and after IVD treatment provides valuable information. First, it allowed inferring the loss of polyphenols during the digestion process and the identifying the compounds present in each sample. So, it was possible to discriminate several compounds that could be correlated to the identified bioactivities. The calculation of the RP was made by equation 2 and it was not applied for the ellagic acid in ellagic acid conjugates and some of ellagitannins possible breakdown products. Some of the compounds may have suffered breakdown in their structure and others didn't even existed in the original matrix before IVD treatment, resulting into RP above 100%, which is not accurate.

For anthocyanins, the HPLC analysis revealed a great loss of anthocyanins after IVD treatment and both IN and OUT fractions showed varying recovery rates. Despite the lower levels of anthocyanins in 0304F6 (compared with Tulameen, for example), this was the extract with the highest RP. The 0123A7 follows 0304F6. Although Tulameen was the extract with the highest values for anthocyanins, the higher percentage of recovery on both fractions was only obtained in compounds, like cyanidin diglucoside. Other compounds such as cyanidin glucosylrutinoside/cyanidin sophoride rhamnoside can be highlighted since they showed the best RP, although they were not the most abundant compound in the matrix. The 0304F6 also showed varying RP with the highest RP for anthocyanins in OUT fraction. 00127A7 and Tulameen revealed better capacity of recovery with similar RP values for most of the compounds.

Flavonols also showed varying RP post IVD, higher than anthocyanins. The raspberry 0304F6 presented the highest RP in OUT fraction but in general this was not the extract with the best RP for all compounds. In general, 00127A7 and Tulameen revealed the best capacity of recovery. Lastly, the flavonols in the yellow raspberry appeared in a larger scale then the anthocyanins, which was already expected given the color of this raspberry. Compounds like quercetin glucuronide showed best RP in 2J19 fractions. Also, data showed that for Q. galactosylrhamnoside / rutinoside 2 in the 2J19 IN fraction had more amount then on the matrix, which can lead to thinking that these compounds can derive from the break of compounds with a complex chain existent in the raspberries before IVD treatment.

Although ellagic acids conjugates were present in low levels in the matrix, the recovery of these compounds was the highest when compared with the two previous ones. These were the only compounds in which the RP showed percentages above 100 %, for certain extracts, as observed for ellagic acid in Glen Ericht, 0304F6 and 00123A7. The extract showing the best RP for ellagic acids conjugates is 2J19. All the other fractions showed similar RP for most of the compounds.

In general, ellagitannins show values close to the ellagic acids, the Sanguiin H6 and H10A appeard in all raspberries, with higher content in Glen Ericht. Sanguiin H10B also appears in all 5 raspberries, with higher contents in the OUT extracts then in the original matrix for Glen Ericht, 0304F6 ans 001237A7. This may be explained by the breakdown of *in planta* compounds into the respective metabolites produced by the *in vitro* digestion. Another example is the sanguiinH6 without 2 x ellagic A and the SanguiinH6 without 2 x ellagic D, which are detectable in the extracts of Glen Ericht, 0304F6 and 00123A7 but only appear in the IN and OUT fractions.

5.2. Total phenols quantification

The Folin-Ciocalteau method was the one used in this study to quantify the polyphenols present in the extracts and fractions. Although this is one of the most common method used to determine total polyphenols in fruit matrix or extracts, it is not specific for it and other classes of compounds that also integrate the matrix can reduce the F-C reagent, tilting the results (Blainski, A. *et al.*, 2013; Schofield, P., *et al.*, 2001). This may explain some of the variations of the results between different assays.

The first assays of polyphenols quantification were made in 2013 with fractions resuspended in ethanol. Comparing these results with the ones made in 2017/2018, it is possible to conclude that water and ethanol, and the mixture of both for Tulameen (in 2017), were the less efficient solvent to dissolve the fractions as total polyphenol content was shown to be lower in these samples. Also, DMSO appears to be most efficient solvent allowing much higher percentage of recovery of total polyphenols, higher values were observed for Glen Ericht. Moreover, both IN and OUT fractions freeze-dried appeared to remain stable throughout the years, not showing great loss of amount of polyphenols. Some results from 2018 assays for IN fractions even showed better levels in many raspberries (such as Glen Ericht, 0304F6 and 00127A7). Although it remains the question whether the stability of the compounds is also maintained.

5.3. Anti-inflammatory potential

The NFAT pathway is central in inflammatory processes, and its mechanism is Ca^{2+} dependent. The yeast model used mimic NFAT-mediated inflammatory pathway by evaluating Crz1 activation (whose gene is the yeast orthologoue to human NFAT gene). The yeast strains were also genetically modified to express de lacZ gene (encoding the β-galactosidase), to facilitate monitoring Crz1 activity. The $MnCl_2$ was used to induce cytosolic Ca^{2+} levels and FK506, an immunosuppressive drug used mainly in medicine to lower the risk of organ rejection, was used as positive control.

