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Extraction of antioxidants from pine bark

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presentado por

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Abstract

Pine bark extracts are a rich source of natural polyphenols with potential antioxidant properties. In this work a preliminary study is conducted to establish the effect of different solvents on extraction yield, total phenolic content, and antioxidant activity of pine bark extracts from *Pinus pinea*.

The extraction method used was the Soxhlet extraction. The samples were extracted with four solvents (hexane, dichloromethane, ethyl acetate and methanol) of different polarity. The different extracts were then subjected to flash column chromatography, followed by HPLC and gas chromatography.

The total phenolic content (TPC) was determined by means of the Folin-Ciocalteu method and the antioxidant activity was determined using the free radical 2,2-Diphenyl-picrylhydrazyl (DPPH). Excellent correlation was found between TPC and antioxidant activity.

Overall, extract obtained from methanol solvent showed the highest phenolic content, as well as the greatest antioxidant activity.

Resumen

Los extractos de corteza de pino son una rica fuente de polifenoles naturales con potenciales propiedades antioxidantes. En este trabajo se realiza un estudio preliminar con extractos de corteza de pino de *Pinus pinea* para establecer el efecto que tienen la utilización de diferentes disolventes sobre el rendimiento de extracción, sobre el contenido fenólico total y sobre la actividad antioxidante.

Se ha utilizado el método de extracción Soxhlet. Las muestras se obtuvieron con cuatro disolventes de diferente polaridad (hexano, diclorometano, acetato de etilo y metanol). A continuación, los diferentes extractos se sometieron a cromatografía en columna, seguida de HPLC y cromatografía de gases.

El contenido fenólico total (TPC) se ha determinado mediante el método de Folin-Ciocalteu y la actividad antioxidante se ha medido mediante el radical libre 2,2-difenil-picrilhidrazilo (DPPH). Se ha encontrado una excelente correlación entre el TPC y la actividad antioxidante.

El extracto obtenido a partir del disolvente metanol muestra el mayor contenido fenólico, así como la mayor actividad antioxidante.

Key words

Pine bark, extracts, antioxidant activity, total phenolic content

Palabras clave

Corteza de pino, extractos, actividad antioxidante, contenido total de fenoles

Contenido

1. Introduction

Lignocellulosic biomass is one of the most widely available sources of renewable matter, as it is currently used in various branches of the food, forestry, construction, energy, pharmaceutical and chemical industries (Bieniek & Mleczek, 2015). Biomass is the only renewable organic carbon source in nature (B. Gomez-Monedero, J. Faria, 2017), which makes it the only alternative to fossil resources for obtaining organic compounds and chemicals.

Biomass from fast-growing trees and shrubs can be used in a multidirectional way, and its exploitation can be carried out by means of different processes depending on the final application. Biomass from forestry sources is mainly constituted by three components, cellulose (40-60%), hemicellulose (10-40%) and lignin (15-30%), whose percentages vary depending on the species, thus its fractionation is of key importance (Brian H. Davison Jerry Parks Mark F. Davis Bryon S. Donohoe, 2013) (Bidlack, J. E., & Dashek, 2017). In general, treatments for processing biomass raw materials in many biorefineries are focused on the separation of their main constituent fractions, and they will vary depending on the type of biorefinery considered (King, D; Inderwildi, Oliver R; Williams, 2010). Amidst the different types of biorefineries, those pertaining to the Biochemical Platform are typically comprised of two consecutive processes (Kumar et al., 2010): i) pretreatments to increase the accessibility of cellulose, hemicelluloses and lignins, ii) enzymatic hydrolysis of the cellulosic components to monomeric sugars that are fermented to ethanol or butanol. In this type of biorefineries, the main focus is put on the cellulose and hemicellulose fractions. Cellulose has found wide application in the production of second-generation biofuels (*i.e.* fuels whose production does not compete with human food production), such as ethanol and 1-butanol, among others. Hemicellulose has found application in the production of platform chemicals such as furfural. Regarding lignin, which is a recalcitrant polymer, more difficulties have been found to develop alternative applications to its main use as low-grade fuel, as its efficient valorization into higher value-added chemicals has not been developed yet. However, the lignin fraction is a promising alternative for replacing fossil-derived chemicals. Indeed, lignin is deemed as the largest renewable source of aromatic building blocks. The development of efficient lignin depolymerization methodologies can contribute not only to the economic and environmental viability of biorefineries, but also to the development of new sustainable strategies for the synthesis of renewable

aromatics, as an alternative to petrochemistry industry (García et al., 2014) (Krzyzaniak et al., 2014) (R. Parajuli, M. Trydeman Knudsen, 2015) (MJ. Stolarski, S. Szczukowski, J. Tworkowski, 2013) (MJ. Stolarski, M. Krzyżaniak, M. Łuczyński, D. Załuski, S. Szczukowski, J. Tworkowski, 2015).

Therefore, lignocellulosic biomass, among all possible renewable energy sources, could contribute to meeting the objectives of renewable energy, production of chemical compounds of bio-based origin and to the development of an EU bioeconomy in 2030. (*The Future of Bio-Based Chemicals in the EU Bioeconomy*, 2019) (Carus et al., 2017) (Kalt & Kranzl, 2011) Owing to the environmental, social and economic benefits of fast-growing trees and shrubs, these raw materials are of great interest for both research organizations and bio-based companies, as they may be one of the main sources of energy and chemical compounds of renewable origin in the near future.

Upon fractionation of forestry biomass, some waste is generally produced, whose recovery would be an advantage from the efficiency point of view in the use of resources and also from the environmental point of view. Specifically, in some processes for the exploitation of the wood from tree species such as pine and poplar, one of the stages of their processing is debarking, in which the bark of the tree is removed and it is generally used as low- value fuel in the drying boilers in the facilities where they are processed.

As in the case of lignin, this energy exploitation of the bark residues is undoubtedly inefficient from the point of view of the quality of the product obtained, since it does not allow the very rich and complex variety of chemical compounds found in the bark to be used. The high-value molecules and functional groups present in the bark, that could be used in the development of organic chemical compounds of very high added value are, therefore, destroyed, resulting only in the release of thermal energy in the combustion. It is therefore necessary to seek for alternatives to exploit the full potential of these residues in a more efficient manner.

The evolution of scientific knowledge on the use of plants by humanity, has occurred over an extended period of time. Primitive civilizations perceived the existence of edible and toxic plants that, when used to combat diseases, empirically revealed their healing potential (Barros, 2008). In this sense, natural products present in plants have enormous complexity and diversity, thus making it necessary to classify them into groups. In the classification by their biosynthetic origin, two groups can be

differentiated: products of the primary metabolism (carbohydrates, lipids, amino acids, peptides and proteins) and products of secondary metabolism (terpenic, phenolic and secondary nitrogenous compounds). These natural products are chemical compounds that can be responsible for pharmacological actions, being called active principles, and are used in therapeutics, such as herbal medicine and medicine, and in the food industry (García-Castillo, E., & Martínez-Solis, 2015)

In the case of pine bark residues, these have a very rich and complex variety of chemical compounds. One of the many compounds found in pine bark are natural polyphenols, which are compounds of the plants´ secondary metabolism, often found in certain plant organs, like leaves or roots. (Vieito et al., 2018) These compounds are characterized by having an aromatic ring and a hydroxyl group. The low O-H bond energy in the homolytic cleavage together with the ability to promote electron delocalization over the aromatic ring make them useful as free radical scavengers. Indeed, phenolic compounds are used as additives in food, fuel and biological systems due to their antioxidant power, as well as in other sectors of fine chemistry (Esparza, I.; Jimenez-Moreno, N.; Bimbela, F.; Ancin-Azpilicueta, C.; Gandia, 2020).

Phenolic compounds are able to remove free radicals which are responsible for the oxidation of lipids, proteins and DNA. This has led to the study of various very high value-added applications in the field of health and biomedicine, with success stories already demonstrated in terms of their application as antioxidant, anti-inflammatory, cardioprotective and anti-cancer compounds of very high effectiveness (Vieito et al., 2018) (L. Botella, M. Sierra, F. Bimbela, 2015) (C W I. Haminiuk, G M. Maciel, M S V. Plata-Oviedo, 2012).

