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Extracção de produtos de valor acrescentado do condensado negro

Extraction of added value products from black condensate

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Industrial e Ambiental, realizada sob a orientação científica do Professor Doutor João Manuel da Costa e Araújo Pereira Coutinho, Professor Associado com agregação do Departamento de Química da Universidade de Aveiro e co-orientação de Doutora Susana Pinto Araújo da Silva Estima Martins do Departamento de I&D da Corticeira Amorim.

Ao meu pai, por tudo o que me ensinou.

o júri

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palavras-chave

Cortiça, condensado negro, vanilina, GC-MS, solubilidade e sistemas aquosos bifásicos

Resumo

Num conceito de biorrefinaria integrada, este trabalho foca a recuperação de compostos de valor acrescentado do condensado negro, um subproduto da indústria corticeira resultante da produção de aglomerado de cortiça expandida.

Numa tentativa de valorizar este subproduto, o trabalho inicia-se com a caracterização por GC-MS da fracção extratável do condensado negro. Este estudo permite identificar quais os compostos mais abundantes e com maior interesse económico. Um dos compostos mais interessantes identificados é a vanilina, a qual é uma biomolécula com muitas aplicações a nível industrial e com um elevado valor de mercado, particularmente quando obtida de origem natural e obtida por processos limpos.

Neste contexto, o principal objectivo deste trabalho é encontrar um método de recuperação da vanilina do condensado negro e dos gases de exaustão que seja seguro, ecológico e que tenha elevados rendimentos e baixos custos associados.

A fim de alcançar este objectivo neste trabalho foram avaliados vários compostos que podiam actuar como hidrótropos e aumentar a solubilidade da vanilina em soluções aquosas, para serem usados como solventes na extracção sólido-liquido. Nesta etapa também foi avaliada a influência da temperatura. Os hidrótopos com mais capacidade para aumentar a solubilidade da vanilina em solução aquosa foram o benzoato de sódio e a dicianimida de 1-etil-3-metilimidazólio, $[C_2mim][N(CN)_2]$. Foi ainda confirmado o aumento da solubilidade da vanilina nas soluções aquosas com o aumento da temperatura.

A extracção sólido-liquido foi realizada com benzoato de sódio a 353 K, tendo-se recuperado uma quantidade considerável de vanilina, no entanto este solvente não é específico para a vanilina e alguns outros compostos são também extraídos.

Para estudar a purificação da vanilina foi efectuada a avaliação de sistemas aquosos bifásicos (ABS) para a recuperação e purificação da vanilina usando acetonitrilo e açúcares. Os resultados de partição nestes sistemas mostram que a vanilina tem maior afinidade para a fase rica em acetonitrilo. Obtiveram-se coeficientes de partição superiores a 3.0 e eficiências de recuperação superiores a 75% num único passo de extração.

Keywords Cork, black condensate, vanillin, GC-MS, solubility and aqueous biphasic systems

Abstract

Within an integrated biorefinery concept, this work focuses on the study of the recovery of value added products from black condensate, a by-product of the cork industry resulting from the production of insulation cork boards.

In an attempt to evaluate this by-product, this work starts with the characterization of the extractives of black condensate, by GC-MS. This study allows the identification of the most abundant compounds with economic value. One of the most interesting compounds found is vanillin, a biomolecule with many applications in industry and with a great economic value, particularly if it is obtained by a natural origin and by clean processes.

In this context, the main goal of this work is to develop a method to recover vanillin from black condensate and from the exhaustion gases that is safe, “greener” and with high yields and low cost.

In order to achieve that goal, this work evaluates various compounds that could act as hydrotropes to increase the solubility of vanillin in aqueous solutions and that can be used as solvents in solid-liquid extraction process. The effect of temperature was also investigated, The enhanced compounds which increase the solubility of vanillin in aqueous solutions were sodium benzoate and 1-ethyl-3-methylimidazolium dicyanimide, [C_2mim] [$N(CN)_2$]. The increase on solubility with the increase of temperature was also confirmed.

Solid-liquid extraction was carried out with sodium benzoate and a considerable amount of vanillin was recovered, but this solvent is not specific for vanillin and some other compounds are concomitantly extracted.

To further study the vanillin purification, aqueous biphasic systems (ABS) composed of acetonitrile and sugars were investigated. The partition cofficients values obtained indicate that vanillin has preferential affinity for the acetonitrile-rich phase. Partition coefficients higher than 3.0 and recovery efficiencies greater than 75 % were obtained in a single-step procedure.

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Notation

List of symbols

wt %	Weight percentage
R^2	Correlation coefficient
K_{van}	Vanillin partition coefficient
K_{ow}	Octanol-water partition coefficient
R_T	Recovery of vanillin
α	Ratio between the top mass and the total mass of the mixture
μ	Viscosity
ρ	Density

List of abbreviations

ABS	Aqueous Biphasic System
ACN	Acetonitrile
AH	After Hydrolysis
BC	Black Condensate
BH	Before Hydrolysis
DCM	Dichloromethane
GC-MS	Gas Chromatography - Mass Spectrometry
ILs	Ionic Liquids
NMR	Nuclear Magnetic Resonance
rpm	Rotations per Minute
TL	Tie-Line
TLL	Tie-Line Length
TMS	Trimethylsilyl
UV	Ultraviolet
[ACN]	Concentration of Acetonitrile
[Carbohydrate]	Concentration of Carbohydrate
$[C_2mim]Cl$	1-ethyl-3-methylimidazolium Chloride
$[C_2mim][N(CN)_2]$	1-ethyl-3-methylimidazolium dicyanimide
[Hydrotrope]	Concentration of Hydrotrope
[Vanillin]	Concentration of Vanillin

1. General Introduction

1.1 Scopes and objectives

Within an integrated biorefinery concept, this work focuses on the recovery of value added products from black condensate, a byproduct of the cork industry resulting from the production of insulation cork boards and which involves the expansion and natural agglomeration of cork granules when due to the submission of cork particles in the temperature range between 523 and 773 K. During this thermal treatment of cork with superheated steam, a gaseous current rich in volatile compounds is created, and later it partly condenses in the autoclave exhaustion ducts, where black condensate is formed. The ducts are regularly cleaned and the black condensate is used as fuel in furnaces.

In an attempt to add value to gaseous effluent, black condensate was studied as a sample of the condensation of this source, due to that the direct studied of gaseous current would be difficult. This work starts with the characterization of the extractives of black condensate, by gas chromatography - mass spectrometry (GC-MS). This study allows us to identify which are the most abundant compounds with economic value. One of the most interesting compounds of black condensate is vanillin, a biomolecule with many applications in industry and with improved economic value. This economic value is particularly important when the vanillin is from natural origin but obtained from clean processes as well. In this context, the main objective of this work is to find an effective extraction method for vanillin from black condensate and from the exhaustion gases that is safe, “greener” and with high yields and of low cost.

In order to achieve this objective, this work evaluates various compounds that could act as hydrotropes, increasing the solubility of vanillin in aqueous solutions, to be used as solvents in the solid-liquid extraction process. Several compounds such as sugars, salts, ionic liquids and citric acid were investigated.

Finally, it was also addressed the extraction of vanillin using liquid-liquid extraction techniques with aqueous biphasic systems (ABS) composed of acetonitrile and sugars.

1.2 Biorefinery's concept

Nowadays, the products resulting from chemical industry based on hydrocarbon platform intermediates, such as benzene, xylene, toluene, butane, ethane and ethylene, are derived from fossil reserves.[1] This is one of the biggest problems of the chemical industry because the price of these intermediates is constantly increasing due to the crude oil price fluctuations. On the other hand, these intermediates and the technology used are often seen as highly polluting, energy intensive and non-sustainable.[1]

In the current economic, social and technological situations, issues such as environment, waste disposal, depletion of non-renewable resources and also the unpredictable crude oil price fluctuations are stimulating the investigation aiming at developing sustainable alternatives to the current fossil-based chemicals.[2] Currently, research is mainly looking for a shift to use industrial feedstocks and green processes to produce these chemicals from renewable biomass resources. In this context, biorefinery appears as a promising alternative to obtain new materials, intermediates and chemical products. A crucial step in developing the future chemical industry is to establish integrated biorefineries capable of converting biomass feedstock into a host of valuable chemicals and energy with minimal waste and emissions.[3] Demirbas[4] describes the biorefinery as "a facility that integrates biomass conversion processes and equipment to produce fuels, power and chemicals from biomass". This concept is analogous to today's crude oil refinery, figure 1, which produces multiple fuels and products from petroleum, but biorefineries intend to use fewer non-renewable resources, to use biomass feedstock, to reduce CO_2 emissions, to create new employment, and to spur innovation using clean and efficient technologies.[5]

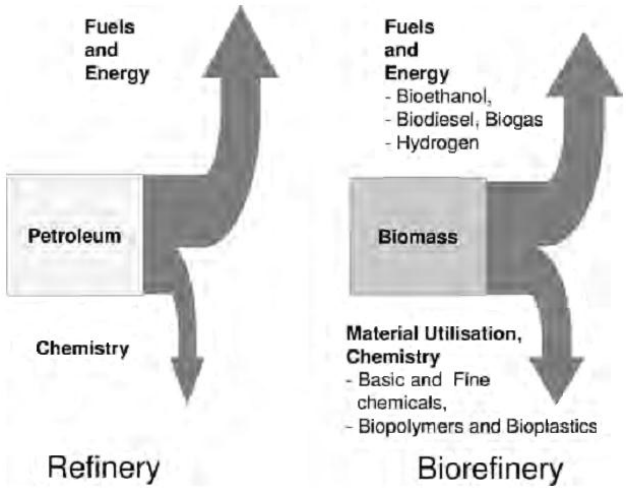

Figure 1: Comparison of the basic-principles of the petroleum refinery and biorefinery.[6]

For these accomplishments it is necessary to integrate the production of higher value chemicals and commodities, as well as low value products, such as fuels and energy, and to optimize the use of resources, maximize profitability and benefits, and minimize wastes.[4, 7] The

range of potential target products includes structures already made by the chemical industry as well as new structures formed from biorefinery building blocks.[8]

Wellish et al.[5] identified the factors which are the key to the question of biorefinery sustainability, such as the type of feedstock, the conversion technologies and their respective conversion and energy efficiencies, the type of products, including co-products, that are manufactured, and what products are substituted by the bioproducts. Among the different types of biorefineries, the lignocellulosic feedstock biorefineries, will probably be the most successful. This is a consequence of the availability of feedstocks, such as agro-food, agro-forest and agro-industrial wastes, which have competitive prices and do not compete with the supply of food, which is one of the most relevant problems facing the biorefineries of first generation.[9] In fact, from the 1.7×10^{11} ton of biomass produced annually, only 6×10^{9} ton are used, and only 3 % is applied in non-food applications.[10, 11]

The implementation of biorefinery concepts in existing agroforest-based activities and the concomitant need to upgrade the byproducts generated in processing agricultural and forest products, represent a short-term response to this goal.[12] The up-grading of the byproducts of the agro-industry constitutes an important challenge on the development of a sustainable economy and of environmentally friendly industrial processes. These byproducts are seen as promising sources of renewable chemicals, materials and fuels and as a response to the inevitable depletion of fossil resources.[11, 13]

The growing of biorefineries has also been the object of thorough appraisals by governments and international institutions, with the result that the funding for basic and applied research in the various relevant areas has been increasing dramatically in the last few years. Forest-related industries, such as cork industry, produce residues that represent a potential source of added value chemicals.

It is thus necessary to develop novel technologies and to perform studies on diverse biomass feedstocks, to identify a core group of chemicals and intermediates, attract investments in research and development to reduce costs and otherwise improve competitiveness with fossil based chemicals,[3, 8, 14] since the sustainable biobased products are the foundation of a successful biorefinery development.[8]

1.3 Cork and industry

Quercus suber L. is the botanical name for a slow growing evergreen oak that flourishes only in specific regions of the Western Mediterranean, figure 2, such as Portugal, Spain, Southern France and part of Italy and North Africa,[15, 16] occupying a worldwide area of 2 119 000 hectares.

The maximum average biological age of the cork oak is approximately 200 years.[17] This species of oak requires a great deal of sunlight and a highly unusual combination of low rainfall and somewhat high humidity.[15] The forest of *Quercus suber* L., "montado" as it is known in Portugal, figure 3, has a very positive impact in Portugal, at both ecological and economic levels. Indeed, the "montado" aids to preserve the soil, to regulate the water cycle and to preserve the biodiversity of flora and fauna.[16, 18]

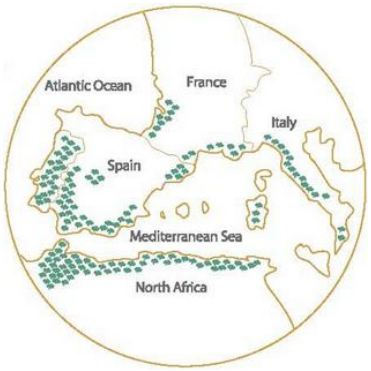

Figure 2: Regions where *Quercus suber* L. flourishes in the world.

The outer bark of *Quercus suber* L. is commonly known as cork. Trees must be from 15 to 30 years old before the first harvesting can occur and the tree trunk must reach 25 cm in diameter. The second harvesting generates somewhat better-quality cork but, in general, only the subsequent cork oak striping generate good quality cork, known as "amadia" cork.[17] Cork is periodically harvested from the tree, figure 4, usually every 9-12 years, depending on the culture region.

Figure 3: Portuguese "montado".

Europe has about 67 % of the total production area and produces more than 88 % of the world's cork.[18] Cork is considered a characteristic Portuguese product and an important driver of the Portuguese economy, since in Portugal, about 100 000 ton of cork/year are produced, which represents about 49.6% of the world production.[18]. Thus the cork industry is of great economic importance, at a time when agriculture and agro-forestry industries are seen as the lever to overcome the national economic crisis.

Figure 4: Cork harvesting.

Cork is normally used in the production of stoppers for wine and other alcoholic beverages, and it has application in thermal and/or acoustic insulation materials[19] such as general purpose agglomerates and agglomerates for wall and floor coverings.[15]

The full exploitation of this resource and specially the detailed study of its chemical composition is a key step towards the valorization of its sub-products. The chemical constitution

of cork has been widely examined[19, 20] and found to depend on factors such as geographic origin, climate and soil conditions, genetic origin, tree dimensions, age and growth conditions. Cork is mostly composed of suberin (40 %, w/w), but also contains lignin (25 %, w/w), polysaccharides (20 %, w/w), extractives (15 %, w/w), and inorganic compounds (1 %, w/w).[9, 12, 20, 21] Cork extractives are mainly composed of aliphatic, phenolic, and triterpenic components. The triterpenic fraction of cork extractives essentially contains betuline, betulinic acid and smaller amounts of sterols. The abundance of phenolic compounds such as vanillin, and triterpenes, such as friedeline, cerine and β-sitosterol in cork, together with their promising applications, directly or as precursors of bioactive components for biomedical and food applications, has prompted the interest in studying its abundance in industrial cork byproducts.[22]

Currently, there is an interest in the development of new products and applications based on cork, since the traditional markets for cork products are facing increasing amounts of concurrent products. These new products could appear from the cork currently used for other applications, because of its peculiar properties,[12, 15] such as high elasticity and low permeability or, what would be even more interesting from an industrial perspective, from the residues or low quality cork. The search for new applications of cork byproducts is particularly attractive within the scope of the biorefinery concept in forest-based industries. Studies aiming at identifying and extracting various added value products from those waste residues are very important to foster an increase in the sustainability of cork-related industries.

2. Black condensate analysis

2.1 Introduction

The abundance of phenolic compounds, such as syringaldehyde and vanillin, and triterpenes, such as friedeline, cerine and β-sitosterol, in cork,[23] together with their promising applications, directly or as precursors of bioactive components for biomedical and food applications, has prompted the interest in studying its abundance in industrial cork byproducts.

Industrial cork processing generates substantial amounts of residues such as "cork powder", "cooking wastewaters" and "black condensate".[22] Cork powder is the main residue, representing 34 000 ton per year in Portugal.[16] This residue is generated mainly during the production of granulated cork for agglomerated materials. It has an inadequate particle size to be suitable for current industrial uses. Cork powder is currently burned to produce energy, but it could represent an important source of suberin (more than 16 000 ton per year).[12]

The cooking of cork planks in boiling water is a key stage in wine stopper production, yielding cooking wastewaters as liquid effluents.[19] These wastewaters have many water soluble compounds, such as phenolic compounds and amino acids, in considerable concentrations.[24]

Black condensate is a residue of the production of insulation cork boards which involves the expansion and natural agglomeration of cork granules after submission of cork particles at temperatures in the range from 523-773 K using superheated steam. During the thermal treatment of cork, compounds are extracted with the steam and later on condensed in the exhaustion ditch and ducts, forming the black condensate. Periodically, this solid byproduct is removed (2 500 tons/year) and burned to produce energy.[16] Although a large number of compounds condense in the ditch, the steam released into atmosphere has also large amounts of extractives, which could become an interesting source of biomolecules.

Nowadays some studies are being carried out in order to upgrade this by-product.[24] Black condensate composition is described in one study[17, 25] that reports the presence of some added value products including triterpenes and phenolic compounds. The interest on these natural compounds relies on the wide variety of relevant properties shown by those families, namely their antioxidant, anti-inflammatory, radical scavenger and antimicrobial properties with importance in food, dietary, health and pharmaceutical industries.[25] The interest in natural phenolic compounds for nutraceutical and cosmetic applications has increased considerably in recent years because of the mentioned properties but also because they do not show adverse effects as it is frequently the case of their synthetic counterparts.[26]

The black condensate, being by nature a volatile fraction of cork, the initial identification of only 16 % of the sample mass is unexpectedly low. The explanation of this fact is related with the presence of esterified lipophilic structures, hydrolysable by conventional hydrolysis reactions. These structures are most probably formed by condensation reactions during the thermal treatment of cork granulates.[17]

Table 1 summarizes the GC-MS results of the analysis of black condensate extractives described in literature.[17] These results reveal that black condensate extract is mainly composed of triterpenes, followed by smaller amounts of alkanols, alkanoic acids, and phenolic compounds.

Table 1: Major compounds identified in the dichloromethane extract of the black condensate, before and after alkaline hydrolysis.[17]

Family of compounds	Compounds
Aliphatic alcohols	Docosanol, C(22:0)
	Tetracosanol, C(24:0)
	Hexacosanol, C(26:0))
Fatty acids	Docosanoic acid, C(22:0)
	Tetracosanoic acid, C(24:0)
	Hexacosanoic acid, C(26:0)
ω-hydroxy fatty acids	18-hydroxyhexadec-9-enoic acid, C(18:1)
	22-hydroxydocosanoic acid, C(22:0)
	24-hydroxtetracosanoic acid, C(24:0)
α,ω -alkanedioic acids	Octadecanedioic acid, C(18:0)
	Octadec-9-enedioic acid, C(18:1)
	Docosanedioic acid, C(22:0)
Phenolic compounds	Ferulic acid
	3-vanillylpropanol
	Vanillylpropanoic acid
	Benzoic acid
Triterpenes	Friedeline
	Betuline
	Betulinic acid
	β-sitosterol

Friedeline is the most abundant compound of the triterpenes family. Although this molecule has not yet commercial application there are some studies on the applicability and extraction of friedeline. Other triterpenes in smaller amounts are also present, namely betuline, betulinic acid and β-sitosterol. Among these triterpenes the only of interest for commercial applications is β-sitosterol in a biomedical treatment of cancer.

After alkaline hydrolysis the increase in the amount of detected compounds is detected with the increase in the contents of alkanols, alkanoic acids and phenolics. Additionally, ω-hydroxyalkanoic and α,ω-alkanedioic acids were only detected in considerable amounts after alkaline hydrolysis. These families of compounds are quite abundant in suberin,[12] and their

presence in black condensate might have resulted from the cleavage of more labile ester functionalities of the suberin macromolecular structure during the thermal treatment.[17]

The development of methodologies to isolate and adequately purify these promising compounds/fractions, instead of simply burning the cork residues, constitutes a stimulating challenge for the valorization of cork as a renewable resource.

2.2 Experimental section

2.2.1 Materials

Black condensates samples (BC 1, BC 2 and BC 3) were supplied by Amorim Isolamentos mill (Portugal). These samples are from two different plants, the first from a chimney of Vendas Novas and the second and third samples from a chimney and ditch of the Silves plant, respectively.

