PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE

ESCUELA DE INGENIERIA

ADSORPTION OF THE MAIN POLYPHENOLS OF PVPP BEER WASTE STREAM IN HIGHLY CROSS-LINKED 12% AGAROSE GEL

JAVIERA E. PÉREZ

Thesis submitted to the Office of Research and Graduate Studies in partial fulfillment of the requirements for the Degree of Master of Science in Engineering

Advisor:

NÉSTOR ESCALONA

Santiago de Chile, (November, 2018)

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Members of the Committee:

NÉSTOR ESCALONA

ROBERTO CANALES

FERNANDO SALAZAR

GLORIA ARANCIBIA

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Dedicated to my mother and father who supported me, no matter what. This is for you

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I would like to start by thanking my family for all the encouragement and comfort they have given me throughout these years. Probably, I would have lost my mind without all the emotional support provided by my mother. Also, the uncountable advises received by my father and his passion for science were fundamental to finish this work.

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RESUMEN

El incesante crecimiento de la población ha generado una serie de problemáticas, incluyendo la enorme producción de desechos de la industria alimentaria. El uso de estos desechos como materia prima para generar nuevos productos ha sido uno de los enfoques usados para abordar este tema. La extracción de polifenoles ha sido de gran interés debido a que reducen el estrés oxidativo y la inflamación en el cuerpo. La industria cervecera, para evitar la formación de turbidez, remueve los polifenoles de la cerveza y los elimina como residuos. Estos residuos poseen gran cantidad de polifenoles, entre ellos, destacan tres compuestos por su abundancia, bioactividad y biodisponibilidad: ácido ferúlico (FA), kaempferol (KAE) y ácido protocatecuico (PCA). Se ha reportado que eficientes separaciones de polifenoles pueden ser llevadas a cabo con agarosa reticulada de alto grado. Sin embargo, no se han caracterizado las interacciones de los polifenoles con este adsorbente ni cómo el solvente afecta el proceso de adsorción, información fundamental para diseñar un proceso óptimo de separación. En este trabajo, se deseó caracterizar por primera vez la adsorción de estos compuestos en agarosa reticulada de alto grado. Para esto, se realizaron experimentos *batch* con diferentes mezclas de agua, etanol y ácido acético como solventes. Los resultados mostraron que la capacidad de adsorción disminuye con el aumento de co-solventes. El modelo de Freundlich representó mejor el equilibrio de adsorción, puesto que sus parámetros fueron más precisos. El análisis termodinámico indicó altas energías de adsorción para el PCA y FA, quizás debido a puentes de hidrógeno simultáneos. Se observó variaciones de la entalpía con respecto a la cobertura de superficie, indicando heterogeneidad de superficie de adsorción para el PCA e interacciones adsorbato-adsorbato para el KAE. Del factor de retención relativo, calculado a partir de las isotermas, se infirió que la separación de estos compuestos es posible usando cromatografía de adsorción con agarosa altamente reticulada. La elución propuesta es comenzando con 15% etanol, 15% ácido acético, seguido de 35% etanol, 35% ácido acético. Sin embargo, no considera los fenómenos cinéticos ni de transferencia de masa, por lo que se deben realizar más estudios para verificar esta propuesta.

ABSTRACT

Over the last decades, there have been several concerns about ongoing population growth resulting in a series of issues, including a vast amount of food waste production. An interesting approach to cope with this issue is using these wastes as raw materials for new products. These products include functional ingredients such as polyphenols, which are a group of compounds known for its protective effects against various oxidative stress diseases. In the brewery industry a standard procedure to avoid haze formation is the removal of polyphenols, generating a considerable amount of polyphenol-rich wastes. From this waste stream, three phenolic compounds stand out for their bioactivity, bioavailability and abundance: ferulic acid (FA), kaempferol (KAE) and protocatechuic acid (PCA). It has been reported that efficient phenolic separations have been accomplished using adsorption chromatography with cross-linked agarose. However, there is no adsorption characterization available, which is fundamental for a proper separation process design. In this study, we aim to study for the first time the adsorption behavior of these phenolics on cross-linked agarose. Different temperatures and solvent mixtures of water, ethanol and acetic acid were assessed using batch experiments. Results showed that the adsorption capacity decreased with increased concentrations of co-solvents. The isotherm model that best described the adsorption equilibrium was Freundlich, since more accurate parameters were obtained. Analysis of the adsorption thermodynamics revealed high adsorption energy for FA and PCA, probably due to simultaneous hydrogen bonding formation. The change of enthalpy with surface coverage indicated heterogeneous adsorption surface for PCA and adsorbate-adsorbate interaction for KAE. A relative retention factor was calculated based on the adsorption isotherms. From these values it was presumed that separation between these phenolics with cross-linked 12% agarose adsorption chromatography, could be achieved through stepwise isocratic elution, starting with 15% ethanol, 15% acetic acid, and followed by 35% ethanol, 35% acetic acid. However, this study does not consider kinetic or mass transfer phenomena, so further analysis should be conducted in order to verify this presumption.

1. INTRODUCTION

1.1. Agroindustrial byproducts

Over the last decades, there have been several concerns about increasing population and consumption resulting in a series of issues, such as food security, increasing environmental deterioration and resource depletion (Holdren et al., 1947). Great efforts are being made to minimize this damage, including the implementation of programs to reduce environmental impact caused by industries and to make a more effective use of our resources. Exploitation of the nutrients found on agroindustrial wastes is an interesting and ecological approach that has been widely studied and adopted to cope with these issues (Mirabella et al., 2014).

In 2012, the ODEPA estimated that agroindustrial residues in Chile were over 7 million m^3/year of liquid wastes and about 1.76 million ton/year of solid wastes. They also showed that these waste streams were either partially exploited and valorized as compost and animal feeding, or were not exploited at all, being disposed in a landfill, increasing the environment burden (Oficina de Estudios y Políticas Agrarias, 2012)

Extensive literature has established that wastes and effluents derived from vegetable and fruit processing contain high amounts of nutrients such as proteins, sugars and lipids, as well as other bioactive compounds that include vitamins, dietary fiber and natural antioxidants (Ayala-Zavala et al., 2011; Federici et al., 2009). Therefore, food wastes can be considered as a cheap and abundant source of fine chemicals and biomaterials (Federici et al., 2009) and, when combined with efficient and green processing technologies, we can provide added-value to agroindustrial residues and reduce their impact to the ecosystem (Mirabella et al., 2014).

Phenolic compounds (or polyphenols) have received special attention over the last decades. These compounds among the most numerous metabolites produced by plants, playing an essential role in their growth and reproduction cycles, and providing protection

against pathogens and predators (Matern et al., 1988). They range from simple phenolic molecules to complex compounds exhibiting molecular weights over 30,000 Da (Bravo, 1998). They also show interesting activities in the human physiology, revealing protective effects against arteriosclerosis, coronary heart disease, cancer and several neurodegenerative diseases (Chiva-Blanch et al., 2013; Kanno et al., 2015; Sławińska-Brych et al., 2016; Suganuma et al., 2016; Yang et al., 2014). Therefore, phenolic compounds are considered attractive additives for functional foods and nutraceuticals.

Many agroindustrial residues have been proposed as rich and economic sources of polyphenols, including apple, grape, tomato and apricot pomace, citrus seeds and peels, mango and onion residues, and olive waste waters, among many others (Mirabella et al., 2014). Recently, Barbosa-Pereira *et al*. discussed that the waste from the brewery process is also an appealing source of phenolic compounds (Barbosa-Pereira et al., 2013).

Beer is one of the most consumed alcoholic beverages. It consists of a complex mixture of nutrients, including carbohydrates, proteins, fatty acids, vitamins and polyphenols. Nevertheless, in many cases these polyphenols are removed from beer since high content of these compounds favors the haze formation. This phenomenon is caused by polyphenol and protein interaction that alters the overall stability of beer. Removal of the phenolic compounds is carried out through polyvinylpolypyrrolidone (PVPP) adsorption, which interacts with polyphenols via hydrogen bonds between their CO-N linkages and phenol groups (Doner et al., 1993; Laborde et al., 2006). This procedure is conducted once during the brewing process, generally after yeast and suspended proteins removal (clarification) (Dadic et al., 1983). This stabilizing method generates a considerable amount of a polyphenol-rich waste that can be an economic source of functional ingredients, providing added value to the industry and reducing its residues. Additionally, this waste stream, compared to other common polyphenol sources, does not compete with the food industry.

1.2. Polyphenols of the PBWS and their pharmacological effects

Phenolic compounds or polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with more than 8,000 phenolic structures currently known. Natural polyphenols can range from simple molecules, such as phenolic acids, to highly polymerized compounds such as tannins (Bravo, 1998). These substances are usually classified into two groups: flavonoids (flavanols, anthocyanidins, flavonols, flavanones) and non-flavonoid compounds (hydroxybenzoic acids, hydroxycinnamic acids and stilbenes). Flavonoids have a common core, consisting of two benzene rings linked by an oxygen-containing a pyran ring (C) ($C_6C_3C_6$), while non-flavonoids are mostly acids (Havsteen, 2002; Oliveira et al., 2011; Rentzsch et al., 2009).

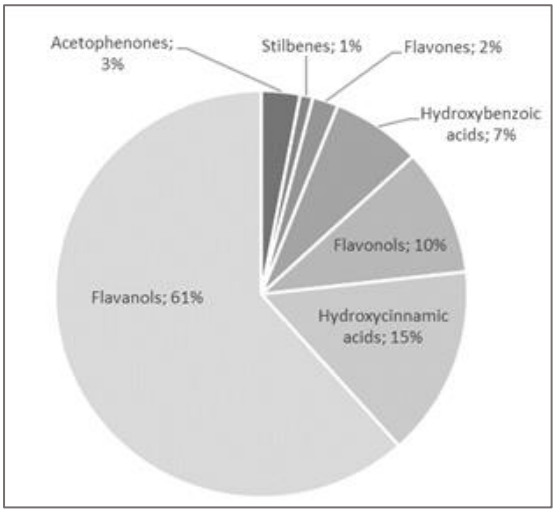
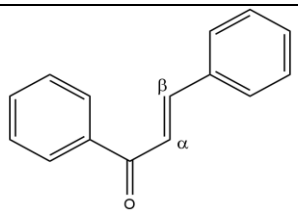
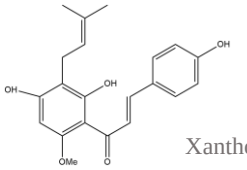
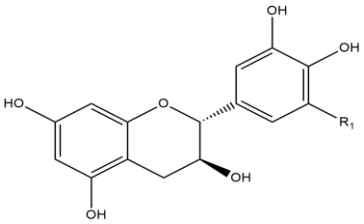
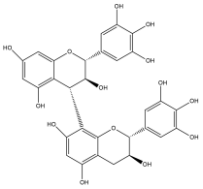
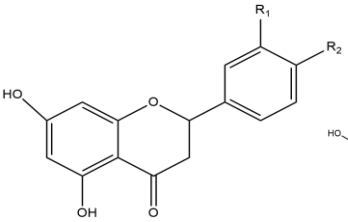
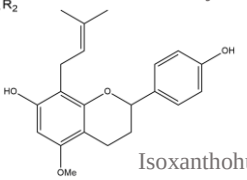
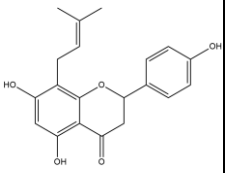
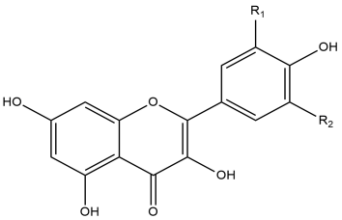
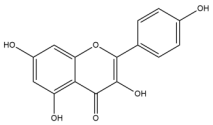
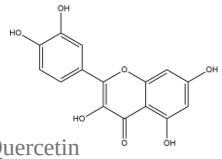
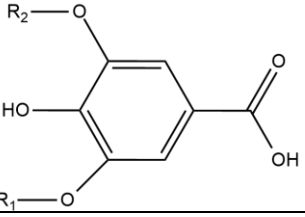
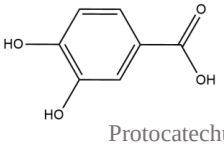


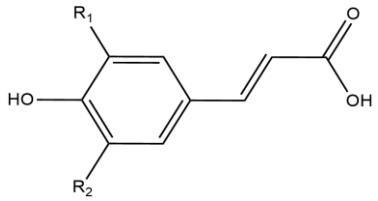
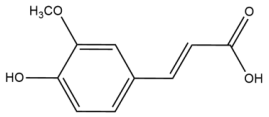
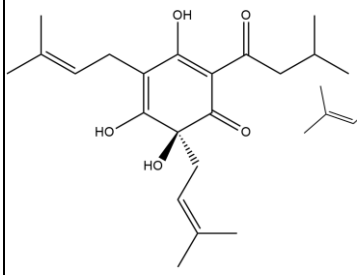
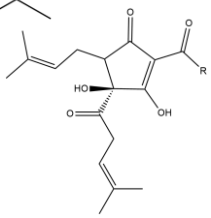
Fig. 1. Distribution of phenolic classes concentrations in the PBWS (Barbosa-Pereira et al., 2013)

The average content of polyphenols in beer ranges between 300 and 600 mg/L, where proanthocyanidins are usually the most abundant (81%), followed by phenolic acids and flavonoids (Frego et al., 2009). The content of phenolic compounds in beer depends on plant species, cultivation technique and the technological procedures used to process the

raw material. Also, environmental factors such as maturity, seasonal conditions and production area also contribute to the variability in their phenolic content and composition (Barbosa-Pereira et al., 2014; Floridi et al., 2003).

Table 1. Estructura química de los compuestos fenólicos principales de la cerveza el PBWS

Chalcones	  Xanthohumol
Flavanols	 Prodelphinidin B3 
Flavanones	 8-Prenylnaringenin  Isoxanthohumol 
Flavonols	 Kaempferol   Quercetin
Hydroxybenzoic acids	  Protocatechuic acid

Hydroxycinnamic acids	  Ferulic acid
Humulones	 Isohumulones  n-isohumulone R = $CH_2CH(CH_3)_2$ *co*-isohumulone R = $CH(CH_3)_2$ *ad*-isohumulone R = $CH(CH_3)CH_2CH_3$

Despite extensive literature identifying and comparing the phenolic composition of several types of beer using different methods of detection (Dvořáková et al., 2007; Quifer-Rada et al., 2015), few studies describe the phenolic profile of the PVPP brewery waste stream (PBWS) and its potential pharmacological effects. Barbosa-Pereira *et al.* (2013) revealed that the polyphenols found in the PBWS corresponded to the main compounds responsible for beer instability, including flavan-3-ols, flavonols and phenolic acids **(Fig. 1)** (Barbosa-Pereira et al., 2013). Another study reported that the PBWS also exhibited bitter acids, such as humulone and cohumulone (α-acids) and lupulone (β-acid) (Munekata et al., 2016). Moreover, a study conducted by Magalhães and coworkers observed that xanthohumol, a highly bioactive polyphenol, can be adsorbed efficiently by PVPP from a hops (*Humulus lupulus L.*) extract (Magalhães et al., 2010). Therefore, it could be speculated that during the beer stabilization process, xanthohumol could also be extracted. The chemical structure of the main phenolic classes found in beer and the PBWS is shown in **Table 1.**

Research related to content of the main phenolic compounds found in the PBWS is summarized in **Table 2**. The most abundant class of polyphenols in this waste stream

Table 2. Phenolic compounds in different beer types and PBWS (mg/L)

Phenolic Classes	Phenolic Compounds	Stout	Ale	Alcohol Free	Lager	Unstabi-lized Lager	PBWS
Chalcones [1,2]	Xanthohumol	0.1 – 0.2	0.1 – 0.2	0.003	0.014 – 0.034	0.015	n.d.
Flavanols [3 - 11]	(+) Catechin	.02	0 – 10.1	0 – 4.5	0 – 5.5	4.2 – 4.3	61.41
	(-) Epicatechin	0.1	0 - 3.8	0 - 0.2	0 - 1.3	1.1 - 1.3	75.22
	Prodelphinidin B3	n.q.	n.q.	n.q.	0.5 - 1.1	2.5 - 2.7	n.q.
	Procyanidin B3	n.q.	n.q.	n.q.	0.3 - 3.6	1.7 - 3.1	n.q.
	Total	**n.q.**	**n.q.**	**n.q.**	**5.5** **	**11.5** **	**403.4** **
Flavanones [1,2,12]	8-Prenylnaringenin	0.008 - 0.2	0 - 0.1	0.003	0 - 0.023	0.013	n.d.
	Isoxanthohumol	0.3 - 2.1	0.8 - 3.4	0.1	0 - 1.0	0.59	0.21
	Total	**0.308 – 2.3**	**0.8 – 3.5**	**0.103**	**0 – 1.023**	**0.603**	**0.21**
Flavonols [3,9,13]	Kaempferol	n.q.	n.q.	n.q.	16.4	n.q.	21.8
	Quercetin	n.q.	n.q.	n.q.	0 - 0.4	n.q.	22.39
	Isoquercetin	n.q.	n.q.	n.q.	n.q.	n.q.	23.52
	Total	**n.q.**	**n.q.**	**n.q.**	**16.8** *	**n.q.**	**68.07** *
Hydroxybenzoic acids [1,3,5,14,15]	4-Hydroxybenzoic acid	0.07	0.3 - 1.8	0.07	0 - 1.2	n.q.	0.63-4.65
	Gallic acid	0.03	0.3 - 1.8	n.q.	0 - 7.0	n.q.	0.5-13.88
	Protocatechuic acid	0.04	0.2 - 1.0	0 - 5.1	0 - 0.8	n.q.	3.7 - 24.7
	Total	**0.14** *	**0.8 – 4.6** *	**0.07 – 5.8** *	**0 – 9.0** *	**n.q.**	**4.83 - 43.23***
Hydroxycinnamic acids [1,3-5,7,14-17]	Caffeic acid	n.q.	0.1	0 - 0.4	0 - 0.5	n.q.	1.0 - 20.27
	Ferulic acid	0.3 - 1.3	0.5 - 7.6	0 - 2.3	0.3 - 14.1	n.q.	13.2 - 58.5
	p-Coumaric acid	0.2 - 0.7	0.9 - 1.9	0.1 - 0.7	0 - 0.22	n.q.	0.36 - 16.89
	Total	**0.5 – 2.0***	**1.5 – 9.6***	**0.1 – 3.4***	**0.3 – 14.8***	**n.q.**	**14.56 – 95.66***
Isohumulones [18 - 21]	Iso-*ad*-humulone	n.q.	n.q.	n.q.	1.5 – 39.3	n.q.	n.q.
	Iso-co-humulone	n.q.	n.q.	n.q.	0.1 – 22.9	n.q.	n.q.
	Iso-n-humulone	n.q.	n.q.	n.q.	0.5 – 46.8	n.q.	n.q.
	Total	**18.9** **	**25.2 – 100** **	**13.4 – 27.1****	**0.7 – 101****	**n.q.**	**n.q.**
Stilbenes [3,22]	trans-Resveratrol	0 - 3.1	0 - 3.3	0 - 0.6	0 - 4.2	n.q.	9.6
	cis-Resveratrol	0 - 1.6	0 - 2.3	0 - 0.47	0 - 0.74	n.q.	n.q.
	Total	**0 – 4.7** *	**0 – 5.6** *	**0 – 1.07** *	**0 – 4.94** *	**n.q.**	**9.6** *

n.d.: not determined; n.q.: not quantified; * Sum of pure compounds above; ** Obtained from reference

References: 1. (Gerhäuser et al., 2002); 2. (Stevens et al., 1999); 3. (Barbosa-Pereira et al., 2013); 4. (Alonso García et al., 2004); 5. (Bartolome et al., 2000); 6. (Cortacero-Ramírez et al., 2004); 7. (Hayes et al., 1987); 8. (Ian McMurrough et al., 1996); 9. (Pavel Jandera et al., 2005); 10. (I. McMurrough et al., 1994); 11. (Arts et al., 2000); (Rong et al., 2000); 13. (Achilli et al., 1993); 14. (Floridi et al., 2003); 15. (Mcmurrough et al., 1984); 16. (Coghe et al., 2004); 17. (Vanbeneden et al., 2006); 18. (Techakriengkrai et al., 2004); 19. (Česlová et al., 2009); 20. (Vanhoenacker et al., 2004); 21. (De Keukeleire, 2000); 22. (Chiva-Blanch et al., 2011)

are flavanols, including (+) catechin and (-) epicatechin, followed by hydroxycinnamic acids, specially ferulic acid. Flavonols are also abundant in PBWS, which are rich in

kaempferol and quercetin. The fourth most abundant phenolic class in PBWS are hydroxybenzoic acids such as protocatechuic acid. Stilbenes and flavanones are the least abundant polyphenol classes in PBWS (Barbosa-Pereira et al., 2013).