The addition of such phenolic compounds as antioxidants (e.g. BHT) to food products can delay the formation of off-flavors and rancidity, thus increasing the shelf-life of the product. (Oreopoulou & Tzia, 2006). Phenolic compounds have been widely used as additives in food products in the past years to improve their quality and appearance. In general, these phenolic compounds are complex molecules that are difficult to synthesize from petrol, so it is cheaper, more practical and safer to extract them from natural products. In addition, consumers have shown a growing concern for these artificial additives in recent years, demanding a more natural alternative. In consequence, food industries have started to seek natural sources for developing food additives. (Vieito et al., 2018)

Therefore, it is necessary to design new utilization strategies that allow to benefit from the enormous variety and complexity of chemical compounds present in these residues. This would contribute to a comprehensive and more efficient use of agroforestry biomass, as well as having a positive impact on the development of the bioeconomy, which could contribute to achieving the EU objectives set for 2030 more easily. (*Plan Nacional Integrado de Energía y Clima 2021-2030 20*, 2020) (Vuorinen, 2018)

Extraction is an important step regarding the isolation and identification of phenolic compounds. The most common extraction methods used to extract polyphenols from natural sources are classic aqueous extraction, ultrasound-assisted extraction, microwave extraction, and supercritical fluid extraction. Choosing the appropriate method requires taking into account different parameters, such as solvents used, the extraction time, the yield and quality of the extracts. (Coșarcă et al., 2019)

Soxhlet extraction has been a standard technique for over a century and the methods based on it remain the primary references in detriment to new leaching methods. Soxhlet extraction is a continuous–discrete technique. It is a very simple methodology that requires little training and can extract more sample mass than most of the latest alternatives (microwave-assisted extraction, supercritical fluid extraction, etc.). (Luque de Castro & Priego-Capote, 2010). In this study, the extraction method used is Soxhlet extraction, owing to its simplicity and low energy consumption. In addition, it avoids the use of any catalyst, thus eliminating the need for recovering the catalyst and reducing the number of unit operations required for obtaining the desired extracts.

In tree bark, the main fraction of polyphenols is composed by hydrolysable tannins and condensed tannins. Hydrolysable tannins are mixtures of simple phenols such as pyrogallol and ellagic acid, and esters of a sugar, mainly glucose, with gallic and digallic acids. Condensed tannins consist of flavonoid units (essentially flavan-3-ols and flavan-3,4-diols) which have suffered varying degrees of condensation and are associated with carbohydrates and traces of amino and amino-acids. (Vieito et al., 2018). In *Pinus* species, some of the most usually found compounds are lignins, oligolignins, flavonoids and pinosylvin, among others. (Pietarinen et al., 2006). The importance of this family of compounds, which constitutes the basis of this study, is their ability to act as antioxidants, which confers them great potential for their

utilization in a variety of applications, as already discussed above. However, in order to obtain these antioxidant compounds from pine bark, extracts are needed. The types of polyphenols recovered, their antioxidant activity and the extraction yield depend on the extraction method and on the solvent used for the extraction.

Therefore, considering the background of the problem described above, the aim of this study is to explore alternative ways for the valorization of agroforestry residues, focusing on the extraction of valuable chemical compounds from pine bark, with particular interest on the phenolic compounds. To that end, different extraction methods are proposed to fractionate and separate compounds of interest, thus obtaining different extracts that must be characterized by different analytical techniques to determine their composition. Lastly, a preliminary study on the potential antioxidant activity of these compounds extracted from pine bark is presented.

2. Objectives

The general objective of this study is to evaluate different alternatives for obtaining, by means of Soxhlet extraction, liquid extracts containing valuable compounds from pine bark, and to characterize the liquid samples obtained, thus evaluating their composition and potential application in the production of antioxidants. Regarding the specific objectives, these are listed below:

- To conduct a preliminary fractionation of the compounds, present in the pine bark.
- To analyze the presence of phenolic compounds in pine bark and in the different fractions.
- To analyze the antioxidant power of said fractions extracts using the 2,2-Diphenyl-picrylhydrazyl (DPPH) method.
- To analyze the phenolic content using the Folin-Ciocalteu method.

3. Materials and methods

3.1 Chemicals and reagents

All the chemicals, solvents and reagents were purchased from Scharlab S.A., Fisher Scientifics, Alfa-Aesar and Sigma Aldrich and were used as received.

Stone pine, *Pinus pinea,* was used as feedstock. It was collected in La Rioja (Spain), in the Sorzano area (42° 20′33″N, 2° 31′41″W, altitude, 722 m). Bark samples were collected in 2015. These were air-dried upon their reception and stored at room temperature for further analysis and use.

3.2 Sample preparation

Stone pine bark was milled using an electric mill (Bosch MKM 6000) and sieved to obtain fractions having different particle sizes (0.5 mm and 0.2 mm sieves). Three different fractions having different particle size (d_p) distributions were thus obtained (d_p <0.2mm, 0.2mm < d_p < 0.5mm, and d_p >5mm). The moisture of the sample was 11.8 % and was determined gravimetrically after drying at 102 ºC for 24 hours.

3.3 Soxhlet Fractionation

20 g of milled pine bark (d_p < 0.2 mm) were placed in a cellulose cartridge (30 x 100 mm and 8-15 μm nominal retention; Scharlab, Part number: CT32530100) and then placed in the Soxhlet extraction system. The samples were subsequently extracted with four solvents (hexane, dichloromethane, ethyl acetate and methanol). The different extracts were then concentrated by rotary distillation and the solvents were thoroughly removed under vacuum.

The resulting oily mixtures were then subjected to flash column chromatography using SiO_2 (Silica Gel 60, 0.04-0.06 mm, 230-400 mesh, Scharlab S.A, PN: GE0048005P) as stationary phase and gradient hexane:ethyl acetate mixtures as mobile phases. Thin layer chromatography was carried using Silica Pre-coated TLC sheets (Alugram Xtra SIL) and revealed at 254 nm. The selected fractions were collected, concentrated by rotary distillation and the solvent was removed under vacuum pump.

3.4 HPLC analysis

HPLC analyses were carried in an Agilent 1100 series equipped with UV detector at λ = 254 nm. Given the nature of the samples, reverse phase chromatography was carried out using three different stationary phases:

(1) Phenomenex Column Kinetex EVO C18 (150 x 4,6 mm)

(2) Phenomenex Column Gemini C18 (250 x 4,6 mm)

(3) Agilent Column Zorbax Eclipse XDB C6 (4,6 x 150 mm).

Aqueous formic acid (0.4 %) and acetonitrile gradients were used as mobile phases in all cases. The extracts or the selected fractions (*ca.* 3 mg) were dissolved in acetonitrile (*ca.* 10 mL), filtered through 0.45 µm PTFE filters (Scharlab, PN PTH2545200) and injected (25 µL).

It must be remarked that the analytical method for HPLC analysis had to be developed for the first time during the elaboration of this study. Owing to this, the use of the three columns is justified, because the method had yet to be developed and optimized, since it had not been done before. The column chosen for the subsequent analyses was Phenomenex Column Kinetex EVO C18 (150 x 4,6 mm).

3.5 GC-FID

Gas chromatography analyses were carried on an Agilent 6890 equipped with a FID detector and a capillary column HP-5MS [(5%-phenyl)-methylpolysiloxane, 60 m × 0.32 mm], with helium as carrier gas at constant pressure (16 psi). The temperature program started at 50 °C and followed by heating to 325 °C at 10 °C min^{-1}. Heating was held at 325 ºC for 15 minutes. Inlet temperature was 230 ºC, with 15 as split ratio. Detector was held at 250 ºC with 45 mL/min of hydrogen flow, 400 mL/min of air flow and 45 mL/min of helium as make-up flow.

Samples were derivatized using *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide prior to GC analyses. The samples (3 mg) were dissolved in 20 µl of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide and 20 µl of anhydrous pyridine as described elsewhere (Van Den Bosch et al., 2015). The samples were then incubated at 80 ºC during 30 min and allowed to cool down. Once the samples reached room temperature, dichloromethane or acetonitrile (0.5 mL) were added, and the samples (1 µL) were analyzed.

3.6 GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) analyses were carried in a Shimadzu GC-2010 chromatograph equipped with a Shimadzu QP-2010 mass-spectrometry detector with the same analysis conditions as those described above for

the GC-FID analyses. However, the same samples that had been used in the GC-FID analyses were used in this case with a 1/100 dilution. Identification of the peaks was done by comparison of the obtained mass spectra against those of compounds compiled in the NIST database library.

3.7 Total phenolic content determination

Total phenolic content in the samples was determined by using the Folin-Ciocalteu method (Singleton, V. L., & Rossi, 1965). The calibration curve was prepared using gallic acid as standard. Briefly, from an aqueous 100 mg/L gallic acid solution, a series of dilutions were prepared with high-purity deionized water to obtain standards concentrations of 1.0, 1.5, 2.0, 3.0 and 4.0 mg/L, respectively. For that purpose, 20, 30, 40, 60 and 80 µL of the standard solution described above were placed in different vials protected from light. Then, 250 µL of 1 N Folin-Ciocalteu reagent was added to each vial, shaken and then 0.75 mL of 7,5% sodium carbonate was added. The resulting mixture was brought to a final volume of 2 mL using high-purity deionized water and allowed to settle for 2h. The absorbance of the standards was measured at $\lambda = 760$ nm in a Selecta V-110D spectrometer.

Excellent correlation between the gallic acid concentration and the absorbance decay in the samples was found (Figure 1).