2.2.2 Experimental procedure

Isolation of extractives by Soxhlet extraction of black condensate

The solid samples were dried at 378 K during 24 h. After drying, extractives from black condensate samples were removed from solid samples (≈ 20 g) by Soxhlet extraction with dichloromethane (DCM) during 6 h (figure 5). The resulting extracts were then dried from the solvent in a rotary evaporator, vacuum-dried, and weighed.

Figure 5: Soxhlet extraction.

The extraction yield of Soxhlet extraction was determined using equation 1:

$$\%\, Yield = \frac{Quantity\ of\ extract\ obtained}{Quantity\ of\ sample\ used} \times 100 \qquad (1)$$

Alkaline hydrolysis of extracts from black condensate

To evaluate the presence of esterified structures in different BC samples, approximately 20 mg samples of extracts were dissolved in 10 mL of a solution of 1 M potassium hydroxide in a 10 % aqueous methanol solution and heated at 373 K under a nitrogen atmosphere during 1 h. The ensuing mixture was cooled to room temperature, acidified with aqueous hydrochloric acid (1 M) until the pH reaches a value of 2, and extracted three times with DCM. Finally, the DCM fraction was evaporated.

Derivatisation of black condensate extracts

Prior to the GC-MS analysis, each sample was silylated. For this purpose, approximately 20 mg of extract was dissolved in 250 µL of pyridine solution of tetracosane (≈ 2 $mg.mL^{-1}$) (internal standard), and components containing hydroxyl and carboxyl groups were converted to their trimethylsilyl (TMS) ethers and esters, respectively, by adding 250 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (derivatization agent) and 50 µL of trimethylchlorosilane (reaction catalyst). The mixture was kept at 343 K during 30 minutes.

Gas chromatography analyses

Figure 6 : Trace GC 2000 gas chromatograph, coupled with a mass selective detector, Finnigan Trace MS.

GC-MS analysis of the TMS-derivatised samples were performed using a Trace GC 2000 gas chromatograph, coupled with a mass selective detector, Finnigan Trace MS, using helium as carrier gas (35 $cm.s^{-1}$) and equipped with a DB-1 J&W capillary column (30 m x 0.32 mm and 0.25 µm film thickness). The chromatographic conditions were as follows: an initial isothermal at 353 K during 5 min, ramped 1 from 353 to 513 K (4 $K.min^{-1}$), ramped 2 from 513 to 558 K (2 $K.min^{-1}$), and finally an isothermal at 558 K until 80 min; injector temperature, 523 K; transfer line temperature, 558 K; split ratio, equal to 1:50. The MS was operated in the electron impact mode with an electron impact energy of 70 eV and collected data at a rate of 0.7364 $scan.s^{-1}$ over a range of m/z 33-700. The ion source was maintained at 523 K.

Chromatographic peaks were identified on the basis of the comparison of their mass spectra with the equipment mass spectra library (Wiley-NIST Mass Spectral Library), their characteristic retention times, obtained under the described experimental conditions, and of their fragmentation profiles with published data.[27]

For quantitative analysis, GC-MS was calibrated with pure reference compounds, representative of the major lipophilic extractive components (namely coniferyl alcohol, octadecanoic acid, nonadecanol and stigmasterol), relative to tetracosane. The respective response factors were calculated as an average of six GC-MS runs. A quantity of each compound was determined for a comparison between pick area of compound and internal standard, and take into account the reference compounds values.

2.3 Results and discussion

2.3.1 Characterization of the extractives from black condensates

The black condensate (BC) composition is described in one study in literature;[17, 25] yet, in this study only a single sample was investigated. However, since black condensate is formed in autoclave pipes in different mills, where different cork and ducts of different length and temperatures are used, the composition of black condensate in different mills and different points of the plant are naturally different. In this context, it is important to study the composition of black condensate in different autoclave exhaustion pipes in order to verify the samples composition variability and which are the major compounds present.

In this work three different samples of black condensate, from different places, were studied: BC 1 (from Vendas Novas mill), BC 2 and BC 3 (from Silves mill). As a first observation (table 2), the BC samples exhibit different characteristics at both visual and physical levels, with heterogeneous colors and different textures.

Table 2: Physical and visual description of the samples.

Sample	Image	Characteristics
BC 1		• Color: dark brown mixed with black • Texture: earthy, very irregular surface • Compact particles, but that easily break • Easy to grind
BC 2		• Color: dark brown mixed with black, with bright areas • Texture: earthy, very irregular surface • Compact particles, but that easily break
BC 3		• Color: dark brown mixed with black, with much of the shiny surface • Texture: doughy, irregular surface • Contains pieces of cork • Compact particles, but that easily break • Hard to grind

Black condensate samples were Soxhlet-extracted with DCM during six hours. The extracts were dried until a constant weight and further characterized by GC-MS.

The extraction yields of black condensate samples are shown in table 3 and were calculated using equation 1.

Table 3: Extraction yields (wt %) of black condensate samples.

Solvent	BC 1	BC 2	BC 3
DCM	54.9±1.0	81.7±0.1	86.5±3.1

The extraction yield of BC 1 is lower than the yields obtained for BC 2 and BC 3 which are closer to the yield described in literature, where the DCM extraction yield is circa 91.9 %.[25] This difference could be explained by the different composition of the samples since the sample BC 1 (collected at Vendas Novas mill) comes from a different plant than samples BC 2 and BC 3, which were collected at Silves mill. These results show a significant difference in the quantity of extractable compounds present in the samples from two different mills.

The different extraction yields observed between BC 2 and BC 3 demonstrates that these two samples have different quantities of extractives; BC 3 has a higher extractive fraction than BC 2. This difference is expected since sample BC 3 was recovered in the ditch of Silves factory and BC 2 was recovered in the chimney of the Silves factory.

Prior to GC-MS analysis the samples were silylated, as described before.[27] This derivatization is important to convert the hydroxyl and carboxyl groups of some compounds to their TMS ethers and esters. The identification of chromatographic peaks was based on the equipment's spectral library and also on the comparison with previously published data, reference compounds, ion fragmentation patterns, and retention times.

In figure 7 it is depicted the GC-MS chromatogram of the BC 1 dichloromethane extract before and after alkaline hydrolysis (BH and AH). The chromatograms of BC 2 and BC 3 samples are presented in appendix A. The chromatograms present the same major compounds, however their relative abundance has a significant change after alkaline hydrolysis.

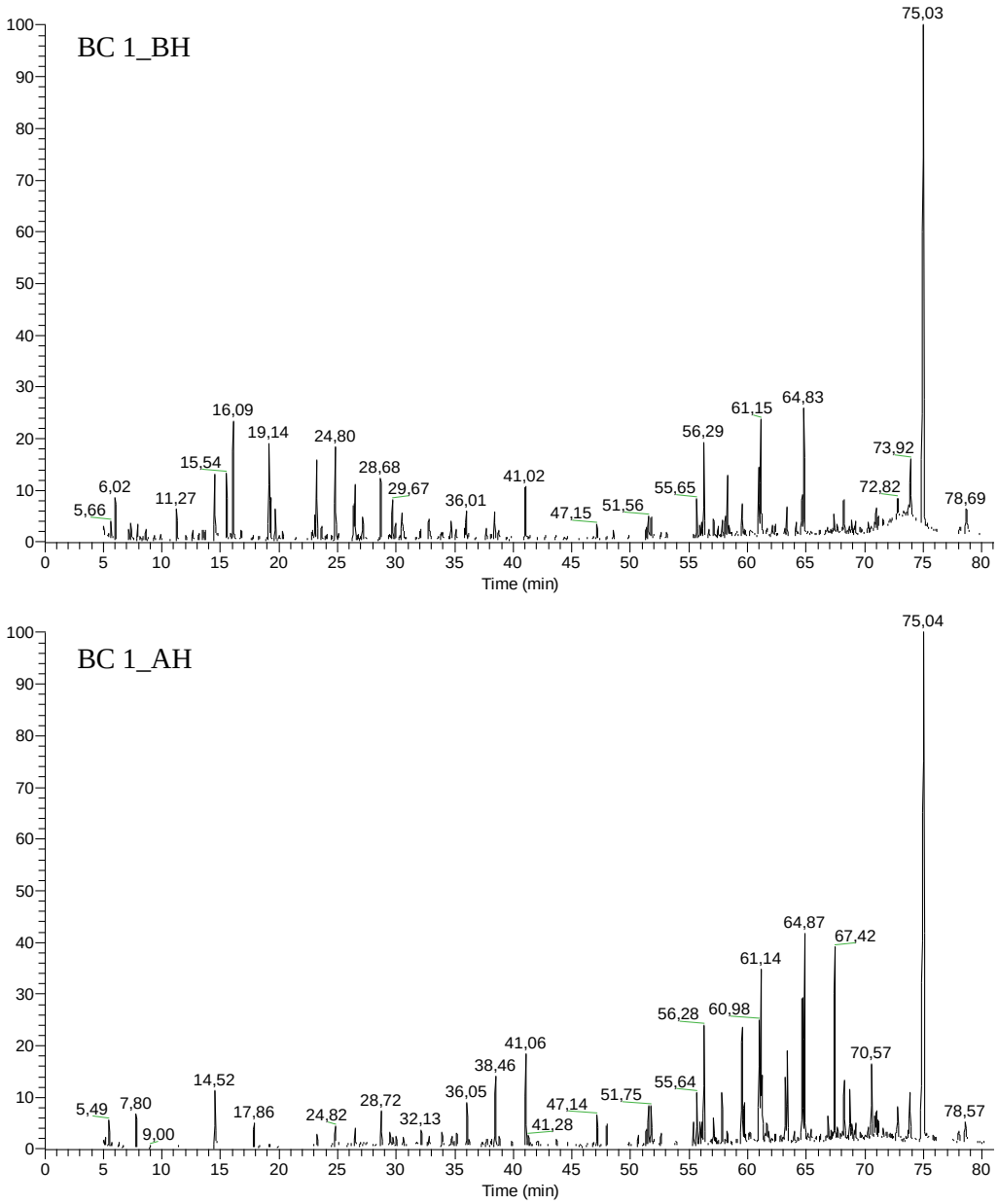

Figure 7: Total ion chromatogram of the derivatized dichloromethane extract of BC 1: BH- before alkaline hydrolysis, AH- after alkaline hydrolysis. Internal standard at 56.28 minutes.

In table 4 the major compounds present in all samples after extraction with dichloromethane, before and after hydrolysis, are reported. An analysis of these results shows that the major compounds are common to the three samples but significant differences in their compositions are also visible. For the BC 1 sample it was identified 54.5 % and 63.8 % of compounds before and after hydrolysis, respectively. From BC 2 it was verified 54.8 % and 60.5 % of compounds before and after hydrolysis, respectively, whereas for BC 3 67.3 % of compounds before hydrolysis and 70.7 % after hydrolysis were identified.

Table 4: Major compounds present in extracts of black condensate after extraction with dichloromethane (grams *per* kilogram*)* before (BH) and after (AH) alkaline hydrolysis.

Family of compounds	Compound	BC 1		BC 2		BC 3	
		BH	**AH**	**BH**	**AH**	**BH**	**AH**
Aliphatic alcohols	C(20:0)						7.02
							7.02
Fatty acids	C(9:0)				3.98		6.73
	C(16:0)						7.09
	C(18:2)		5.08			4.57	
	C(18:1)		5.01			4.54	6.57
	C(22:0)	13.65	13.30	23.46	18.27	20.40	24.73
	C(23:0)		8.95	12.24	14.12	12.72	25.99
	C(24:0)	16.96	22.20	26.69	19.28	28.56	34.23
		30.62	54.54	62.39	55.66	70.80	105.34
ω-hydroxy fatty acids	C(18:1)		7.47		4.99		8.82
			7.47		4.99		8.82
α,ω-alkanedioic acids	C(18:1)		23.80		9.70		17.06
	C(20:0)		21.21		19.88		42.60
	C(22:0)		9.09		8.74		19.99
			54.10		38.32		79.64
α-hydroxy acid	Glycolic acid	4.99					
		4.99					
Phenolic compounds	Catechol	19.07				12.43	
	Vanillin	16.06		11.91		14.21	6.71
	Syringaldehyde	6.38				3.92	
	Isoeugenol					5.09	
	Vanillic acid		5.41			5.22	8.00
	3-vanillylpropanol		9.11			4.47	12.60
	Vanillylpropanoic acid	9.72	12.87				9.40
	Ferulic acid						7.39
		51.23	27.39	11.91		45.33	44.10
Triterpenes	β-sitosterol	6.33		12.71	8.39	12.63	19.83
	Stigmastan-3,4-diene					2.66	10.68
	n. i. Friedeline deriv.	10.43	7.00	18.15	6.83	12.39	
	Friedeline	142.70	134.43	207.80	114.46	105.54	122.33
		159.46	141.43	238.66	129.68	133.22	152.84
Others/ Unidentified		97.10	66.54	58.08	45.13	114.55	109.10

According to the results obtained and shown in figure 8, BC could be considered an abundant source of triterpenic compounds, and particularly of friedeline, which is known to have promising applications (table 5), directly or as a precursor of bioactive components for biomedical applications.[28, 29] This large amount of triterpenic compounds is according to the results described in literature.[22]

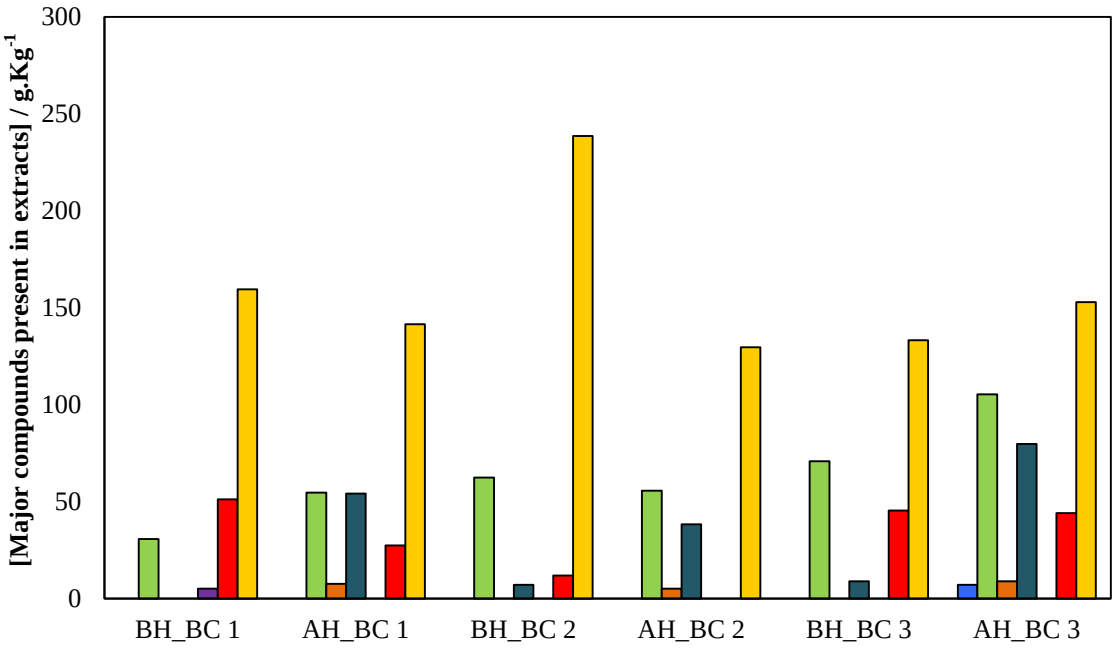

Figure 8: Contents of the major families of compounds identified by GC-MS in the DCM extract of the samples of BC before (BH) and after (AH) alkaline hydrolysis: aliphatic alcohols, fatty acids, ω-hydroxy fatty acids, α- hydroxy fatty acids, α,ω-alkanedioic acids, triterpenes, phenols.

The results obtained show an increase in the amounts of detected compounds after alkaline hydrolysis. However, contrarily to what expected the amount of triterpenes does not increase, but friedeline continues to be the most abundant compound, followed by smaller amounts of β-sitosterol. In the three samples, there are some fatty acids, which increase significantly after alkaline hydrolysis, such as docosanoic, tricosanoic and tetracosanoic acids. This growth means that fatty acids are present in esterified forms in BC samples. These results are in agreement with literature.[22]

Phenols are other important group of compounds present in this byproduct. In BC 1 and BC 3 there are significant amounts of catechol, vanillin and vanillylpropanoic acid. On the other hand, in sample BC 2 vanillin is the only phenolic compound present. After alkaline hydrolysis do not be observed increases in amount of phenols. This result is important because the need to use alkaline hydrolysis in process of extraction removes the natural status of these products. Vanillin, the main component of vanilla, which is the world's most popular flavoring material and therefore it has an increased economic value, is one of the most important phenolic compounds present in the extracts, and which has extensive applications (table 5) in food, beverages, and in the perfumery and pharmaceutical industries.

Table 5: Applications of major compounds present in the black condensate samples.

Family of compounds	Compound	Applications
Fatty acids	C(16:0)	Production of soaps and cosmetics.
	C(18:2)	Precursor of arachidonic acid. Used in the manufacturing of fast-drying oils.
	C(18:1)	Used as sodium salt in soap production.
	C(22:0)	Used in hair conditioners, in lubricating oils and as antifoam in the production of detergents.
ω-hydroxy fatty acids	C(18:1)	Synthesis of polymeric materials, polyurethanes synthesis and polyesters.
α,ω-alkanedioic acids	C(18:1)	
	C(20:0)	
	C(22:0)	
α-hydroxy acid	Glycolic acid	Used in cosmetics and detergents.
Phenolic compounds	Catechol	Precursor of pesticides, flavors and fragrances.
	Vanillin	Used in pharmaceuticals, food, cosmetics and cleaning products. It is also used as a chemical intermediate in the production of drugs and biocides.
	Syringaldehyde	It has the same applications of vanillin, but a lower commercial value.
	Isoeugenol	It is used as an intermediate in the production of vanillin, and is widely used in fragrances and as a flavoring additive.
	Vanillic acid	Used as flavoring agent and as an intermediate in the production of vanillin.
	Ferulic acid	It is used as a precursor in the manufacture of other aromatic compounds.
Triterpenes	β-sitosterol	Used in medical treatments (phytotherapy).
	Friedeline	It is reported as inhibiting growth of tumor cells, but has not yet been used.

The results obtained in this work have a relevant difference in their composition when compared with literature[22] since these samples don't have large quantities of ω-hydroxyl fatty acids and α, ω-alkanedioic acids after alkaline hydrolysis.[22] This variation could be related with the sampling local, because the higher the samples are collected in the chimney, more volatile compounds and less hydrolysable compounds are present, as can be confirmed by our samples.

The results obtained demonstrate that black condensates can be valuable resources of chemicals, and the development of methodologies to isolate and adequately purify those compounds/fractions instead of simply burning the residues, will be a relevant contribution to the valorization of cork as a renewable resource.

2.4 Conclusions

The results obtained demonstrate that black condensate can be a valuable resource of added value chemicals, mainly triterpenes and phenolic compounds. In this context, the search of new techniques for extracting added value compounds from this cork by-product is particularly attractive within the scope of the biorefinery concept.

Among the several identified extractives, interesting amounts of vanillin were found, making black condensate a promising source of that compound. Vanillin stands out for its high commercial value. The development of methodologies to isolate and adequately purify these promising compounds/fractions, instead of simply burning the cork residues, constitutes a stimulating challenge for the valorization of cork byproducts as a renewable resource.

3.Solubility of vanillin

3.1 Introduction

Vanillin, 4-hydroxy-3-methoxybenzaldehyde, presents the chemical formula $CH_3O(OH)C_6H_3CHO$, and its molecular structure is depicted in figure 9. Vanillin is an aromatic aldehyde, belonging to the group of simple phenolic compounds. Structurally, vanillin has three functional groups, including an aldehyde, ether, and phenol groups.[30] This phenolic compound is generally recognized as safe[31] and is the major component of natural vanilla[32] which is one of the most widely used and important flavoring materials.[33]

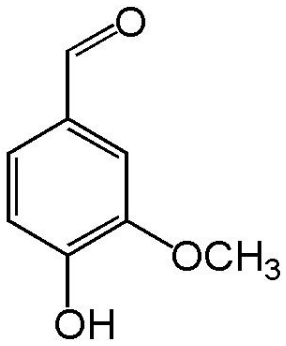

Figure 9: Structure of vanillin.