Many health benefits have been attributed to the polyphenols found in PBWS. Ferulic acid, kaempferol and protocatechuic acid stand out for their bioactivities related to inflammation, cancer, cardiovascular diseases diabetes and, most of all, for their high bioavailability in the human body (Williamson et al., 2018).

1.2.1. Ferulic acid

Ferulic acid (FA) is a hydroxycinnamic acid widely found on wheat at 72.21 mg/100 g fresh weight (FW), dark chocolate at 24 mg/100 g FW and dried dates at 18.36 mg/100 g FW (Al-Farsi et al., 2005; Counet et al., 2006; Lempereur, 1997). This compound stands out by its protection against neurodegenerative diseases and antiaging effects. FA also exhibits protective effects against diabetes, hypertension and some types of cancer. Several neurodegenerative *in vivo* studies with rats have suggested that long-term administration of FA reduces neurotoxicity induced by β-Amyloid peptide, proposing this compound as a protective agent against Alzheimer's disease (Joshi et al., 2006; H.-S. Kim et al., 2004; Sultana et al., 2005; Yan et al., 2001). Recently, Sgarbossa and coworkers reviewed the potential of FA as a therapeutic drug for neurodegenerative diseases and encouraged carrying out further studies on this topic (Sgarbossa et al., 2015).

The antiaging effects of FA has been observed in vitro excised human skin and in vivo healthy human volunteers. The results show that FA is able to reach deep cutaneous layers and efficiently protect phosphatidylcholine liposomes from UV C-induced peroxidation (Saija et al., 1999). Moreover, FA was able to reduce UV B-induced skin erythema (Saija et al., 2000). Incorporation of this compound in a topical solution improved chemical stability of L-ascorbic and α-tocopherol (vitamins C and E) and doubled photoprotection to solar-stimulated irradiation of skin, suggesting that topical application of FA can successfully protect against photoaging and skin cancer (Lin et al., 2005).

The protective effects of FA against diabetes, hypertension and cancer have been observed in mice models. Evidence of the antidiabetic effects of FA were observed through reduced blood glucose, plasma triglycerides, free fatty acids, cholesterol, phospholipids and lipid peroxidation. Further evidence shows that treatment with FA in diabetic rats raised the levels of reduced glutathione and the activity of some antioxidant enzymes as well as reduced kidney function loss (nephropathy) (Balasubashini et al., 2003, 2004; Choi et al., 2011). Protection of FA against hypertension include attenuation of blood pressure rise and mitigation of nicotine-induced toxicity. These results were associated to nitric oxide-mediated vasodilatation and modulation of antioxidant status (Sudheer et al., 2005; Suzuki et al., 2002; Toda et al., 1991). Studies related to FA chemoprevention have reported cytotoxic and antiapoptotic effects in cervical, prostate, colon and breast cancer cell lines (Janicke et al., 2011; Kampa et al., 2004; Srinivasan et al., 2007; Zhou et al., 2016).

1.2.2. Kaempferol

Kaempferol (KAE) is a polyphenol that belongs to the subgroup of flavonols. This phenolic compound is widely distributed in plants and vegetables. Particularly, high concentrations have been detected in spices, including capers (104.29 mg/100 g FW), cumin (38.6 mg/100 g FW), cloves (23.8 mg/100 g FW) and caraway (16.4 mg/100 g FW) (Inocencio et al., 2000; Shan et al., 2005). Extensive literature indicates that KAE exhibits anti-inflammatory and anticancer activities, as well as protection against cardiovascular diseases (CVD)(Beg et al., 1996; García-Mediavilla et al., 2007; Lee et al., 2004).

It has been observed that the anti-inflammatory effects of KAE is mainly related to the regulation of the transcription factor NF-κB. This factor, when activated induces the transcription of other proteins related to inflammatory conditions, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Activation of NF-κB can result from the presence of other inflammation proteins, including interleukin 1β, protein kinase C and tumor necrosis factor (TNF-α) (Beg et al., 1996; García-Mediavilla et al., 2007; Lee et al., 2004). Many *in vitro* and *in vivo* studies have shown that KAE blocks NF-κB

activities along with iNOS and COX-2 levels (Comalada et al., 2005; García-Mediavilla et al., 2007; Kim et al., 2010).

It is widely known that tumor development requires that tumor cells acquire apoptosis resistance, increased angiogenesis (generation of new blood vessels to cover the increasing nutrients and oxygen demand) and expansion to other parts of the body (metastasis) (Calderón-Montaño et al., 2011). KAE have exhibited protection against these three stages *in vitro*. Firstly, KAE induced apoptosis has been observed in glioblastoma, lung cancer, pancreatic, human osteosarcoma, colon cancer and breast cancer cell. Proposed mechanisms of action include increased oxidative damage and alteration of signaling pathways associated to the cell cycle (Huang et al., 2010; Li et al., 2009; Nguyen et al., 2004; Sharma et al., 2007; Yoshida et al., 2008; Zhang et al., 2008). Secondly, inhibition of angiogenesis by KAE has been observed in human umbilical vein endothelial cells (HUVECs) and ovarian cancer cells. Studies *in vitro* reveal that the antiangiogenic affects are related to the reduction of the vascular endothelial growth factor (VEGF) expression through various signaling pathways (VEGF is the main positive regulator of angiogenesis) (Ahn et al., 2009; Luo et al., 2009, 2012). Finally, metastasis is reduced by KAE through the blockage of metalloproteinases (MMPs), since the presence of these proteins is positively correlated to malignant tumors. MMPs activities reduction by KAE has been observed in human glioma, osteosarcoma, oral cancer and breast cancer cells (Chen et al., 2013; Lin et al., 2010; Michaud-Levesque et al., 2012; Phromnoi et al., 2009; Shen et al., 2006).

There is extensive literature on the protective effects of KAE against CVD. These effects include three main activities: modulation of endothelial dysfunction, avoidance of hypertension and reduction of platelet aggregation (Perez-Vizcaino et al., 2010). First, the regulation of endothelial dysfunction is associated to the increment of nitric oxide production through the improvement of endothelium-dependent vasorelaxation, reduction of asymmetric dimethylarginine plasma concentrations and increased expression of aortic endothelial nitric oxide synthase (eNOS). This property is also associated to the delay of

low density lipoproteins (Hirano et al., 2001; Hou et al., 2004; Xiao et al., 2009). Second, KAE antihypertensive effects have been attributed to the inhibition of angiotensin-converting enzyme (ACE), a protein that increases blood pressure (Balasuriya et al., 2011; Loizzo et al., 2007; Oh et al., 2004). Also, it has been observed that KAE reduces oxidative stress in the vessel wall of vascular smooth muscle cells through inhibition of NADPH oxidase activity (Jimenez et al., 2015). Lastly, KAE has inhibited collagen-induced aggregation. Studies suggest that this phenolic inhibits multiple components of the glycoprotein signaling pathway, as well as kinases from the MAPK/ERK, JNK and Akt/PKB pathway (Choi et al., 2015; Wang et al., 2015).

1.2.3. Protocatechuic acid

Protocatechuic acid (PCA) belongs to one of the most widely distributed phenolic classes, the hydroxybenzoic acids. The greatest sources of PCA are star anise (32.2 mg/100 g FW), red and green chicory (21.79 and 16.78 mg/100 g FW, respectively), and black olives (7 mg/100 g FW) (Boskou, 2010; Rossetto et al., 2005; Shan et al., 2005). This compound is mostly known for its chemopreventive effects, yet antibacterial properties and protection against oxidative damage have also been observed for PCA (Kakkar et al., 2014). Moreover, it was found that the catabolism of several flavonoids by the gut microbiota leads to the formation of this compound (Williamson et al., 2018).

The chemopreventive activities of PCA has been observed in various rat models with chemically induced carcinogenesis. The results show that PCA administration effectively inhibited the development of different cancer types, including oral cavity, colon, pancreas liver, urinary, bladder and skin cancer (Tanaka et al., 2011). Diverse mechanisms of actions have been proposed for PCA anticancer effects, including the up-regulation of several apoptosis inductors and the prevention of DNA mutation caused by oxidative damage (Lin et al., 2007; Tanaka et al., 2011; Tseng et al., 2000). Moreover, it has been suggested that PCA modulates enzymes involved in phase I and phase II carcinogenesis, in other words, PCA can modulate the activation and detoxification of chemical

carcinogens (Baer-Dubowska et al., 1998; Szaefer et al., 2003; Tanaka et al., 2011). These findings suggest that PCA as a potent candidate for chemoprevention (Kakkar et al., 2014).

The antibacterial effects of PCA have been observed for several food spoilage bacteria. These bacteria include Salmonella typhimurium DT104, Escherichia coli O157:H7, Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Klebsiella pneumoniae and some molds (Aziz et al., 1998; Dhingra et al., 2017; Fernández et al., 1996). So, it has been proposed that PCA might be an effective additive to prevent food contamination (Chao et al., 2009). PCA has been further related to beneficial effects related to its antioxidant activity, providing protection against neurotoxicity and hepatotoxicity (Liu et al., 2002; Scazzocchio et al., 2011; Shi et al., 2006). Studies in diabetic mice have shown that PCA exhibits triglyceride-lowering, anticoagulatory, antioxidative and anti-inflammatory protection (Lin et al., 2009).

1.3.Separation of phenolic compounds

1.3.1. Liquid chromatography

Liquid column chromatography is the main process used to separate and purify polyphenols (Valls et al., 2009). According to IUPAC, chromatography refers to “a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction” (Ettre, 1993). Particularly, in liquid chromatography the mobile phase comprises one or more liquid solvents. The compounds being separated are subjected to several sorption–desorption processes throughout the column. Isolation of the components is based on differentiated sorption onto the stationary phase. This difference causes the compounds to move through the column at different speeds, which eventually leads to their separation (Sharma, 2000). Differentiated migration speeds can be obtained by diverse mechanisms, such as surface interactions (London forces, electrostatic forces, hydrogen bond formation), size exclusion (smaller

molecules are further delayed as they penetrate deeper inside the pores of the stationary phase), adsorbate–adsorbent ion exchange, or a combination of these. The diverse mechanisms involved in this process allows the application of chromatography to solve many problems, including fractionation of highly complex mixtures (Sharma, 2000).

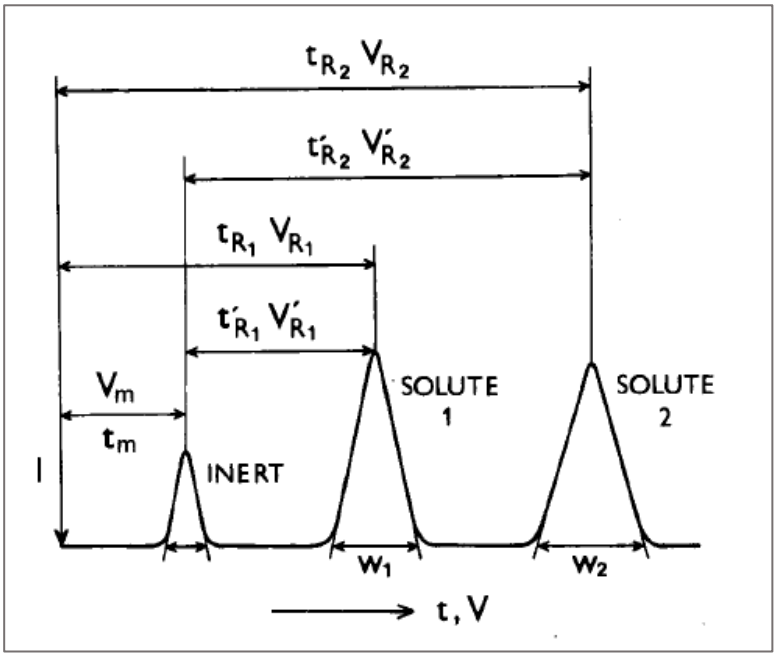


Fig. 2. Representation of a chromatogram of liquid chromatography (absorbance intensity vs time or volume of eluate from the column) (Jandera et al., 1985)

Chromatography is characterized by various parameters. Some of them can be measured directly from the chromatogram, in which the elution peaks are treated as a Gaussian curve (**Fig. 2**). The retention of the solutes can be represented by the retention time (t_R) or by the retention volume (V_R), defined as the time (or volume of eluate) from the sample injection on the column to the peak of the compound in the elution curve. These terms are usually corrected (t_R', V_R') by the column dead time (t_m) or dead volume (V_m), which corresponds to the elution of an inert compound that is not retained under the operating conditions. The expressions that correlates these terms are the following:

$$V_R = t_R F_m \qquad (eq.1)$$

$$V'_R = t'_R F_m \qquad (eq.2)$$

$$V'_R = V_R - V_m \qquad (eq.3)$$

$$t'_R = t_R - t_m \qquad (eq.4)$$

Where F_m is the volume flow-rate of the mobile phase (Jandera et al., 1985). A more fundamental parameter that represents the degree of solute retention is the capacity factor (k') that, contrarily to the time or volume retention, it does not depend on flow-rate or the column dimensions (Ahuja et al., 2005). The capacity factor is defined as:

$$k' = \frac{V'_R}{V_m} = \frac{t'_R}{t_m} \qquad (eq.5)$$

The capacity factor is proportional to the partition coefficient (K_D) and to the free energy of adsorption (P Jandera et al., 1985; Kloubek, 1992). The partition coefficient is the equilibrium constant that governs the distribution between the solute in the stationary and the mobile phases (Jandera et al., 1985). As K_D and the free energy of adsorption are related to equilibrium, both can be calculated from or associated to adsorption isotherms (which are discussed below) (Harrison et al., 2003).

Since chromatography is employed to separate compounds, one of the most important term is the resolution (R_s), used to quantify the separation between two solutes, 1 and 2. The resolution is calculated as the distance of the two elution peaks divided by the average bandwidths (ω) of the solutes:

$$R_S = \frac{2\left(V'_{R_2} - V'_{R1}\right)}{\omega_1 + \omega_2} \qquad (eq.6)$$

Thus, the resolution is increased by increasing the difference in the retention volumes and by reducing the bandwidths of the elution peaks. This term can be rearranged as an expression of the capacity factor and the theorical number of plates (n), as explained in Jandera & Chucácek (1985) (Jandera et al., 1985):

$$R_S = \frac{\sqrt{n_2}}{4} \cdot \frac{k_2' - k_1'}{1 + k_2'} \qquad (eq.7)$$

Suggesting that significantly different retention capacities leads to increased resolution. In eq. 6 and eq. 7 it is considered that the second compound (2) elutes after the first compound (1).

1.3.2. Liquid chromatography for polyphenol separation

The vast majority of the literature on separation and purification of polyphenols is focused on analytical methods to characterize the phenolic profile of natural products (Tsao et al., 2004) and research related to large scale purification is limited. Still, different chromatographic techniques have been developed to achieve high purity and yields for different phenolic groups such as catechins, isoflavones and flavanols. These techniques include low pressure chromatography, preparative reversed-phase liquid chromatography (RPLC), counter-current chromatography (CCC) and centrifugal partition chromatography (CPC). Nevertheless, these techniques have been described as tedious, time and solvent consuming, require multiple separation steps, columns deteriorate rapidly, and some of them can be difficult to scale-up (Gu et al., 2006a; Valls et al., 2009).

Agarose is a hydrophilic polymer derived from red seaweed that when reacted with epichlorohydrin under alkaline conditions, yields a heavily cross-linked gel media. This gel media, also known as Superose®, contains various polar groups such as ether bonds, hemiacetals and hydroxyl groups (Gu et al., 2006b). It has been observed that, adsorption chromatography with highly cross-linked 12% agarose exhibits strong separation and purification capacities of low molecular weight aromatic substances (Tan et al., 2010). It has been reported that Superose® is highly selective for phenolic compounds and stands well harsh cleaning conditions (Xu et al., 2007). Moreover, one-step purification of some phenolic acids and (-) epigallocatechin gallate have been achieved through this method with stepwise isocratic elution (variation of the solvent composition throughout the elution process) (Gu et al., 2008; D. Liu et al., 2011; Xu et al., 2007).

The mechanism of adsorption and separation of phenolics with cross-linked 12% agarose, has been shown to be of mixed-mode type, prevailing hydrophobic and hydrophilic interactions, the latter being the most dominant via hydrogen bond formation (Gu et al., 2006a; Tan et al., 2010). High yields and purity of different polyphenol extracts have been achieved through isocratic or step-wise isocratic elution with aqueous solvent mixtures containing ethanol and acetic acid (Gu et al., 2006a, 2008; Xu et al., 2007). Hence, this method can be applied to separate and purify the main polyphenols found in PBWS, leading to a one-step efficient process.

An efficient one-step separation with adsorption chromatography is usually accomplished through a trial and error approach, trusting the experience and intuition of the process designer (Nyiredy, 2004). However, this procedure can be time consuming, expensive and will probably not result in optimal performance. Alternatively, a model-based optimization leads to a definite optimum in an efficient manner (Engell et al., 2004) . For liquid adsorption chromatography, mathematical models generally include equations related to axial dispersion, interfacial mass transfer between mobile and stationary phases, intraparticle diffusion and adsorption equilibrium (Gu, 2015). A successful process optimization strongly relies on accurate parameter estimation, particularly the ones associated to adsorption equilibrium (Gu, 2015).

Adsorption equilibrium is studied through adsorption isotherms, which are the distribution of adsorbate molecules in the solid and liquid phases at equilibrium and constant temperature. Adsorption isotherms can provide information related to the adsorbate-adsorbent interaction, adsorption mechanism and the nature of the process (Giles et al., 1974; Inamuddin et al., 2017). Equilibrium parameters and further information is gathered when isotherm data points are fitted to an isotherm model. These experimental data can be obtained either through static-batch techniques or by column studies. In each case, the results should be the same since neither the adsorption equilibrium nor the thermodynamic behavior changes with the process configuration (Loebenstein, 1962). Batch experiments are usually more simple, inexpensive and less time-consuming than column procedures,

therefore, this technique is generally preferred to carry out adsorption studies (Loebenstein, 1962).

A wide variety of isotherm models are available to describe the adsorption process, some of them based on thermodynamic and kinetic principles (Foo et al., 2010). The most commonly used models are Langmuir and Freundlich, which describe different systems. Langmuir represents ideal systems characterized by a monolayer adsorption (the thickness of the adsorbed layer is one molecule), fixed and identical number of adsorption sites, no lateral interaction between adsorbed molecules and homogeneous adsorption (all sites have equal enthalpies or affinities for the adsorbate) (Foo et al., 2010). Contrarily, the Freundlich model represents more complex systems in which an infinite number of molecules can be adsorbed and where adsorption sites are heterogeneous (Allen et al., 2004). Generally, linearized forms of these models are used to fit model parameters, however, it has been shown that more appropriate results are obtained with a combination of non-linear regression and statistical analysis (Kumar et al., 2008).

Despite the promising results obtained for polyphenol separation using Superose® adsorption chromatography, to our knowledge, there is no research regarding adsorption equilibrium of polyphenols on cross-linked agarose. Moreover, the importance of these studies on designing solid-liquid separation processes reveal the need to be conducted. Hence, the aim of this study is to carry out a first characterization of the adsorption in highly cross-linked agarose of three bioactive polyphenols, representative of some of the most abundant phenolic classes of the PBWS (FA, PCA and KAE). Adsorption isotherms are used to evaluate the effect of temperature and the composition of water, ethanol and acetic acid on adsorption capacity. Moreover, accurate and significant equilibrium parameters are obtained for a posterior development of a chromatographic model. The adsorption process is further assessed through thermodynamic analysis. Finally, a preliminary proposal is presented of a sequence of solvent mixtures to achieve an adequate separation of the studied polyphenols through adsorption chromatography with stepwise elution.

Particularly, through this study we intend to answer the following questions: Do the polyphenols on cross-linked agarose adsorb ideally or is there a degree of surface heterogeneity? Which isotherm model and isotherm parameters best describes the adsorption equilibrium of this system? How does the concentration of water, ethanol and acetic acid in the solvent affects the adsorption process? Is the adsorption ruled by physisorption or chemisorption? Is there a solvent mixture of water, ethanol and acetic acid in which adsorption of the three phenolics is different enough to be separated using adsorption chromatography?