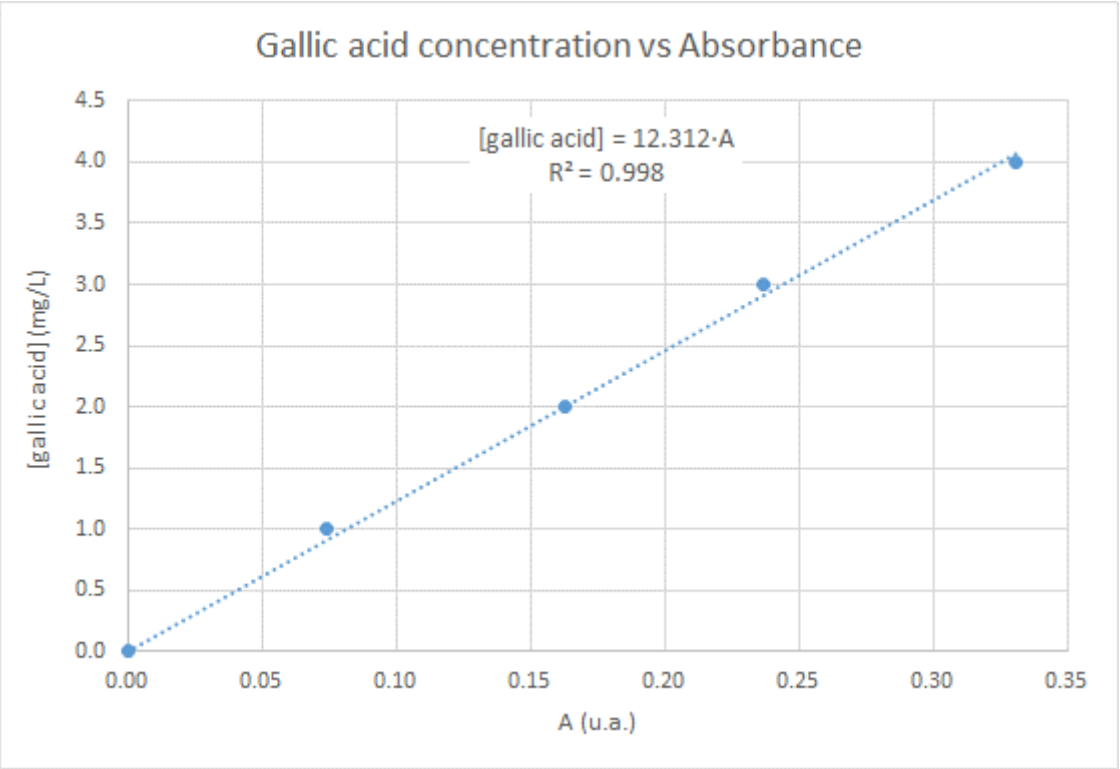


Figure 1. Calibration curve of the gallic acid concentration using the Folin-Ciocalteu method.

Regarding the analysis of the different samples containing the extracts obtained from pine bark, the procedure was similar. 2 mg of sample was diluted in 50 mL of high-purity deionized water. 0.5 mL of the sample solution was then mixed with the Folin-Ciocalteu reagent (0.75 mL, 1 N) and a solution of sodium carbonate (7.5%, 0.75 mL). The resulting mixture was allowed to settle for 2 h in the dark, and the absorbance was measured at $\lambda = 760$ nm.

3.8 Determination of the antioxidant activity

Antioxidant activity of phenolic compounds was measured by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method as described elsewhere (Brand-Williams et al., 1995). The calibration curve was prepared by using ascorbic acid as the calibration standard. Briefly, 0.5 mL of ascorbic acid standard at 10, 20, 30, 35, 40 mg/L was mixed thoroughly with 1.5 mL of DPPH–methanol reagent (4.5 mg of DPPH in 50 mL of methanol) and allowed to stay at room temperature in dark for 5 min prior to measuring the solution absorbance at $\lambda = 517$ nm in a Selecta V-110D spectrometer.

A good correlation between the ascorbic acid concentration and the absorbance decay in the samples was found (Figure 2).

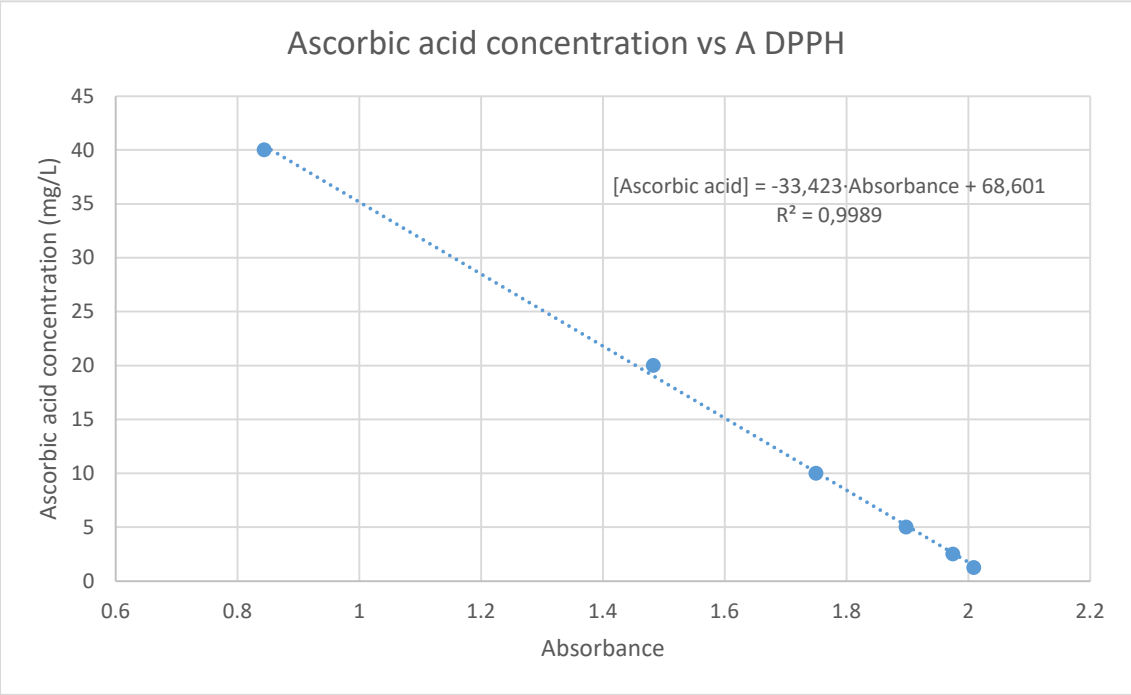


Figure 2. Calibration curve of the ascorbic acid anti-radical activity against DPPH

Once the calibration curve was stablished, 0.5 mL of diluted extracts (60 mg/L) was mixed with 1.5 mL of the DPPH–methanol reagent. The absorbance of the sample at λ = 517 nm was measured at different times.

4. Results and discussion

4.1 Soxhlet extraction

There are hundreds of chemical compounds in the pine bark. Therefore, it is impossible to analyze and isolate them without making a previous fractionation. One of the most straightforward methodologies to carry this mixture of compounds consists on Soxhlet extraction using different polarity solvents, and in different polarity sequences. Four different solvents, hexane, dichloromethane, ethyl acetate and methanol were selected according to their different polarity and dielectric constant (Table 1) and having in mind the additional possibility of creating hydrogen bonds in the case of methanol. These differences will account for the differences in the respective fractions' properties.

Besides dipole moment and dielectric constant, it is important to notice that all the selected solvents presented low or relatively low boiling points (40 ºC to 80 ºC, Table 1). This is of paramount importance when evaluating the energy efficiency of the process, its potential for scaling-up as well as for further recovery of the solvent by distillation.

Table 1. Physical properties of the selected solvents.

Solvent	Boling point (ºC)	Dipole moment (Db)	Dielectric constant
Hexane	69	0	2.02
Dichloromethane	40	1.60	9.08
Ethyl acetate	78	1.78	6.02
Methanol	68	1.70	33.00

To optimize the extraction of the target compounds, the contact between the sample and the extract must be as close as possible. Therefore, from the different particle size distributions obtained by sieving, the smallest one (d_p > 0.2 mm) was used for the extraction experiments, since it can be expected that the smaller particle size, the better contact surface with the solvent should be achieved, which in turn would provide higher extraction yields.

Four rounds of Soxhlet extraction were performed (Table 2) using sequences of solvents having different polarity orders to determine the effect of the polarity gradient during the overall fractionation process. Two rounds of Soxhlet extraction were performed during 4h, using solvents in increasing order of polarity (named as Soxhlet 1,

hexane, dichloromethane and ethyl acetate) and also in decreasing order of polarity (Soxhlet 2, methanol, ethyl acetate, dichloromethane and hexane). Another round of Soxhlet extraction was performed, for 4 h, using solvents in increasing order of polarity including methanol (Soxhlet 3, hexane, dichloromethane, ethyl acetate and methanol). Finally, the effect of the extraction time was evaluated with a final round performed using the same gradient used in Soxhlet 3, but with 24 h of total extraction time instead of 4 h.