Vanilla finds extensive applications in food, beverages, perfumery and pharmaceutical industry.[34] Natural vanilla is a complex mixture of flavor components extracted from the cured pods of different species of plant genus Vanilla, the tropical Vanilla orchid: *Vanillus planifolia* and *Vanillus tahitensis*.[35] The most valuable source of vanilla is *Vanillus planifolia*[33], figure 10, because of its pod quality and yield.

In recent years, researchers have been exploring vanillin's properties as an antioxidant, antimicrobial[36] and anticarcinogenic agent.[37] Thus, owing to its medicinal properties, besides being a flavoring agent, vanillin has a tremendous potential to be used as a food preservative and health food agent.[34] Also, in the food industry there is a growing interest in naturally occurring flavor compounds that exhibit antioxidant and antimicrobial activities against both Gram-positive and Gram-negative food-spoilage bacteria and have been shown to be effective against both yeasts and moulds in fruit purees and laboratory growth media.[36, 38]

Figure 10: *Vanillus planifólia*.

Vanillin is also used as a chemical intermediate in the production of pharmaceuticals and fine chemicals for use in biocides and specialty chemicals in technical applications.[39, 40]

The flavor profile of vanilla extract contains more than 200 components. Commercially, natural vanilla extract is sold as a dilute 35 – 40 % ethanolic extract containing about 0.1 – 0.2 % vanillin,[41] and it is used in the food, beverage, pharmaceutical, tobacco and fragrance industries. Although the aroma and flavor of vanilla extract is attributed mainly due to presence of vanillin,[42, 43] many other volatile compounds that are present also contribute to its flavor, such as vanillic acid, vanillyl alcohol and p-hydroxybenzaldehyde.[44]

Vanillin occurs in trace amounts in other plants such as tobacco[45] and cork[19]. However, the pods of the Vanilla orchid still remain the only commercial source of natural vanillin. True vanilla pods possess a pure delicate spicy flavor that cannot be duplicated exactly by synthetic

routes. Also, the flavor quality of vanilla extracts vary considerably, depending upon the origin, curing technique used, storage conditions, extraction methods, and age of the vanilla extract itself.[34] For this reason, and because of limited supply, natural vanilla is able to command a premium price, leading to numerous efforts of its blending and adulteration. As a consequence, the analytical techniques required to detect these adulterations have themselves become more and more sophisticated. The most recent technique allows the determination of the ^{13}C abundance of each carbon site thanks to quantitative ^{13}C nuclear magnetic resonance spectroscopy (^{13}C-NMR).[46]

Although more than 12 000 tons of vanillin are produced each year,[33] about 97 % of vanillin sold in the market comes from synthetic sources using coniferin, eugenol, safrole, guaiacol and lignin.[35] Some of these compounds are coming from natural sources, however the use of chemical processes to obtain vanillin removes the status of natural to this compound.

Even though vanillin produced by these means is able to meet the global annual demand, it suffers from serious drawbacks, because of its lower quality.[35] First, the aroma of synthetically produced vanillin is not comparable with that of natural vanillin. Secondly, chemical synthesis involves the use of hazardous chemicals, and hence under current USA and European legislations cannot be used in natural flavors, resulting in decreased consumer appeals.

Vanillin is a white crystalline powder with an intense and pleasant odor and it is mildly soluble in water at room temperature. The properties of vanillin are reported in table 6.

Table 6: Thermophysical properties of vanillin.[30]

Molar mass $g.mol^{-1}$	Density $g.cm^{-3}$	Melting point K	Boiling point K	pKa	Solubility in water $g.L^{-1}$
152,15	2,056*	353 - 354	558	8,2*	10*

*at 298K

Taking into account the limited supply and high price of natural vanilla and the predominance of chemically synthesized vanillin, there is an incentive to explore and develop biological sources of ‘‘natural’’ vanillin that could be marketed as a realistic alternative to the chemically-synthesized substance.[33] Furthermore, extraction and purification of vanillin from agro-industrial residues, which have been described as a good source to recover “natural” phenolic compounds, are very attractive within the scope of the biorefinery concept. In this context, it is important to study alternative methods of extraction and purification of vanillin from these sources in order to develop “greener” methods with low costs.

Nowadays, there is an emergent interest in the use of "greener" solvents for separation processes. New and more benign solvents and techniques for the selective extraction of vanillin from agricultural and industrial wastes should be explored.

Solubility of vanillin

The solubility of solid compounds in pure solvents and mixed solvents plays a vital role in crystallization processes and has a significant impact in extraction and purification steps in industrial applications. The solubility of vanillin is an important characteristic to take into account in studies of extraction and purification of this biomolecule from a complex solution. It is known that the solubility of vanillin is higher in mildly polar solvents, such as ethanol and methanol, and lower in the case of non-polar solvents, such as hexane.[47] Thus, alcoholic solvents have been commonly employed to extract phenolic compounds from natural sources, because they give a relatively high yield of total extract, even though they are not highly selective for phenols. Particularly, mixtures of alcohols and water have revealed to be more efficient in extracting phenolic constituents than the corresponding pure solvent systems.[48] Vanillin's solubility is also strongly temperature dependent, for example, in water is 1 g in 100 mL at 293 K, and 16 g in 100 mL at 353 K.[49]

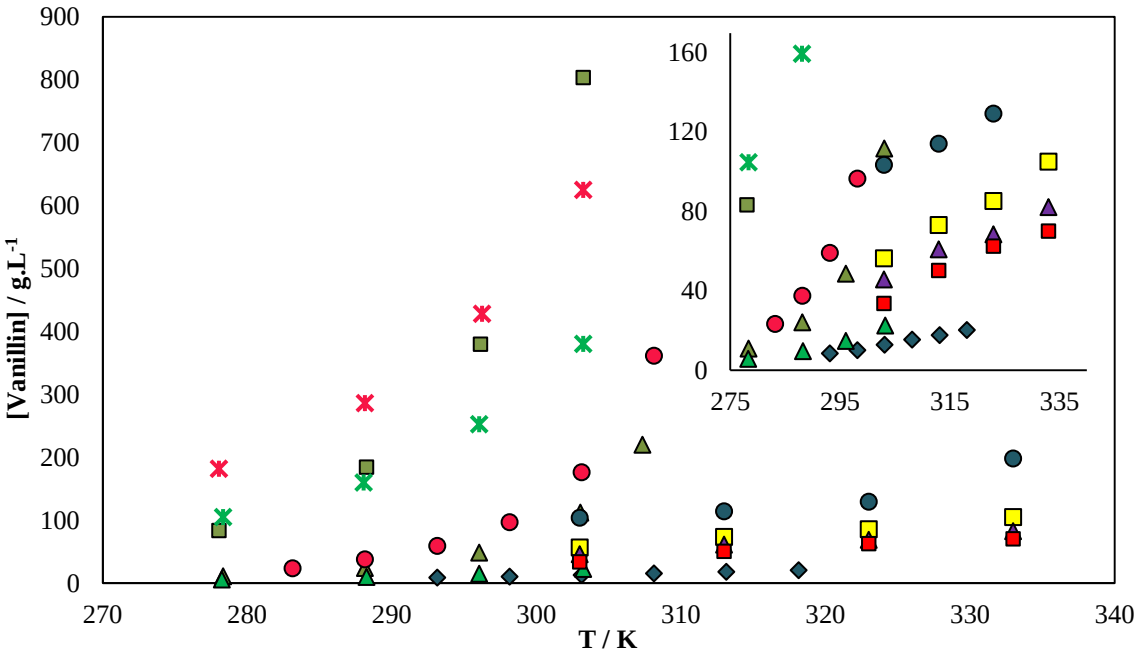

Figure 11: Vanillin solubility in ◆ water,[51] and in aqueous solutions of ▲ ethanol (20 wt %),[53] ■ ethanol (40 wt %),[53] ● 2-propanol (20 wt %),[52] ✱ 2-propanol (95 wt %),[52] ▲ ethylene glycol (20 wt %),[53] ✱ ethylene glycol (95 wt %),[53] ● nicotinamide (32 wt %),[50] ■ sodium salicylate (38 wt %),[50] ▲ resorcinol (24 wt %)[50] and ■ citric acid (42 wt %)[50].

The effect of the temperature, nature and concentration of various hydrotropes, a class of highly water soluble salts / molecules with an ability to dramatically increase the solubility of

sparingly soluble hydrophobic compounds in water,[50, 51] and alcohols, in the vanillin solubility is shown in figure 11.

The solubility of vanillin increases in the presence of hydrotropes, such as, nicotinamide,[50] sodium salicylate,[50] resorcinol[50] and citric acid,[50] and in the presence of alcohols, such as 2-propanol[52] and ethanol.[53] The solubility of vanillin also increases with an increase in temperature.[50] The maximum solubility of vanillin is verified with aqueous solutions of ethanol, resorcinol and 2-propanol, and it is even higher at higher temperatures. These solvents have hydroxyl groups which can hydrogen bond with vanillin and enhance its solubility.

In this work, we aim to study the increase on the solubility of vanillin in aqueous solutions of sugars, polyols, sodium benzoate and citric acid. Two ionic liquids were also investigated in order to infer on their hydrotropicity potential.

3.2 Experimental section

3.2.1 Materials

In this work it was evaluated the ability of several hydrotropes to enhance the vanillin solubility in aqueous media. The compounds studied are: citric acid, glucose, sodium benzoate, sorbitol, sucrose, xylitol, and two ionic liquids (ILs), 1-ethyl-3-methylimidazolium chloride, $[C_2mim]Cl$, and 1- ethyl-3-methylimidazolium dicyanimide, $[C_2mim][N(CN)_2]$.

Citric acid, 100 wt % pure, was supplied by Fisher Scientific. Anhydrous glucose, extra pure, was supplied by Schalab. Sodium benzoate, > 99.0 wt % pure, was supplied by Merk. Sorbitol, > 99.0 wt % pure, was supplied by Fluka. Sucrose, > 99.5 wt % pure, was supplied by Himedia. Xylitol, > 99.0 wt % pure, was supplied by Sigma. These compounds were used as received.

The ILs, $[C_2mim]Cl$ and $[C_2mim][N(CN)_2]$, were supplied by Iolitec. To reduce the water and volatile compounds content to negligible values, ILs individual samples were dried under constant agitation at vacuum and moderate temperature (333 K) for a minimum of 48 h. After this procedure, the purity of each IL was further checked by ^{1}H and ^{13}C NMR and found to be > 98 wt %.

Vanillin, > 99 wt % pure, was supplied by Aldrich.

The water employed was double distilled, passed across a reverse osmosis system, and further treated with a Milli-Q plus 185 water purification apparatus.

3.2.2 Methods

Vanillin was added in excess amounts to each hydrotrope aqueous solutions (20, 15, 10 and 5wt %) and equilibrated on an air oven under constant agitation using an Eppendorf Thermomixer Comfort equipment. The equilibrium temperatures were 303, 313 and 323 (± 0.5) K. Previously optimized equilibration conditions were established: stirring velocity of 750 rpm and at least for 72 h. After the saturation conditions all samples were centrifuged in a Hettich Mikro 120 centrifuge to properly separate the macroscopic phases during 20 minutes at 4500 rpm.

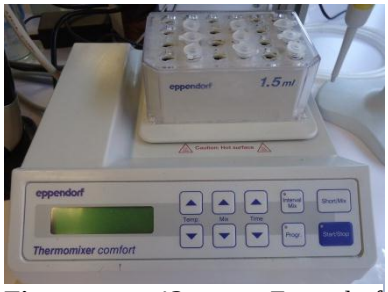

Figure 12: Eppendorf Thermomixer Comfort equipment.

After centrifugation, samples were put in an air bath equipped with a Pt 100 probe and PID controller at the temperature used in assays during 1 h to establish the equilibrium. And samples of the liquid phase were carefully collected and the amount of vanillin was quantified through UV-spectroscopy, using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, at a wavelength of 280 nm. A proper calibration curve was previously established. At least three individual samples of each aqueous solution, and at each concentration of hydrotrope, were

quantified in order to determine the average solubility of vanillin and the respective standard deviation.

3.3 Results and discussion

The solubility of vanillin in water was chosen for the validation of the experimental method. Figure 13 shows that the results gathered in this work are in good agreement with the results described in literature.[50, 51, 53-55] Therefore, the optimized operational conditions can be used for the remaining vanillin solubility studies.

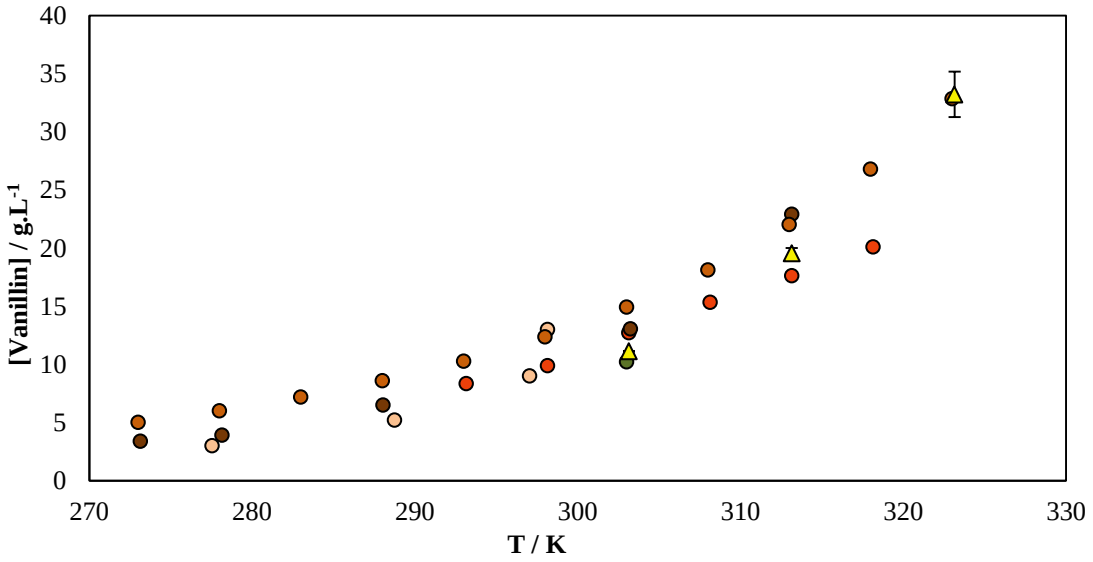

Figure 13: Solubility of vanillin in water. Comparison between the results reported in literature (●[50] ●[51] ●[53] ●[54] ●[55]) and the data gathered in this work(▲).

Taking into account the solubility of vanillin in alcohol and glycerol aqueous solutions, depicted in figure 11, it seems that the hydrogen bonding plays an important role on the hydrotrope ability to increase the solubility of the biomolecule. Thus, it was decided to explore the effect of other polyols and sugars on the solubility of vanillin in aqueous solutions. Glucose, sucrose, sorbitol and xylitol were here studied and the results obtained are reported in table 7 and plotted in figure 14.

Table 7: Solubility of vanillin in aqueous solutions of sugars (glucose and sucrose) and polyols (sorbitol and xylitol) at 303 K.

Hydrotrope		**wt % of hydrotrope in aqueous solution**			
		20	**15**	**10**	**5**
Glucose	**[Vanillin] g.L^{-1}**	5.4±0.2	7.1±0.3	9.0±0.9	9.2±0.2
Sucrose		6.3±0.7	7.1±0.3	8.9±0.9	8.7±0.5
Sorbitol		4.9±0.7	6.8±0.1	10.4±0.5	10.7±0.1
Xilitol		6.8±0.3	8.8±0.7	8.8±0.9	10.7±0.2

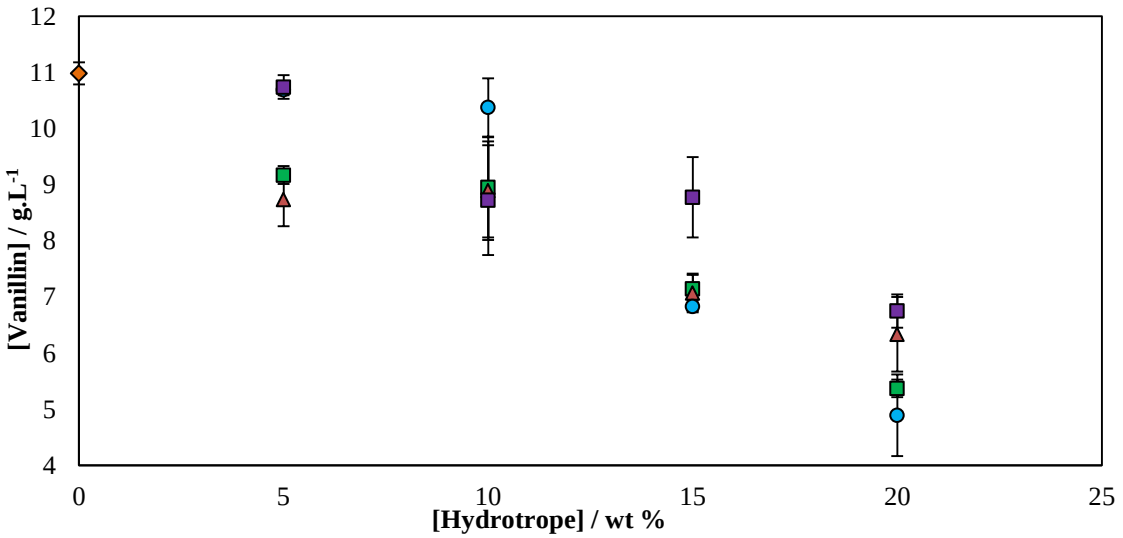

Figure 14: Solubility of vanillin at 303 K in ◆ H_2O, and aqueous solutions of ■ glucose, ▲ sucrose, ● sorbitol and ■ xylitol.

The results depicted in figure 14 show a decrease on the vanillin solubility with the increase on the concentration of sugars and polyols. This decrease could be explained with the lack of an amphiphilic character by these molecules that, albeit able to hydrogen bond with vanillin, possess a limited ability to establish other interactions such as dispersion forces.

Aiming at further exploring the effect of hydrotropes, we further studied the impact on the vanillin solubility of citric acid, sodium benzoate, $[C_2mim]Cl$ and $[C_2mim][N(CN)_2]$ which are described such as enhanced solvents for vanillin.[50, 56]

In figure 15 the results obtained in aqueous solutions of this second series of compounds are depicted. The detailed results are presented in table 8.

All the studied compounds enhance the solubility of vanillin in aqueous media (when the concentration of the hydrotrope in aqueous solution increases from 5 to 20 wt %). This is in good agreement with the results reported in literature for other hydrotropes, such as nicotinamide and sodium salicylate.[50]

Table 8: Solubility of vanillin in aqueous solutions of hydrotropes (citric acid, $[C_2mim]Cl$, $[C_2mim][N(CN)_2]$ and sodium benzoate) at 303 K.