1.4. Hypothesis

Ferulic acid, kaempferol and protocatechuic acid (relevant polyphenols of the PBWS) adsorb differently on cross-linked 12% agarose depending on the composition of the mobile phase. Hence, a suitable strategy could be designed to separate them using gradient elution chromatography.

1.5. Objectives

1.5.1. Main objective

Assess the effect of mobile phase composition and temperature on the adsorption on cross-linked 12% agarose of some of the main polyphenols found in the brewery PVPP waste stream.

1.5.2. Specific objectives

To accomplish the main goal of this study, three specific objectives were defined:

(1) Assess the effect of different water, ethanol and acetic acid mixtures on the adsorption equilibrium of the polyphenols studied and decide the isotherm model that fits the experimental data best.

(2) Determine the thermodynamics of the adsorption of the defined polyphenols through the analysis of the effect of temperature on the adsorption equilibrium.

(3) Propose a chromatographic operating strategy, based on the adsorption behavior of the selected polyphenols, that would maximize their separation.

2. MATERIALS AND METHODS

2.1. Materials

The phenolic compounds, ferulic acid, protocatechuic acid and kaempferol were purchased from Xi'an Hao-xuan Biotech Co., Ltd. (Xi'an, China), their purity is shown in **Table 2-1**. Highly cross-linked agarose, Superose® 12 Prep Grade, was obtained from GE healthcare Bio-sciences (Uppsala, Sweden). In addition, acetic acid glacial and ethanol were used in this study and were of analytical grades supplied by Merck S.A. (Darmstadt, Germany) and Fisher Scientific (New York, USA), respectively. Finally, type II purified water (ASTM standard) purchased from Agualey Ltda. was used for experimental procedures.

2.2. Apparatus and experimental procedure

Experiments were performed for each polyphenol using different mixtures of water, ethanol, and acetic acid, listed in **Table 3**. As the solvent mixtures are expressed in terms of volume (v/v), to avoid errors caused by temperature changes, the mass corresponding to each solvent volume at 20°C was calculated. These calculated amounts were added to the mixture using a balance. Adsorption isotherms were obtained using the batch method in the system shown in Cuevas *et al.* This system involves a Carrousel 12 Plus ™ Reaction Station (Radleys, Saffron Walden, UK) with 12 borosilicate stirred glass tubes of 20 mL each, allowing multiple simultaneous experiments. In addition, temperature is controlled with a thermoregulated bath (PolyScience cooling/heating circulator model 9506A, Illinois, USA) (Cuevas-Valenzuela et al., 2015). The solvent mixtures used for the experiments differ between the phenolics due to the difference in their solubility. The solubilities found in literature for each compound and the solvent mixtures used for the experiments are shown in **Table 3**.

To perform the adsorption experiments, 0.005 g dry weight of cross-linked 12% agarose was measured using an analytical balance with a resolution of 0.0001 g and added to the glass tubes. Once the Carrousel system reached a temperature of 20°C, 5 mL of the

phenolic solutions were poured into the corresponding flask. All glass tubes were carefully capped to avoid evaporation. The solution-adsorbent mixture was kept under agitation (500 rpm) at 20°C for 1 h. A similar procedure was carried out to assess the effect of temperature in polyphenols adsorption from a solvent mixture of water: ethanol: acetic acid (70:15:15 v/v). In this case, the experiments were performed at 10°C and 30°C, keeping the rest of the operating conditions invariable. Isotherm curves were set up using 6 different initial concentrations of each polyphenol; experiments were performed in triplicate.

Once the adsorption equilibrium was reached, the liquid phase and solid phase were quickly separated using a polytetrafluoroethylene (PTFE) filter with a pore size of 0.45 μm and a diameter of 13 mm (Bonna-Agela Technologies, Delaware, USA). The filtrates were properly diluted, so polyphenol concentrations could be measured using an ultraviolet-visible spectrophotometer (Reyleigh UV-1601, Beijing Beifen-Ruili Analytical Instrument Co. Ltd., Beijing, China). The absorbance of each polyphenol was measured in a 200 to 600 nm spectrum to establish the maximum wavelengths for spectrophotometric measurement (**Table 3**). Calibration curves ($R^2 < 0.99$) for each polyphenol in each solvent mixture were prepared to correlate absorbance and concentration. Increases in concentration during absorbance determination due to evaporation, were prevented by using cell caps. After the concentration of the phenolic solutions were calculated, C_e (mg/L), the equilibrium adsorption capacity, q_e (mg/g), was calculated using a mass balance,

$$q_e = \frac{(C_0 - C_e) \cdot V}{A} \qquad (eq. 8)$$

Where V is the volume of the phenolic solution, A is the weight of dry agarose (g) and C_0 is the concentration of the solution prior to the adsorption process (mg/L).

Table 3. Some characteristics of the polyphenols and specifications for adsorption experiments and absorbance reading

Compound	Purity	Water solubility at 25°C (mg/L)	Wavelength (nm)	Solvent mixtures (water: ethanol: acetic acid v/v)
Ferulic acid	> 98%	0.5251 – 0.5273 [a, b]	325	90:5:5 (W90) 70:15:15 (W70) 50:25:25 (W50) 30:35:35 (W30)
Protocatechuic acid	> 99%	18726 – 29400 [c, d]	260	100:0:0 (W100) 70:15:15 (W70) 50:25:25 (W50) 30:35:35 (W30)
Kaempferol	> 98%	1.25 – 1.34 [e, f]	265	70:15:15 (W70) 50:25:25 (W50) 30:35:35 (W30)

[a] (Haq et al., 2017); [b](Shakeel et al., 2017); [c](Noubigh et al., 2007); [d] (Srinivas et al., 2010);[e] (Telange et al., 2016); [f] (Zhang et al., 2015)

2.3. Analysis

2.3.1. Adsorption isotherm models

The adsorption isotherms describe the relationship between the amount of polyphenol adsorbed in agarose (q_e, mg/g) and the polyphenol diluted in the solvent (C_e, mg/mL) at equilibrium. The most commonly used isotherm models, Langmuir and Freundlich, were applied in our study to find which one represents better the adsorption process. The Langmuir model assumes monolayer adsorption and fixed number of adsorption sites. It also assumes that all adsorption sites are equal and there is no interaction between adsorbed molecules (Foo et al., 2010). This model can be described as:

$$q = \frac{q_{max} K_L C}{1 + K_L C} \qquad (eq. 9)$$

Where q_{max} is the maximum adsorption capacity (mg/g) and K_L is the adsorption equilibrium constant which is related to the free energy of adsorption. This expression is often linearized to find the model constants, one of its linearized forms correlates 1/q vs 1/C as follows:

$$\frac{1}{q} = \frac{1}{C} + \frac{1}{K_L q_{max} C} \qquad (eq. 10)$$

Contrarily, the Freundlich model is often used to represent non-ideal adsorption. It can be applied on processes with multilayer formation, as well as adsorption with heterogenous surfaces and irregular heat adsorption distributions (Adamson et al., 1997). The equation that describes the Freundlich isotherm is shown below:

$$q = K_F C^{1/n} \qquad (eq. 11)$$

Where, K_F and n are the Freundlich parameters associated to adsorption capacity and adsorption intensity (Qiu et al., 2007). Additionally, 1/n has been related to the degree of surface heterogeneity, being more heterogeneous when its value is closer to zero (Foo et al., 2010). The linearized expression for the Freundlich model can be written as:

$$\log(q_e) = \log(K_F) + \frac{1}{n}\log(C_e) \qquad (eq. 12)$$

2.3.2. Model fitting

Model parameters were estimated through linear and non-linear regression. Linear regression was performed by minimization of the sum of the square errors between the linearized equations (eq. 10 and eq. 12 for the Langmuir and Freundlich model, respectively) and the logarithm of the experimental adsorption capacity, $\log(q_e)$. The coefficient of determination was calculated as shown in eq. 15. For non-linear regression,

model parameters were estimated by minimizing the error between the experimental measurement of the adsorbed polyphenol (q_{exp}) and the adsorbed polyphenol calculated by the respective model (q_{calc}). Optimization was performed using the Microsoft Excel solver add-in. Two objective functions for model fitting were tested:

Sum of square errors (SSE)

It is the most frequently used objective function and it can be described by the equation:

$$\sum_{i=1}^{N} \left(q_{calc} - q_{exp}\right)_i^2 \qquad (eq.13)$$

Where N represents the number of data points. Normally, parameters obtained using the SSE objective function tend to provide a better fit for higher adsorbed solute concentrations where the magnitude of the square errors are higher (Mane et al., 2007).

Average relative error (ARE)

This objective function is given by:

$$\frac{100}{N} \sum_{i=1}^{N} \left| \frac{q_{calc} - q_{exp}}{q_{exp}} \right|_i \qquad (eq.14)$$

Since this function is normalized by experimental data, the relative error is more distributed, providing a better (relative) fitting in the entire concentration range (Foo et al., 2010).

First, the fitting of the models obtained was compared using the adjusted coefficient of determination (R^2). This value represents the ratio between the explained variance and the total variance (Kumar et al., 2008), adjusted by the number of parameters (p) and number of experimental data (N) and it can be calculated as follows:

$$R^2 = 1 - \frac{RSS}{TSS} = 1 - \frac{\sum_{i=1}^{N}\left(q_{exp} - q_{calc}\right)^2}{\sum_{i=1}^{N}\left(q_{exp} - \overline{q_{exp}}\right)^2} \qquad (eq.15)$$

Where RSS is the residual sum of squares, TSS is the total sum of squares and $\overline{q_{exp}}$ is the mean of the experimental measurement of the adsorbed polyphenol.

2.3.3. Confidence interval

Many authors discourage the use of the coefficient of determination as the single criteria to select the best-fit model when nonlinear regression is being evaluated (Ayoob et al., 2008; Boulinguiez et al., 2008). Therefore, the significance of model parameters was examined to help discriminate between the Langmuir and Freundlich models. The 95% confidence intervals were obtained as described by Sánchez *et al*. and are presented in the following form (Sánchez et al., 2014):

$$IC(0.95) = \theta \pm 1.96 \cdot \sigma \qquad (eq.16)$$

A parameter is considered statistically significant when the following criteria is fulfilled:

$$t - value = \left|\frac{\theta}{\sigma}\right| \geq 2 \qquad (eq.17)$$

Where θ is the parameter and σ is the standard deviation of the estimated parameter.

In addition, given the confidence intervals obtained for the model parameters, the simultaneous prediction confidence intervals were also calculated. These prediction intervals refer to the confidence intervals of the simulated values of the dependent variable (equilibrium adsorption capacity, q_e). The function *nlparci* of Matlab 8.2.0.701 (R2013b) was used to obtain the simultaneous prediction intervals, in which a linear approximation is made in order to apply the Scheffe's S-method for linear regression, as described by Vecchia *et al*. (Vecchia et al., 1987).

2.3.4. Thermodynamic analysis

Further understanding of the adsorption process of the polyphenols in agarose can be obtained through the analysis of thermodynamic parameters. The enthalpy change (ΔH, kJ/mol), Gibbs free energy change (ΔG, kJ/mol) and the entropy change (ΔS, kJ/mol K) can provide information related to the energy changes occurred in the agarose after adsorption and the mechanisms involved in this process (Gao et al., 2013). Enthalpy change was obtained according to the linearized van´t Hoff equation:

$$\ln(C_e) = \frac{\Delta H}{RT} + \ln(K) \qquad (eq.18)$$

Where Ce is the polyphenol concentration in the liquid phase at equilibrium (mg/mL), T is the temperature (K), R is the ideal gas constant and ΔH is the isosteric adsorption enthalpy change. Enthalpy change was calculated through the plot of $\ln(C_e)$ vs 1/T, where C_e must be at the same adsorption capacity (isosteric concentration). The Gibbs energy change calculation depends on the model that represents best the polyphenol adsorption. If the Freundlich model has the best fit, the following expression is used:

$$\Delta G^0 = -nRT \qquad (eq.19)$$

Where n is the Freundlich index parameter. Contrarily, if Langmuir is the best-fit model, the Gibbs energy change is calculated using the equation:

$$\Delta G^0 = -\ln(K_L)RT \qquad (eq.20)$$

Where K_L is the adsorption equilibrium constant of the Langmuir model. In addition, the entropy change was calculated as follows:

$$\Delta S = \frac{\Delta H - \Delta G^0}{T} \qquad (eq.21)$$

Each parameter is presented with the corresponding uncertainty (Farrance et al., 2012). Details on the calculation of the expanded uncertainty is shown in **Appendix A**.

2.3.5. Column retention

In liquid chromatography, selection of a suitable mobile phase is essential to accomplish successful separations of complex mixtures. Application of gradient elution techniques allows further improvements in the separation process (Jandera et al., 1985). Ideally, the retention factors of the solutes should be different enough to move through the column at different speeds, allowing a high-resolution separation. However, unnecessarily large differences in retention might lead to long elution times, which is undesirable in commercial scale processes. Thus, in chromatography we seek for the maximum resolution in the shortest possible time.

In adsorption chromatography, the retention factor is proportional to the adsorption equilibrium constant, and when approximated to linear adsorption it can be expressed as (Horváth et al., 1983):

$$K = \frac{q_e}{C_e} \qquad (eq. 22)$$

Additionally, from the definition of the retention factor it is obtained that the retention factor and the equilibrium constant are directly proportional, leading to the following relationship (Horváth et al., 1983):

$$k' = \phi \cdot \frac{q_e}{C_e} \qquad (eq. 23)$$

Where ϕ is the ratio between the volumes of the stationary phase and mobile phase in the column. In this expression q_e and C_e must have the same units (mg/g), since the retention factor is dimensionless. Conversion of the units of C_e to mg/g required the density of the solvents, and since these solvents are a mixture of liquids, the density was obtained using

the UNIFAC model from Aspen Plus V10. The order of elution of FA, KAE and PCA was inferred based on the retention factor. However, assuming that in preparative chromatography the samples injected to the column are highly concentrated, due to solubility differences, the concentration of the compounds in the sample will differ considerably. Therefore, these values were normalized by a retention factor calculated at high concentrations for each compound (k'_{max}):

$$k'_{max} = \phi \cdot \frac{q_{e,max}}{C_{e,max}} \qquad (eq.24)$$

Where $C_{e,max}$ (mg/g) is the maximum C_e used for the equilibrium studies and $q_{e,max}$ (mg/g) is the adsorption capacity at $C_{e,max}$. Then, a relative retention factor was calculated (k_r'):

$$k'_r = \frac{k'}{k'_{max}} = \frac{q_e/q_{e,max}}{C_e/C_{e,max}} \qquad (eq.25)$$

that could be used to compare the compounds retention for the different solvents studied.

3. RESULTS AND DISCUSSION

3.1. Adsorption isotherms

The adsorption isotherms of the polyphenols on agarose were studied for different solvent mixtures of water, ethanol and acetic acid at 20°C, the results are presented in **Fig. 3 (A) – (C)** and experimental data is presented in **Appendix B**. For all three polyphenols, the adsorption on the solvents W70, W50 and W30 were studied. However, solubility problems arose for some solutes when higher water concentrations were applied. Therefore, solvents with higher water proportions were only studied on FA (W90) and PCA (W100). The effect of the solvent composition on the adsorption capacity is the same for the three phenolics. At high concentrations of water (W100 for PCA, W90 for FA and W70 for KAE) the highest adsorption capacities were observed. However, for lower

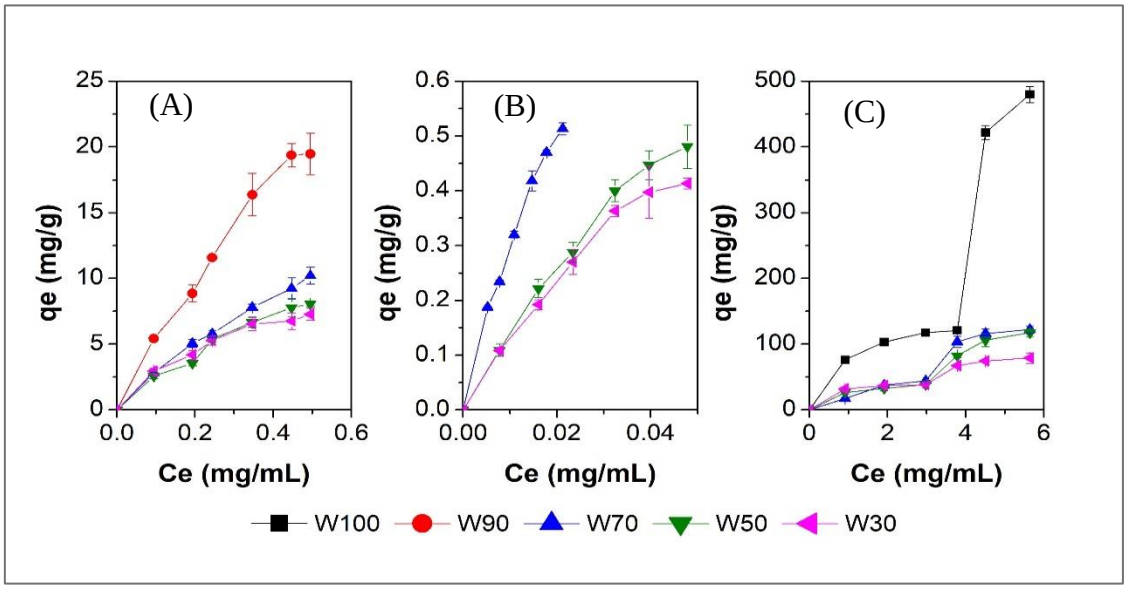


Fig. 3. Effect of the solvent on adsorption isotherms of (A) ferulic acid, (B) kaempferol, and (C) protocatechuic acid on cross-linked agarose. The solvents studied were for all phenolics were W70, W50 and W30. Solvents with higher water proportion were only studied for FA (W90) and PCA (W100) since they possess higher water solubilities.

concentrations of water, adsorption capacities were almost independent of water

composition (W70, W50 and W30 for phenolic acids; W50 and W30 for KE). Although the relative errors shown in **Fig. 3** are low (<10%), a few outliers were obtained. Those measures were eliminated and repeated. Discussion on the replicability and reproducibility of the procedure is presented in **Appendix F.**

The reduction of adsorption capacity with reduced water concentration was also observed by Silva *et al.* (2007), who suggest that this response is related to solvophobicity originated by the presence of a solute on a highly structured aqueous solution that tends to self-aggregate via hydrogen bond interactions (Ahuja, 1989; Silva et al., 2007). The authors indicate that increasing solvophobicity of a compound in a given solvent, increases the tendency of the solvent molecules to push the solute towards the adsorbent. The authors also mention that solvophobicity of polyphenols is higher in solvents with high dielectric constants (water, ε_{H2O} = 78.5) than in solvents with low dielectric constants (ethanol and acetic acid, ε_{etOH} = 24.3 and ε_{AA} = 6.15) (Silva et al., 2007). Gu and coworkers argue that high acetic acid concentrations reduce the available adsorption sites for polyphenols, decreasing their adsorption capacity. The authors also mention that interaction of acetate ions and delocalized electrons from the surface might also contribute to the reduction of the adsorption capacity (Gu et al., 2006b).

According to the isotherm classification system defined by Giles & Smith (1974), all curves follow the L-type shape, indicating that as more sites in the substrate are filled in, it becomes much more difficult for an adsorbate molecule to find a vacant site available (Giles et al., 1974). Also, the adsorbate is either horizontally adsorbed or there is no strong competition from the solvent (Giles et al., 1960). However, these isotherms differ on the subgroup of the classification; FA and KAE mainly exhibit L1 shapes, while PCA presents L4 shapes. In FA and KAE adsorption curves, completion of the surface coverage has not been reached and a clear plateau is not observed. Probably, the plateau could be reached for higher polyphenol concentrations,

but (i) higher concentrations cannot be obtained due to their low solubility, and (ii) they are not relevant for the current study since these high concentrations have not been found in PBWS. PCA adsorption curves are characterized by a second rise and a second plateau. This behavior can appear when a re-orientation of the adsorbed molecules happens (horizontal orientation at the first plateau and vertical orientation at the second), or when a second layer is formed on top of the first (Giles et al., 1960). In the case of PCA both situations could be occurring. Further analysis will only consider the first rise of PCA adsorption since the rest of the curve show concentrations not relevant for this study.