Table 2. Extraction rounds and mass yields. Mass yields are referred to dry material

	Solvent	Extraction time (h)	Mass Yield (%)	Total mass yield (%)
Soxhlet 1	Hexane	4	3.3	4.8
	Dichloromethane	4	0.5	
	Ethyl acetate	4	1.0	
Soxhlet 2	Methanol	4	57.6	63.5
	Ethyl acetate	4	0.6	
	Dichloromethane	4	0.5	
	Hexane	4	4.9	
Soxhlet 3	Hexane	4	1.7	46.4
	Dichloromethane	4	0.6	
	Ethyl acetate	4	1.0	
	Methanol	4	43.2	
Soxhlet 4	Hexane	24	4.6	74.1
	Dichloromethane	24	0.7	
	Ethyl acetate	24	3.0	
	Methanol	24	65.9	

The relative mass yields (*i.e.*, with respect to the mass of the original sample) obtained with all the solvent in the different rounds are presented in Table 2. As it can be seen, the total mass yield is relatively low in Soxhlet 1, where only hexane, dichloromethane and ethyl acetate are used, whereas the overall yield drastically increased when methanol was also used. Indeed, it can be observed that in Soxhlet 2, Soxhlet 3 and Soxhlet 4 most of the mass yield was achieved upon the extraction step with methanol.

The order of the solvents also seems to influence the yield of the extraction process. In Soxhlet 2, where the most polar solvent (methanol) was first used, the overall yield was higher than in Soxhlet 3, where the most non-polar solvent (hexane) was first used instead. In Soxhlet 2, it can be assumed that most of the polar compounds and some of the non-polar compounds that are present in the bark are extracted upon

methanol fractionation, thus leaving the solid matrix with fewer compounds yet to be extracted for the latter solvents. However, by starting with the most non-polar solvent, and despite obtaining a lower mass yield, it can be assumed that non-polar compounds are extracted in the leaching rounds using hexane and dichloromethane.

As it could be expected, when the extractions were run for 24 hours, the recovered mass was noticeably higher, because the sample was much longer in contact with the solvent, and therefore the amount of recovered mass is subsequently greater.

4.2 Flash column chromatography

Soxhlet extraction partly separates the compounds according to their polarity and affinity to the different solvents. Even though they are fractioned, there is still a mixture of many compounds in the extract. Thus, the aim of the column chromatography is to fractionate even more the extracts obtained in the Soxhlet extraction, and to allow for a better separation of these compounds, which will ease their isolation and further identification and quantification by gas chromatography.

In the present work, column chromatography was applied to the different extracts obtained upon Soxhlet extraction with the different solvents; one column was applied to the hexane extract, another to the dichloromethane (DCM) extract, and another one to the methanol extract.

With the different fractions obtained from the column chromatography, a thin layer chromatography (TLC) was done to group the fractions that appear to have the same compounds, and to determine the fractions of interest for the successive analyses. To determine the fractions of interest, several criteria were used. On the one hand, the absorption presented by the TLL in UV at a wavelength of 254 nm was taken into account. This criterion is useful in this case because phenols absorb UV light, since they are aromatic compounds. On the other hand, the TLC plates were revealed using anisaldehyde that, under heating, oxidizes the organic compounds, producing “spots” that are sometimes not visible even using the UV light. With the revelation of the plaques, more “spots / compounds” could be observed. Based on the different spots observed, the fractions that appear to have more interesting compounds were chosen.

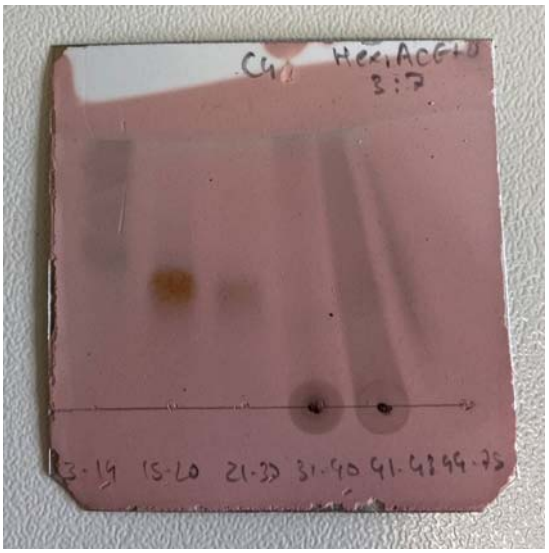


Figure 3. TLC of the fraction obtained by column chromatography applied to the DCM extract, revealed with anisaldehyde.

As an example, the revelation of TLC plaques using anisaldehyde is shown for the TLC carried out using the fraction obtained by column chromatography applied to the DCM extract (Figure 3). Different spots can be observed at different heights that depend on the polarity of the compounds present in the sample. The higher the polarity of the compound, the more it interacts with the solvent (which is polar in this case), hence the higher the spot.

In the first fraction, it can be seen that there are different spots at different retention in the stationary phase, Rf. Each spot corresponds to different compounds. This means that multiple compounds are likely to be present in that fraction. It is also noticeable that in the second and the third fractions spots are located at the same height. This means that most likely they will contain the same chemical compounds. Based on these observations, both fractions were combined and analyzed in the following analyses.

4.3 HPLC analysis

Isolation of the as-extracted compounds is a difficult task given the complexity of the resulting mixtures. Preparative HPLC is, undoubtedly, one of the most useful techniques for this purpose. With the aim of evaluating the possibility of using preparative HPLC, the different fractions obtained from Soxhlet 4 (Table 2) were subjected to HPLC analysis.

The analysis method was previously optimized by using a mixture of phenols and phenol ethers, reversed-phase stationary phases, and gradient aqueous formic acid (0.4 % v/v) / acetonitrile as mobile phase (see Appendix 1). Given that the desired compounds are aromatic, the wavelength of the UV detector was fixed at 254 nm. The selected analysis conditions are compiled in Table 3.

Table 3. Selected conditions for the preliminary HPLC analyses

Time (min)	% Aqueous formic acid (0.4 % v/v)	% Acetonitrile	Flow (mL/min)	Detector wavelength, λ (nm)
0	100	0	1	254
5	100	0		
50	0	100		
70	0	100		
75	100	0		
80	100	0		
Stationary phase		Phenomenex Column Gemini C18 (250 x 4.6 mm)		

Although HPLC experiments were preliminary and the UV absorption detector does not provide information about the identity of the products, some interesting information can be obtained from the extract's chromatograms (Figure 4).

First, it has to be considered that the absorption wavelength is 254 nm, the same as in the case of the TLC analyses. Therefore, it can be deduced that all the detected compounds are aromatic. Second, a C18 chromatography column is used as stationary phase, and a gradient with increasing non-polar phase is used as mobile phase. Therefore, it can be expected that shorter retention times correspond to polar compounds whereas longer reaction times correspond to non-polar compounds.

Indeed, it can be seen in the chromatogram from the hexane fraction that, except for one peak at *ca.* 10 min., all the most important peaks appeared at 30.3, 35.9, 37,7 and 39.2 min, respectively. Therefore, it can be assumed that these correspond to non-polar compounds, as expected. All these peaks are also present in the rest of fractions but with decreasing relative intensity with respect to increasing polarity order of the solvents.

Two important peaks at shorter retention times, 21.8 and 26.1 min. appear in the dichloromethane extract, and their relative intensity increase in the ethyl acetate and the

methanol extracts, suggesting that these are polar compounds. In the case of the methanol fraction, it can be seen that a shoulder appears between 14 and 26.1 min, this is covering the retention time of most of the standards (Appendix 1). This indicates that a vast majority of this extract corresponds to polar compounds, but the resolution of this mixture was obviously not optimal.

Therefore, it can be concluded that polar aromatic compounds, presumably phenols, have been extracted. However, the resolution of the method should be improved prior to its application in preparative HPLC.