Hydrotrope		**wt % of hydrotrope in aqueous solution**			
		5	**10**	**15**	**20**
Citric acid	**[Vanillin] $g.L^{-1}$**	12.3±0.1	15.2±0.6	18.6±0.3	21.0±0.4
$[C_2mim]Cl$		17.6±0.2	26.7±0.3	38.9±1.7	45.2±5.6
$[C_2mim][N(CN)_2]$		24.5±0.6	50.0±1.0	82.1±2.3	120.7±6.3
Sodium benzoate		20.8±0.3	31.7±0.7	53.0±1.3	73.0±8.8

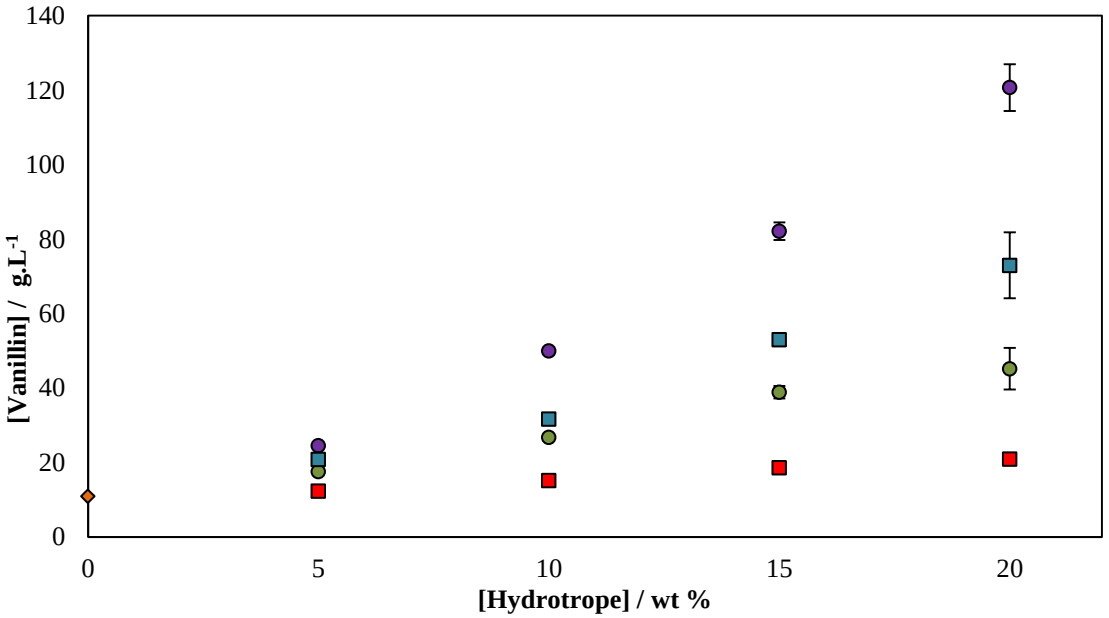

Figure 15: Solubility of vanillin in ◆ H_2O and in aqueous solution of ■ citric acid, ● $[C_2mim]Cl$, ■ sodium benzoate and ● $[C_2mim][N(CN)_2]$, at 303 K.

The increase in solubility of vanillin with hydrotrope solutions is justified by Masilamani et al.[50] as a collective molecular phenomenon, possibly occurring by the aggregation of a solute with the hydrotrope aggregates or by improved hydrogen-bonding.

The amount of vanillin dissolved in an aqueous solution of $[C_2mim][N(CN)_2]$ has an increase with the ionic liquid concentration of about one order of magnitude. In fact, this IL is the best solvent studied for the dissolution of vanillin. The solubilization of a solute is influenced by the hydrophobic part of a stable co-aggregate formed between the solute molecules and the hydrotrope and is also influenced by the chain length of an alkyl group of an hydrotrope. Another good hydrotrope is sodium benzoate which increases the solubility of vanillin 7 times when compared with water, and has the advantage of having a lower cost and being actually used as a food additive. The recovery of vanillin from the hydrotrope solution can be easily achieved by a simple dilution with distilled water, since vanillin solubility is highly sensitive to the hydrotrope concentration.

In order to study the influence of temperature in the solubility of vanillin, the two enhanced hydrotropes, $[C_2mim][N(CN)_2]$ and sodium benzoate, were used. These results are reported in table 9 and plotted in figure 16. Figure 16 shows a significant increase in the solubility of vanillin with the increase in hydrotrope concentration and also with temperature. A similar trend was observed in literature for other hydrotropes.[51] This increase is more relevant when compared with the increase of the solubility of vanillin in water with temperature, which is quite low.

Table 9: Influence of temperature and hydrotrope concentration in vanillin's solubility.

Hydrotrope	wt % of hydrotrope in aqueous solution	[Vanillin] g.L^{-1}	T / K		
			303	313	323
[C_2mim][N(CN)$_2$]	10		50.0±1.0	80.7±3.3	
	20		120.7±6.3	159.8±6.9	366.1±22.9
Sodium benzoate	10		31.7±0.7	42.9±0.8	181.9±16.9
	20		73.0±8.8	110.3±8.1	432.1±27.8

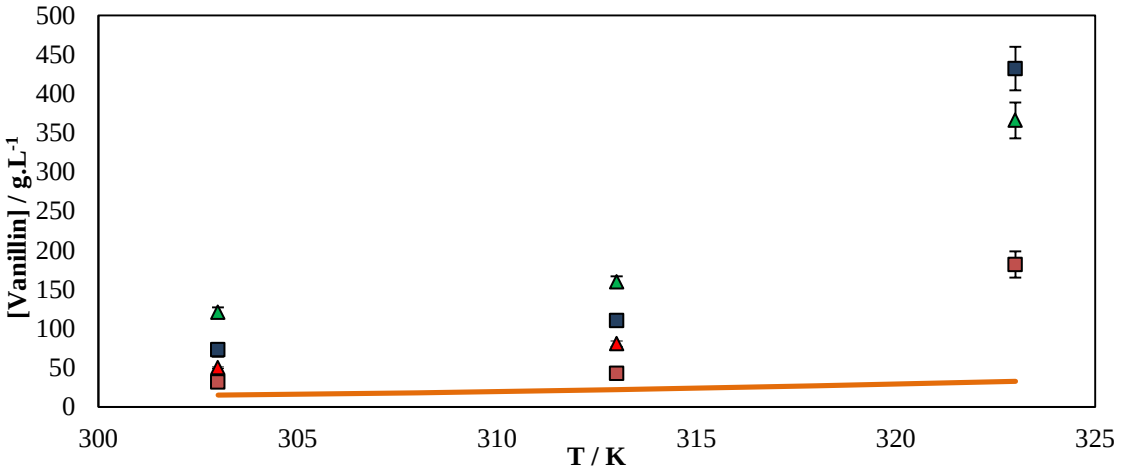

Figure 16: Influence of temperature in the vanillin's solubility in — water[51] and in aqueous solutions of ■ 10 wt % of sodium benzoate, ▲ 10 wt % of [C_2mim][N(CN)$_2$], ■ 20 wt % of sodium benzoate and ▲ 20 wt % of [C_2mim][N(CN)$_2$].

The increase in solubility of vanillin with temperature is more important in aqueous solution of sodium benzoate than in the aqueous solution of [C_2mim][N(CN)$_2$] at 20 wt %. The aqueous solution of 10 wt % of [C_2mim][N(CN)$_2$] at 323 K formed an aqueous biphasic system and cannot be used in this type of application.

3.4 Conclusions

The solubility of vanillin which is poorly soluble in water has been shown to increase in the presence of hydrotrope solutions. It increases with both the increase of hydrotrope concentration and temperature.

In this study, an aqueous solution of 20 wt % of $[C_2mim][N(CN)_2]$ proved to be the best solvent studied. However, at 323 K, sodium benzoate is found to be the best hydrotrope for the enhancement of the solubility of vanillin.

These conclusions are very interesting, since vanillin can be recovered from the hydrotrope solution by a simple dilution with distilled water or by a decrease in temperature because its solubility is highly sensitive to the hydrotrope concentration and temperature of equilibrium.

4. Extraction of vanillin from black condensate

4.1 Introduction

As shown in chapter 2, black condensate is an interesting source of natural compounds. Vanillin is the most interesting compound present in the studied samples because it has a high economic value and it is extensively used at an industrial level.

Nowadays, only vanilla is used as a natural source of vanillin. Vanillin occurs in trace amounts in other plants, including commercial products such as tobacco.[45] However, the pods of the Vanilla orchid still remain the only commercial source of natural vanillin, because the extraction of vanillin from other plants have low yields.

The conventional methods employed for the extraction and purification of natural vanilla include heat treatment, homogenization, percolation, maceration, and solid-liquid extraction.[34] Extracts are prepared mainly by percolating or macerating chopped vanilla pods with ethyl alcohol and water. Distillation is not used because it destroys the fragrance of aromatic compounds. Commercial vanilla extraction could be divided in two categories: the percolation method and the oleoresin method. The percolation method consists of a circulating mixture of ethanol and water containing 35-50 % of alcohol during 48-72 h.[53] The oleoresin method consists of pulverizing whole pods and then circulating ethanol over the pods under vacuum at about 318 K. The excess ethanol is removed by evaporation and the extraction is done during 8-9 days. Using the oleoresin process, a higher strength vanilla extract can be prepared than with the percolation method.[34] Commercially, natural vanilla extract is sold as a dilute ethanolic extract containing about 1.0 $g.L^{-1}$ of vanillin.

The conventional extraction procedures have some disadvantages since they involve several unit operations and are expensive.[57] Moreover these methods of extraction suffer from a number of drawbacks which include low extraction yields, large extraction time and high solvent consumption.[34] Recovery procedures represent, nowadays, the major cost associated to the extraction of biomolecules from natural sources.[58] For that reason it would be important to study extraction methods with the objective of making them sustainable.

In chapter 3, we have shown other solvents which have the ability to dissolve vanillin in aqueous solution. The best solvents studied were $[C_2mim][N(CN)_2]$ and sodium benzoate. We have further shown that sodium benzoate is the best solvent studied at high temperatures. Due to these facts, in this chapter we intend to study the extraction of vanillin from black condensate with a solid-liquid method, using sodium benzoate as solvent.

4.2 Experimental section

4.2.1 Materials

In this section black condensate particles with sizes in the range (0.4 mm < d < 1.0 mm) were used to study the extraction of vanillin from black condensate using sodium benzoate aqueous solutions.

4.2.2 Methods

Vanillin extraction

Pieces of black condensate (sample BC 1, because it is the sample with the highest amount of vanillin) were grinded and manually sift in two sieves (1.0 mm ≤ d and d ≥ 0.4 mm) to exclude the particles with more than 1.0 mm in diameter and the particles with less than 0.4 mm.

The vials containing the mixture were placed under agitation (250 rpm) for several periods of time and at 353 K. The temperature was maintained by means of 353 K.

The extract was vacuum filtered at room temperature and then the sample was alkalinized with sodium hydroxide to ensure that the sodium benzoate is not transformed into benzoic acid, since it is soluble in dichloromethane and can interferes with the GC-MS analysis. After alkalization, the samples were submitted to a liquid-liquid extraction in a separating funnel at room temperature with dichloromethane. The amount of vanillin in dichloromethane fraction was quantified through GC-MS.

Assays

The variables studied in the extraction of vanillin from black condensate were the concentration of sodium benzoate, contact time and solid-liquid ratio.

Table 10: Operational conditions used in the extraction of vanillin from black condensate.

Assay	Sodium benzoate concentration (wt %)	Contact time (min)	Solid-liquid ratio
1	20	90	1/10
2	10	90	1/10
3	20	30	1/10
4	20	90	1/20

Derivatisation of black condensate extracts

Prior to GC-MS analysis, each sample was trimethylsilylated (TMS). For this purpose, approximately 20 mg of extract was dissolved in 250 µL of pyridine solution of tetracosane (≈ 2

$mg.mL^{-1}$) (internal standard), and components containing hydroxyl and carboxyl groups were converted to their TMS ethers and esters, respectively, by adding 250 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (derivatization agent) and 50 µL of trimethylchlorosilane (reaction catalyst). The mixture was kept at 343 K during 30 minutes.

Gas chromatography analyses

GC-MS analysis of the TMS-derivatised samples was performed using a Trace GC 2000 gas chromatograph, coupled with a mass selective detector, Finnigan Trace MS, using helium as carrier gas (35 $cm.s^{-1}$) and equipped with a DB-1 J&W capillary column (30 m x 0.32 mm and 0.25 µm film thickness). The chromatographic conditions were the same as described before in chapter 2.

Chromatographic peaks were identified on the basis of the comparison of their mass spectra with the equipment mass spectra library (Wiley-NIST Mass Spectral Library), their characteristic retention times, obtained under the described experimental conditions, and of their fragmentation profiles with published data.[27]

For quantitative analysis, GC-MS was calibrated with pure reference compounds, representative of the major lipophilic extractive components (namely coniferyl alcohol, octadecanoic acid, nonadecanol and stigmasterol). The respective response factors were calculated as an average of six GC-MS runs. A quantity of each compound was determined for a comparison between pick area of compound and internal standard, and take into account the reference compounds values.

4.3 Results and discussion

Taking into account the processes used for the extraction of vanillin from vanilla pods,[59] the extraction of caffeine from guarana previously studied by the Path group[60] and the results obtained in chapter 3, three variables were chosen (sodium benzoate concentration, contact time and solid-liquid ratio) for the extraction of vanillin from black condensate.

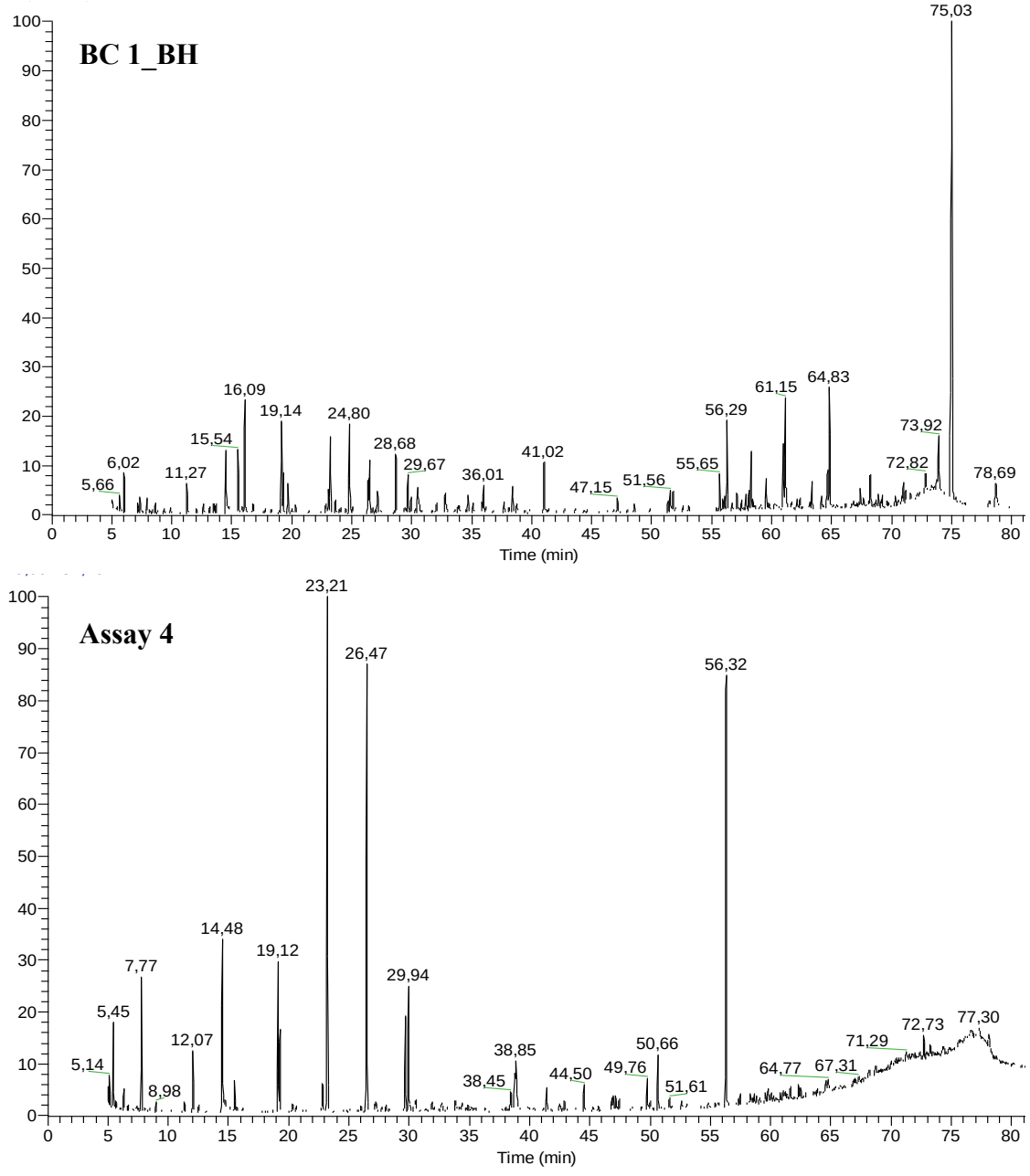

Figure 17: Total ion chromatogram of the derivatized dichloromethane extract of sample 1 of black condensate before alkaline hydrolysis (BC 1_ BH), and total ion chromatogram of the derivatized sodium benzoate extract of assay 4. Pyridine at 23.21 minutes and internal standard at 56.28 minutes.

The comparison between the results obtained for assays 1 to 4 after the solid-liquid extraction with sodium benzoate and liquid-liquid extraction with DCM shows a similar composition for all assays. The chromatograms of assays 1, 2 and 3 are reported in appendix B, and the chromatogram of assay 4 is reported in figure 17. Regarding the assay 1 48.9 % of the compounds were quantified, in assay it was quantified 57.0 % of the compounds, from assay 3 it was quantified 50.6 % of the compounds, and from assay 4 it was quantified 53.7 % of the compounds.

On the other hand, when comparing the chromatogram of sample BC 1 before alkaline hydrolysis and the chromatogram of one of the assays (figure 17) it can be seen a significant difference between both. The assays 1 to 4 have a decrease in the number of compounds when compared with the extract of sample BC 1 obtained by the soxhlet extraction with DCM (chapter 2). These results indicate that this solid-liquid extraction is therefore selective for the extraction of some compounds.

The major compounds present in the extraction assays are shown in table 11.

Table 11: Major compounds present in assays of sodium benzoate extraction at 353 K (grams of compound *per* kilogram of black condensate*)*.

Compound	**Assay**			
	1	**2**	**3**	**4**
Vanillin	0.1367	0.0202	0.0358	0.1114
Syringaldehyde	0.8138	0.1703	0.3703	0.6741
Methyl homovanillate	0.2226	0.0335	0.0739	0.1879
Others / unidentified	1.0970	0.3799	0.9930	0.9883

The gathered results show that this process of extraction (solid-liquid extraction with aqueous solution of sodium benzoate followed by a liquid-liquid extraction with DCM) is selective for the extraction of vanillin and derivatives. However, the concentration of each compound varies the same way between the assays indicating that the conditions of solid-liquid extraction have a similar influence on the extraction of all compounds.

The analysis of the results presented in table 12 suggests that the solid-liquid ratio is the variable with less influence on the extraction, since the assay with lower solid-liquid ratio (assay 4) has a lower decrease on the concentration of all extracted compounds when compared with the assay at the same conditions, but with twice solid-liquid ratio (assay 1). The other two variables, time of contact and concentration of sodium benzoate, have a visible influence on extraction, once the concentration of extracted compounds has a significant decrease when compared with the assay 1. It was also found that the variable with higher influence on vanillin extraction is the concentration of sodium benzoate.

Table 12: Concentration of vanillin (grams of compound *per* kilograms of extract*)* on sample BC1 after soxhlet extraction with DCM, And on assays 1 to 4 after solid-liquid extraction with aqueous solutions of sodium benzoate at 353 K followed by a liquid-liquid extraction with DCM.

		[Compound] g.kg^{-1}	
		Vanillin	**Syringaldehyde**
DCM soxhlet extraction		28.7098	11.4122
Assay	1	10.0524	59.8319
	2	1.6857	14.2146
	3	3.9555	40.9279
	4	6.4441	39.0027

One interesting aspect of these results is the extraction of syringaldehyde (precursor of the synthesis of vanillin and one of the compounds present in vanilla extracts), since this is one of the major compounds present on the extracts. The combined extraction of vanillin and syringaldehyde from black condensate can be an interesting feature since both have commercial interest.

The assay with the best yield for vanillin and syringaldehyde extraction (table 12) was assay 1, carried out with an aqueous solution of 20 wt % of sodium benzoate, 90 minutes of contact time with black condensate particles, a solid-liquid of 0.1, at 353 K and at 250 rpm.

4.4 Conclusions

In this chapter the extraction of vanillin from black condensate using aqueous solutions of sodium benzoate was studied. It can be concluded that this process is selective for vanillin and derivatives and has interesting yields of extraction. On the other hand, it was verified that this is a good process to extract other related compounds, namely syringaldehyde.

To optimize the extraction of vanillin, three variables were studied (sodium benzoate concentration, contact time and solid-liquid ratio). The most important parameter is the sodium benzoate concentration because the change of this variable varies significantly the concentration of extraction products at the final of extraction.

5. Extraction of vanillin using ABS

5.1 Introduction

In this section aqueous biphasic systems (ABS) composed of sugars and acetonitrile (ACN) were studied as model liquid-liquid extraction techniques. These systems can be valuable to extract vanillin from a complex extract such as black condensate.