3.2. Model fitting

The parameters estimated for each isotherm with linear and nonlinear regression for the Langmuir and Freundlich models are presented in **Tables 4 – 6**; the parameters are presented with the corresponding error and coefficient of determination. In some cases, parameter estimation for the same model present rather different values for the different objective functions. For example, estimation of q_{max} for FA adsorption with W70 with linear regression resulted in a value 2.5 times higher than the one obtained with non-linear

Table 4. Langmuir and Freundlich parameters for the adsorption of FA on agarose at different solvent mixtures

Solvent mixture	Function	LANGMUIR				FREUNDLICH			
		K_L	q_{max}	Error	R^2	K_F	n	Error	R^2
W90	L-SSE	1.23	50.90	3.83	0.9853	36.30	1.23	2.89	0.9889
	NL-SSE	0.87	67.05	2.34	0.9910	35.79	1.25	0.15	0.9890
	NL-ARE	1.26	50.60	4.54	0.9868	35.71	1.25	4.16	0.9846
W70	L-SSE	0.90	64.87	6.48	0.9984	17.91	1.29	0.10	0.9984
	NL-SSE	1.28	25.92	0.10	0.9987	17.47	1.33	0.01	0.9988
	NL-ARE	1.21	27.08	0.08	0.9978	17.88	1.29	1.71	0.9984
W50	L-SSE	1.32	25.20	0.83	0.9661	13.89	1.37	0.78	0.9816
	NL-SSE	1.24	26.51	1.58	0.9815	14.01	1.36	0.10	0.9817
	NL-ARE	2.14	14.88	1.43	0.9748	13.61	1.42	5.61	0.9806
W30	L-SSE	1.27	21.32	0.78	0.9893	11.33	1.74	0.36	0.9887
	NL-SSE	1.97	16.45	6.16	0.9921	11.06	1.81	0.05	0.9892
	NL-ARE	1.39	19.93	11.61	0.9901	11.17	1.76	3.32	0.9886

regression using SSE (**Table 4**). In addition, estimation of q_{max} for PCA adsorption with W70 with linear regression was almost 3.2 times higher than the one obtained with nonlinear regression using SSE (**Table 6**). However, in most isotherms, results were very similar (if not the same), independent of the estimation method used.

Table 5. Langmuir and Freundlich parameters for the adsorption of KAE on agarose at different solvent mixtures

Solvent mixture	Function	LANGMUIR				FREUNDLICH			
		K_L	q_{max}	Error	R^2	K_F	n	Error	R^2
W70	L-SSE	34.53	1.19	1E-03	0.9935	9.68	1.32	8E-04	0.9946
	NL-SSE	27.60	1.41	6E-04	0.9960	9.25	1.34	8E-04	0.9947
	NL-ARE	27.42	1.39	2.77	0.9948	9.25	1.34	2.34	0.9938
W50	L-SSE	8.26	1.82	2E-03	0.9902	6.55	1.20	2E-03	0.9854
	NL-SSE	12.55	1.32	8E-04	0.9947	5.06	1.32	2E-03	0.9897
	NL-ARE	9.54	1.58	2.57	0.9918	7.33	1.15	3.50	0.9836
W30	L-SSE	15.75	1.00	2E-03	0.9860	4.54	1.32	3E-03	0.9713
	NL-SSE	18.45	0.91	2E-03	0.9870	3.48	1.46	3E-03	0.9768
	NL-ARE	17.10	0.94	3.15	0.9854	5.25	1.26	3.51	0.9596

To compare the results, and as a first approach to select the best-fit model, the coefficient of determination was used despite being discouraged (Miaou et al., 1996). The models studied only differ on the equation or the parameters values, but no difference on the number of the parameters exists so other comparison methods, such as Akaike Information Criterion (AIC) or BIC, are not suitable. Analysis of the R^2 shows that in almost all cases nonlinear regression with SSE results in a better parameter estimation, followed by ARE in Langmuir models and linear in Freundlich models. Despite attaining a relatively clear distinction between parameter estimation method with R^2, the distinction between Langmuir and Freundlich is not evident. In many cases, the Langmuir model tend to give a slightly higher R^2, but the difference does not seem high enough to select this model.

Analysis of the significance of the model parameters and the simultaneous prediction interval were performed to carry out a more appropriate selection of the best-fit model.

Table 6. Langmuir and Freundlich parameters for the adsorption of PCA on agarose at different solvent mixtures

Solvent mixture	Function	LANGMUIR				FREUNDLICH			
		K_L	q_{max}	Error	R^2	K_F	n	Error	R^2
W100	L-SSE	1.13	150.79	3.72	0.9995	80.40	3.02	37.24	0.9953
	NL-SSE	1.15	150.04	3.58	0.9996	81.39	3.16	35.13	0.9956
	NL-ARE	1.11	151.79	0.47	0.9995	78.78	3.03	1.95	0.9940
W70	L-SSE	0.05	409.99	0.55	0.9631	18.61	1.15	0.05	0.9693
	NL-SSE	0.19	129.08	0.33	0.9818	20.24	1.32	0.04	0.9754
	NL-ARE	0.10	188.70	2.43	0.9669	17.84	1.19	0.44	0.9606
W50	L-SSE	1.26	48.21	0.82	0.9954	26.94	3.00	0.00	0.9984
	NL-SSE	1.20	49.01	0.74	0.9953	26.94	3.00	0.00	0.9984
	NL-ARE	0.60	60.30	0.81	0.9939	26.95	3.00	0.01	0.9984
W30	L-SSE	2.96	43.26	1E-05	1.0000	32.35	5.68	0.27	0.9997
	NL-SSE	2.96	43.26	3E-07	1.0000	32.39	5.74	0.27	0.9997
	NL-ARE	2.96	43.26	3E-03	1.0000	32.24	5.80	0.47	0.9994

The 95% confidence intervals of the parameters estimated with nonlinear regression using SSE is presented in **Table 7**. Parameters estimated with the Freundlich model are significant for every isotherm in all compounds studied, while parameters obtained with

the Langmuir model are mostly significant except for the W70 isotherm of FA and PCA. Since significant parameters were achieved with the Freundlich model for these two phenolics, and not by Langmuir, it could suggest that Freundlich manages to represent better their adsorption on agarose. However, the parameters for KAE are significant for both models, but with slightly higher t-Values for Freundlich.

Further evaluation of the goodness of fit of both models was carried out through the evaluation of the simultaneous prediction intervals (Anderson et al., 2015), the results are presented in **Fig. 4 – Fig. 6**. From **Fig. 4** and **Fig. 5** it is observed that for ferulic acid and kaempferol confidence intervals of the model predictions are relatively narrow and practically the same for Langmuir and Freundlich. Although, protocatechuic acid isotherms (**Fig. 6**) show narrow prediction confidence intervals in W100 and W30 isotherms, Langmuir model predictions are more reliable. Model predictions with both

models are less reliable in W70 and W50 isotherms than all isotherms discussed above, since their confidence intervals are much wider.

Table 7. Significance of Langmuir and Freundlich model parameters

Compound, solvent mixture		LANGMUIR				FREUNDLICH			
		K_L (t-Value)		q_{max} (t-Value)		K_F (t-Value)		n (t-Value)	
FA	W90	0.87 ± 0.76	(2.3)	67.05 ± 43.80	(3.1)	35.79 ± 5.64	(12.7)	1.25 ± 0.24	(10.6)
	W70	1.16 ± 0.31	(7.4)	27.86 ± 5.27	(10.6)	17.47 ± 0.88	(39.8)	1.33 ± 0.08	(32.5)
	W50	**1.27 ± 1.27**	**(2.0)**	21.37 ± 14.58	(2.9)	14.02 ± 2.78	(10.1)	1.36 ± 0.34	(8.0)
	W30	3.08 ± 1.34	(4.6)	12.16 ± 2.63	(9.2)	11.06 ± 1.50	(14.8)	1.81 ± 0.38	(9.5)
KAE	W70	27.60 ± 12.65	(4.4)	1.41 ± 0.45	(6.2)	9.25 ± 3.80	(4.9)	1.34 ± 0.18	(15.2)
	W50	12.55 ± 7.02	(3.6)	1.32 ± 0.51	(5.2)	5.06 ± 2.40	(4.2)	1.32 ± 0.24	(10.8)
	W30	18.45 ± 12.89	(2.9)	0.91 ± 0.39	(4.7)	3.48 ± 2.26	(3.1)	1.46 ± 0.41	(7.1)
PCA	W100	1.15 ± 0.19	(11.9)	150.04 ± 7.18	(41.8)	81.39 ± 9.03	(18.0)	3.15 ± 1.08	(5.9)
	W70	**0.19 ± 0.59**	**(0.6)**	**129.08 ± 288.22**	**(0.9)**	20.25 ± 12.86	(3.1)	1.32 ± 1.21	(2.2)
	W50	1.20 ± 0.60	(4.0)	49.01 ± 7.64	(12.8)	26.94 ± 0.04	(1321.2)	3.00 ± 0.02	(362.6)
	W30	2.96 ± 0.01	(797.3)	43.26 ± 0.02	(4704.7)	32.39 ± 1.46	(44.4)	5.75 ± 1.88	(6.1)

* Non-significant parameters are marked with bold numbers

It is worth mentioning that subtle differences are obtained between the two models; hence, both can be used to represent the adsorption isotherm of these polyphenols in agarose. Previous studies have also shown good agreement between equilibrium data and the two models (Gao et al., 2013; Qiu et al., 2007; Ribeiro et al., 2002). In our study we opted for the Freundlich model (since more accurate parameters were obtained), therefore, further analysis in this study was carried out considering this model.

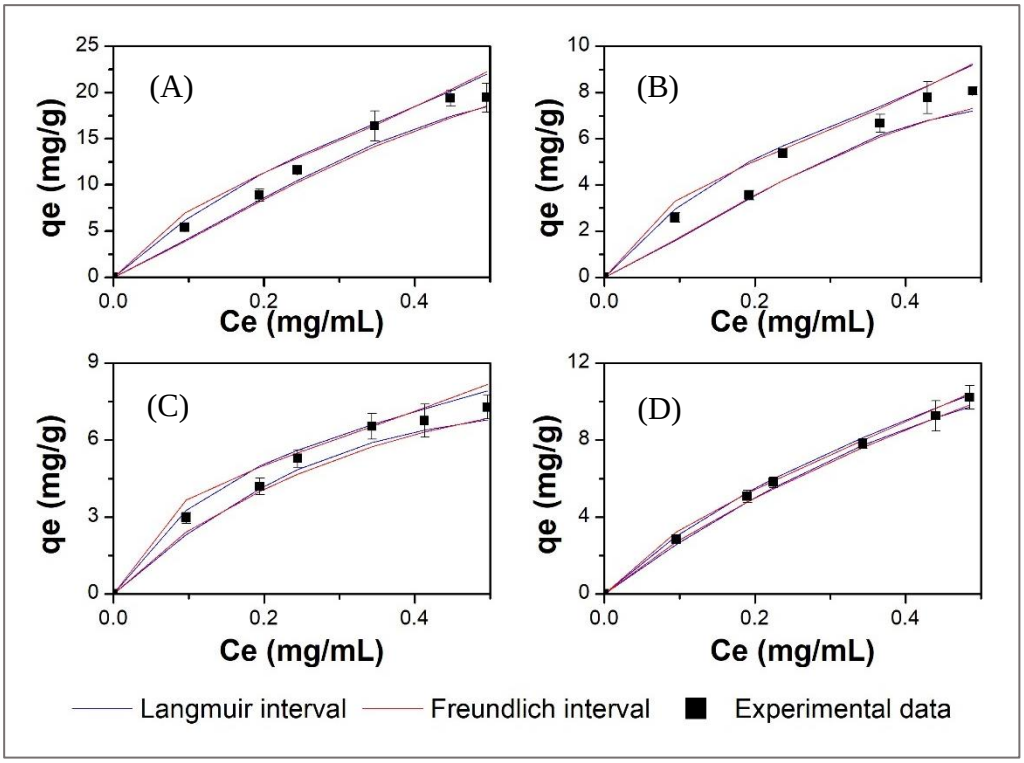


Fig. 4. Simultaneous confidence prediction intervals for the adsorption capacities of FA at different solvent mixtures: (A) W90, (B) W70, (C) W50, (D) W30

The value of the parameter n in the Freundlich model can indicate the type of isotherm: irreversible ($n = 0$), very favorable ($0.1 < n < 0.5$), moderately favorable ($0.5 < n < 1$) and unfavorable ($n > 1$) (Hamdaoui, 2006; Vasiliu et al., 2011). From **Table 3-4** it can be observed that FA and KAE isotherms are moderately favorable and PCA isotherms are mostly very favorable. In addition, the $n < 1$ values also indicate that the surface of agarose may exhibit some level of heterogenicity in all cases. The adsorption capacities (K_F) are considerably higher for PCA, followed by FA and KAE. The K_F values for these last two compounds decrease when the proportion of ethanol and acetic acid is increased, verifying that adsorption is higher when the liquid phase has greater water proportion, as previously discussed. For PCA this effect on K_F is not evident but, considering the confidence intervals, the adsorption capacity tends to decrease with higher co-solvent proportion.

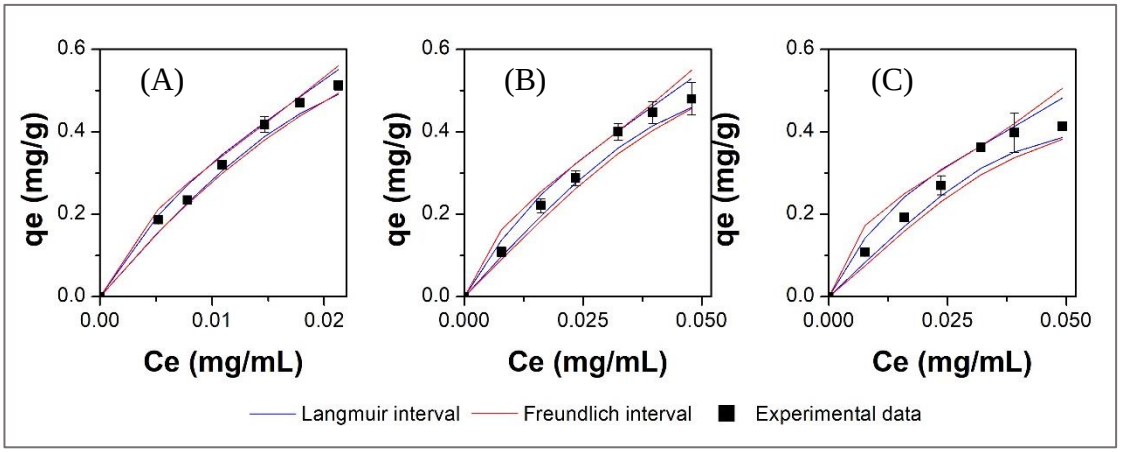


Fig. 5. Simultaneous confidence prediction intervals for the adsorption capacities of KAE at different solvent mixtures: (A) W90, (B) W70, (C) W50, (D) W30

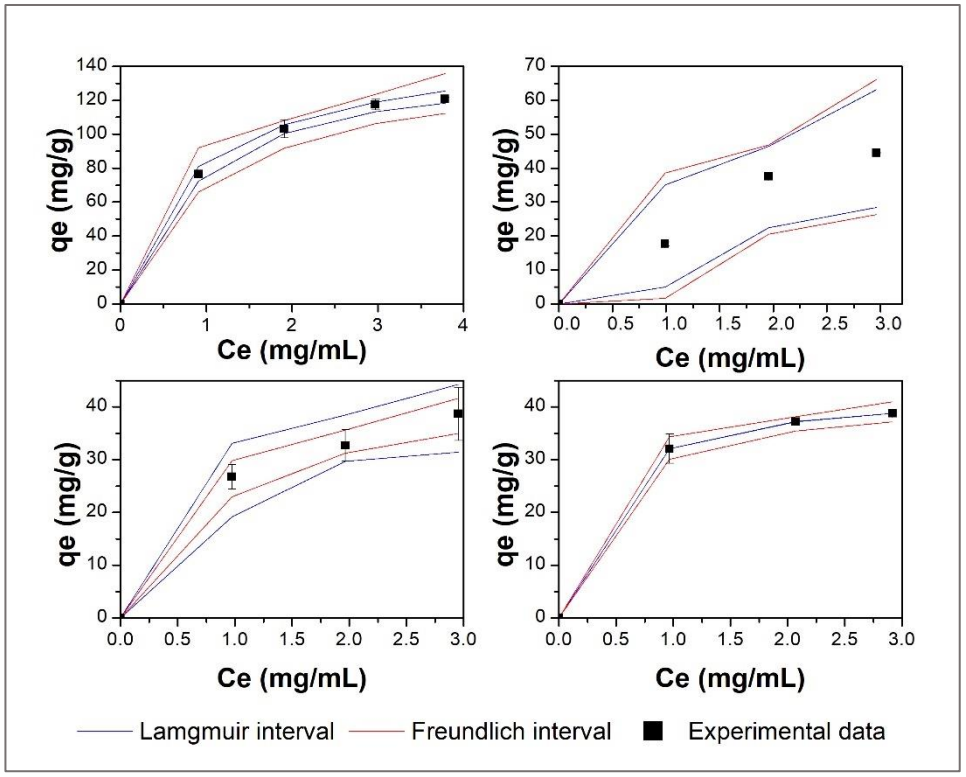


Fig. 6. Simultaneous confidence prediction intervals for the adsorption capacities of PCA at different solvent mixtures: (A) W100, (B) W70 (C) W50, (D) W30.

3.3. Thermodynamic analysis

Experiments on the adsorption of the phenolic compounds on agarose from aqueous solvent with 15% co-solvent was studied at three different temperatures: 10°C, 20°C and 30°C (**Fig. 7**). For the three polyphenols, it can be observed that adsorption decreases with increasing temperature, confirming that the adsorption process is exothermic, as expected for almost any adsorption process. The same L4 type curve, with two curve rises, is observed for protocatechuic at the three temperatures studied.

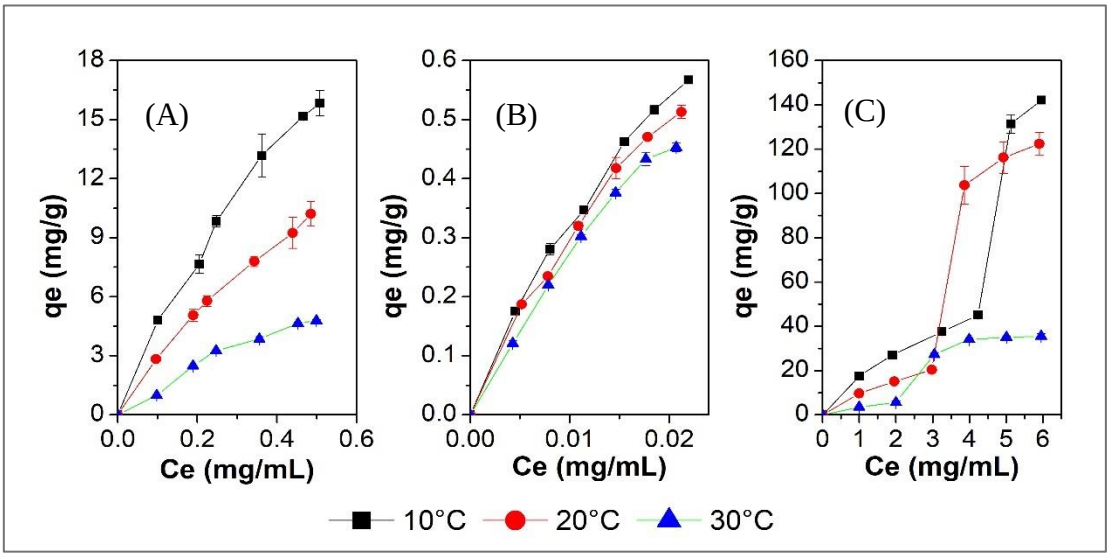


Fig. 7. Temperature effect on adsorption isotherms on cross-linked agarose with solvent W30, for: (A) FA, (B) KAE, (C) PCA. The temperatures evaluated are 10°C, 20°C and 30°C

The Gibbs energy (ΔG^0) of adsorption was calculated with eq. 19 for all phenolics. The values obtained for each temperature and with their respective expanded uncertainty is presented in **Table 8**. The enthalpy (ΔH) and entropy of adsorption (ΔS) are presented in **Table 9**, these values also appear with their respective expanded uncertainty. The negatives values of ΔG and ΔH suggest that, for all cases, the adsorption process is spontaneous and exothermic, respectively. These values also indicate if the process is ruled by chemisorption ($|\Delta H|$ and $|\Delta G^0| > 80$ kJ/mol) or physisorption ($|\Delta H|$ and $|\Delta G^0| <$

20 kJ/mol) (Liu et al., 2010; Quintero et al., 2013). From **Tables 8** and **9**, the only process that is undoubtedly ruled by physisorption is the adsorption of KAE. Contrarily, considering that the enthalpy of adsorption for FA and PCA are higher than the values established for physisorption, it can be established some degree of chemisorption for these compounds. However, Huang and coworkers also obtained high enthalpy values for the adsorption of tea polyphenols in three polymeric adsorbents (PMVBA, PMVBU and PMA). They suggested that, since hydrogen bonding energy is 5 – 50 kJ/mol, the high values could be related to multiple hydrogen interactions (Huang et al., 2007; Wendler et al., 2010). It is possible that in the adsorption of FA and PCA simultaneous hydrogen bonding with agarose is also involved. Indeed, studies on the interaction between cross-linked 12% agarose and phenolic compounds revealed that agarose provides a large number of hydrogen bond acceptor sites for the hydroxyl groups of polyphenols and proposed that hydrogen bonding are the dominating adsorption factor in this adsorbate-adsorbent system (Tan et al., 2010; Xu et al., 2006). Additionally, since hydrogen bonding energy strongly depends on the distance of the involved atoms (Wendler et al., 2010), it may also be possible that the hydrogen bonding formed for the phenolic acids are much closer to the surface than the ones formed by KAE. Hence, high enthalpy values could be related to simultaneous hydrogen interactions implicating that physisorption is the leading adsorption mechanism. The latter suggests that no structural changes occur on agarose and that no desorption limitations are involved (Gao et al., 2013; Quintero et al., 2013). However, the possibility of chemical adsorption for PCA or FA cannot be ruled out.