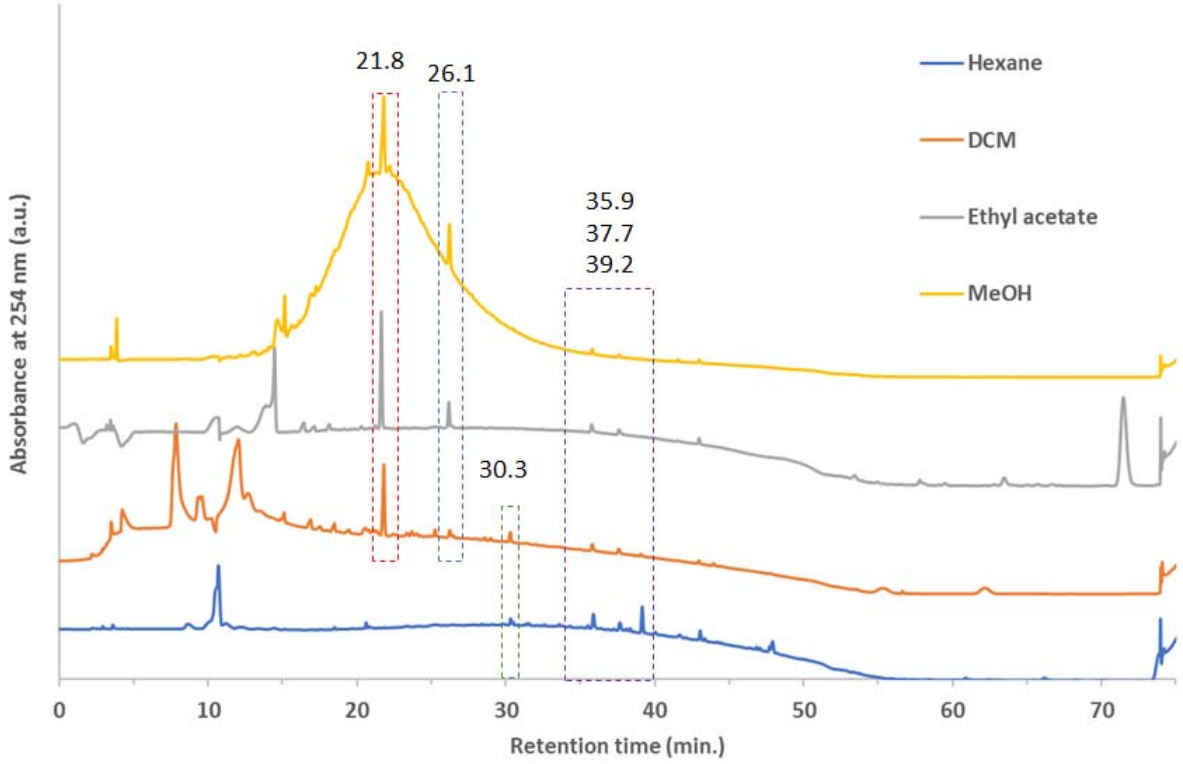
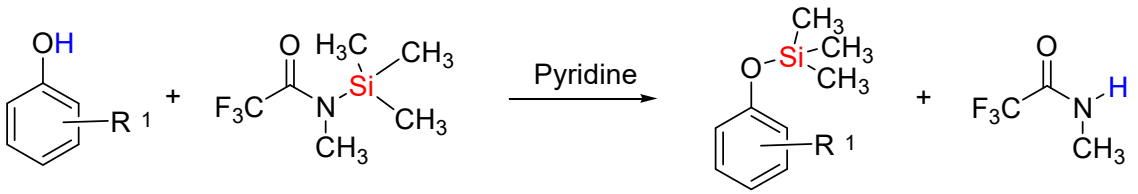


Figure 4. HPLC chromatograms of the extracts in different solvents from Soxhlet 4. Figures correspond to the retention time.

4.4 Gas Chromatography

The fractions obtained from Soxhlet 4 were derivatized following the protocol described by Van der Bosch *et al.* (Van Den Bosch et al., 2015) using *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide in the presence of pyridine. This protocol is based on the reaction of hydroxyl groups, either aliphatic or aromatic, with a silyl amide resulting in a silylated ether according to Scheme 1. As a result, the reaction products are not able to form hydrogen bonds that could increase their volatility and subsequently enable GC analysis.



Scheme 1. Reaction mechanism between phenols and N-methyl-N-(trimethylsilyl)-trifluoroacetamide.

All the extracts were derivatized and further analyzed by GC-FID using an HP-5 column that is commonly used in the analysis of phenols and phenolic dimers (Van Den Bosch et al., 2015) (Cornejo et al., 2020). The GC-FID chromatograms presented significant differences between the polar and non-polar solvents (Figure 5) showing that in the polar solvents a much higher number of compounds were detected.

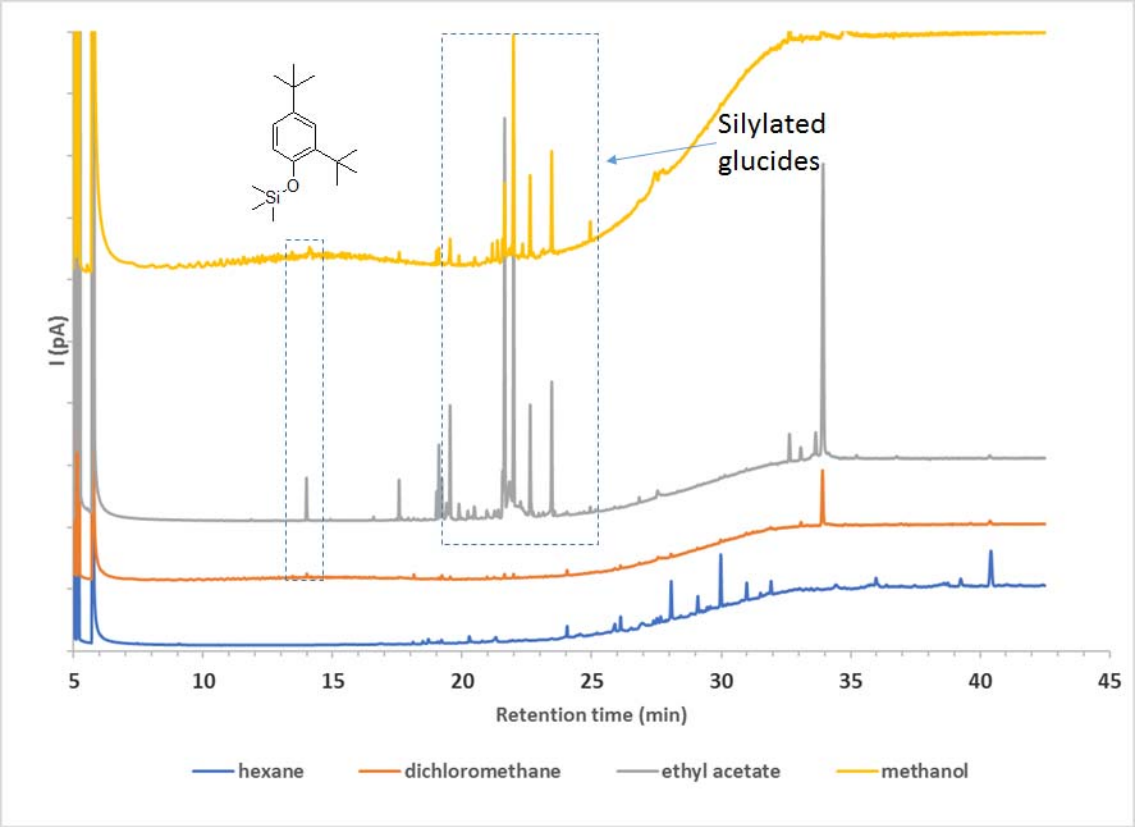


Figure 5. GC-FID analysis of the extracts in different solvents from Soxhlet 4. Figures correspond to the retention time.

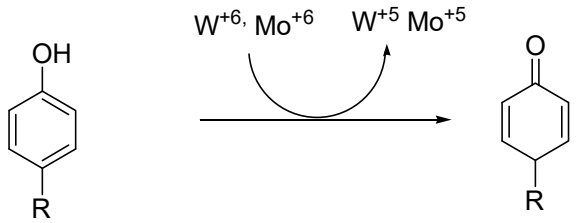
These samples were also analyzed by GC-MS chromatography (See Appendix 2) using similar analysis conditions. In view of the GC-FID results obtained, focus was

only put on the longest retention times in the GC-MS analyses. Unfortunately, most of the detected compounds corresponded to silylated glucides and only 2,4-ditert-butylphenoxytrimethylsilane could be clearly detected as one of our target compounds in the ethyl acetate extract, although low quantities were also detected in the dichloromethane and methanol extracts.

Considering the results from the HPLC analyses, the total phenolic content analyses and the analyses of the antioxidant activity (*vide infra)*, this clearly indicates that both the derivatization methodology and the analytical method must be strongly improved.

4.5 Total phenolic content determination

Total phenolic content was determined by using the Folin-Ciocalteu method. It is based on the fact that phenolic compounds react with the Folin-Ciocalteu reagent at basic pH values, resulting in a blue coloration capable of being determined spectrophotometrically at λ = 765 nm. This reagent, which contains a mixture of sodium tungstate and sodium molybdate in phosphoric acid, reacts with the phenolic compounds present in the sample. Phosphomolybdic tungstic acid (formed by the two salts in acid medium) is yellow, but by being reduced by phenolic groups, it results in a complex of intense blue color, whose intensity is measured to evaluate the polyphenol content. (Scheme 2).



Scheme 2. Reaction mechanism between phenols and the Folin-Ciocalteu reactant.

The oxidation of the polyphenols causes the appearance of a bluish coloration that has an absorption maximum at λ =765 nm and is quantified by spectrophotometry, based on a gallic acid standard line. This analysis of total polyphenols is frequently used in the study of antioxidant properties, since it shows a close correlation with the different methods of measuring antioxidant activity. (García Martínez et al., 2015)

Based on the results obtained in the HPLC and gas chromatography for the different extracts, seven samples were selected to determine the total phenolic content: one sample from each extraction solvent in Soxhlet 4 and one sample from each column chromatography (except the ethyl acetate column, since the results of the chromatography were not clear).