Aqueous biphasic systems

Aqueous biphasic systems are liquid–liquid extraction methods based on two aqueous phases formed by mixing two different water-soluble polymers, such as polyethylene glycol/dextran,[57] or one water-soluble polymer and a salt such as polyethylene glycol/potassium phosphate.[61] After mixing, the phase separation is accomplished either by settling under gravity or by centrifugation. Each phase contains predominantly water and one of the polymers or salt.[62] ABS as extractive techniques were first introduced by Albertsson in 1958.[63] ABS are a simple and benign technique because more than 80% of the phases is water which means that biomolecules are not easily denatured on these systems. Other advantages are a good combination between the recovery and purification steps,[64, 65] due to the rapid mass transfer and selective separation, and an easy scale up.[57] Furthermore, at an industrial level, ABS do not present major problems because engineering and existing equipment are easily adapted to the requirements of the technique.

ABS have been shown to be an efficient alternative, and a clean approach, for the separation and purification of a broad array of biomolecules through their partitioning between the two aqueous liquid phases.[57, 62] Therefore, ABS have applications in the field of biotechnology for the separation and purification of biological materials, such as plant and animal cells, microorganisms, viruses, membranes, proteins, nucleic acids, enzymes and other added value biomolecules, such as vanillin.[62]

The selection of ABS depends on the type of biomolecule and economic considerations, because the partitioning of a biomolecule in ABS can vary depending on several factors, such as the biomolecule size, surface properties, molecular weight, temperature, pH and net charge.[61] It is also important to take into account the interactions between a biomolecule and the distinct phases, that could involve hydrogen bonds, van der Waals, dispersive and electrostatic-interactions, as well as steric and conformational effects.[65]

For the design of ABS as extraction processes it is required the phase diagrams and respective tie-lines. The phase diagrams are unique under a particular set of conditions such as pH and temperature. They provide information about the concentration of phase forming components required to form a biphasic system and the concentration of the phase components in the top and bottom phases. The binodal curve represents the separation between the miscible

and immiscible region. Below the binodal curve it is the one phase whereas for concentrations above the curve two immiscible aqueous phases are formed.

ABS of sugars and acetonitrile

The literature has devoted little attention to the use of carbohydrates as substituents of inorganic salts and polymers in ABS and on their ability to improve the routes of extraction and purification of biomolecules. The use of sugars in the formulation of ABS was addressed by Wang et al.[66] in combination with acetonitrile, as well as by Wu et al.[67] and Freire et al.[68] in combination with ionic liquids.

Acetonitrile (CH_3CN) is a colorless aprotic solvent, which is fully miscible in water at temperatures close to room temperature. The acetonitrile molecules do not strongly interact with themselves and tend to form a hydrogen bond network with water molecules.[69] Acetonitrile is an important chemical widely used in the perfumes, rubber products, pesticides, or used to synthesize pharmaceuticals. It is also applied as a solvent to extract fatty acids from animal and vegetable oils.[70]

Carbohydrates, with the general formula $(CH_2O)_x$, are a large and diverse group of organic compounds. These molecules are non-charged, biodegradable, nontoxic, and a renewable feedstock. They are classified into monosaccharides, oligosaccharides (2–10 monossacharides linked, *e.g.* sucrose = glucose + fructose) and polysaccharides (> 10 monosaccharides linked, e.g. starch).[71] Carbohydrates are polyhydroxy aldehydes or ketones with a high affinity for water since several –OH groups, with a dual donor/acceptor character, can be involved in hydrogen bonding, and thus, present an inherent salting-out aptitude(also known as sugaring-out effect). Therefore, carbohydrates are potential substitutes to conventional salts used in the formation of ABS.

This work is focused on the study of the impact of the structure of various carbohydrates: monosaccharides (glucose, mannose, galactose, xylose, arabinose and fructose), disaccharides (sucrose and mannose) and those sold commercially and used in food industries (sucrose, fructose and glucose), on the formation of ABS using acetonitrile at 298.15 K and the extractive potential of these systems in the extraction of vanillin was evaluated.

5.2 Experimental section

5.2.1 Materials

The ABS studied in this work were formed by several carbohydrates and acetonitrile. The carbohydrates used were Sucrose > 99.5 wt % pure, was supplied from Himedia, D-(+)-Maltose ≥ 98.0 wt % pure, was supplied from Sigma, D-(+)-Glucose > 99.5 wt % pure, was supplied from Scharlau, D-(+)-Mannose > 99.0 wt % pure, was supplied from Aldrich, D-(+)-Galactose > 98.0 wt % pure, was supplied from GPR Rectapur, D-(+)-Xylose ≥ 99.0 wt % pure, was supplied from Carlo Erba and L-(+)-Arabinose > 99.0 wt % pure, was supplied from BHD Biochemicals, D-(-)-Fructose > 98.0 wt % pure , was supplied from Panreac. The acetonitrile, HPLC grade with a purity of 99.9 wt %, was purchased from Sigma. The vanillin > 99 wt % pure, was supplied by Aldrich. Commercial fructose, sucrose and glucose are of food grade and were obtained in a local supermarket at Aracaju, Sergipe, Brazil. Distilled and deionized water was used in all experiments.

5.2.2 Methods

Phase diagrams

The studied systems comprised acetonitrile and different carbohydrates, and which can be divided in monosaccharides (D-(+)-glucose, D-(+)-mannose, D-(+)-galactose, D-(+)- xylose, L-(+)-arabinose and D-(-)-frutose) and disaccharides (sucrose and D-(+)-maltose). The ternary phase diagrams were determined at 298 (± 1) K and at atmospheric pressure by the cloud-point titration method. Stock solutions of the carbohydrates (≈ 40 - 70 wt %, depending on the carbohydrate solubility saturation in water) and acetonitrile (≈ 80 wt %) were previously prepared and used for the determination of the phase diagrams. Repetitive drop-wise addition of the carbohydrate solution to the aqueous solution of acetonitrile was carried out until the detection of a cloudy solution, followed by the drop-wise addition of ultra-pure water until the detection of a monophasic region (clear and limpid solution). All these additions were carried out under continuous stirring.

Tie-lines

The tie-lines (TLs) were obtained through the gravimetric method originally described by Merchuck *et al*..[72] For the calculation of TLs, a mixture at the biphasic region of each ternary system was prepared, vigorously stirred and allowed to reach equilibrium, by the separation of both phases, for a minimum of 18 h, and at 298 (± 1) K. After the equilibration step, both top and bottom phases were separated and weighted using a Metller Toledo AL-204 balance (± 0.0001 g). Each individual TL was determined by application of the lever arm rule, which

describes the relationship between the weight of the top phase and the overall system composition. For that purpose, the binodal curves were correlated using equation 2.

$$Y = A \times \exp\left(BX^{0.5} - CX^{3}\right) \quad (2)$$

where X and Y are the carbohydrate and acetonitrile weight percentages, respectively, and *A*, *B* and *C* are constants obtained by the regression.

The determination of the TLs was then accomplished by solving the following system of four equations (equations 3 to 6 for the four unknown values of Y_T, Y_B, X_T and X_B.

$$Y_T = A \times \exp\left(BX_T^{0.5} - CX_T^{3}\right) \quad (3)$$

$$Y_B = A \times \exp\left(BX_B^{0.5} - CX_B^{3}\right) \quad (4)$$

$$Y_T = \left(Y_M / \alpha\right) - \left(\left(1-\alpha\right)/\alpha\right)Y_B \quad (5)$$

$$X_T = \left(X_M / \alpha\right) - \left(\left(1-\alpha\right) - \alpha\right)X_B \quad (6)$$

where subscripts *M*, *T* and *B* denote, respectively, the initial mixture, and the top and bottom phases. The value of α is the ratio between the mass of the top phase and the total mass of the mixture. The system solution results in the acetonitrile and carbohydrate concentration in the top and bottom phases, and thus, TLs can be simply represented.

The tie-line length (TLL) was determined through the application of equation 7,

$$\mathrm{TLL} = \sqrt{(\mathrm{X_T} - \mathrm{X_B})^2 - (\mathrm{Y_T} - \mathrm{Y_B})^2} \quad (7)$$

Partitioning of Vanillin

The partitioning systems for vanillin were prepared in graduated glass centrifuge tubes weighing the appropriate amounts of carbohydrate, acetonitrile and an aqueous solution containing vanillin. Vanillin was at a concentration of 0.4 $g.L^{-1}$ in the initial aqueous solution. After the complete mixing of all components, each system was centrifuged at 3.000 x *g* for 10 minutes to favor the phase separation, and then each tube was placed in thermostatic bath at 298 (± 1) K for at least 18 h. The volume of each phase was initially measured. After, both phases were carefully separated for the quantification of vanillin and for the determination of their density, viscosity and pH values.

The density and viscosity of the bottom phase (carbohydrate-rich) were determined in the temperature range from 298 to 323 K, and at atmospheric pressure, using an automated SVM 300 Anton Paar rotational Stabinger viscosimeter-densimeter. The pH values (± 0.02) of the top and bottom phases were measure at 298 K using a HI 9321 Microprocessor pH meter (HANNA Instruments).

The concentration of vanillin at each aqueous phase was quantified through UV-spectroscopy, using a Varian Cary 50 Bio UV-Vis spectrophotometer, and at a wavelength of 280 nm making use of a calibration curve previously established.

The concentration of vanillin was determined taking into account the concentration of the antioxidant in each phase and according to equation 8,

$$K_{van} = \frac{C_T}{C_B} \tag{8}$$

where K_{van} is the partition coefficient of vanillin, *C* represents the vanillin concentration, and the subscripts *T* and *B* denote the top (acetonitrile-rich) and bottom (carbohydrate-rich) phases, respectively. The recoveries of vanillin (R_T) for the top phase was evaluated using the equation 9:

$$R_T = \frac{C_T}{\left(C_T + C_B\right)} \times 100 \tag{9}$$

where *C* and the subscripts *T* and *B* are described above.

5.3 **Results and Discussion**

Phase diagram and tie lines

The systems investigated in this work are formed by acetonitrile and a large array of carbohydrates. The molecular structures of the studied carbohydrates are depicted in figure 18.

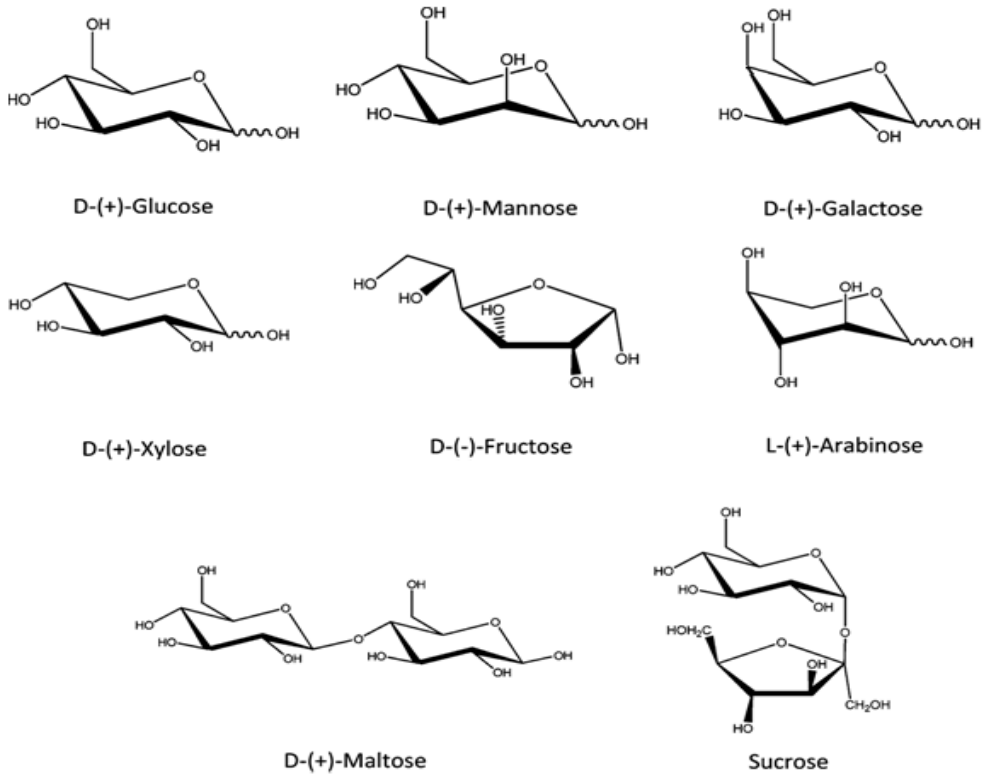

Figure 18: Chemical structure of the monosaccharides and disaccharides studied.

The experimental phase diagrams for each monosaccharide (D-(+)-glucose, D-(+)-mannose, D-(+)-galactose, D-(+)-xylose, L-(+)-arabinose and D-(-)-fructose), disaccharide (sucrose and D-(+)-maltose) and commercial carbohydrate (glucose, fructose and sucrose), were determined at 298 K and atmospheric pressure. The corresponding phase diagrams are presented in figures 19 to 21 and allow analysis of the carbohydrate potencial to induce an ABS. All binodal curves are represented in molality units to avoid disparities in the evaluation of the carbohydrate potential in inducing the liquid-liquid demixing and which could simple result from their distinct molecular weights. The experimental weight fraction data are provided in appendix C, tables C.1 to C.4.

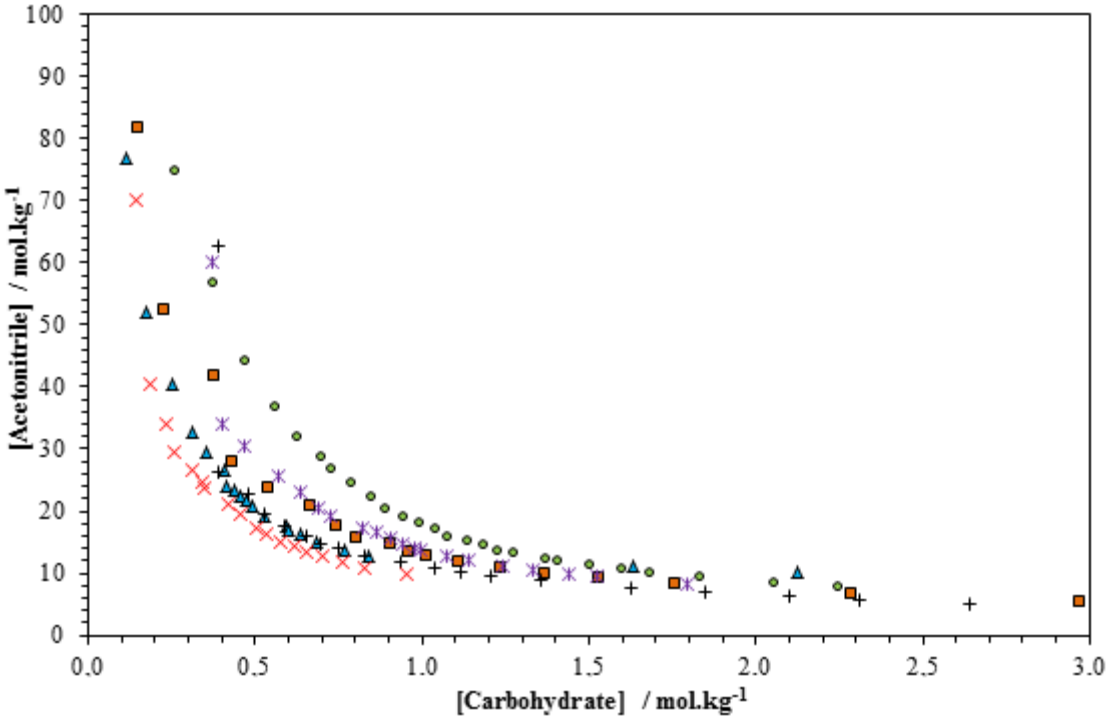

Figure 19: Phase diagrams for the ternary systems composed by acetonitrile + carbohydrate + water at 298 K. ■ D-(-)-Fructose, ▲ D-(+)-Glucose, ● D-(+)-Xylose, × D-(+)-Galactose, ✳ L-(+)-Arabinose, + D-(+)-Mannose.

The addition of a concentrated carbohydrate aqueous solution to acetonitrile leads to phase separation: a top acetonitrile-rich phase and a bottom carbohydrate-rich phase. According to Galema *et al.*[73] the hydration of carbohydrate depends on the ratio between axial and equatorial hydroxyl groups. Thus, the carbohydrates can be classified into three groups of decreasing hydration: (a) both OH(2) and OH(4) are axial ; (b) OH(4) is equatorial and OH(2) is either axial (D-(+)-mannose) or equatorial (D-(+)-glucose); (c) OH(4) is axial and OH(2) is equatorial (D-(+)-galactose). The binodal curves for the systems with acetonitrile, and the various monosaccharidies, and depicted in figure19, show indeed an increasing tendency of phase separation proportional to their hydration ability: D-(+)-xylose < L-(+)-arabinose ≈ D-(-)-fructose < D-(+)-glucose < D-(+)-mannose < D-(+)-galactose.

Aldoses with 5 carbon atoms, such as D-(-)-fructose, are less effective in promoting ABS formation, due to the lower number of hydroxyl groups and, consequently, a lower hydration ability and less favorable conformation for hydrogen bounding with water.

The comparison between the isomers D-(+)-glucose (an aldose with a 6-sided ring) and D-(-)-fructose (a ketose with a 5-sided ring) suggests that ketoses are less effective in inducing the formation of two aqueous phases. Epimers of aldoses with 6 carbon atoms, which are distinguished by the position of the hydroxyl group at carbon 2, epimers D-(+)-glucose and D-(+)-mannose, have similar abilities to induce ABS formation. However, the position of the hydroxyl group at carbon 4, epimers D-(+)-glucose and D-(+)-galactose, facilitates the phase

formation with D-(+)-galactose. Therefore, the orientation of the hydroxyl at carbon 4 plays an important role in the ABS formation ability.

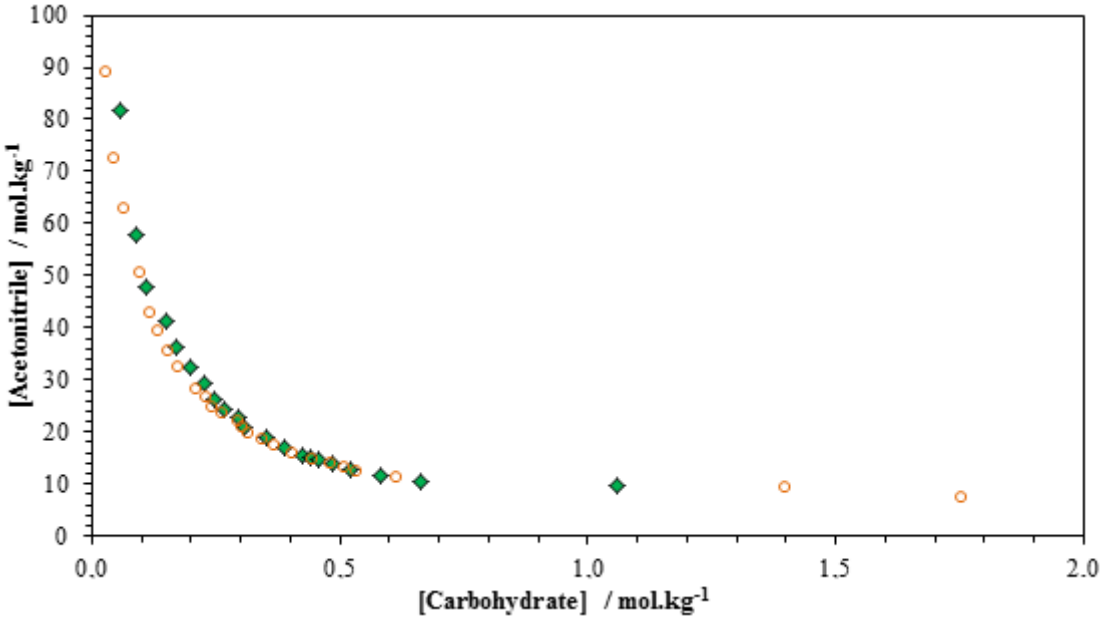

Figure 20: Phase diagrams for the ternary systems composed by acetonitrile + carbohydrate + water at 298 K. ◆ Sucrose; ○ D-(+)-Maltose.