Table 8. Gibbs free energy of adsorption of FA, KAE and PCA on highly cross-linked agarose

	$\Delta G^0 \pm U(\Delta G^0)$ (kJ/mol) *		
	10°C	20°C	30°C
FA	-3.18 ± 0.88	-3.23 ± 0.40	-3.70 ± 1.1
KAE	-3.20 ± 0.71	-3.28 ± 0.86	-3.41 ± 1.6
PCA	-3.69 ± 0.63	-3.57 ± 0.68	-3.53 ± 2E-07

From **Tables 8** and **9**, it is also worth noticing that despite the large differences on enthalpy change, the free energy change is not significantly different. This result could be explained by the so-called enthalpy-entropy compensation, in which high enthalpy changes are compensated with high entropy changes, reducing the effect on the overall free energy change (Horváth et al., 1983). This effect has been observed in a wide range of fields, including adsorption, hydrogen bonding, liquid chromatography and various biochemical processes (Forrey et al., 2012; L. Liu et al., 2001; Sugihara et al., 2000). The positive correlation between ΔH and ΔS (or compensation effect) might exist since stronger intermolecular interaction (associated to enthalpy) results in larger decrease in configurational freedom or in a system with greater order (associated to higher entropy change) (Forrey et al., 2012). There has been controversy and misunderstanding about this phenomenon, however, a model that manages to explain this compensation, associates this effect to two interaction: solvent reorganization and molar shift (Grunwald et al., 1999; Liu et al., 2001). In adsorption, the mechanism of compensation is led by molar shift, in which reorganization of solutes molecules occur on distinguishable adsorption sites (L. Liu et al., 2001). Due to this phenomenon, the entropy change for KAE is lower than for the phenolic acids. From the latter it could be speculated that lower ΔS of KAE is related to the higher -OH moieties of KAE, in which more spatial configurations are available for the adsorption of this compound on the surface. Hence, the order of the system does not change as much as when only one or two spatial configurations are available (as in the case of the phenolic acids).

Additionally, from **Table 9** the absolute value of the PCA enthalpy decreases when adsorption capacity (or surface coverage) increases. This is the most common behavior for heterogeneous surfaces since the higher energy sites of the surface are the ones occupied first, resulting in higher enthalpy magnitudes. When the surface coverage increases, molecules are forced to occupy the lower energy sites, explaining the lower enthalpy magnitudes (Huang et al., 2007). For KAE, the absolute value of enthalpy increases with increasing adsorption capacity, this is generally explained by a strong influence in positive adsorbate-adsorbate interaction (Martínez et al., 2002). The latter

can also be observed from the Do's model when the parameter μ (representing the adsorbate-adsorbate interaction) is significantly higher than the contribution of the enthalpy change by surface heterogeneity (Do et al., 1997). Contrarily, for FA little influence of the surface coverage on enthalpy is observed, indicating that the adsorption sited for this phenolic are similar and homogeneous (Foo et al., 2010).

Table 9. Enthalpy and entropy change of adsorption of FA, KAE and PCA on highly cross-linked agarose

	Q (mg/g)	ΔH ± U(ΔH) (kJ/mol)	ΔS ± U(ΔS) (kJ/mol K)		
			10°C	20°C	30°C
FA	0.2	-43.62 ± 6.3	0.143 ± 0.045	0.138 ± 0.043	0.132 ± 0.042
	0.25	-44.56 ± 2.0	0.146 ± 0.015	0.141 ± 0.014	0.135 ± 0.015
	0.3	-45.33 ± 0.26	0.149 ± 0.0065	0.144 ± 0.0032	0.137 ± 0.0074
	0.35	-45.98 ± 0.063	0.151 ± 0.0062	0.146 ± 0.0028	0.139 ± 0.0072
	0.4	-46.54 ± 0.82	0.153 ± 0.0085	0.148 ± 0.0062	0.141 ± 0.0090
	0.45	-47.04 ± 2.2	0.155 ± 0.017	0.149 ± 0.015	0.143 ± 0.016
KAE	0.1	-5.803 ± 0.014	0.009 ± 0.0050	0.009 ± 0.0059	0.008 ± 0.010
	0.2	-6.38 ± 0.010	0.011 ± 0.0050	0.011 ± 0.0059	0.010 ± 0.010
	0.3	-7.053 ± 0.0044	0.014 ± 0.0050	0.013 ± 0.0059	0.012 ± 0.010
	0.4	-7.847 ± 0.0017	0.016 ± 0.0050	0.016 ± 0.0059	0.015 ± 0.010
	0.5	-8.798 ± 3.4E-04	0.020 ± 0.0050	0.019 ± 0.0059	0.018 ± 0.010
	0.6	-9.957 ± 9.4E-06	0.024 ± 0.0050	0.023 ± 0.0059	0.022 ± 0.010
PCA	0.05	-116.6 ± 2.7	0.399 ± 0.020	0.386 ± 0.019	0.373 ± 0.018
	0.1	-112.5 ± 3.5	0.384 ± 0.025	0.372 ± 0.024	0.359 ± 0.023
	0.2	-108.3 ± 4.4	0.370 ± 0.032	0.357 ± 0.031	0.346 ± 0.029
	0.3	-105.9 ± 5.1	0.361 ± 0.036	0.349 ± 0.035	0.338 ± 0.033
	0.4	-104.2 ± 5.5	0.355 ± 0.039	0.343 ± 0.038	0.332 ± 0.036
	0.5	-102.8 ± 5.9	0.350 ± 0.042	0.339 ± 0.041	0.328 ± 0.039

3.4. Column retention

The retention of FA, KAE and PCA in highly cross-linked agarose using mixtures of water, ethanol and acetic acid as mobile phase was inferred based on the equilibrium adsorption studies. To infer the retention, a relative retention factor was calculated for the

solvent mixtures studied at different phenolic concentrations, as indicated in the Materials and Methods section. The results are shown in **Fig. 8 (A) – (C)**, in this case, results for W100 or W90 are not considered since retention in these solvent mixtures cannot be compared between all three phenolics. For each compound, it is observed that retention decreases when polyphenol concentration rises. This reduction is considerably more pronounced for PCA than for FA and KAE. The retention of the three phenolics is reduced when water concentration is reduced from W70 to W50. However, when the water concentration is further reduced (from W50 to W30) the relative retention keeps decreasing for PCA but rises for KAE and FA. Additionally, it can be observed that for all solvent mixtures at high polyphenol concentrations ($C_e/C_{e,max} > 0.5$) KAE is the compound with higher retention, followed by FA and PCA. Although, for W50 the curves of KAE and FA are practically the same. At low concentrations ($Ce/C_{e,max} < 0.1$), PCA exhibits considerably higher relative retention values for all solvent mixtures.

The reduction of retention with increasing solute concentrations indicates that, in a chromatographic system, the elution may cause asymmetric peaks. It is commonly observed that when adsorption equilibrium has a concave downward form (such as Langmuir and Freundlich with $1/n < 1$), the resulting chromatographic peak has a sharp front and a tail at the end as shown in **Appendix C(a)** (Harrison et al., 2003). Since the retention of PCA is more variable with solute concentration, compared to FA and KAE, the tail of its peak will be longer. It is possible that when the retention curves of two phenolics intersect at some point, part of the tail of the fist eluted compound elutes at the same time as the second compound (**Appendix C(b)**). Consequently, to achieve a better separation it would be advisable to avoid solvents in which adsorption curves intersect, although this may not always be possible.

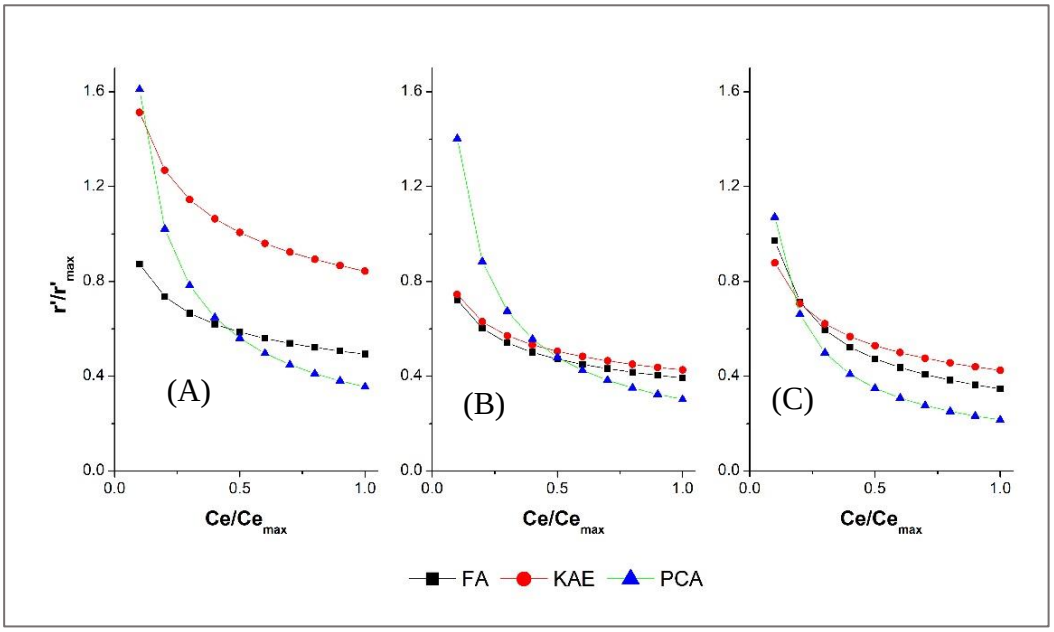


Fig. 8. Relative retention factor for FA, KAE and PCA in different solvents: (A) W70, (B) W50, (C) W30

Fig. 8 indicates that PCA would elute first under isocratic operation with W70 or W30, followed by FA and then KAE. However, with W50, after the elution of PCA, FA and KAE would elute simultaneously. Gu and coworkers studied the separation of different phenolic classes using adsorption chromatography with cross-linked agarose as adsorbent and water, ethanol and acetic acid mixtures as mobile phase. They concluded that the order of elution was associated to the number of hydroxyl groups, in which the compound with the most -OH moieties was the last to elute (Gu et al., 2006b). From **Fig. 9**, it is observed that only the elution of KAE agrees with this result, while, according to the number of -OH moieties, the elution of PCA and FA should be inverted. Nonetheless, the authors also observed that the elution pattern is also affected by intramolecular interaction between hydroxyl groups and adjacent functional groups, including other hydroxyl as well as carboxyl or methyl groups (Gu et al., 2006b). Both phenolic acids present intramolecular interaction with a carboxylic group. Additionally, PCA has a hydroxyl-hydroxyl interaction and FA presents hydroxyl-methyl interactions. Probably the intramolecular

interactions in FA are weaker than in PCA, thus leaving the hydroxyl groups of FA more available to interact with the adsorption surface.

Separation with W70 would result in almost complete isolation of KAE, although the separation of PCA and FA would probably be more difficult. In this case, the peak tail of PCA would elute at the same time as the front of FA. With W50 separation of any compound would be difficult since FA and KAE have almost the same retention and the retention curve of PCA intercepts with both. In this case, two eluted solutions would be obtained, one containing isolated PCA and another containing the three phenolics. Isocratic elution with W30 would probably produce a satisfactory separation of PCA but the separation between KAE and FA would probably be deficient.

Unless a very long column is employed, a suitable separation of these polyphenols via isocratic elution cannot be accomplished. Alternatively, the application of stepwise isocratic elution could achieve better results. According to the relative retentions calculated and the discussion above, it would be better start with W70 to delay the elution of KAE (to separate it from FA and PCA) and then continue with W30 to achieve a better resolution between FA and PCA. With this approach, like isocratic elution with W70, it is possible that the peak of FA might be eluted with part of the tail of PCA, although in a considerably lower degree.

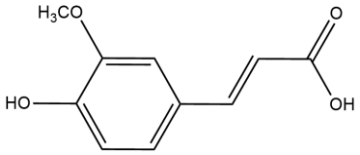
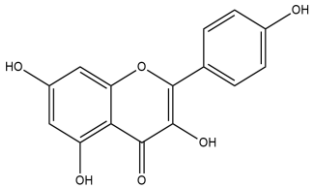
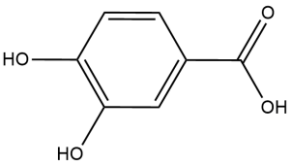
Ferulic acid	Kaempferol	Protocatechuic acid
		

Fig. 9. Chemical structure of FA, KAE and PCA

It is important to realize that this is a preliminary estimation based only on the equilibrium of the studied polyphenols, the adsorbent and the solvents. This estimation does not

consider a possible interaction between the polyphenols or competition for the adsorbent. Moreover, these studies were not conducted on a chromatographic column so kinetic or mass transfer phenomena were not assessed. Consequently, further studies require experimentation on a column to examine how these parameters affect the separation and if the proposed elution technique can separate the compounds with the expected resolution.

4. CONCLUSIONS

The present study provides the characterization of the adsorption behavior on highly cross-linked 12% agarose of three PBWS bioactive polyphenols, FA, KAE and PCA. Equilibrium studies showed that PCA had the highest affinity for the adsorbent, FA had an intermediate affinity, and KAE presented the lowest affinity for cross-linked agarose gel. Moreover, it was observed that both isotherm models, Freundlich and Langmuir, fitted well the experimental data, although, Freundlich showed more significant parameters. In all cases, it was observed a tendency to reduce adsorption capacity of the studied polyphenols with increased concentrations of ethanol and acetic acid on the mobile phase. FA and PCA showed high adsorption energies, probably due to simultaneous hydrogen bond interactions. It seems that the adsorption process presents the enthalpy-entropy compensation effect, since the adsorption enthalpies of the studied phenolics were significantly different and their free energy change were similar.

A relative retention factor was calculated that helped us infer the elution order of the studied phenolics. It was proposed that a separation between the three phenolics can be achieved with a stepwise isocratic elution, starting with 15% ethanol, 15% acetic acid, and followed by 35% ethanol, 35% acetic acid. With this system, protocatechuic acid would elute first, followed by ferulic acid, and then kaempferol. However, future studies on a chromatographic column are required to assess kinetic and mass transfer phenomena.

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A P P E N D I X E S

APPENDIX A: EXPANDED UNCERTAINTY CALCULATION

When a parameter, *y*, is calculated from another parameters, the uncertainties of the input variables derives uncertainties in the new parameter, *y* (Farrance et al., 2012). This is the case of the adsorption Gibbs energy change (ΔG^0) and the adsorption entropy change (ΔS). The relationship that allows the calculation of the uncertainty of the new parameter from the input parameters depend on the relationship between these parameters.

For the Gibbs energy change the general form of eq. 17 is:

$$\Delta G^0 = A \cdot B \cdot x_1 \qquad eq.A1$$

Where x_1 is 1/n parameter from the Freundlich model and A, B are constants. Then, from the rules of the evaluation of standard uncertainty through relationships with the input variables (Farrance et al., 2012), the uncertainty of ΔG^0 is:

$$u(\Delta G^0) = \sqrt{(A \cdot B)^2 \cdot u(x_1)^2} \qquad eq.A2$$

Similarly, the general form of eq. 19 is:

$$\Delta S = A \cdot x_1 + A \cdot x_2 \qquad eq.A3$$

Where x_1 is ΔG^0 and x_2 is $-\Delta H$, A is a constant. The uncertainty of ΔS is obtained as follows:

$$u(\Delta S) = \sqrt{A^2 \cdot u(x_1)^2 + A^2 \cdot u(x_2)^2} \qquad eq.A4$$

APPENDIX B: EXPERIMENTAL DATA FOR ADSORPTION CAPACITIES

Table 10. Experimental data of adsorption capacities of FA at different solvent mixtures at 20°C

W90			W70			W50			W30		
Ce	*qe*	SD	*Ce*	*qe*	SD	*Ce*	*qe*	SD	*Ce*	*qe*	SD
0	0	0	0	0	0	0	0	0	0	0	0
0.095	5.428	0.094	0.096	2.829	0.069	0.094	2.580	0.212	0.096	2.958	0.215
0.194	8.872	0.655	0.190	5.064	0.301	0.193	3.554	0.204	0.194	4.191	0.329
0.244	11.575	0.065	0.225	5.790	0.273	0.237	5.370	0.197	0.245	5.278	0.338
0.347	16.377	1.626	0.343	7.802	0.264	0.366	6.666	0.388	0.343	6.529	0.511
0.447	19.360	0.890	0.440	9.247	0.793	0.429	7.781	0.683	0.413	6.757	0.640
0.495	19.465	1.565	0.485	10.212	0.620	0.489	8.065	0.180	0.496	7.272	0.453

Table 11. Experimental data of adsorption capacities of KAE at different solvent mixtures at 20°C

W70			W50			W30		
Ce	*qe*	SD	*Ce*	*qe*	SD	*Ce*	*qe*	SD
0	0	0	0	0	0	0	0	0
0.0052	0.1874	0.0014	0.0078	0.1092	0.0115	0.0076	0.1085	0.0036
0.0078	0.2345	0.0027	0.0161	0.2214	0.0170	0.0159	0.1923	0.0089
0.0109	0.3200	0.0062	0.0234	0.2879	0.0182	0.0236	0.2702	0.0230
0.0147	0.4179	0.0182	0.0324	0.3998	0.0199	0.0320	0.3630	0.0097
0.0178	0.4703	0.0046	0.0396	0.4468	0.0260	0.0390	0.3974	0.0476
0.0212	0.5130	0.0115	0.0478	0.4805	0.0397	0.0492	0.4132	0.0097

Table 12. Experimental data of adsorption capacities of PCA at different solvent mixtures at 20°C

W100			W70			W50			W30		
Ce	*qe*	SD	*Ce*	*qe*	SD	*Ce*	*qe*	SD	*Ce*	*qe*	SD
0	0	0	0	0	0	0	0	0	0	0	0
0.91	76.37	1.28	0.991	17.66	0.581	0.974	26.71	2.294	0.968	32.06	2.756
1.91	103.22	5.31	1.957	37.58	0.683	1.966	32.70	3.022	2.070	37.18	0.363
2.97	117.50	3.11	2.963	44.50	0.111	2.953	38.65	5.051	2.920	38.77	0.002
3.79	120.79	0.90	3.866	103.6	8.515	4.047	82.55	3.051	4.274	67.60	0.299
4.50	421.88	10.47	4.923	116.1	7.124	4.894	106.1	9.252	5.036	74.49	0.181
5.64	479.74	12.15	5.899	122.4	5.326	6.071	117.5	6.043	5.970	79.24	7.978

Table 13. Experimental data of adsorption capacities of FA at 10°C and 30°C using W70 as solvent

10°C			30°C		
Ce	*qe*	SD	*Ce*	*qe*	SD
0	0	0	0	0	0
0.100382	4.827452	0.055474	0.097706	0.987852	0.006213
0.204628	7.660668	0.458754	0.189221	2.497482	0.027169
0.247734	9.832924	0.282792	0.247077	3.271094	0.027958
0.36196	13.1885	1.082666	0.355722	3.86258	0.115681
0.465258	15.15772	0.151139	0.452999	4.648291	0.045282
0.507695	15.84738	0.64011	0.49922	4.78624	0.050893