As it can be expected, the more non-polar extracts were not soluble in water, and indeed the hexane and dichloromethane extracts were not soluble in deionized water. Thus, these samples were diluted in methanol-acetone (1:1) whereas methanol and ethyl acetate extracts and purified extracts were dissolved in water (Table 4).

Table 4. Solvents used for the dilution of the samples

Sample	Solvent
Methanol	Water
Ethyl acetate	Water
DCM	Methanol-Acetone (1:1)
Hexane	Methanol-Acetone (1:1)
Methanol column	Water
DCM column	Methanol-Acetone (1:1)
Hexane column	Methanol-Acetone (1:1)

The absorbance values of each sample composed of 2.0 mg of extract in 50 mL of solvent were measured at λ = 760 nm, and the total phenolic content, determined as concentration of gallic acid (mg/L), was estimated with the calibration curve. The obtained results are shown in Table 5.

Table 5. Absorbance and TPC of the samples

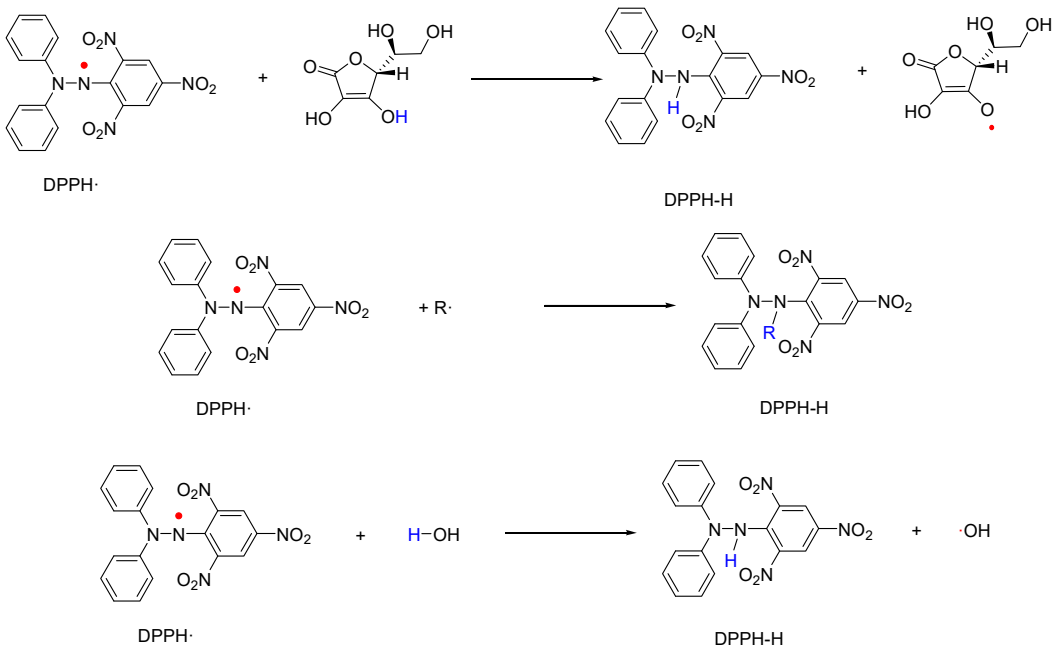
Sample	Absorbance (λ =760 nm)	TPC (mg/L gallic acid)
Methanol	0.773	9.517
Ethyl acetate	0.626	7.707
DCM	0.170	2.093
Hexane	0.069	0.850
Methanol column	0.585	7.203
DCM column	0.347	4.270
Hexane column	0.081	0.997

As can be seen in the results, the TPC increases with the polarity of the extracting solvent from 0.850 mg/L in the hexane extract to 9.517 mg/L in the methanol

extract. Phenols are chemical compounds consisting of one or more hydroxyl groups (-OH) bonded directly to an aromatic hydrocarbon group that provides high polarity to the molecules. There are many different types of phenols, being either simple monomers or more complex phenolic compounds that may be composed of a different number of aromatic rings. Depending on the complexity of the molecule, its polarity changes. The more aromatic rings and less hydroxyl groups the molecule has, the lower the polarity is. Therefore, methanol and ethyl acetate solvents drag the majority of the phenols, leaving the most non-polar solvents (DCM and hexane) with small amounts of phenols.

4.6 Determination of the antioxidant activity

To evaluate the antioxidant activity of extracts, they are allowed to react with a stable radical, 2,2-Diphenyl-picrylhydrazyl (DPPH) in a methanol solution. The reduction of DPPH, as indicated below, is followed by monitoring the decrease in its absorbance at a characteristic wavelength during the reaction. In its radical form, DPPH presents a specific absorbance at λ = 517 nm, but upon reduction by an antioxidant (AH) or a radical species ($R^{\cdot}$), the absorption disappears. This is because DPPH presents a purple coloration in methanolic medium. As a consequence of the donation of an electron or a proton by a compound with antioxidant power, the hue disappears. (Gaviria Montoya et al., 2009) This methodology is actually based on the reactivity of the radical species with the most protic acid form of the ascorbic acid, leading to its homolytic cleavage to produce DPPH-H. DDPH radicals can also react with other radical species, giving rise to DPPH-R coupling and, ultimately, with one of the hydrogen atoms from methanol and water (Scheme 3).



Scheme 3. Reaction pathways for DPPH deactivation.

First, the decay of the absorbance of DPPH· was evaluated in the absence of any extract and/or ascorbic acid (Figure 6). Blanks samples were prepared with the solvent used in the preparation of the extracts (with water or methanol-acetone) and the DPPH radical. This mixture should not have any antiradical activity since it does not contain any antioxidants. However, from minute 40 onward, the absorbance starts to decay, giving the notion that antioxidant activity is present in the mixture. Noteworthy, this decay in absorption is expressed in ascorbic acid concentration equivalents, as obtained from the corresponding calibration curve (Figure 2). This decay in the absorbance is due to the reaction of the DPPH radical with the proton of the solvent, establishing a balance between chemical loads. As it can be expected, the decay in absorbance is faster in water than in methanol-acetone because of its higher protic character. This interaction between the DPPH and the solvents of the samples may affect the absorbance of the extracts.

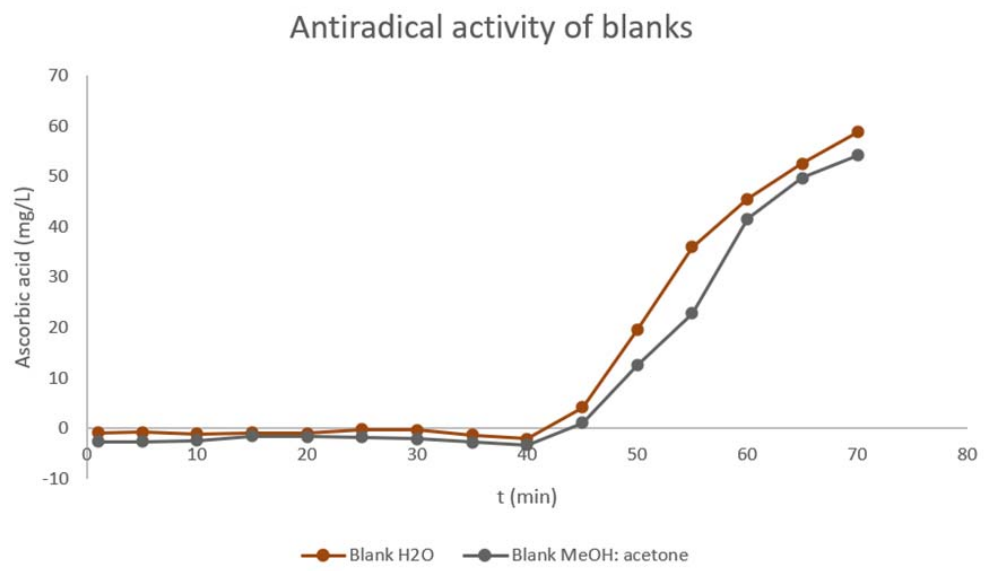


Figure 6. Antiradical activity in blank solutions

It has been mentioned before that the decay of DPPH was measured in water and methanol-acetone mixtures. As it can be expected, the same solubility problems than in the determination of the phenolic compounds were found and, therefore, the same solvent and solvent mixture were used (Table 4).

With the calibration curve of the DPPH analysis in hand, the procedure for preparing the samples had to be fine-tuned in order to determine the most adequate concentration of the sample required to get an optimal absorbance value. At this point, it was important to make the absorbance of the samples in the quantification range of the calibration curve and, therefore, different sample concentrations were used. The sample used to set the correct concentration was the methanolic extract from Soxhlet 4. Aqueous solutions with different concentrations were prepared (5, 10, 20, 40, 60, 80 mg/L). Then, 0.5 mL of the sample was mixed with 1.5 mL of DPPH, as mentioned before, and their absorbance was measured at λ = 517 nm. The concentration that gave an optimal absorbance value was that of 60 mg/L. Once the concentration was set, the different samples were prepared using these parameters. The analyzed samples corresponded with those analyzed in the total phenolic content, so as to see if there is a relation between the total phenolic content and the antioxidant activity. This is the measured antioxidant activity in ascorbic acid, which corresponds to a sample whose concentration is 60 mg of sample /L, as already detailed in the materials and methods section.