The phase diagrams shown in figure 20 display the effect of disaccharides through the formation of ABS. Sucrose consists of glucose and fructose linked by a glycosidic bond while maltose is formed by two glucose units. These disaccharides have similar capabilities for ABS formation in a system with acetonitrile at 298 K.

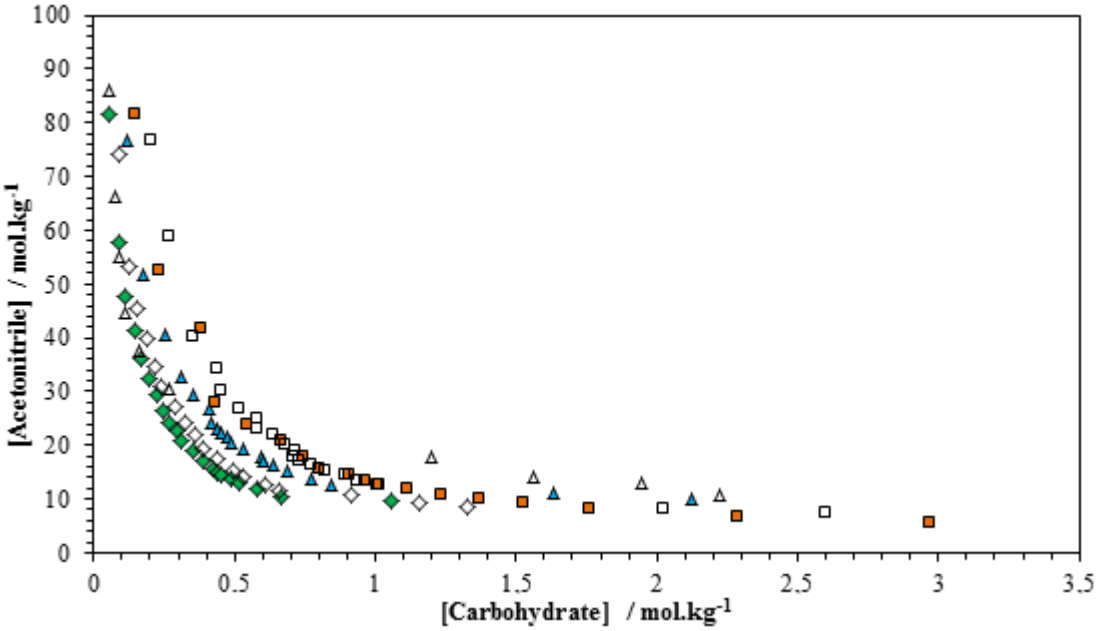

Figure 21: Phase diagrams for the ternary systems composed by acetonitrile + carbohydrate + water at 298 K. ◆ Sucrose; ◇ Commercial sucrose, ■ D-(-)-Fructose; □ Commercial fructose, ▲ D-(+)-Glucose, △ Commercial glucose.

Figure 21 shows the comparison between the high purity and commercial forms of sucrose, glucose and fructose towards the ABS formation. The binodal curves show a decreasing order in inducing ABS according to: sucrose > commercial sucrose > glucose > fructose ≈ commercial fructose > commercial glucose. The use of commercial carbohydrates leads to a decrease of the biphasic region envelope which may be a result of a low purity level and to the presence of impurities. The difference was more pronounced when using commercial glucose (corn syrup, glucose) due to the presence of other sugars such as isomaltose, maltose and maltotriose and as already pointed out by Pontoh and Low.[74]

All the binodal curves were fitted using equation 2. The regression coefficients (R^2) and the fitted parameters *A*, *B* and *C*, estimated by least-squares regression, are reported in table 13.

Table 13: Adjusted parameters (± 10^{-4}) obtained from the regression of Merchuck equation for ternary system acetonitrile + carbohydrate at 298 K and atmospheric pressure.

Carbohydrate	Regression Parameters			
	A	**B**	**C**	**R^2**
Sucrose	114.5 ± 2.2	-0.280 ± 0.008	$2.8 \times 10^{-5} \pm 5.4 \times 10^{-6}$	0.9964
D-(+)-Maltose	102.0 ± 1.3	-0.245 ± 0.006	$3.8 \times 10^{-5} \pm 5.6 \times 10^{-6}$	0.9962
D-(+)-Glucose	122.6 ± 2.7	-0.332 ± 0.011	$4.4 \times 10^{-5} \pm 1.3 \times 10^{-5}$	0.9962
D-(+)-Mannose	127.6 ± 5.8	-0.356 ± 0.014	$2.8 \times 10^{-16} \pm 1.7 \times 10^{-6}$	0.9954
D-(+)-Galactose	123.3 ± 3.0	-0.375 ± 0.011	$1.1 \times 10^{-5} \pm 9.0 \times 10^{-6}$	0.9978
D-(-)-Fructose	134.6 ± 2.2	-0.342 ± 0.006	$7{,}1 \times 10^{-16} \pm 1.1 \times 10^{-6}$	0.9978
D-(+)-Xylose	177.7 ± 6.2	-0.394 ± 0.012	$3.4 \times 10^{-6} \pm 3.2 \times 10^{-6}$	0.9960
L-(+)-Arabinose	151.6 ± 5.6	-0.393 ± 0.006	$4.1 \times 10^{-7} \pm 4.1 \times 10^{-6}$	0.9965

Figure 22 presents four examples of the correlation of the data for the systems composed of acetonitrile and carbohydrate (D-(-)-fructose, sucrose, L-(+)-Arabinose or D-(+)-galactose) and water. The results for remaining systems are presented in appendix C, figure C.1 to C.4. To complete the phase diagrams, several TLs and respective TLLs were further calculated, and their values are reported in table C.5 of appendix C. Some examples of the TLLs representation are shown in figure 22.

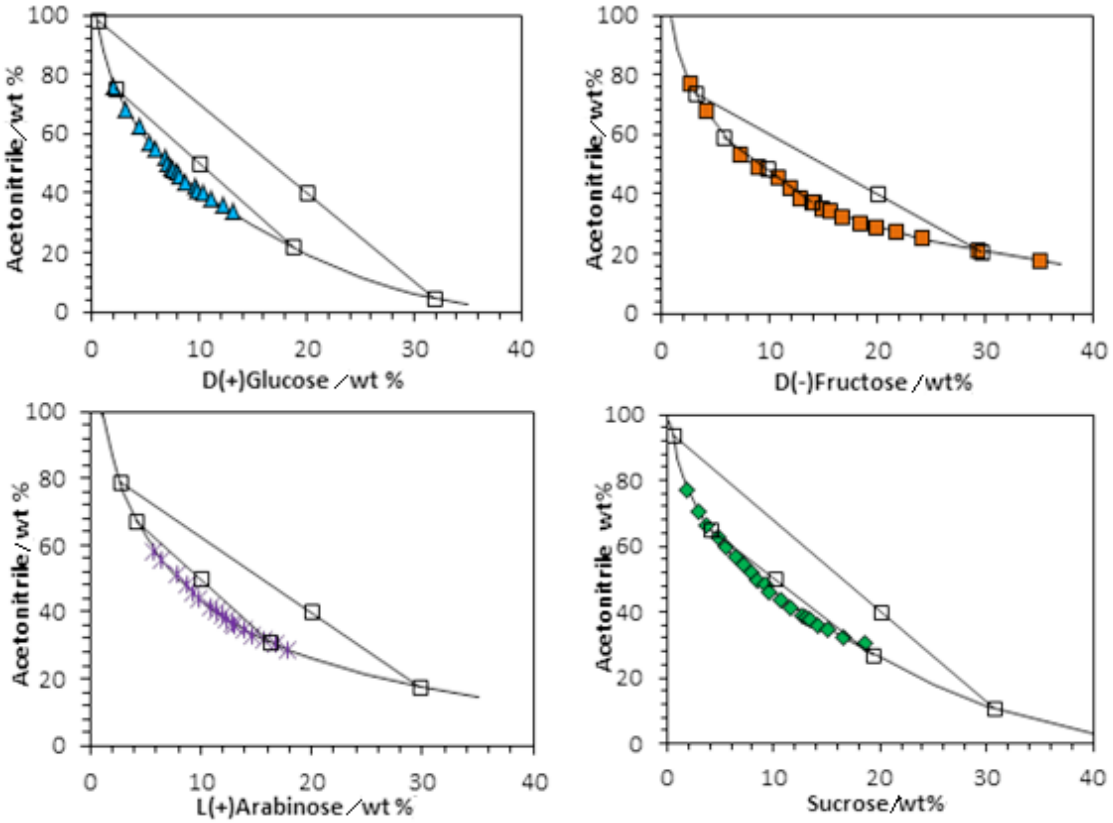

Figure 22: Phase diagrams for the ternary systems composed by acetonitrile + carbohydrate at 298.15 K (▲ D-(+)-Glucose, ■ D(-)Fructose, ✳ D-(+)-Arabinose and ◆ Sucrose), □ TL data, (—) binodal adjusted data through equation 01.

The application of ABS in industrial processes for biomolecules extraction and purification also depends on their physical properties. Particularly, large differences in the densities of both phases favor the phase separation whereas low viscosities increase the mass transfer coefficients. Hence, the characterization of the densities and viscosities for the sugar-rich phase were here determined. It should be remarked that acetonitrile, at 298 K, presents a density of 0.7766 g.cm^3 and a viscosity of 0.3369 mPa.s.[75] These values are below the values of pure water at the same temperature (0.9991 g.cm^3 and 1.0 mPa.s)[76] and thus the properties of the acetonitrile-rich phase were not determined due to a lack of proper equipment to measure densities and viscosities within this range. Furthermore, the sugar composition (the more dense and viscous compound) in the acetonitrile-rich phase is always below 7 wt % (tables C.1 to C.4, appendix C).

Table 14: Experimental value of densities (ρ) and viscosities (μ) of bottom phase of acetonitrile and carbohydrate based aqueous two-phase systems at 298.15 and 323.15 K.

Carbohydrate	System	ρ / g.cm^{-3}		μ / mPa.s^{-1}	
		298 K	323 K	298 K	323 K
Sucrose	A	1.0984	1.0808	3.5606	1.8145
	B	1.0535	1.0343	2.2117	1.1956
D-(+)-Maltose	A	1.0968	1.0793	3.5977	1.8345
	B	1.0678	1.0495	2.5711	1.3809
D-(+)-Glucose	A	1.0991	1.0825	3.2582	1.6831
	B	1.0358	1.0173	1.8355	1.0510
D-(+)-Mannose	A	1.0990	1.0813	3.0513	1.6009
	B	1.0314	1.0121	1.7401	0.9794
D-(+)-Galactose	B	1.0429	1.0243	1.8812	1.0643
	C	1.0004	0,9797	1.3995	0.8233
D-(+)-Xylose	A	1.0738	1.0550	2.5039	1.3760
	B	1.0091	0.9872	1.4637	0.8606
L-(+)-Arabinose	A	1.0919	1.0729	2.6091	1.4102
	B	1.0288	1.0083	1.6412	0.9372
D-(-)-Frutose	A	1.0977	1.0782	2.8406	1.4917
	B	1.0228	1.0021	1.6096	0.9216

For the carbohydrate-rich phase the densities range from 1.0004 g.cm^3 (galactose) to 1.0991 g.cm^3 (glucose) whereas the viscosities are between 1.3995 mPa.s (galactose) and 3.5977 mPa.s (maltose). The densities and viscosities at 298 K and 323 K for the carbohydrate-rich phase of different systems are presented in table 14. These values are significantly lower than the viscosities obtained for ABS constituted by polymers such as polypropylene glycol (polymer-rich phase: 18.1 – 64.7 mPa.s and Na_2SO_4 –rich phase: 1.91 – 3.73 mPa.s)[77] or ionic liquids (ionic liquid-rich phase: 8.0 – 1.03 mPa.s) ABS.[56] The low viscosity of acetonitrile-carbohydrate ABS favor thus the mass transfer on extraction processes as well as the phases handling if a scale-up is needed.

Partitioning of vanillin

The application of the investigated systems as alternative extractive techniques was studied with the partitioning of vanillin. The vanillin was chosen as a model biomolecule because it is widely used as a flavoring agent.[33] For each system, two different compositions were investigated: 20 wt % carbohydrate + 40 wt % acetonitrile and 10 wt % carbohydrate + 50 wt % acetonitrile. The pH values of both phases of each ABS are presented in table C.6, appendix C. These values range between 5.48 and 7.06. Therefore, vanillin is mainly present as

a neutral molecule under these conditions.[78] The influence of the pH in the chemical structure of vanillin is shown in figure C.5 of appendix C.

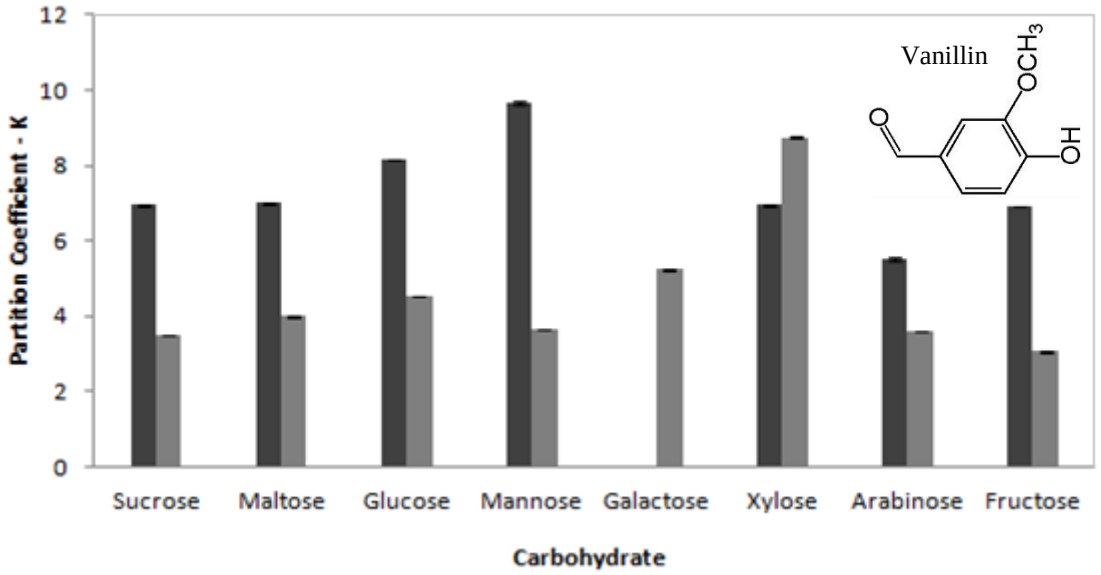

Figure 23: Partition coefficient of vanillin between the acetonitrile and the carbohydrate-rich phase at 298 K. ■ system 40-20 wt % acetonitrile-carbohydrate and ■ system 50-10 wt % acetonitrile-carbohydrate.

For all the systems the partition coefficients of vanillin are higher than 1 and demonstrate the preferential affinity of vanillin towards the acetonitrile-rich phase (figure 23). This preferential migration is in good agreement with the octanol-water partition coefficient of vanillin (log K_{ow} = 1.19),[39] which indicates the preferential affinity of vanillin for more hydrophobic phases. Acetonitrile (log K_{ow} = -0.17) is indeed more hydrophobic than carbohydrates (-2.30 < log K_{ow} < -4.70) and support the trend observed.

The effect of system composition, namely the TLL, on the extraction ability was studied by changing the point of initial mixture, acetonitrile-carbohydrate, from 40 - 20 wt % to 50 - 10 wt %. The composition of each phase is described in table 15. A large decrease in the partition coefficient was observed with the system composed of mannose (K_{van} = (9.67 ± 0.04) and (3.66 ± 0.01)) with decrease in TLL. An opposite pattern was verified with the system constituted by xylose and for which the partition coefficient increases from (6.95 ± 0.01) to (8.74 ± 0.03) with a decrease in the TLL. It should be remarked that Gu and Zhang[79] studied the partitioning of various biomolecules in system composed of acetonitrile and water at sub-zero temperatures (263 K). Most compounds preferentially partitioned for the water-rich phase[79] contrarily to what was observed here.

Table 15: Weight fraction compositions (TLs) at the top (*T*) and bottom (*B*) phases, initial mixture composition (*M*), and respective TLLs for the several systems composed of acetonitrile (*Y*) and carbohydrate (*X*) at 298 K and atmospheric pressure.

Carbohydrate	100 x weight fraction / wt %						
	Y_M	X_M	Y_T	X_T	Y_B	X_B	TLL
Sucrose	39.97	20.07	93.91	0.50	10.71	30.68	88.51
	49.94	10.14	64.86	4.09	27.15	19.38	40.69
D-(+)-Maltose	40.05	19.96	92.15	0.17	6.59	32.66	91.52
	49.95	10.02	65.24	3.31	18.13	23.97	51.44
D-(+)-Glucose	39.98	19.99	98.37	0.44	4.56	31.86	58.98
	49.98	10.00	75.42	2.14	22.09	18.61	55.81
D-(+)-Mannose	40.04	20.00	77.14	1.99	17.62	30.88	66.15
	49.92	9.98	69.29	2.93	28.34	17.84	43.58
D-(+)-Galactose	49.99	10.01	73.92	1.87	21.46	19.73	55.42
	45.00	8.01	32.02	12.54	54.21	4.80	23.50
D-(-)-Fructose	40.03	20.03	73.66	3.11	20.96	29.62	58.98
	48.65	9.76	59.28	5.76	37.61	13.92	23.15
D-(+)-Xylose	39.95	20.05	79.31	4.20	20.77	27.78	63.11
	49.99	10.02	50.06	10.26	50.06	10.26	0.00
L-(+)-Arabinose	39.97	19.91	79.15	2.73	17.59	29.72	67.21
	50.01	10.00	67.51	4.24	30.97	16.28	38.47

The K_{van} rank at different mixtures is similar to the order of formation of ABS previously noted. For instance, for the mixture composition constituted by 20 wt % of carbohydrate and 40 wt % of acetonitrile, the order of partition coefficients is according to,

Aldoses with 6C: D-(+)-glucose < D-(+)-mannose

Aldoses with 5C: L-(+)-arabinose < D-(+)-xylose

Monossacharides: Aldoses with 5C ≈ D-(-)-fructose (Ketose) < Aldose with 6C

Dissacharides: Sacarose ≈ D-(+)-maltose

In addition, for the mixture point composed of 10 wt % of carbohydrate and 50 wt % of acetonitrile, the partition coefficient values increase according to,

Aldoses with 6C: D-(+)-mannose < D-(+)-glucose < D-(+)-galactose

Aldoses with 5C: L-(+)-arabinose < D-(+)-xylose

Monossacharides: Aldoses with 5C ≈ D-(-)-fructose (Ketose) < Aldose with 6C

Dissacharides: Sacarose < D-(+)-maltose

All the results indicate that the hydration capacity of the carbohydrate leads to an exclusion effect through the biomolecule towards the acetonitrile-rich phase and confirms the *sugaring-out* effects reported by other authors.[66, 80] In addition, for aldoses with 6 carbons atoms, the order is inversely proportional to the dielectric constant of each carbohydrate: D-(+)-mannose (4.25) ≈ D-(+)-glucose (4.27) > D-(+)-galactose (3.28).[81]

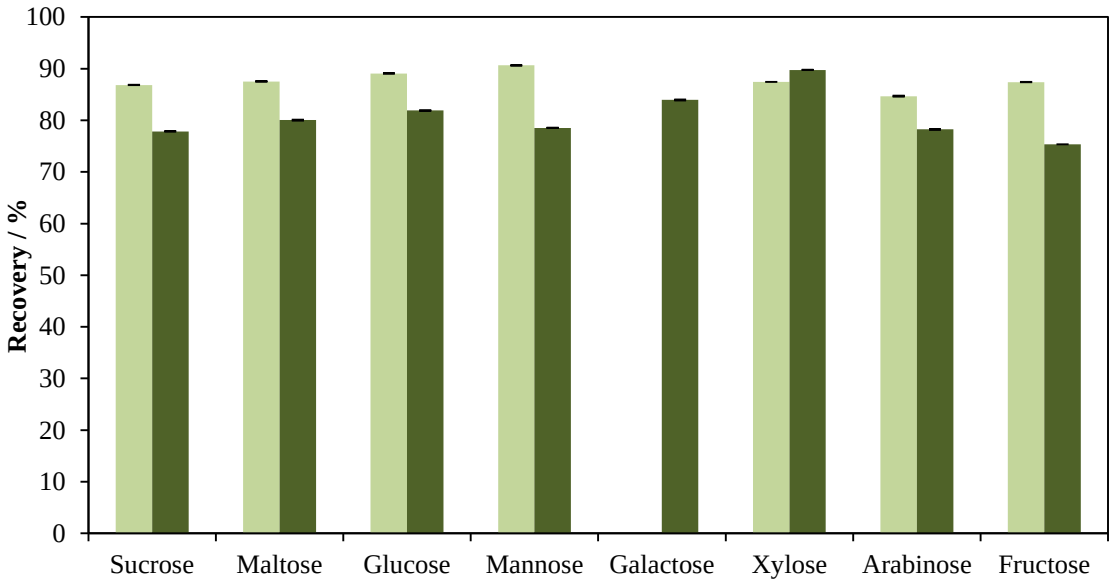

Figure 24: Recovery of vanillin on the top phase for systems acetonitrile + carbohydrate at 298 K. ■ system 40 - 20 wt % acetonitrile-carbohydrate and ■ system 50 - 10 wt % acetonitrile-carbohydrate.