Table 14. Experimental data of adsorption capacities of KAE at 10°C and 30°C using W70 as solvent

10°C			30°C		
Ce	*qe*	SD	*Ce*	*qe*	SD
0	0	0	0	0	0
0.00454084	0.17589587	0.00459492	0.0042776	0.1211735	0.0047455
0.00802876	0.2806882	0.00920163	0.00785623	0.220	0.00100814
0.01141727	0.34676326	0.00186786	0.01114967	0.30249891	0.00282311
0.01548944	0.46279399	0.00390596	0.01462386	0.37614374	0.00615985
0.01849074	0.51666501	0.00705311	0.01765973	0.43337184	0.01143973
0.0219423	0.56727582	0.00406026	0.02072297	0.45318603	0.00687286

Table 15. Experimental data of adsorption capacities of PCA at 10°C and 30°C using W70 as solvent

10°C			30°C		
Ce	*qe*	SD	*Ce*	*qe*	SD
0	0	0	0	0	0
1.00007947	17.5532874	0.1068469	1.00007107	3.45861043	0.07693856
1.90201417	27.1087308	1.48065077	2.00029269	5.67603195	0.59527508
3.2476862	37.5707988	0.4916142	3.04100273	27.2547396	0.94375293
4.23068646	45.0124	0.97086025	3.99451858	34.0561917	0.81731401
5.12589163	131.523285	4.2114754	5.00374257	35.0747433	0.57766199
5.9582702	142.143705	0.38935104	5.95262063	35.4751997	1.19856742

APPENDIX C: ASYMMETRIC CHROMATOGRAPHIC PEAKS

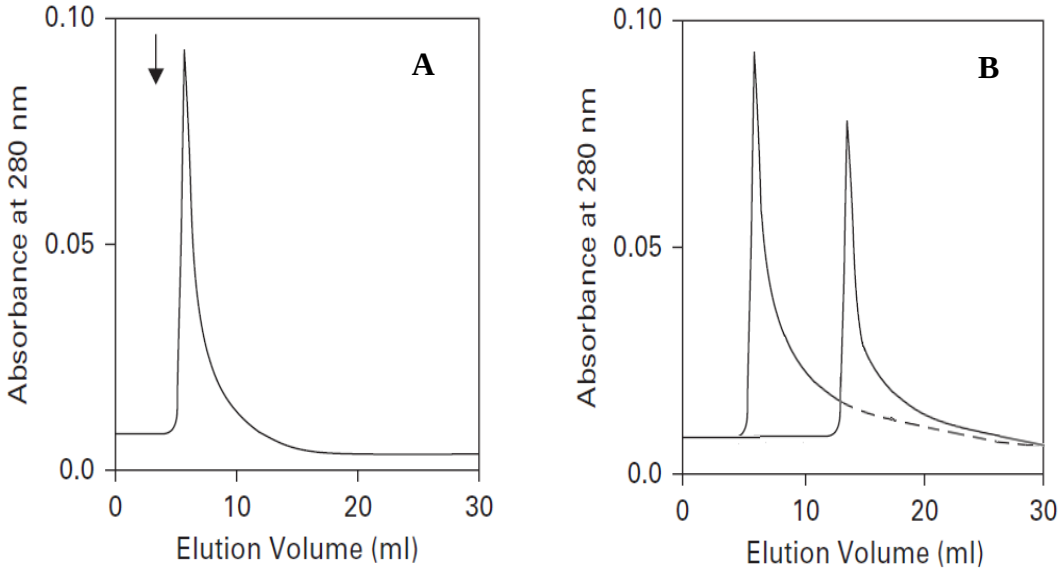

Fig. 10. Representation of the elution of (A) a solute with a concave downward shape adsorption isotherm (Harrison et al., 2003), (B) two solutes with a concave downward shape adsorption isotherm

APPENDIX D: RESULTS FOR THE RELATIVE RETENTION CALCULATION

Table 16. Relative retention for FA at different solvent mixtures

	Relative retention factor (r'/r'_{max})			
Ce/Ce_{max}	W90	W70	W50	W30
0.1	1.585	0.874	0.723	0.973
0.2	1.380	0.736	0.602	0.713
0.3	1.272	0.665	0.541	0.595
0.4	1.201	0.619	0.501	0.523
0.5	1.149	0.586	0.472	0.473
0.6	1.108	0.560	0.450	0.436
0.7	1.074	0.539	0.432	0.407
0.8	1.046	0.522	0.417	0.384
0.9	1.021	0.507	0.404	0.364
1	1.000	0.493	0.393	0.347

Table 17. Relative retention for KAE at different solvent mixtures

	Relative retention factor (r'/r'_{max})		
Ce/Ce_{max}	W70	W50	W30
0.1	1.513	0.746	0.878
0.2	1.269	0.630	0.706
0.3	1.145	0.571	0.621
0.4	1.065	0.533	0.568
0.5	1.006	0.505	0.529
0.6	0.961	0.483	0.499
0.7	0.924	0.465	0.476
0.8	0.893	0.450	0.456
0.9	0.867	0.438	0.440
1	0.844	0.427	0.425

Table 18. Relative retention for PCA at different solvent mixtures

	Relative retention factor (r'/r'_{max})			
Ce/Ce_{max}	W100	W70	W50	W30
0.1	4.562	1.610	1.403	1.071
0.2	2.843	1.022	0.884	0.662
0.3	2.155	0.783	0.674	0.499
0.4	1.771	0.648	0.557	0.408
0.5	1.521	0.560	0.480	0.350
0.6	1.343	0.497	0.425	0.308
0.7	1.209	0.449	0.383	0.277
0.8	1.104	0.411	0.351	0.252
0.9	1.018	0.381	0.324	0.232
1	0.948	0.355	0.302	0.216

APPENDIX E: NOMENCLATURE

C_e	Equilibrium concentration
$C_{e,max}$	Maximum equilibrium concentration of the isotherms for each compound
E_i	Number of theorical plates for compound i
F_m	Volume flow-rate of the mobile phase
K	Adsorption equilibrium constant
K_F	Freundlich adsorption capacity parameter
K_L	Langmuir adsorption equilibrium constant
k_i'	Retention capacity factor for compound i
k_{max}'	Retention capacity factor at high polyphenol concentrations
k_r'	Relative retention capacity factor
N	Number of data points
n	Freundlich adsorption intensity parameter
q_{calc}	Adsorption capacity calculated with isotherm model
q_e	Equilibrium adsorption capacity
$q_{e,max}$	Adsorption capacity at $C_{e,max}$
q_{exp}	Adsorption capacity obtained experimentally
q_{max}	Langmuir maximum adsorption capacity parameter
R_s	Chromatographic resolution
T	Temperature
t_m	Column dead time
t_R	Retention time
t_R'	Corrected retention time
V_m	Column dead volume
V_R	Retention volume
V'_R	Corrected retention volume
ΔG^0	Change of Gibbs energy
ΔH	Change of enthalpy
ΔS	Change of entropy
θ	Estimated parameter
σ	Standard deviation of estimated parameter
ϕ	Ratio between stationary phase and mobile phase volumes
ω_i	Bandwidth of the chromatographic peak for compound i

APPENDIX F: REPRODUCIBILITY OF THE ADSORPTION PROCESS

The procedure to obtain the adsorption isotherms is presented in Materials and Method and the results are presented in **Fig. 3-1** and **Fig. 3-5.** From these figures, it can be observed that experimental data show low relative errors (<10%). However, in few cases, measurements that highly escaped from the mean value were obtained (outliers). These outliers were deleted, and the procedure was repeated to obtain a new measurement. There are many experimental factors that affect the results and that could have been the source of these outliers (Roy, 1993). So, in order to get meaningful and reproducible data, special considerations have to be made with the following aspects:

(a) **Adsorbent preparation.** Since storage of cross-linked agarose has to be in a solution of ethanol and water, the treatment previous to its use consists on washing the agarose gel with distilled water and then dry the gel on a Büchner funnel connected to a side-arm flask subjected to vacuum. Probably, the humidity was not the same in all cases, changing the adsorptive behavior of the agarose gel.

(b) **Temperature.** Although the Carrousel equipment has a temperature control, it is possible that the controller was not precisive enough. Hence, changes in the environmental temperature affected the temperature of the samples and, consequently, adsorption equilibrium. Moreover, changes in temperature could also have occurred during agarose and solvent separation (after equilibrium was reached).

(c) **Phase separation.** Once adsorption equilibrium is reached, the solid phase (agarose gel with adsorbed polyphenols) has to be separated from the solvent-polyphenol solution. Depending on the material of the filter used for this procedure, considerable amounts of solute could be retained or reactions between the filter and the solvent could occur. In fact, first experiments were conducted using nylon filters and the polyphenols diluted in the solvent were higher than the initial amount of polyphenol (impossible). When a new filter material was applied (polytetrafluoroethylene), results improved.

APPENDIX G: SUBMITTED REASERCH ARTICLE

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Manuscript Draft

Manuscript Number:

Title: ADSORPTION OF THE MAIN POLYPHENOLS OF THE POLYVINYLPOLYPYRROLIDONE (PVPP) BEER WASTE STREAM IN CROSS-LINKED 12% AGAROSE GEL

Article Type: Full length article

Section/Category: C. Adsorption, Catalysis and Electrochemistry

Keywords: Isotherm adsorption, Brewery waste stream; Ferulic acid; Kaempferol; Protocatechuic acid

Corresponding Author: Dr. Nestor Escalona, Dr.

Corresponding Author's Institution: Pontificia Universidad Católica de Chile

First Author: Javiera Perez

Order of Authors: Javiera Perez; Pamela Rivera; Nestor Escalona, Dr.; Ricardo Perez

Abstract: The adsorption behavior of 12% cross-linked agarose gel was studied for three polyphenols normally found in brewery waste streams: ferulic acid (FA), kaempferol (KAE) and protocatechuic acid (PCA). Adsorption was evaluated in batch experiments at different temperatures in aqueous solutions containing ethanol and acetic acid. The order of affinity towards the adsorbent was PCA > FA > KAE. Results showed that adsorption capacity decreased with increased concentrations of ethanol and acetic acid and that the Freundlich model had a slightly better overall fit to experimental data, since more accurate parameters were obtained. Changes of enthalpy with surface coverage indicated a slightly heterogeneous adsorption surface for PCA and adsorbate-adsorbate interaction for KAE and FA. This work provides fundamental knowledge related to the adsorption mechanism and how it is affected by solvent composition. Thus, this information may contribute to optimize the separation of phenolics using Superose 12 as the stationary phase. Moreover, based on the adsorption isotherms, separation between these three phenolics using adsorption chromatography with cross-linked agarose as stationary phase is attainable using 15% ethanol, 15% acetic acid, followed by 35% ethanol, 35% acetic acid as eluents. However, further analysis should be conducted to assess kinetic and mass transfer phenomena.

Suggested Reviewers: Siew-Young Quek
University of Auckland
sy.quek@auckland.ac.nz

Jianhan Huang
Central South University
xiaomeijiangou@yahoo.com.cn

Nong-xue Qiu

Shaanxi Normal University
qiunx@163.com

Herminia Domínguez
Universidad de Vigo
herminia@uvigo.es

Agustín Marsal
Instituto de Química Avanzada de Cataluña
agusti.marsal@iqac.csic.es

1: Cover Letter

Néstor Escalona Burgos
Pontificia Universidad Católica de Chile
4860, Avda. Vicuña Mackenna
Macul, Santiago, Chile
(56 2) 2 354 4237
neescalona@ing.puc.cl

Martin Malmsten, Ph.D
Editor-in-Chief
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Dear Dr. Malmsten:

I am pleased to submit an original research article entitled "Adsorption of the Main Polyphenols of the PVPP Beer Waste Stream in Cross-Linked 12% Agarose Gel" for consideration for publication in the *Journal of Colloid and Interface Science*. We characterized the adsorption behavior of Superose 12 on three bioactive polyphenols found in a brewery waste stream (ferulic acid, kaempferol and protocatechuic acid). We present fundamental data that contribute to the understanding of the separation process of phenolics through this gel, as well as significant isotherm parameters that would allow a better process design using model-based optimization.

Given the large amounts of polyphenols disposed during the brewing process and the interesting pharmacological effects they possess, we felt compelled to study the adsorption equilibrium of model polyphenols from this waste stream on an adsorbent widely used for phenolics separation, Superose 12. We believe our work will appeal to process engineers searching for information that will help them separate phenolics efficiently. Moreover, our research will provide your readers with knowledge of fundamental principles related to cross-linked agarose gel adsorption of polyphenols, and how this process can be applied to generate nutraceuticals or food ingredients from brewery wastes.

This manuscript has not been published and is not under consideration for publication elsewhere. If you feel that the manuscript is appropriate for your journal, we suggest the following reviewers:

Siew-Young Quek. E-mail: sy.quek@auckland.ac.nz (University of Auckland, New Zealand)
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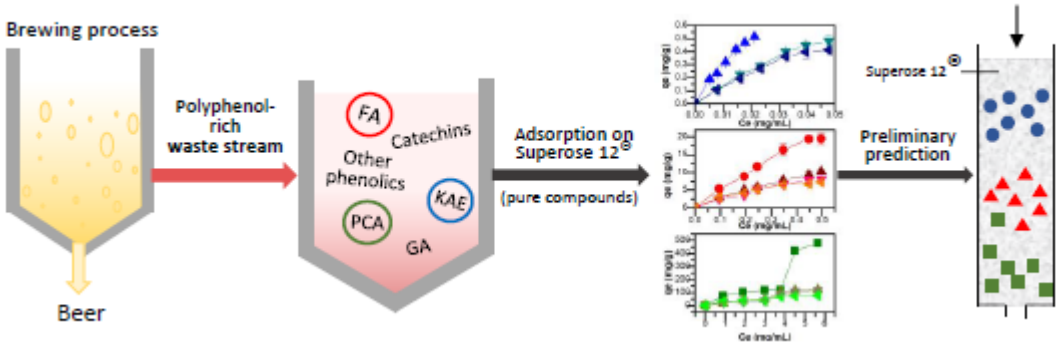
Nong-xue Qiu. E-mail: qiunx@163.com (Shaanxi Normal University, China)
Herminia Domínguez González. E-mail: herminia@uvigo.es (Universidad de Vigo, España)
Agustín Marsal. E-mail: agusti.marsal@iqac.csic.es (Instituto de Química Avanzada de Cataluña, España)

Thank you for your consideration!

Sincerely,

Néstor Escalona Burgos, PhD
Associate Professor, Department of Chemical Engineering and Bioprocessing
Pontificia Universidad Católica de Chile

*3: Graphical Abstract



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ADSORPTION OF THE MAIN POLYPHENOLS OF THE POLYVINYLPOLYPYRROLIDONE (PVPP) BEER WASTE STREAM IN CROSS-LINKED 12% AGAROSE GEL

Javiera Pérez-Manríquez[a1], Pamela Rivera[a2], José Ricardo *(first name)* Pérez-Correa[a3] and Néstor Escalona[a*]

[a] *Department of Chemical and Bioprocess Engineering, School of Engineering, Pontificia Universidad Católica de Chile, Vicuña Mackenna 4860, Macul, Santiago, Chile.*

[a1] jeperez1@uc.cl
[a2] pgrivera@uc.cl
[a3] perez@ing.puc.cl
[a*] Corresponding author. E-mail: neescalona@ing.puc.cl.
Tel: +56 2 2354 4516

Conflict of interest: The authors confirms that this article content has no conflict of interest.

Abstract

The adsorption behavior of 12% cross-linked agarose gel was studied for three polyphenols normally found in brewery waste streams: ferulic acid (FA), kaempferol (KAE) and protocatechuic acid (PCA). Adsorption was evaluated in batch experiments at different temperatures in aqueous solutions containing ethanol and acetic acid. The order of affinity towards the adsorbent was PCA > FA > KAE. Results showed that adsorption capacity decreased with increased concentrations of ethanol and acetic acid and that the Freundlich model had a slightly better overall fit to experimental data, since more accurate parameters were obtained. Changes of enthalpy with surface coverage indicated a slightly heterogeneous adsorption surface for PCA and adsorbate-adsorbate interaction for KAE and FA. This work provides fundamental knowledge related to the adsorption mechanism and how it is affected by solvent composition. Thus, this information may contribute to optimize the separation of phenolics using Superose 12 as the stationary phase. Moreover, based on the adsorption isotherms, separation between these three phenolics using adsorption chromatography with cross-linked agarose as stationary phase is attainable using 15% ethanol, 15% acetic acid, followed by 35% ethanol, 35% acetic acid as eluents. However, further analysis should be conducted to assess kinetic and mass transfer phenomena.

Keywords: Isotherm adsorption, Brewery waste stream; Ferulic acid; Kaempferol; Protocatechuic acid.

Abbreviations. FA: Ferulic acid; KAE: Kaempferol; PCA: Protocatechuic acid; PVPP: Poly(vinylpolypyrrolidone); PBWS: PVPP brewery waste stream.

Symbols. C_e: Equilibrium concentration; $C_{e,max}$: Maximum equilibrium concentration of the isotherms for each compound; K: Adsorption equilibrium constant; K_F: Freundlich adsorption capacity parameter; K_L: Langmuir adsorption equilibrium constant; k': Retention factor; k'_{max}: Retention factor at high polyphenol concentrations; k'_r: Relative retention factor; N: Number of data points; n: Freundlich adsorption intensity parameter; q_{calc}: Adsorption capacity calculated with isotherm model; q_e: Equilibrium adsorption capacity; $q_{e,max}$: Adsorption capacity at $C_{e,max}$; q_{exp}: Experimental adsorption capacity;

1. Introduction

Over the last decades, there have been several concerns about ongoing population growth resulting in a series of issues, such as food security, increasing environmental deterioration and resource depletion [1]. Great efforts are being made to minimize this damage, including the implementation of programs to reduce environmental impact caused by industries and making a more effective use of our resources. The recovery of food ingredients found in agroindustrial wastes is an interesting and ecological approach that has been widely studied and adopted to cope with these issues [2].

Phenolic compounds (or polyphenols) have received special attention as functional food ingredients and nutraceuticals due to their interesting activities on the human physiology, including protective effects against arteriosclerosis, coronary heart disease, cancer and several neurodegenerative diseases [3–7].

Many agroindustrial residues have been proposed as rich and economic sources of polyphenols [2]. Recently, Barbosa-Pereira *et al*. considered the waste from the brewery process as an appealing source of phenolic compounds [8] since in many brewing processes polyphenols are removed from beer to avoid haze formation (accumulation of protein-polyphenol complexes) by polyvinylpolypyrrolidone (PVPP) as an adsorbent. These phenolic compounds are then thrown into the PVPP brewery waste stream (PBWS). Thus, a considerable amount of polyphenol-rich waste is produced, becoming an economic source of

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ΔG^0: Gibbs energy change; ΔH: Enthalpy change; ΔS: Entropy change; θ: Estimated parameter; σ: Standard deviation of estimated parameter; ϕ: Stationary and mobile phase volume ratio.

functional ingredients. Its exploitation would provide added value to the industry and reduce its residues.

Since these compounds have such different properties and mechanisms of action, separation and purification of the PBWS polyphenols provide the opportunity to exploit these compounds by their distinctive characteristics. Hence, obtention of purified polyphenols is highly desired.

Techniques developed to separate polyphenols generally include variations of liquid chromatography, although these methods have been described as tedious, time and solvent consuming, as well as difficult to scale-up [9,10]. However, a technique consisting in adsorption chromatography over cross-linked 12% agarose gel (Superose 12®) has been highly recommended for polyphenol purification; this is not only because one-step separation can be achieved or it is easily scalable, but because it is chemically stable, allowing harsh cleaning procedures [11–14]. Moreover, higher yields and purity are obtained for step-wise isocratic elution using mobile phases containing different proportions of water, ethanol and acetic acid [10,14].

Despite the promising results obtained for polyphenol separation using Superose 12® adsorption chromatography, to our knowledge, there is no research regarding adsorption equilibrium of polyphenols on cross-linked agarose. The importance of these studies on designing industrial scale solid-liquid separation processes reveals the need to conduct such experiments. Hence, the aim of this study is to carry out a first characterization of the adsorption mechanism in highly cross-linked agarose of three bioactive polyphenols, representative of some of the most abundant phenolic classes found in PBWS (ferulic acid

(FA), kaempferol (KAE) and protocatechuic acid (PCA)). Adsorption isotherms are used to evaluate the effect of temperature and the composition of water, ethanol and acetic acid on adsorption capacity. Moreover, accurate and significant equilibrium parameters are obtained which will be useful to develop a chromatographic model for process design and optimization. The adsorption process is further assessed through thermodynamic analysis. Finally, a preliminary proposal is presented of a sequence of solvent mixtures to achieve an adequate separation of the studied polyphenols through adsorption chromatography with stepwise elution.