The absorbance of each sample was allowed to stabilize for 1 minute and then, its absorbance was measured each 5 minutes for 70 minutes at $\lambda = 517$ nm. This was repeated during 3 consecutive days. The antioxidant activity, expressed as concentration of ascorbic acid (mg/L), was estimated with the calibration curve for each sample. The results obtained are represented in different graphs.

The antiradical activity of the extracts from Soxhlet extraction is shown in Figure 7. There is a clear increase in the antiradical activity with the increase of the polarity of the extracting solvent. The antiradical activity (expressed as ascorbic acid concentration) increases from 0 mg/L for hexane, 8.8 mg/mL in dichloromethane, 31.8 mg/mL in ethyl acetate and up to 56.8 mg/ L in methanol after 30 minutes. This is because the antiradical activity can be attributed to the presence of phenols, which are polar molecules that interact with polar solvents. Thus, the higher the polarity of the solvent, the higher the antiradical activity. In the case of the hexane, it can be observed that there is no antiradical activity. This is because it is a very non-polar solvent that drags mainly long carbonated chains with very little oxygen presence. Phenols, however, are polar molecules that are expected to interact with polar solvents.

It can be observed how beyond 40 minutes, even the hexane extract appears to have antiradical activity. As it was mentioned, this is because of the degradation of the DPPH, and not due to the antiradical activity of the hexane extract.

It can be appreciated that the antioxidant concentration has a slight increase over time in all cases except for the hexane extract, which barely has any antiradical activity. This is because not all compounds have the same chemical reaction speed. Some compounds react with the DPPH at minute 5 or some at minute 30, which gives an increase in the antiradical activity of the sample as time evolves.

Another factor that is shown in the degradation curves for DPPH is the standard deviation (SD) of each extract corresponding to the measurements carried out per triplicate using three aliquots of the different extracts. It can be observed that SD increases from minute 40 on. Before minute 40, the SD is mostly low, since there is little variation between measurements taken in different days, which shows the robustness of the method. SD is slightly higher in the dichloromethane extracts, that is attributed to the use of methanol-acetone solvent mixture. However, from minute 40 on, with the deterioration of the DPPH the results begin to vary more, since the DPPH also interferes with the solvent as well as with the phenols. The error bars, which have been

depicted in Figure 7 as mean ± standard deviation, suggest that there are significant differences between the different extracts analyzed, despite the overlapping seen in some cases between error bars of some of the experimental data corresponding to different solvents.

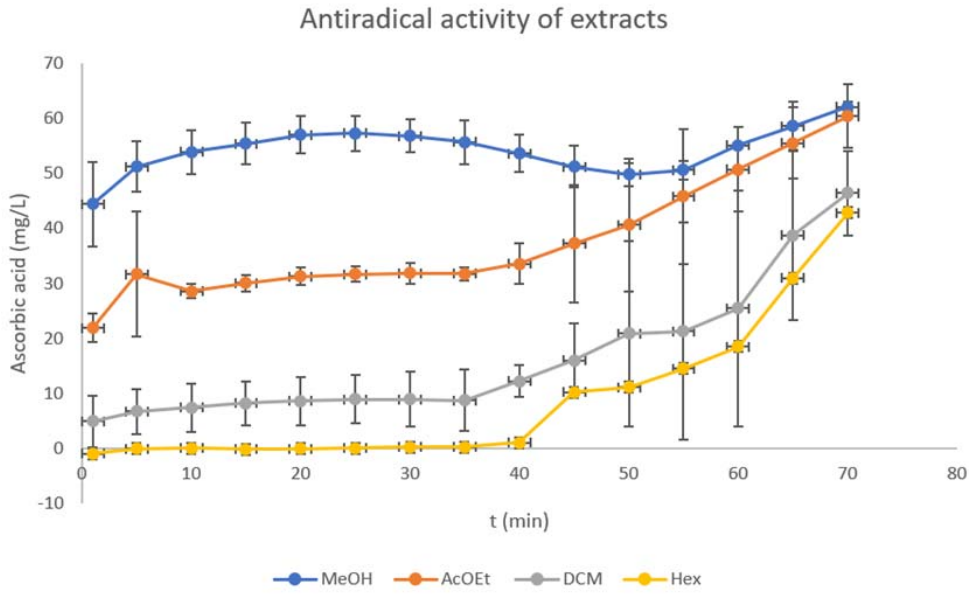


Figure 7. Evolution of the antiradical activity of the extracts from Soxhlet 4 referred to ascorbic acid concentration. Error bars correspond to the standard deviation of three measurements.

In Figures 8, 9 and 10, the antiradical activity of each extract with their corresponding column are shown. In the case of methanol (Figure 8), it can be seen that the evaluated samples present almost similar antiradical activity regardless of applying column chromatography or not. The sample obtained after applying column chromatography has slightly lower activity than the initial extract, meaning that some of the compounds with the most activity was probably recovered in other fractions of the extract that were not analyzed.

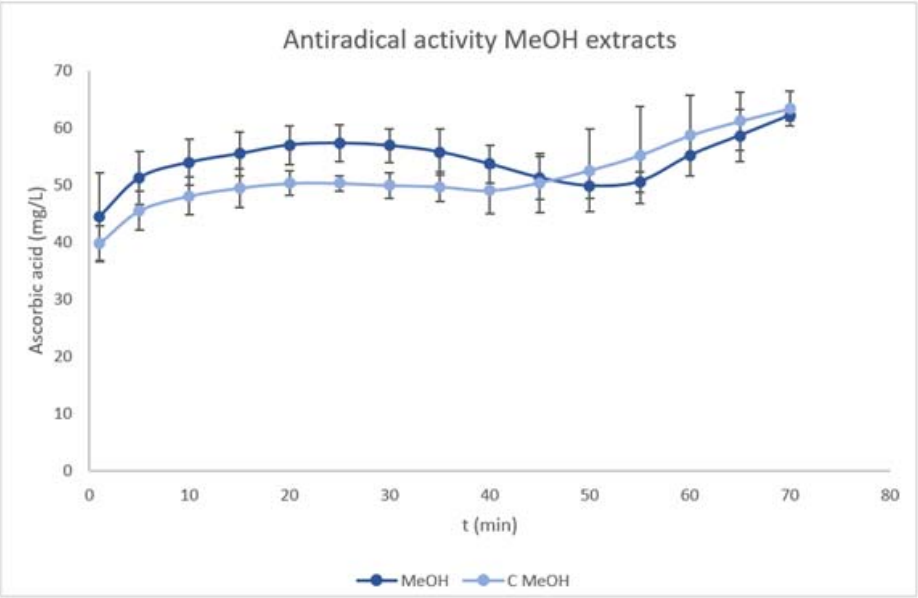


Figure 8. Evolution of the antiradical activity of the methanol extracts, before and after applying column chromatography, from Soxhlet 4. Antiradical activity is expressed as ascorbic acid concentration. Error bars correspond to the standard deviation of three measurements carried out with different sample aliquots.

In the case of the dichloromethane samples (Figure 9), the difference between the initial extract and its column is significant. Indeed, the activity of the extract after 30 minutes is 37.3 mg/L, against 31.8 mg/L in the initial extract. This means that the column has helped to get rid of some less inactive chemical compounds and therefore concentrated the phenols of the sample.

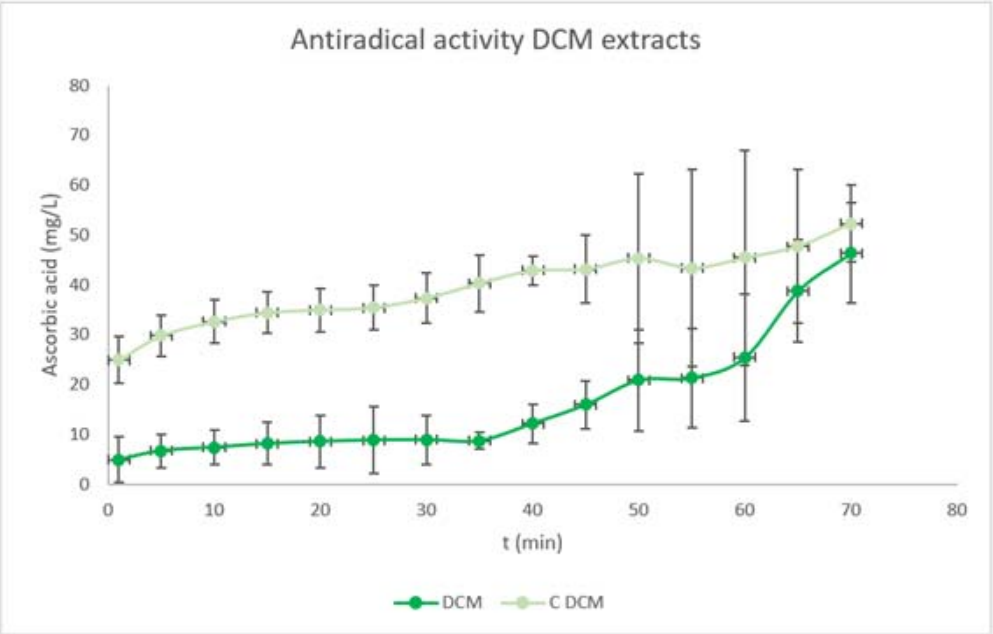


Figure 9. Evolution of the antiradical activity of the dichloromethane extracts, before and after applying column chromatography, from Soxhlet 4. Antiradical activity is expressed as ascorbic acid concentration. Error bars correspond to the standard deviation of three measurements carried out with different sample aliquots.