Based on the quantification of vanillin and on the volume of each phase, the recoveries of vanillin for the acetonitrile-rich phase were also determined and are presented in figure 24. As observed with the partition coefficients, the recovery values indicate a preferential migration of vanillin for the acetonitrile-rich phase. The recovery of vanillin ranges between (75.35 ± 0.06)%, with the system formed by acetonitrile and fructose and (90.63 ± 0.06)%, with the system formed by acetonitrile and mannose. In general, high recovery efficiencies are attained in a single step procedure.

5.4 Conclusions

This study reports novel ATPS formed by acetonitrile and a large array of carbohydrates (monosaccharides and disaccharides). The ternary phase diagrams, tie-lines and tie-line lengths were determined at 298 K and at atmospheric pressure. Based on the phase diagrams behavior it was shown that the ATPS formation is a main result of the hydration capacity of each sugar. Besides high purity carbohydrates, commercial food grade sugars were also investigated and shown to be less able to form ATPS.

To explore the applicability of the investigated systems, the partitioning of vanillin was conducted in several ATPS and at two different mixture compositions. In all the extraction essays vanillin preferentially migrated for the acetonitrile-rich phase. The trend on the partition coefficients is also dependent on the hydration capacity of each carbohydrate. The recovery of vanillin in the acetonitrile-rich phase ranged between 73 and 95% in a single step procedure.

6. Final remarks

6.1 Conclusions

From the perspective of an integrated biorefinery, black condensate from the Amorim cork industry, was studied with the purpose of valorizing this residue. It was found that this cork by-product is an interesting source of biomolecules, mainly triterpenes and phenolic compounds. Essentially, vanillin was found as a main constituent of high commercial value.

With the objective of extracting vanillin from gaseous effluent, black condensate were studied as a sample of the condensation of this source. For the extraction of vanillin, the solubility of vanillin in hydrotrope aqueous solutions was studied. In general, the solubility of vanillin increases with the increase on the hydrotrope concentration and with temperature. The best hydrotrope studied was sodium benzoate and because of that it was further used in the direct extraction of vanillin from black condensate. The extraction of vanillin directly from black condensate with sodium benzoate demonstrated to be selective for a specific type of compounds, vanillin and its derivatives.

As a last approach, novel ABS composed of sugars and acetonitrile were studied for the extraction and recovery of vanillin. In all systems vanillin preferential migrated for the acetonitrile-rich phase, and recovery efficiencies higher than 73% were observed in a single-step procedure.

6.2 Future work

In the future, it would be interesting to study more hydrotrope compounds in order to better understand their influence in the solubility of vanillin in aqueous solutions.

It is also important to make a detailed study of the extraction of vanillin from black condensate, using aqueous solutions of sodium benzoate, resorting to a factorial plan 2^3.

Moreover, it is also interesting to study the purification of vanillin extracted from black condensate using the ABS proposed.

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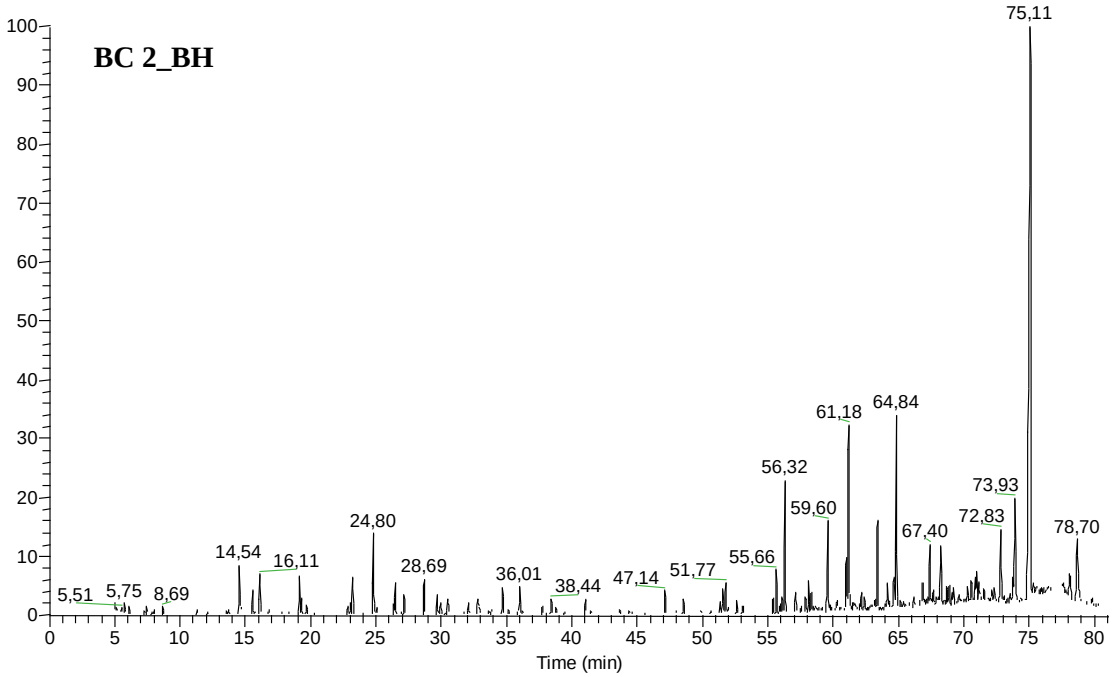
List of Publications

Co-author in:

- Teresa Mourão, Ana Filipa M. Cláudio, Isabel Boal-Palheiros, Mara G. Freire, João A.P. Coutinho, *Evaluation of the impact of phosphate salts on the formation of ionic-liquid-based aqueous biphasic systems*. The Journal of Chemical Thermodynamics, **54** (2012) 398-405.
- Ana Rosa Silva, Teresa Mourão, João Rocha, *Oxidation of cyclohexane by transition-metal complexes with biomimetic ligands*. Catalysis Today (2012). doi:10.1016/j.cattod.2012.07.043.
- Gustavo B. Cardoso, Teresa Mourão, Fernanda M. Pereira, Mara G. Freire, Alini T. Fricks, Cleide M. F. Soares, Álvaro S. Lima, *Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin*. Separation and Purification Technology (2012), doi: 10.1016/j.seppur.2012.11.001.
- Carlos F. C. Marques, Teresa Mourão, Catarina M. S. S. Neves, Álvaro S. Lima, Isabel Boal-Palheiros, João A.P. Coutinho and Mara G. Freire. *Aqueous biphasic systems composed of ionic liquids and sodium carbonate as enhanced routes for the extraction of tetracycline*. Biotechnology Progress (2012), submitted for publication.
- Jorge F. B. Pereira, Teresa Mourão, Luís Paulo N. Rebelo, Robin D. Rogers, Mara G. Freire and João A. P. Coutinho. *A new class of aqueous two-phase systems composed of polymers and biocompatible ionic liquids based on the choline cation,* in preparation.

Appendix

Appendix A


BC 2_BH
75,11
61,18
64,84
56,32
73,93
59,60
72,83
67,40
78,70
24,80
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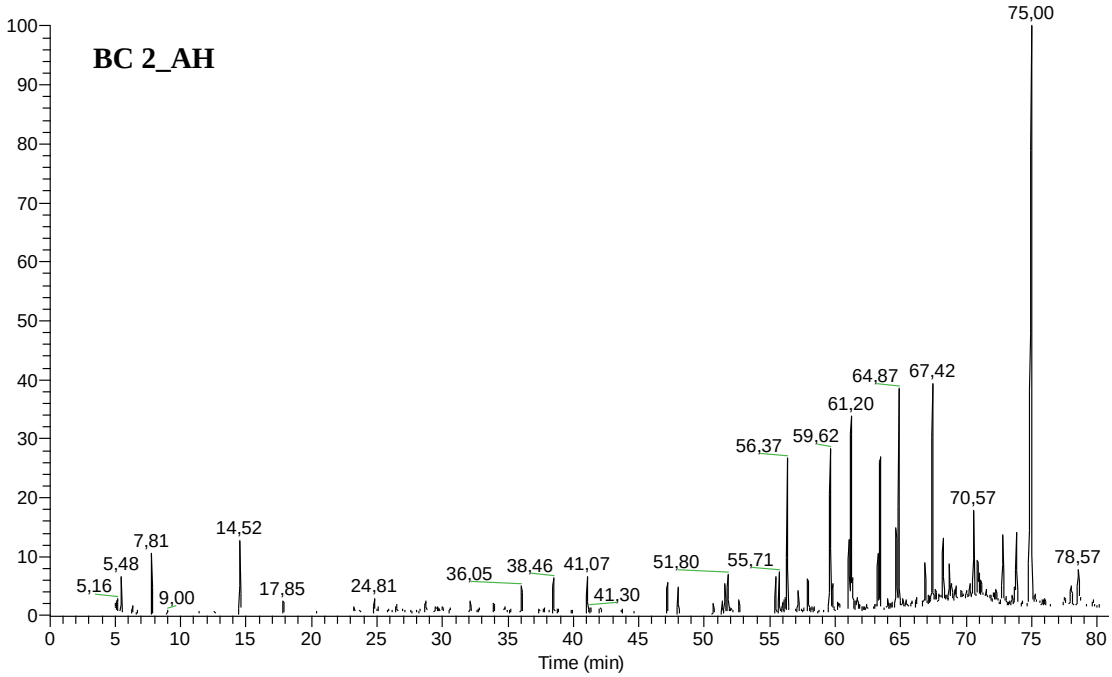
36,01
38,44
47,14
51,77
55,66
5,51
5,75
8,69
Time (min)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64,87
67,42
61,20
56,37
59,62
70,57
14,52
7,81
5,48
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41,07
41,30
51,80
55,71
78,57
Time (min)

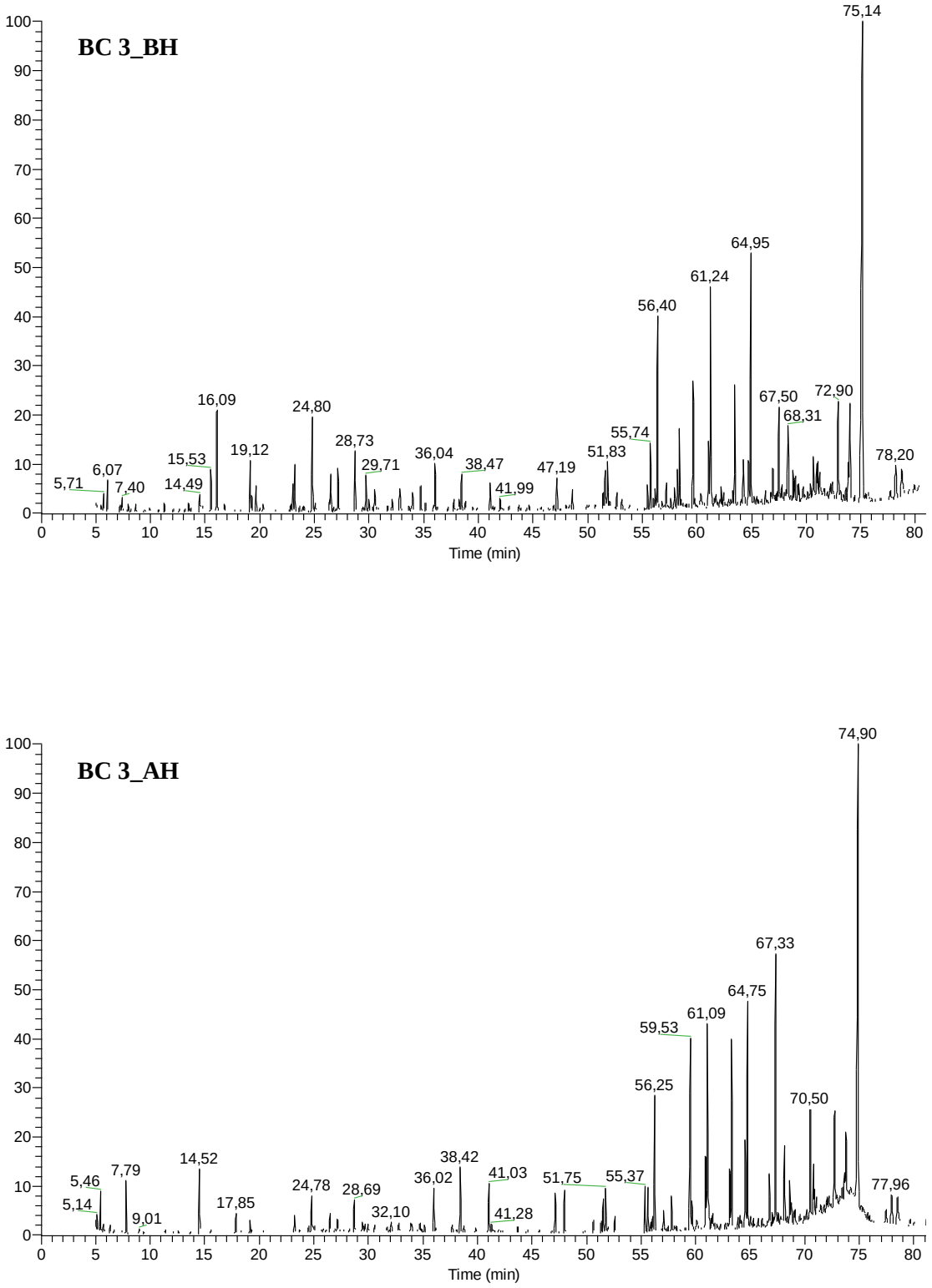
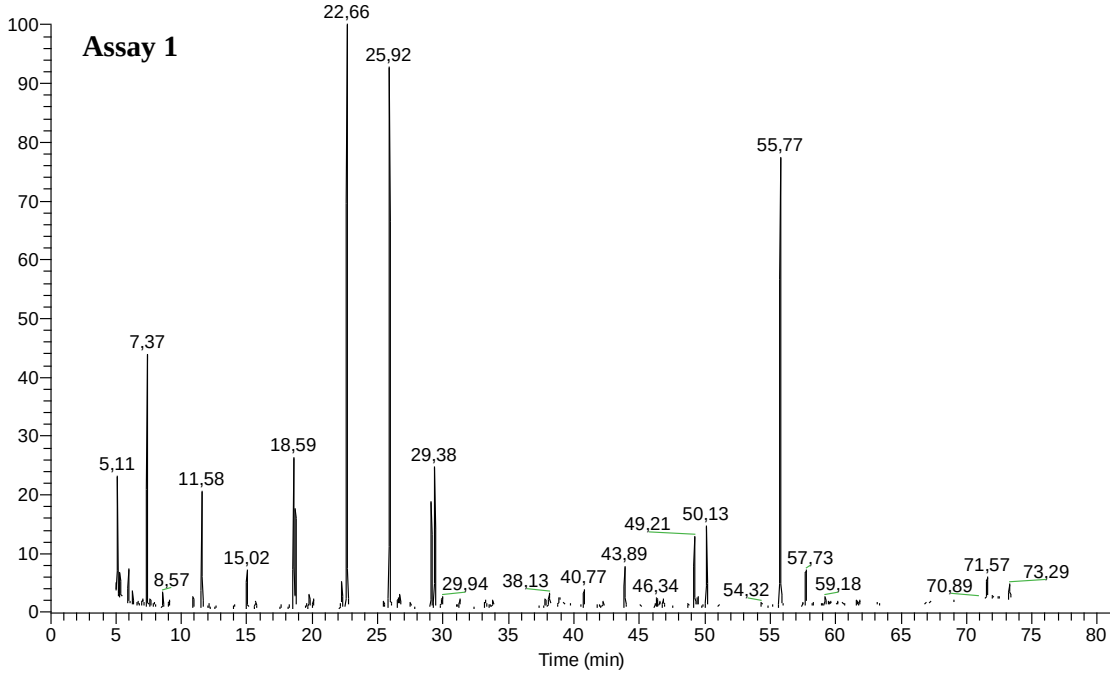


Figure A.1: GC-MS chromatogram of the dichloromethane extracts BC 2, BC 3, both before and after alkaline hydrolysis.

Appendix B


Assay 1
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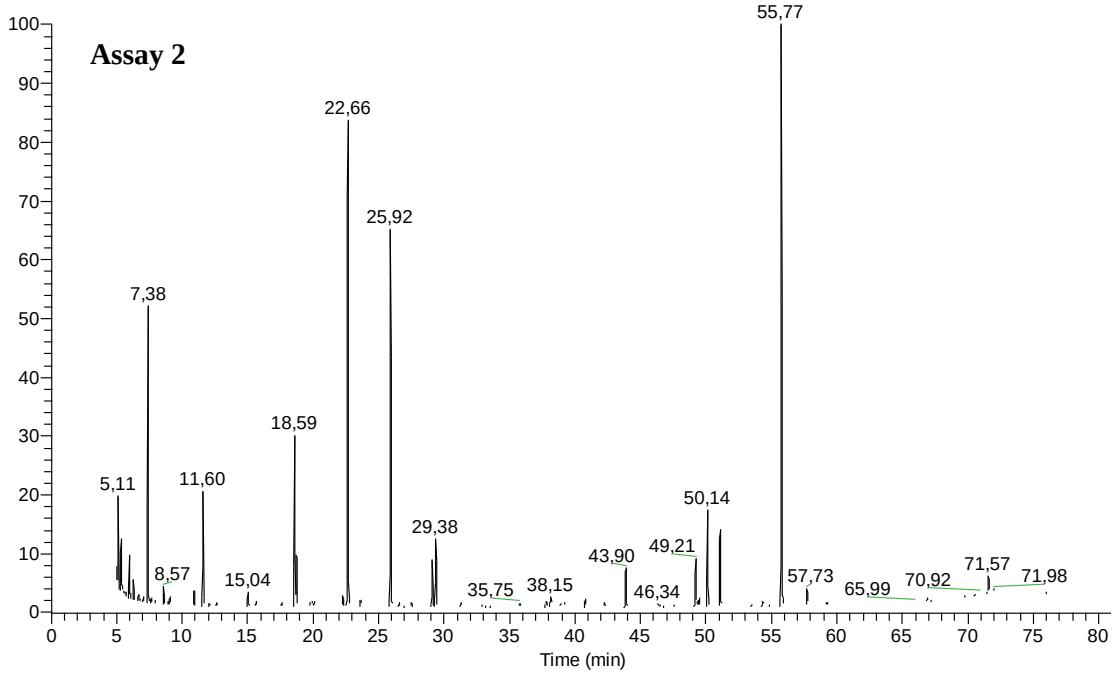
25,92
55,77
7,37
18,59
29,38
5,11
11,58
49,21
50,13
43,89
15,02
57,73
71,57
73,29
8,57
29,94
38,13
40,77
46,34
54,32
59,18
70,89
Time (min)


Assay 2
55,77
22,66
25,92
7,38
18,59
5,11
11,60
50,14
29,38
43,90
49,21
71,57
8,57
15,04
57,73
71,98
35,75
38,15
46,34
65,99
70,92
Time (min)

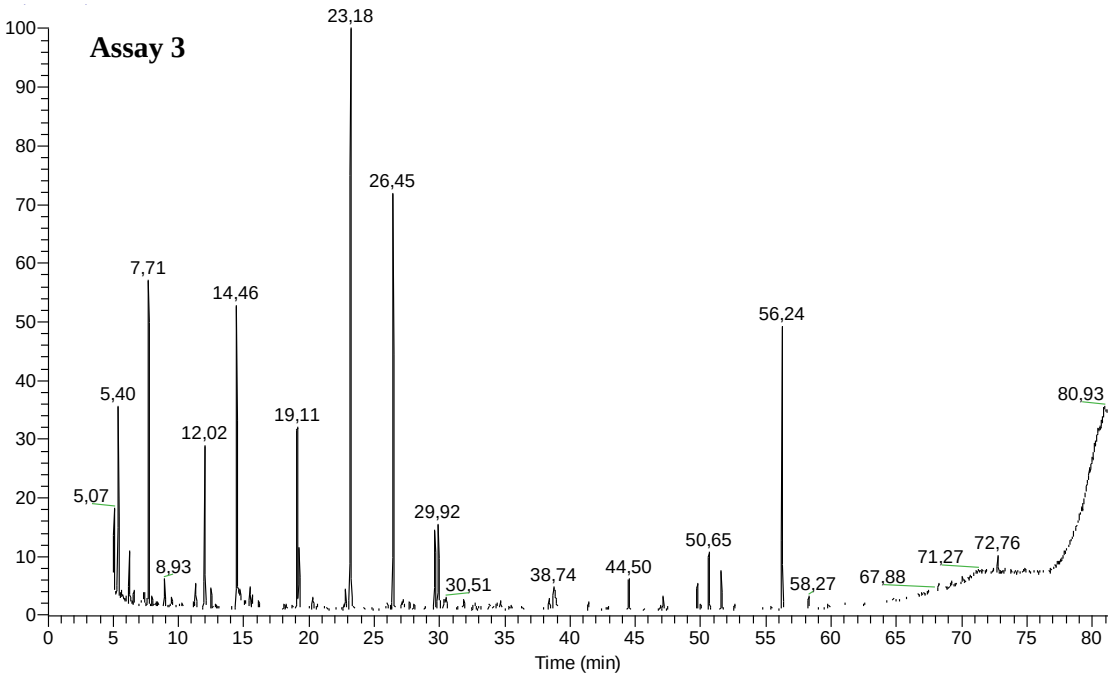


Figure B.1: GC-MS chromatogram of the assays 1, 2, 3, after solid-liquid extraction with aqueous solution of sodium benzoate and liquid-liquid extraction with DCM.