2. Materials and methods

2.1. Materials

The phenolic compounds, ferulic acid, protocatechuic acid and kaempferol were purchased from Xi'an Hao-xuan Biotech Co., Ltd. (Xi'an, China); their purity is shown in Table 1. Highly cross-linked agarose, Superose® 12 Prep Grade, was obtained from GE healthcare Bio-sciences (Uppsala, Sweden). In addition, analytical grade acetic acid glacial and ethanol used in this study were supplied by Merck S.A. (Darmstadt, Germany) and Fisher Scientific (New York, USA), respectively.

2.2. Apparatus and experimental procedure

Experiments were performed for each polyphenol listed in **Table 1** using different mixtures of water, ethanol, and acetic acid. Adsorption isotherms were obtained using a batch method in a Carrousel system described in Cuevas *et al*. (Cuevas-Valenzuela, González-Rojas, Wisniak, Apelblat, & Pérez-Correa, 2015). The solvent mixtures used for the experiments differ between the phenolics due to the difference in their solubility. The solubilities found

in literature for each compound and the solvent mixtures used for the experiments are shown in **Table 1**.

To perform the adsorption experiments, 0.005 g dry weight of cross-linked 12% agarose was measured, using an analytical balance with a resolution of 0.0001 g, and then added to the glass tubes. Once the Carrousel system reached a temperature of 20°C, 5 mL of the phenolic solutions were poured into the corresponding flask. All glass tubes were carefully capped to avoid evaporation. The solution-adsorbent mixture was kept under agitation (500 rpm) at 20°C for 1 h. A similar procedure was carried out to assess the effect of temperature in polyphenols adsorption from a solvent mixture of water: ethanol: acetic acid (70:15:15 v/v). In this case, the experiments were performed at 10°C and 30°C, keeping the rest of the operating conditions invariable. Isotherm curves were set up using 6 different initial concentrations of each polyphenol; experiments were performed in triplicate.

Once the adsorption equilibrium was reached, the liquid phase and solid phase were quickly separated using a polytetrafluoroethylene (PTFE) filter with a pore size of 0.45 µm and a diameter of 13 mm (Bonna-Agela Technologies, Delaware, USA). The filtrates were properly diluted, so polyphenol concentrations could be measured using an ultraviolet-visible spectrophotometer (Reyleigh UV-1601, Beijing Beifen-Ruili Analytical Instrument Co. Ltd., Beijing, China). The absorbance of each polyphenol was measured in a 200 to 600 nm spectrum to establish the maximum wavelengths for spectrophotometric measurement (Table 1). Calibration curves ($R2 < 0.99$) for each polyphenol in each solvent mixture were prepared to correlate absorbance and concentration. Increases in concentration during absorbance determination due to evaporation, were prevented by using cell caps. After the concentration

of the phenolic solutions were calculated, C_e (mg/L), the equilibrium adsorption capacity, q_e (mg/g), was calculated using a mass balance,

$$q_e = \frac{(C_0 - C_e) \cdot V}{A} \qquad (eq.1)$$

Where V is the volume of the phenolic solution, A is the weight of dry agarose (g) and C_0 is the concentration of the solution prior to the adsorption process (mg/L).

2.3. Analysis

2.3.1. Adsorption isotherm models

The adsorption isotherms describe the relationship between the amount of polyphenol adsorbed in agarose (q_e, mg/g) and the polyphenol diluted in the solvent (C_e, mg/mL) at equilibrium. The most commonly used isotherm models, Langmuir and Freundlich, were applied in our study to find which one represents better the adsorption process. The Langmuir model assumes monolayer adsorption and fixed number of adsorption sites. It also assumes that all adsorption sites are equal and there is no interaction between adsorbed molecules [15]. This model can be described as:

$$q = \frac{q_{max} K_L C}{1 + K_L C} \qquad (eq.2)$$

Where q_{max} is the maximum adsorption capacity (mg/g) and K_L is the adsorption equilibrium constant which is related to the Gibbs energy of adsorption. To find the model constants, this expression is often put in linear form as follows:

$$\frac{1}{q} = \frac{1}{C} + \frac{1}{K_L q_{max} C} \qquad (eq.3)$$

The Freundlich model, often used to represent non-ideal adsorption, is characterized by multilayer formation, heterogenous surface and irregular heat adsorption distributions [16]. The equation that describes the Freundlich isotherm is:

$$q = K_F C^{1/n} \qquad (eq. 4)$$

Where, K_F and n are model parameters associated with adsorption capacity and adsorption intensity, respectively [17]. Additionally, 1/n has been related to the degree of surface heterogeneity, being more heterogeneous when its value is closer to zero [15]. The linear form of the Freundlich model is:

$$\log(q_e) = \log(K_F) + \frac{1}{n}\log(C_e) \qquad (eq. 5)$$

2.3.2. Model fitting

Model parameters were estimated through linear and non-linear regression. Linear regression was performed by minimization of the sum of the square errors of the linear form of the adsorption models (eq. 3 and eq. 5 for the Langmuir and Freundlich model, respectively). For non-linear regression, model parameters were estimated by minimizing the error between the experimental measurement of the adsorbed polyphenol (q_{exp}) and the adsorbed polyphenol calculated by the respective non-linear model (q_{calc}). Optimization was performed using the Microsoft Excel solver add-in. Two objective functions for model fitting were tested:

(1) <u>Sum of square errors (SSE):</u>

It is the most frequently used objective function, and it can be described by the following equation:

$$\sum_{i=1}^{N}\left(q_{calc}-q_{exp}\right)_i^2 \qquad (eq.6)$$

Where N represents the number of data points. Normally, parameters obtained using the SSE objective function tend to provide a better fit for higher adsorbed solute concentrations where the magnitude of the square errors are higher [18].

(2) Average relative error (ARE)

This objective function is given by:

$$\frac{100}{N}\sum_{i=1}^{N}\left|\frac{q_{calc}-q_{exp}}{q_{exp}}\right|_i \qquad (eq.7)$$

Since this function is normalized by experimental data, the relative error is more distributed, providing a better overall fitting [15].

Comparison of the fitted models was carried out using the coefficient of determination (R^2). This value represents the ratio between the explained variance and the total variance [19], and it can be calculated as follows:

$$R^2 = 1-\frac{RSS}{TSS} = 1-\frac{\sum_{i=1}^{N}\left(q_{exp}-q_{calc}\right)^2}{\sum_{i=1}^{N}\left(q_{exp}-\overline{q_{exp}}\right)^2} \qquad (eq.8)$$

However, many authors discourage the use of the coefficient of determination as the single criteria to select the best-fit model when nonlinear regression is being evaluated [20]. Therefore, the significance of model parameters was examined to help discriminate between the Langmuir and Freundlich models. The 95% confidence intervals were obtained as described by Sánchez *et al.*[21] and are presented in the following form:

$$IC(0.95) = \theta \pm 1.96 \cdot \sigma \qquad (eq.9)$$

A parameter is considered statistically significant when the following criteria is fulfilled:

$$t - value = \left|\frac{\theta}{\sigma}\right| \geq 2 \qquad (eq.10)$$

Where θ is the parameter and σ is the standard deviation of the estimated parameter.

In addition, given the confidence intervals obtained for the model parameters, the simultaneous prediction confidence intervals were also calculated. These prediction intervals refer to the confidence intervals of the simulated values of the dependent variable (equilibrium adsorption capacity, q_e). The function *nlparci* of Matlab 8.2.0.701 (R2013b) was used to obtain the simultaneous prediction intervals, in which a linear approximation is made in order to apply the Scheffe's S-method for linear regression, as described by Vecchia *et al.* [22].

2.3.3. Thermodynamic analysis

Further understanding of the adsorption process of the polyphenols in agarose can be obtained through the analysis of thermodynamic parameters. The enthalpy change (ΔH, kJ/mol), Gibbs energy change (ΔG, kJ/mol) and the entropy change (ΔS, kJ/mol K) can provide information related to the energy changes occurred in the agarose after adsorption and the mechanisms involved in this process [23]. Enthalpy change was obtained according to the linear form of the van´t Hoff equation:

$$\ln(C_e) = \frac{\Delta H}{RT} + \ln(K) \qquad (eq.11)$$

Where T is the temperature (K), R is the ideal gas constant and ΔH is the isosteric adsorption enthalpy change. Enthalpy change was calculated through the plot of ln(*Ce*) vs 1/T, where *Ce* must be at the same adsorption capacity (isosteric concentration). The Gibbs energy change calculation depends on the model that best represents the polyphenol adsorption. If the Freundlich model has the best fit, the following expression is used [24]:

$$\Delta G^0 = -nRT \qquad (eq.12)$$

Where n is the Freundlich index parameter. Contrarily, if Langmuir is the best-fit model, the Gibbs energy change is calculated using the equation [25]:

$$\Delta G^0 = -\ln(K_L)RT \qquad (eq.13)$$

Where K_L is the adsorption equilibrium constant of the Langmuir model. In addition, the entropy change was calculated as follows:

$$\Delta S = \frac{\Delta H - \Delta G^0}{T} \qquad (eq.14)$$

Each parameter is presented with the corresponding uncertainty calculated as described by Farrance & Frenkel [26].

2.3.4. Column retention

In liquid chromatography, selection of a suitable mobile phase is essential to accomplish successful separations of complex mixtures. Application of gradient elution techniques allows further improvements in the separation process [27]. Ideally, the retention factors of the solutes should be different enough to move through the column at different speeds, allowing a high-resolution separation. However, unnecessarily large differences in retention

might lead to long elution times, which is undesirable in commercial scale processes. Thus, in chromatography we seek for the maximum resolution in the shortest possible time.

In adsorption chromatography, the retention factor (k') is related to the adsorption equilibrium constant (K). If the adsorption process is approximated by a linear model, the equilibrium constant can be expressed as [28]:

$$K = \frac{q_e}{C_e} \qquad (eq.15)$$

Additionally, the relationship between the retention factor and the equilibrium constant is directly proportional [28], leading to the following expression:

$$k' = \phi \cdot \frac{q_e}{C_e} \qquad (eq.16)$$

Where ϕ is the ratio between the volumes of the stationary phase and mobile phase in the column. In this expression, q_e and C_e must have the same units (mg/g), since the retention factor is dimensionless. Conversion of the units of C_e to mg/g required the density of the solvents, and since these solvents are a mixture of liquids, the density was obtained using the UNIFAC model from Aspen Plus V10. The order of elution of FA, KAE and PCA was inferred based on the retention factor. However, considering that in preparative chromatography the samples injected to the column are highly concentrated due to solubility differences, the concentration of the compounds in the sample will differ significantly. Therefore, these values were normalized by a retention factor calculated at high concentrations for each compound (k'_{max}):

$$k'_{max} = \phi \cdot \frac{q_{e,max}}{C_{e,max}} \qquad (eq.17)$$

Where $C_{e,max}$ (mg/g) is the maximum C_e used for the equilibrium studies and $q_{e,max}$ (mg/g) is the adsorption capacity at $C_{e,max}$. Then, a relative retention factor was calculated (k_r'):

$$k_r' = \frac{k'}{k'_{max}} = \frac{q_e/q_{e,max}}{C_e/C_{e,max}} \qquad (eq.\ 18)$$

that could be used to compare the compounds retention for the different solvent mixtures studied.

3. Results and discussion

3.1. Adsorption isotherms

The adsorption isotherms of the polyphenols on agarose were studied for different solvent mixtures of water, ethanol and acetic acid at 20°C; the results are presented in **Fig. 1 (A) – (C)**. This Figure shows that the effect of the solvent composition on the adsorption capacity is the same for the three phenolics. At high concentrations of water (W100 for PCA, W90 for FA and W70 for KAE), the highest adsorption capacities were observed. However, for lower concentrations of water, adsorption capacities were almost independent of water composition (W70, W50 and W30 for phenolic acids; W50 and W30 for KE). The same behavior with water composition and polyphenol adsorption was observed by Silva *et al.* [29], who suggest that this response is related to solvophobicity originated by the presence of a solute on a highly structured aqueous solution that tends to self-aggregate via hydrogen bond interactions [29,30]. The authors indicate that increasing solvophobicity of a compound in a given solvent increases the tendency of the solvent molecules to push the solute towards the adsorbent. The authors also mention that solvophobicity of polyphenols is higher in solvents with high dielectric constants (water, ε_{H2O} = 78.5) than in solvents with low dielectric constants (ethanol and acetic acid, ε_{etOH} = 24.3 and ε_{AA} = 6.15) [29]. Gu and

coworkers argue that high acetic acid concentrations reduce the available adsorption sites for polyphenols, decreasing their adsorption capacity. The authors also mention that the interaction of acetate ions and delocalized electrons from the surface might also contribute to the reduction of the adsorption capacity [10].

According to the isotherm classification system defined by Giles & Smith, all curves follow the L-type shape, indicating that as more sites in the substrate are filled in, it becomes much more difficult for an adsorbate molecule to find a vacant site available [31]. Also, the adsorbate is either horizontally adsorbed or there is no strong competition from the solvent [32]. However, these isotherms differ on the subgroup of the classification; FA and KAE mainly exhibit L1 shapes, while PCA presents L4 shapes. In FA and KAE adsorption curves, completion of the surface coverage has not been reached and a clear plateau is not observed. Probably, the plateau could be reached for higher polyphenol concentrations, but (i) higher concentrations cannot be obtained due to their low solubility, and (ii) they are not relevant for the current study since these high concentrations have not been found in PBWS. PCA adsorption curves are characterized by a second rise and a second plateau. This behavior can appear when a re-orientation of the adsorbed molecules happens (horizontal orientation at the first plateau and vertical orientation at the second), or when a second layer is formed on top of the first [32]. In the case of PCA, both situations could be occurring. Further analysis will only consider the first rise of PCA adsorption since the rest of the curve shows concentrations not relevant for this study.

3.2. Model fitting

The parameters estimated for each isotherm with linear and nonlinear regression for the Langmuir and Freundlich models are presented in Supplementary material (**Tables S1-S3**);

the parameters are presented with the corresponding error and coefficient of determination. In some cases, estimated parameters for the same model present rather different values for the different objective functions. For example, estimation of q_{max} for FA adsorption with W70 with linear regression resulted in a value 2.5 times higher than the one obtained with non-linear regression using SSE (**Table S1**). In addition, estimation of q_{max} for PCA adsorption with W70 with linear regression was almost 3.2 times higher than the one obtained with nonlinear regression using SSE (**Table S3**). However, in most isotherms, results were very similar (if not the same), independent of the estimation method used.

To compare the results, and as a first approach to select the best-fit model, the coefficient of determination was used despite being discouraged [33]. The models studied only differ on the equation or the parameters values, but no difference in the number of the parameters exists; therefore the other comparison methods, such as Akaike Information Criterion (AIC) or BIC, are not suitable. Analysis of the R^2 shows that in almost all cases nonlinear regression with SSE results in a better parameter estimation, followed by ARE in Langmuir models and linear in Freundlich models. Despite attaining a relatively clear distinction between parameter estimation method with R^2, the distinction between Langmuir and Freundlich is not evident. In many cases, the Langmuir model tends to give a slightly higher R^2, but the difference does not seem high enough to select this model.

Analysis of the significance of the model parameters and the simultaneous prediction interval were performed to carry out a more appropriate selection of the best-fit model. The 95% confidence intervals of the parameters, estimated with nonlinear regression using SSE, are presented in **Table 2**. Parameters estimated with the Freundlich model are significant for all isotherms and for all compounds studied, while parameters obtained with the Langmuir

model are mostly significant, except for the W70 isotherm of FA and PCA. Since significant parameters were achieved with the Freundlich model for these two phenolics, and not by Langmuir, it could be argued that Freundlich manages to better represent their adsorption on agarose. However, the parameters for KAE are significant for both models, but with slightly higher t-Values for Freundlich.

Further evaluation of the goodness of fit of both models was carried out through the evaluation of the simultaneous prediction intervals [34]. **Fig. S1** and **Fig. S2** show that for ferulic acid and kaempferol, confidence intervals of the model predictions are relatively narrow and practically the same for Langmuir and Freundlich. Although, protocatechuic acid isotherms (**Fig. S3**) show narrow prediction confidence intervals in W100 and W30 isotherms, Langmuir model predictions are more reliable. Model predictions with both models are less reliable in W70 and W50 isotherms than all isotherms discussed above, since their confidence intervals are much wider.

It is worth mentioning that subtle differences are obtained between the two models; hence, both can be used to represent well the adsorption isotherm of these polyphenols in agarose. Previous studies have also shown good agreement between equilibrium data and the two models [17,23,25]. In our study, we opted for the Freundlich model (since more accurate parameters were obtained); therefore, further analysis in this study was carried out considering this model.

The value of the parameter n in the Freundlich model can indicate the type of isotherm: irreversible ($n < 10$), very favorable ($2 < n < 10$), moderately favorable ($1 < n < 2$) and unfavorable ($n < 1$) [35,36]. From **Table 2,** it can be observed that FA and KAE isotherms are moderately favorable and PCA isotherms are very favorable in most cases. In addition,

$1/n > 1$ values also indicate that in all cases the surface of agarose may exhibit some degree of heterogeneity. The adsorption capacities (K_F) are considerably higher for PCA, followed by FA and KAE. The K_F values for these last two compounds decrease when the proportion of ethanol and acetic acid is increased, verifying that adsorption is higher when the liquid phase has greater water proportion, as previously discussed. For PCA, this effect on K_F is not evident but, considering the confidence intervals, the adsorption capacity tends to decrease with higher co-solvent proportion.

3.3. Thermodynamic analysis

Experiments on the adsorption of the phenolic compounds on agarose from aqueous solvent with 15% co-solvent were studied at three different temperatures: 10°C, 20°C and 30°C (**Fig. 2**). For the three polyphenols, it can be observed that adsorption decreases with increasing temperature, confirming that the adsorption process is exothermic, as expected for almost any adsorption process. The same L4 type curve, with two curve rises, is observed for protocatechuic at the three temperatures studied.

The Gibbs energy (ΔG^0) of adsorption was calculated from eq. 12 for all phenolics. The values obtained for each temperature and with their respective expanded uncertainty is presented in **Table 3**. The enthalpy (ΔH) and entropy of adsorption (ΔS) are presented in **Table 4**; these values also appear with their respective expanded uncertainty. For all cases the negative values of $\Delta G°$ and ΔH suggest that the adsorption process is spontaneous and exothermic. These values also indicate if the process is ruled by chemisorption ($|\Delta H|$ and $|\Delta G^0| > 80$ kJ/mol) or physisorption ($|\Delta H|$ and $|\Delta G^0| < 20$ kJ/mol) [37,38].

From **Tables 3** and **4**, the only process that is undoubtedly ruled by physisorption is the adsorption of KAE. Contrarily, considering that the enthalpy of adsorption for FA and PCA

are higher than the values established for physisorption, some degree of chemisorption is presumed for these compounds. However, Huang and coworkers also obtained high enthalpy values for the adsorption of tea polyphenols in three polymeric adsorbents (PMVBA, PMVBU and PMA) [39]. They suggested that, since hydrogen bonding energy is 5 – 50 kJ/mol, the high values could be related to multiple hydrogen interactions [39,40]. It is possible that in the adsorption of FA and PCA, simultaneous hydrogen bonding with agarose is involved. Indeed, studies on the interaction between cross-linked 12% agarose and phenolic compounds revealed that agarose provides a large number of hydrogen bond acceptor sites for the hydroxyl groups of polyphenols and proposed that hydrogen bonding is the dominating adsorption factor in this adsorbate-adsorbent system [41,42]. Additionally, since hydrogen bonding energy strongly depends on the distance of the involved atoms [40], it may also be possible that the hydrogen bonding formed for the phenolic acids is much closer to the surface than the ones formed by KAE. Hence, high enthalpy values could be related to simultaneous hydrogen interactions, implicating that physisorption is the leading adsorption mechanism; this suggests that no structural changes occur on agarose and that no desorption limitations are involved [23,38]. However, the possibility of chemical adsorption for PCA or FA cannot be ruled out.