Overall, the assays revealed a remarkable inhibition of the Crz1 activation in cells pre-treated with the extracts (both IN and OUT) and induced with 3 mM of $MnCl_2$, with significant differences when compared with the cells induced with $MnCl_2$. Remarkably, inhibition of Crz1 activation, i.e. potential anti-inflammatory activity, was seen both in the OUT than the IN fractions. The rank of potential anti-inflammatory activity for the OUT and IN fractions (from

the lowest to the highest) is the following, respectively: 0304F6 < 00123A7 < 2J19 < Tulameen < Glen Ericht; and 00123A7< 2J19 < 0304F6 < Tulameen < Glen Ericht.

In both cases the Glen Ericht and Tulameen were the ones that exhibited the highest anti-inflammatory potential. It is reported that the protective action of polyphenols are not only related to anthocyanins and flavonols, but also with hydroxybenzoic acids (ellagic acids and ellagitannins) (Proteggente, A.R., *et al.*, 2003). Ellagitannins and ellagic acids showed better RP than some anthocyanins and flavonols, so these compounds not only resist more to the IVD, but also maybe pointed out as the compounds with the most interest and bioactivity in the raspberries extract. Also, the interactions between fibers, proteins and polyphenols can be related to their survival capacity during IVD and also their capacity of attenuation, and therefore their % of protection.

Glen Ericht was the raspberry with the highest amount of protein, a relatively high level of polyphenols and the one with the best RP for the ellagic acids and ellagitannins and possible breakdowns, so perhaps the % protection of this extract is related to all this together. Tulameen was not rich in proteins but had relatively high levels of fiber and average RP for Ellagic acids an Ellagitannins and possible breakdowns. Extract 00123A7 was the extract that had the highest level of fiber and also one of the highest content of phenolic compounds, but most of his content was anthocyanins. The results obtained for the yellow raspberry (2J19) were very curious, this one did not have anthocyanins, was rich in protein and showed the best RP for several ellagic acid compounds was 2J19 which validates the conclusion that Ellagic acids may be the most important bioactive compounds in the raspberries fractions.

The OUT fraction of 0304F6 was the less protective one while the IN fraction was ranked in the third position indicating that there may exist compounds present in the IN fraction with bioactivity towards attenuation of the Crz1 activation.

6. Conclusions

Overall, it is possible do conclude that the digested raspberries fractions obtained from the 5 different near-isogenic raspberries have great anti-inflammatory potential, therefore all of them significantly attenuated the Crz1 activation.

Glen Ericht and Tulameen were considered the one with the highest level of attenuation for both fractions, both had relatively high content of fiber and protein, but Tulameen had much higher content of polyphenols than Glen Ericht. It is in the ellagic acids and conjugates and in the amount of ellagitannins that these two raspberries exhibit more pronounced differences.

The extract 0304F6 had the lowest anti-inflammatory potential for the OUT fraction while the IN fraction it was in third place may give us the information that there may exist compounds that integrate the bio-accessible fractions of the serum that may have effect in the attenuation of Crz1 activation. The yellow raspberry (2J19) was the one with no anthocyanins and despite that it showed a very interesting result validating the conclusion that Ellagic acids conjugates and ellagitannins (and possible breakdown products) may be the most important bioactive compounds in the raspberries fractions. They appear mostly in the fractions and not on the raspberry before IVD, and some only were detected in the fractions.

Also, the synergistic effects can be viewed as the key for the anti-inflammatory capacity promoted by these fractions, and not only the composition in each group of polyphenols.

Moreover, it is clear that fiber and protein influence how these compounds react upon digestion, although so far these mechanics/interactions are not entirely known, and it is not possible to correlate the extent of their interaction with polyphenols bioavailability. Thus, understanding these factors could be a key factor for deciphering the health benefits of polyphenols consumption.

This study can be considered as one first step to better understand the most bioactive compounds of the raspberries. In the future, the protective activity of these fractions and some of the highlighted compounds should be tested in other inflammatory pathways such as the NF-kB. Also, tests using human cell lines such as myocardial cells and keratocytes and dermal fibroblasts (human skin), could be interesting and bring more responses regarding the potential anti-inflammatory activity of the raspberries extracts. Some studies have already been done where the NFAT via of inflammation was pointed to have an important role in regulation of keratocytes and dermal fibroblasts. (Putt., M.E., *et al.*, 2009; Al-Daraji, W.I. *et al.*, 2010). It is equally important to unravel polyphenol pathways/mechanism of action and also unknot the physiochemical changes that can occur in the stomach and/or in the small intestine during digestion.

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