Appendix C

Table C.1. Experimental binodal mass fraction data for the system composed of Acetonitrile (1) + Commercial Sugar (2) + Water (3) at 298 K

Glucose		**Fructose**		**Sucrose**	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
77.955	0.992	75.929	3.565	75.312	2.930
73.117	1.332	70.796	4.587	68.674	4.123
69.413	1.601	62.311	5.985	65.089	5.089
64.753	1.927	58.516	7.274	62.059	6.017
60.610	2.852	55.238	7.578	58.653	6.925
55.732	4.584	52.229	8.510	56.018	7.599
42.308	17.803	50.546	9.507	52.900	8.960
35.050	25.957	48.522	9.519	49.785	9.972
30.650	28.612	47.248	10.300	47.305	10.908
36.676	21.988	45.014	10.963	44.484	11.790
		43.599	11.431	41.952	12.999
		42.194	11.313	38.318	14.561
		41.174	11.636	36.867	15.419
		40.065	12.199	34.267	17.212
		38.777	12.870	32.261	18.409
		37.135	13.868	30.423	23.871
		35.726	14.458	27.963	28.338
		34.102	15.392	25.781	31.236
		25.484	26.725		
		23.501	31.916		

Table C.2. Experimental binodal mass fraction data for the system composed of Acetonitrile (1) + Aldose with 6 carbon atoms (2) + Water (3) at 298 K

D-(+)-Glucose		D-(+)-Mannose		D-(+)-Galactose	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
75.952	2.037	51.956	6.583	62.487	3.269
68.083	3.043	48.270	7.973	58.263	4.061
62.418	4.352	44.567	8.729	54.868	4.461
57.228	5.301	41.834	9.598	52.307	5.282
54.799	5.973	39.811	10.516	50.454	5.816
52.322	6.851	37.683	11.150	49.167	5.938
49.768	6.963	36.625	11.888	46.324	7.030
48.810	7.322	34.530	13.026	44.471	7.564
47.971	7.567	32.636	14.398	41.386	8.350
47.079	7.885	30.837	15.761	40.031	8.749
45.882	8.105	29.327	16.774	38.243	9.449
44.101	8.732	27.856	17.821	36.978	9.987
42.067	9.635	26.561	19.642	35.764	10.520
41.117	9.763	23.620	22.664	34.582	11.242
39.985	10.301	22.110	24.985	32.843	12.081
38.321	10.997	20.200	27.473	31.061	13.009
35.813	12.171	19.129	29.368	28.840	14.641
34.133	13.153	17.564	32.218		

Table C.3. Experimental binodal mass fraction data for the system composed of Acetonitrile (1) + Aldose with 5 carbon atoms (2) + Water (3) at 298 K.

L-(+)-Arabinose		**D-(+)-Xylose**	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**
58.229	5.681	69.999	5.309
55.546	6.552	64.610	6.597
51.164	7.855	60.156	7.746
48.547	8.696	56.955	8.573
45.838	9.363	52.365	9.804
44.123	9.857	50.217	10.544
41.656	10.996	47.913	11.279
40.337	11.509	45.866	11.742
38.932	12.008	44.228	12.376
37.758	12.366	42.857	12.935
36.755	12.812	41.296	13.485
35.885	13.001	39.737	13.895
34.467	13.873	38.693	14.526
33.191	14.591	36.147	15.548
31.389	15.737	35.281	16.056
30.270	16.695	33.211	17.442
28.908	17.774	31.921	18.413
		30.689	19.317
		29.593	20.171
		26.277	23.548

Table C.4. Experimental binodal mass fraction data for the system composed of Acetonitrile (1) + Ketose – D-(-)-Fructose or Disaccharides (2) + Water (3) at 298 K

D-(-)-Fructose		D-(+)-Maltose		Sucrose	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
77.014	2.614	78.541	0.935	77.006	1.872
68.306	3.962	74.816	1.553	70.338	3.028
53.262	7.224	72.090	2.141	66.216	3.663
49.396	8.900	67.466	3.193	62.917	4.866
46.157	10.736	63.792	3.824	59.724	5.505
42.142	11.853	61.734	4.336	57.000	6.383
39.186	12.645	59.387	5.006	54.619	7.155
37.405	14.025	57.168	5.570	52.039	7.837
35.601	14.756	53.783	6.752	49.833	8.404
34.451	15.428	52.310	7.338	48.374	9.123
32.813	16.674	50.501	7.708	46.237	9.576
30.814	18.200	49.086	8.214	43.735	10.733
29.103	19.775	47.287	9.155	41.303	11.693
27.463	21.597	46.097	9.455	39.031	12.733
25.323	24.062	44.889	9.776	38.148	13.060
21.494	29.154	43.329	10.525	37.394	13.473
18.159	34.844	41.764	11.187	36.138	14.234
		39.588	12.195	34.652	15.108
		37.891	13.228	32.513	16.617
		35.132	14.818	30.174	18.503
		34.018	15.436		

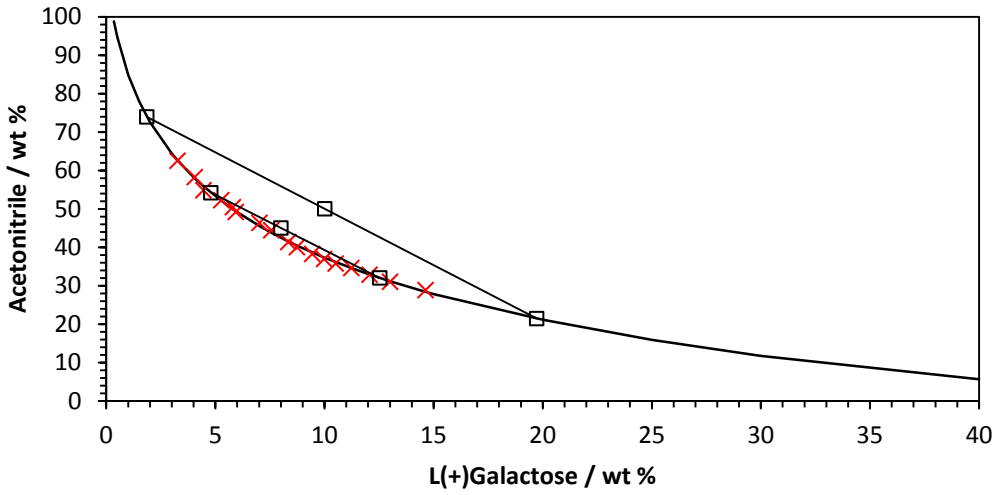


Figure C.1. Phase diagram for the ternary system composed of acetonitrile + L(+)-Galactose at 298 K (×), □- TL data, (—) binodal adjusted data through equation 2.

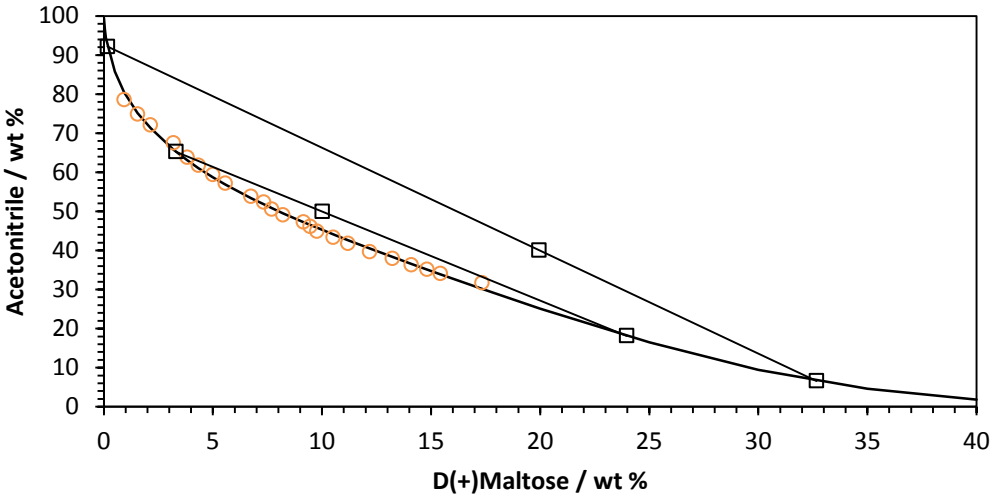


Figure C.2. Phase diagram for the ternary system composed of acetonitrile + D(+)-Maltose at 298 K (○), □- TL data, (—) binodal adjusted data through equation 2.

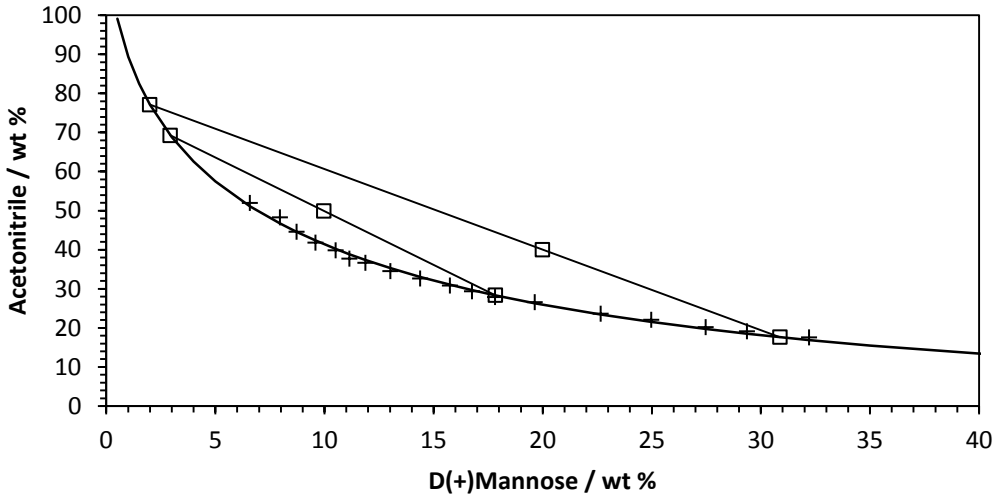


Figure C.3. Phase diagram for the ternary system composed of acetonitrile + D(+)-Mannose at 298 K (+), □- TL data, (—) binodal adjusted data through equation 2.

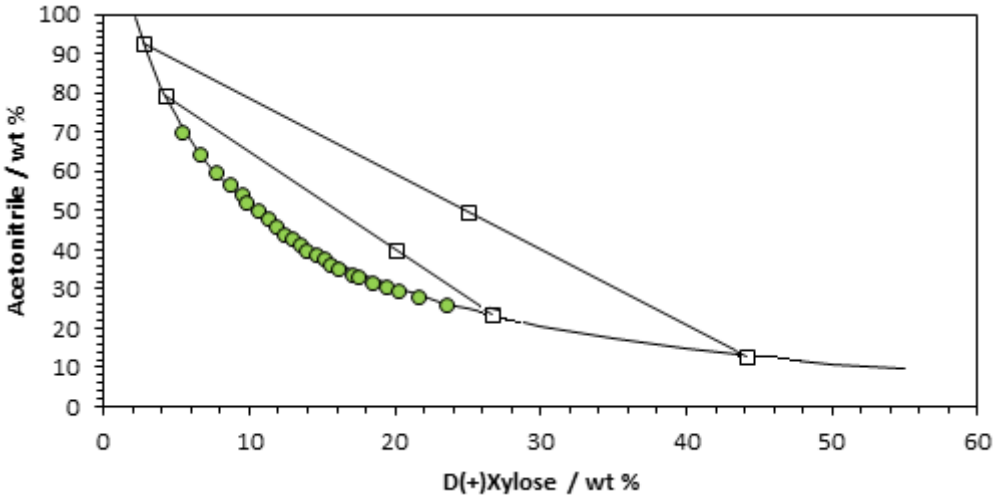


Figure C.4. Phase diagram for the ternary systems composed of acetonitrile + D(+)-Xylose at 298 K (●), □- TL data, (—) binodal adjusted data through equation 2.

Table C.5: Mass fraction composition for the TLs and respective TLLs, at the top (T), bottom (B) phase, and initial biphasic composition of the mixture (M), composed of acetonitrile (Y) and carbohydrates (X) at 298 K and atmospheric pressure.

Carbohydrate	mass fraction / wt %						
	Y_M	X_M	Y_T	X_T	Y_B	X_B	TLL
Sucrose	39.97±0.03	20.07±0.05	93.91±0.01	0.50±1.99	10.71±0,09	30.68±0.03	88.51
	49.94±0.02	10.14±0.10	64.86±0.02	4.09±0.24	27.15±0.04	19.38±0.05	40.69
D-(+)-Maltose	40.05±0.03	19.96±0.05	92.15±0.01	0.17±5.84	6.59±0.15	32.66±0.03	91.52
	49.95±0.02	10.02±0.10	65.24±0.02	3.31±0.30	18.13±0.06	23.97±0.04	51.44
D-(+)-Glucose	39.98±0.03	19.99±0.05	98.37±0.01	0.44±2.26	4.56±0.03	31.86±0.22	58.98
	49.98±0.02	10.00±0.10	75.42±0.02	2.14±0.47	22.09±0.05	18.61±0.05	55.81
D-(+)-Mannose	40.04±0.03	20.00±0.05	77.14±0.02	1.99±0.50	17.62±0.06	30.88±0.03	66.15
	49.92±0.02	9.98±0.10	69.29±0.02	2.93±0.34	28.34±0.04	17.84±0.06	43.58
D-(+)-Galactose	49.99±0.02	10.01±0.10	73.92±0.02	1.87±0.54	21.46±0.01	19.73±0.05	55.42
	45,00±0.02	8.01±0.12	32.02±0.03	12.54±0.08	54.21±0.02	4.80±0.21	23.50
D-(-)-Fructose	40.03±0.03	20.03±0.05	73.66±0.02	3.11±0.32	20.96±0.03	29.62±0.05	58.98
	48.65±0.02	9.76±0.10	59.28±0.02	5.76±0.17	37.61±0.03	13.92±0.07	23.15
D-(+)-Xylose	39.95±0.03	20.05±0.05	79.31±0.01	4.20±0.24	20.77±0.01	27.78±0.04	63.11
	49.99±0.02	10.02±0.10	50.06±0.02	10.26±0.10	50.06±0.02	10.26±0.10	0.00
L-(+)-Arabinose	39.97±0.03	19.91±0.05	79.15±0.02	2.73±0.37	17.59±0.01	29.72±0.03	67.21
	50.01±0.02	10.00±0.02	67.51±0.02	4.24±0.24	30.97±0.03	16.28±0.06	38.47

Table C.6. pH values of the acetonitrile(top)- and carbohydrate(bottom)-rich phases at 298 K.

	System A		System B	
Carbohydrate	**Top phase**	**Bottom Phase**	**Top phase**	**Bottom Phase**
Sucrose	7.06	6.35	6.97	6.76
D-(+)-Maltose	6.55	5.97	6.92	6.64
D-(+)-Glucose	6.84	5.69	6.98	6.28
D-(+)-Mannose	7.00	6.28	6.89	6.41
D-(+)-Galactose	-	-	6.81	6.09
D-(+)-Xylose	6.64	5.83	5.96	5.95
L-(+)-Arabinose	6.78	5.73	6.34	6.14
D-(-)-Fructose	6.36	5.80	6.34	5.48

A: 40 wt % acetonitrile + 20 wt % carbohydrate; B: 50 wt % acetonitrile + 10 wt % carbohydrate.

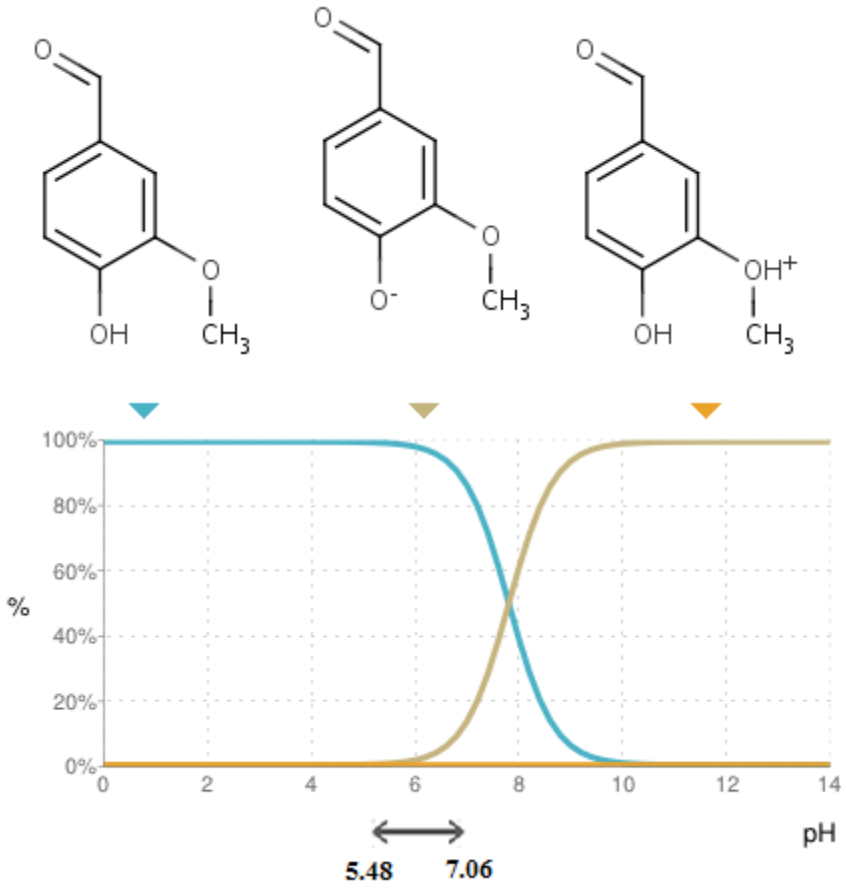


Figure C.5. Chemical structure of vanillin at different pH values. This content was adapted from the Chemspider chemical database (http://www.chemspider.com/).