From **Tables 3** and **4**, it is also worth noticing that despite the large differences on enthalpy change, the Gibbs energy change does not vary significantly. This result could be explained by the so-called enthalpy-entropy compensation, in which high enthalpy changes are compensated with high entropy changes, reducing the effect on the overall Gibbs energy change [28]. This effect has been observed in a wide range of fields, including adsorption, hydrogen bonding, liquid chromatography and various biochemical processes [43–45]. The

positive correlation between ΔH and ΔS (or compensation effect) might exist since stronger intermolecular interaction (associated to enthalpy) results in a reduction of the configurational freedom or in a more ordered system (associated to higher entropy change) [44]. There has been controversy and misunderstanding about this phenomenon; however, a model that manages to explain this compensation, associates this effect with two interactions: solvent reorganization and molar shift [43,46]. In adsorption, the mechanism of compensation is led by the molar shift, in which reorganization of solute molecules occur on distinguishable adsorption sites [43]. Due to this phenomenon, the entropy change for KAE is lower than for the phenolic acids. It could therefore be speculated that lower ΔS of KAE is related to the higher -OH moieties of KAE, in which more spatial configurations are available for the adsorption of this compound on the surface. Hence, the order of the system does not change as much as when only one or two spatial configurations are available (as in the case of the phenolic acids).

Additionally, **Table 4** indicates that, for all phenolics, there is a slight change in ΔH values with increasing adsorption capacity (or surface coverage). For PCA, the enthalpy decreases when the adsorption capacity increases. This is the most common behavior for heterogeneous surfaces since the higher energy sites of the surface are the ones occupied first, resulting in higher enthalpy magnitudes. When the surface coverage increases, molecules are forced to occupy the lower energy sites, explaining the lower enthalpy magnitudes [39]. For KAE and FA, the absolute value of enthalpy increases with increasing adsorption capacity; this is generally explained by a strong influence in positive adsorbate-adsorbate interaction [47]. This interaction can also be observed in the Do's model [48] where the parameter μ

(representing the adsorbate-adsorbate interaction) is significantly higher than the contribution of the enthalpy change by surface heterogeneity.

3.4. Column retention

In highly cross-linked agarose, using mixtures of water, ethanol and acetic acid as mobile phases, the retention of FA, KAE and PCA was estimated based on the equilibrium adsorption studies. For this purpose, a relative retention factor was calculated for the solvent mixtures studied at different phenolic concentrations, as indicated in the Materials and Methods section. Estimation results are shown in **Fig. 3 (A) – (C)**; W100 and W90 are not considered, since retention in these solvent mixtures cannot be compared among all three phenolics. For all compounds, it is observed that retention decreases when polyphenol concentration rises. This reduction is considerably more pronounced for PCA than for FA and KAE. The retention of the three phenolics is reduced when water concentration is reduced from W70 to W50. However, when the water concentration is further reduced (from W50 to W30) the relative retention keeps decreasing for PCA but rises for KAE and FA. Additionally, it can be observed that for all solvent mixtures at high polyphenol concentrations ($C_e/C_{e,max} > 0.5$), KAE is the compound with higher retention, followed by FA and PCA. Although, for W50 the curves of KAE and FA are practically the same. At low concentrations ($Ce/C_{e,max} < 0.1$), PCA exhibits considerably higher relative retention values for all solvent mixtures.

The reduction of retention with increasing solute concentrations indicates that, in a chromatographic system, the elution may cause asymmetric peaks. It is commonly observed that when adsorption equilibrium has a concave downward form (such as Langmuir and Freundlich with $1/n < 1$), the resulting chromatographic peak has a sharp front and a tail at

the end as shown in **Fig. S4(a)** [49]. Since the retention of PCA is more variable with solute concentration, compared to FA and KAE, the tail of its peak will be longer. It is possible that when the retention curves of two phenolics intersect at some point, part of the tail of the first eluted compound elutes at the same time as the second compound (**Fig. S4(b)**). Consequently, to achieve a better separation it would be advisable to avoid solvents that have adsorption curves that intersect, although this may not always be possible.

Fig. 3 indicates that PCA would elute first under isocratic operation with W70 or W30, followed by FA and then KAE. In the case of W50, PCA elutes first and then FA and KAE elute simultaneously. Gu and coworkers studied the separation of different phenolic classes using adsorption chromatography with cross-linked agarose as adsorbent and water, ethanol and acetic acid mixtures as the mobile phase. They concluded that the order of elution was associated with the number of hydroxyl groups, in which the compound with the most -OH moieties was the last to elute [10]. From **Fig. 4**, it is observed that only the elution of KAE agrees with this result, while, according to the number of -OH moieties, the elution of PCA and FA should be inverted. Nonetheless, the authors also observed that the elution pattern is also affected by intramolecular interaction between hydroxyl groups and adjacent functional groups, including other hydroxyl as well as carboxyl or methyl groups [10]. Both phenolic acids present intramolecular interaction with a carboxylic group. Additionally, PCA has a hydroxyl-hydroxyl interaction and FA presents hydroxyl-methyl interactions. Probably the intramolecular interactions in FA are weaker than in PCA, thus leaving the hydroxyl groups of FA more available to interact with the adsorption surface.

Separation with W70 would result in almost complete isolation of KAE, although the separation of PCA and FA would probably be more difficult. In this case, the peak tail of

PCA would elute at the same time as the front of FA. With W50, complete separation of the three compounds will be difficult since FA and KAE have almost the same retention. In addition, the retention curve of PCA intercepts with the retention curves of FA and KAE. Consequently, two eluted solutions would be obtained, one containing isolated PCA and another containing the three phenolics. Isocratic elution with W30 might produce a satisfactory separation of PCA but the separation between KAE and FA will be deficient.

Unless a very long column is employed, a suitable separation of these polyphenols via isocratic elution cannot be accomplished. Alternatively, the application of stepwise isocratic elution could achieve better results. According to the relative retentions calculated and the discussion above, it would be better start with W70 to delay the elution of KAE (to separate it from FA and PCA) and then continue with W30 to achieve a better resolution between FA and PCA. With this approach, like isocratic elution with W70, it is possible that the peak of FA might be eluted with part of the tail of PCA, although in a considerably lower degree.

It is important to consider that this is a preliminary estimation based only on the equilibrium of the studied polyphenols, the adsorbent and the solvents. This estimation does not consider a possible interaction between the polyphenols or competition for the adsorbent. Moreover, these studies were not conducted on a chromatographic column; hence, adsorption kinetics and mass transfer were not assessed. Consequently, further studies require experimentation on a column to examine how these parameters affect the separation and if the proposed elution technique can separate the compounds with the expected resolution.

4. Conclusions

Separation of polyphenols with adsorption chromatography, using Superose 12® as the stationary phase, has shown very promising results, however little research was available regarding the adsorption mechanisms of phenolics on this gel media. The present study provides a first characterization of the adsorption process of three major polyphenols found in the polyvinylpolypyrrolidone brewery waste stream using Superose 12®. Equilibrium studies coupled with significance analysis showed that, on average, the Freundlich model fit the experimental data well for the different solvent mixtures studied. It was observed that the adsorption capacity of the studied polyphenols was reduced with increased concentrations of ethanol and acetic acid on the mobile phase. Ferulic and protocatechuic acids showed high enthalpy changes, probably due to simultaneous hydrogen bond interactions. It seems that the adsorption process presents the enthalpy-entropy compensation effect, since the adsorption enthalpies of the studied phenolics were significantly different and their Gibbs energy changes were similar.

A relative retention factor was calculated based on the adsorption isotherms, suggesting that a separation with good resolution could be achieved with a stepwise isocratic elution, starting with 15% ethanol, 15% acetic acid, and followed by 35% ethanol, 35% acetic acid. With this system, protocatechuic acid would elute first, followed by ferulic acid, and then kaempferol. Finally, this work provides fundamental knowledge related to the adsorption mechanism and how it is affected by solvent composition. This information may contribute to optimize the separation of phenolics using cross-linked agarose gel as the stationary phase.

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7. References

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8. Figure captions

Fig. 1. Effect of solvent mixture on polyphenol adsorption for (A) ferulic acid, (B) kaempferol, (C) protocatechuic acid.

Fig. 2. Temperature effect on adsorption on agarose with 15% co-solvent for: (A) FA, (B) KAE, (C) PCA.

Fig. 1. Relative retention for each phenolic in (A) W70, (B) W50, (C) W30.

Fig. 4. Chemical structure of (A) ferulic acid, (B) kaempferol, and (C) protocatechuic acid

9. Tables

Table 1. Some characteristics of the polyphenols and specifications for adsorption experiments and absorbance reading.

Compound	Purity	Water solubility at 25°C (mg/L)	Wavelength (nm)	Solvent mixtures (water: ethanol: acetic acid v/v)
Ferulic acid	> 98%	0.525 – 0.527 [50,51]	325	90:5:5 (W90) 70:15:15 (W70) 50:25:25 (W50) 30:35:35 (W30)
Protocatechuic acid	> 99%	18726 – 29400 [52,53]	260	100:0:0 (W100) 70:15:15 (W70) 50:25:25 (W50) 30:35:35 (W30)
Kaempferol	> 98%	1.25 – 1.34 [54,55]	265	70:15:15 (W70) 50:25:25 (W50) 30:35:35 (W30)

Table 2. Significance of Langmuir and Freundlich model parameters.

Compound, solvent mixture		LANGMUIR						FREUNDLICH					
		K_L	(t-Value)		q_{max}	(t-Value)		K_F	(t-Value)		n	(t-Value)	
FA	W90	0.87 ± 0.76	(2.3)		67.05 ± 43.80	(3.1)		35.79 ± 5.64	(12.7)		1.25 ± 0.24	(10.6)	
	W70	1.16 ± 0.31	(7.4)		27.86 ± 5.27	(10.6)		17.47 ± 0.88	(39.8)		1.33 ± 0.08	(32.5)	
	W50	**1.27 ± 1.27**	**(2.0)**		21.37 ± 14.58	(2.9)		14.02 ± 2.78	(10.1)		1.36 ± 0.34	(8.0)	
	W30	3.08 ± 1.34	(4.6)		12.16 ± 2.63	(9.2)		11.06 ± 1.50	(14.8)		1.81 ± 0.38	(9.5)	
KAE	W70	27.60 ± 12.65	(4.4)		1.41 ± 0.45	(6.2)		9.25 ± 3.80	(4.9)		1.34 ± 0.18	(15.2)	
	W50	12.55 ± 7.02	(3.6)		1.32 ± 0.51	(5.2)		5.06 ± 2.40	(4.2)		1.32 ± 0.24	(10.8)	
	W30	18.45 ± 12.89	(2.9)		0.91 ± 0.39	(4.7)		3.48 ± 2.26	(3.1)		1.46 ± 0.41	(7.1)	
PCA	W100	1.15 ± 0.19	(11.9)		150.04 ± 7.18	(41.8)		81.39 ± 9.03	(18.0)		3.15 ± 1.08	(5.9)	
	W70	**0.19 ± 0.59**	**(0.6)**		**129.08 ± 288.22**	**(0.9)**		20.25 ± 12.86	(3.1)		1.32 ± 1.21	(2.2)	
	W50	1.20 ± 0.60	(4.0)		49.01 ± 7.64	(12.8)		26.94 ± 0.04	(1321.2)		3.00 ± 0.02	(362.6)	
	W30	2.96 ± 0.01	(797.3)		43.26 ± 0.02	(4704.7)		32.39 ± 1.46	(44.4)		5.75 ± 1.88	(6.1)	

* Non-significant parameters are marked with bold numbers

Table 3. Gibbs free energy of adsorption of FA, KAE and PCA on highly cross-linked agarose

	$\Delta G^0 \pm U(\Delta G^0)$ (kJ/mol) *		
	10°C	20°C	30°C
FA	-3.18 ± 0.88	-3.23 ± 0.40	-3.70 ± 1.1
KAE	-3.20 ± 0.71	-3.28 ± 0.86	-3.41 ± 1.6
PCA	-3.69 ± 0.63	-3.57 ± 0.68	-3.53 ± 2×10^{-7}

(*) The reported expanded uncertainty, U(x), is based on a standard uncertainty multiplied by a coverage factor k = 2, providing a coverage probability of approximately 95%

Table 4. Enthalpy and entropy change of adsorption of FA, KAE and PCA on highly cross-linked agarose

	Q (mg/g)	$\Delta H \pm U(\Delta H)$ (kJ/mol)	$\Delta S \pm U(\Delta S)$ (kJ/mol K)		
			10°C	20°C	30°C
FA	0.2	-43.62 ± 6.3	0.143 ± 0.045	0.138 ± 0.043	0.132 ± 0.042
	0.25	-44.56 ± 2.0	0.146 ± 0.015	0.141 ± 0.014	0.135 ± 0.015
	0.3	-45.33 ± 0.26	0.149 ± 0.0065	0.144 ± 0.0032	0.137 ± 0.0074
	0.35	-45.98 ± 0.063	0.151 ± 0.0062	0.146 ± 0.0028	0.139 ± 0.0072
	0.4	-46.54 ± 0.82	0.153 ± 0.0085	0.148 ± 0.0062	0.141 ± 0.0090
	0.45	-47.04 ± 2.2	0.155 ± 0.017	0.149 ± 0.015	0.143 ± 0.016
KAE	0.1	-5.803 ± 0.014	0.009 ± 0.0050	0.009 ± 0.0059	0.008 ± 0.010
	0.2	-6.38 ± 0.010	0.011 ± 0.0050	0.011 ± 0.0059	0.010 ± 0.010
	0.3	-7.053 ± 0.0044	0.014 ± 0.0050	0.013 ± 0.0059	0.012 ± 0.010
	0.4	-7.847 ± 0.0017	0.016 ± 0.0050	0.016 ± 0.0059	0.015 ± 0.010
	0.5	-8.798 ± 3.4×10^{-4}	0.020 ± 0.0050	0.019 ± 0.0059	0.018 ± 0.010
	0.6	-9.957 ± 9.4×10^{-6}	0.024 ± 0.0050	0.023 ± 0.0059	0.022 ± 0.010
PCA	0.05	-116.6 ± 2.7	0.399 ± 0.020	0.386 ± 0.019	0.373 ± 0.018
	0.1	-112.5 ± 3.5	0.384 ± 0.025	0.372 ± 0.024	0.359 ± 0.023
	0.2	-108.3 ± 4.4	0.370 ± 0.032	0.357 ± 0.031	0.346 ± 0.029
	0.3	-105.9 ± 5.1	0.361 ± 0.036	0.349 ± 0.035	0.338 ± 0.033
	0.4	-104.2 ± 5.5	0.355 ± 0.039	0.343 ± 0.038	0.332 ± 0.036
	0.5	-102.8 ± 5.9	0.350 ± 0.042	0.339 ± 0.041	0.328 ± 0.039

5: Figure

Figure 1

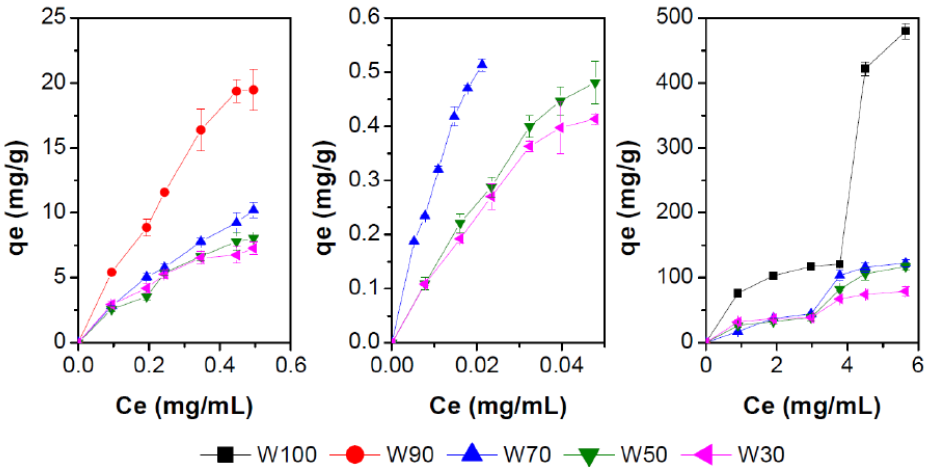

Figure 2

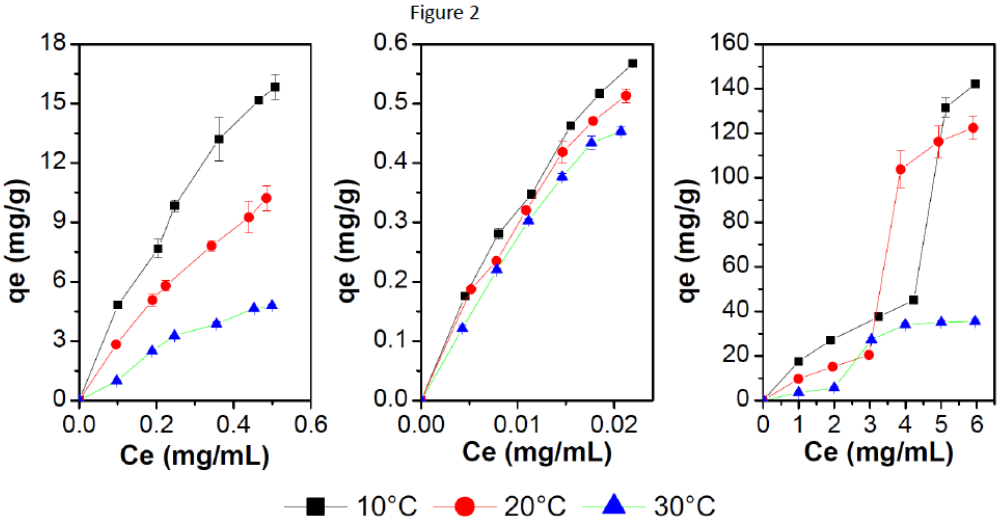

Figure 3

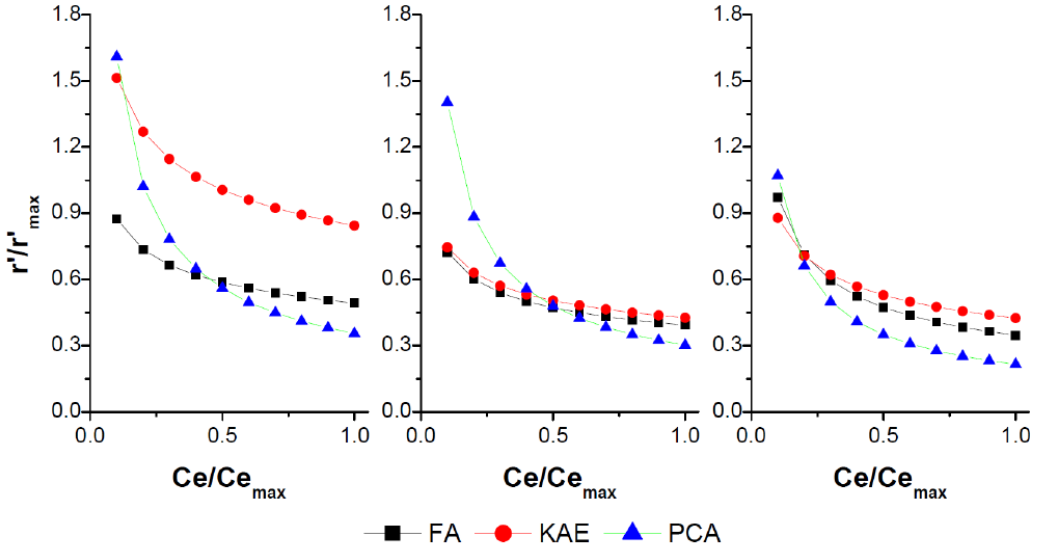
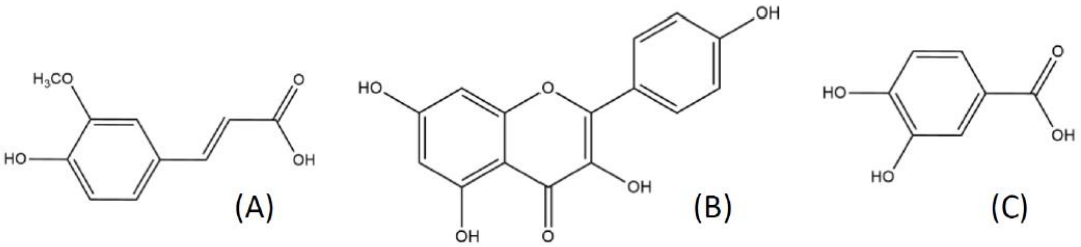
Figure 4