Similarly, the activity of the hexane extract before and after the chromatography step is compared in Figure 10. In this case, none of the samples presents any antiradical activity. This suggests that the concentration of phenols before and after the column was conducted is similar and that no compound with antiradical activity was extracted from pine bark with hexanes.

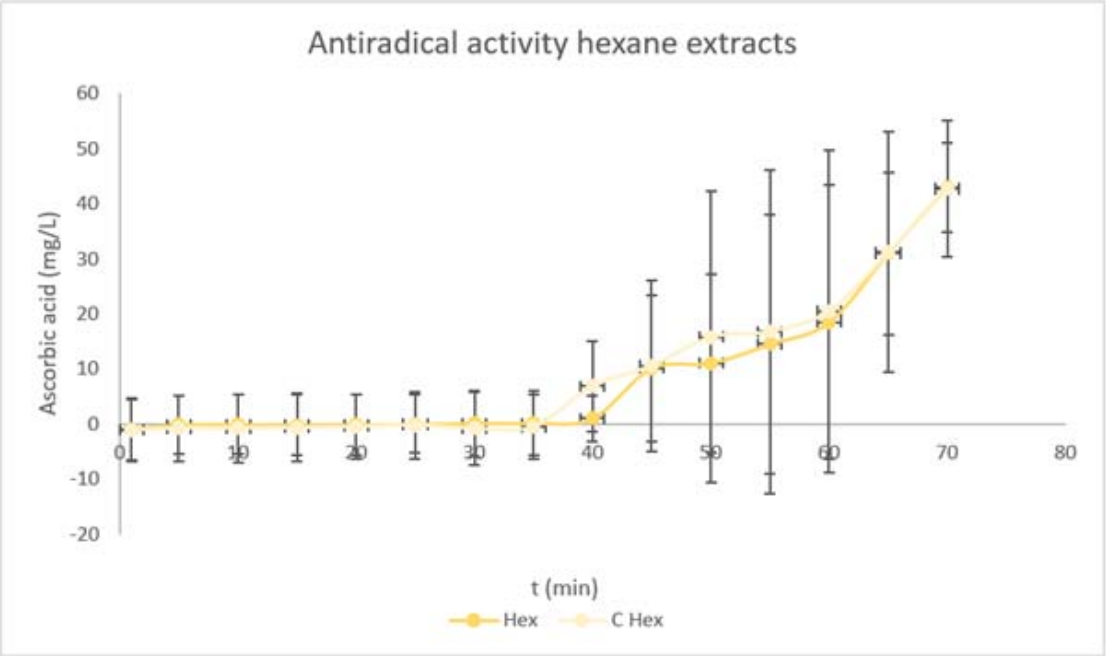


Figure 10. Evolution of the antiradical activity of the hexane extracts, before and after applying column chromatography, from Soxhlet 4. Antiradical activity is expressed as ascorbic acid concentration. Error bars correspond to the standard deviation of three measurements carried out with different sample aliquots.

As it can be observed in Table 6, the results for the TPC and the antiradical activity are consistent, as the higher the measured TPC, the higher the antioxidant activity. As shown in Figure 11, both parameters present a good correlation. This is also consistent, as it was mentioned before, with the preliminary HPLC measurements.

In the HPLC measurements, it was established that in the extracts from the most polar solvents (methanol and ethyl acetate), the most important peaks appeared at shorter retention times. Since the column used had a gradient with increasing non-polar phase as mobile phase, the most polar compounds had shorter retention times, whereas the less polar compounds had higher retention times. As mentioned before, phenols are polar compounds, thus the polar extracts should contain most of the phenols extracted from the solid sample. This is verified with the TPC, showing that the greatest phenol content appears in the methanol extract and the lowest phenol content is that corresponding to the hexane extract.

Lastly, comparisons were done between the results from the TPC and those from the antiradical activity measurements. Phenols have antiradical activity; they act as antioxidants. Seeing the results of the antioxidant activity, it can be concluded that, in fact, the extract with the greatest amount of phenols has the highest antiradical activity.

Table 6. Comparison of TPC and antiradical activity results.

Sample	TPC (mg/L gallic acid)	Antiradical activity after 30 min (mg/L ascorbic acid)
Methanol	9.517	56.9
Ethyl acetate	7.707	31.9
DCM	2.093	8.8
Hexane	0.850	0.0
Methanol column	7.203	49.9
DCM column	4.270	37.3
Hexane column	0.997	0.8

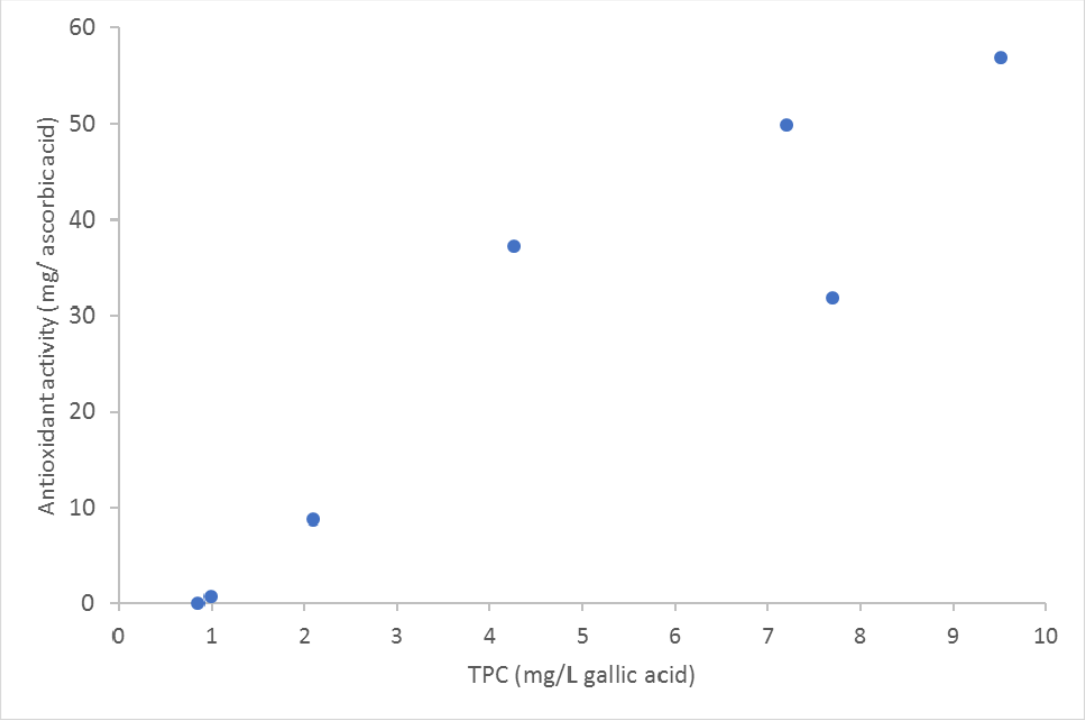


Figure 11. Comparison of the antioxidant activity (mg/L ascorbic acid) results against TPC results (mg/L gallic acid)

5. Conclusions

Pine bark is constituted by a complex mixture of chemical compounds that could be fractionated by means of Soxhlet extraction using different solvents of varying polarity. The samples were then subjected to HPLC and gas chromatography to separate and isolate the compounds present in the samples. The resolution of the analytical method should be improved in order to be able to isolate and identify specific compounds.

The extracts were then studied to establish the presence of phenolic compounds. Total phenolic content was determined by using the Folin-Ciocalteu method. From the extracts studied in this work, it can be inferred that phenolic compounds are mostly found in samples extracted with polar solvents rather than non-polar ones. This is confirmed with the results from the TPC analyses. The TPC of the sample extracted with methanol was 9.517 mg/L of gallic acid, whereas the TPC of the sample extracted with hexane was 0.85 mg/L of gallic acid.

The samples were analyzed to determine their antiradical activity by using a stable radical DPPH method. A high correlation between the TPC of the extracts and their antioxidant capacities was established.

Overall, the determination of antiradical activity was established, but further analyses are needed to isolate and identify these said compounds.

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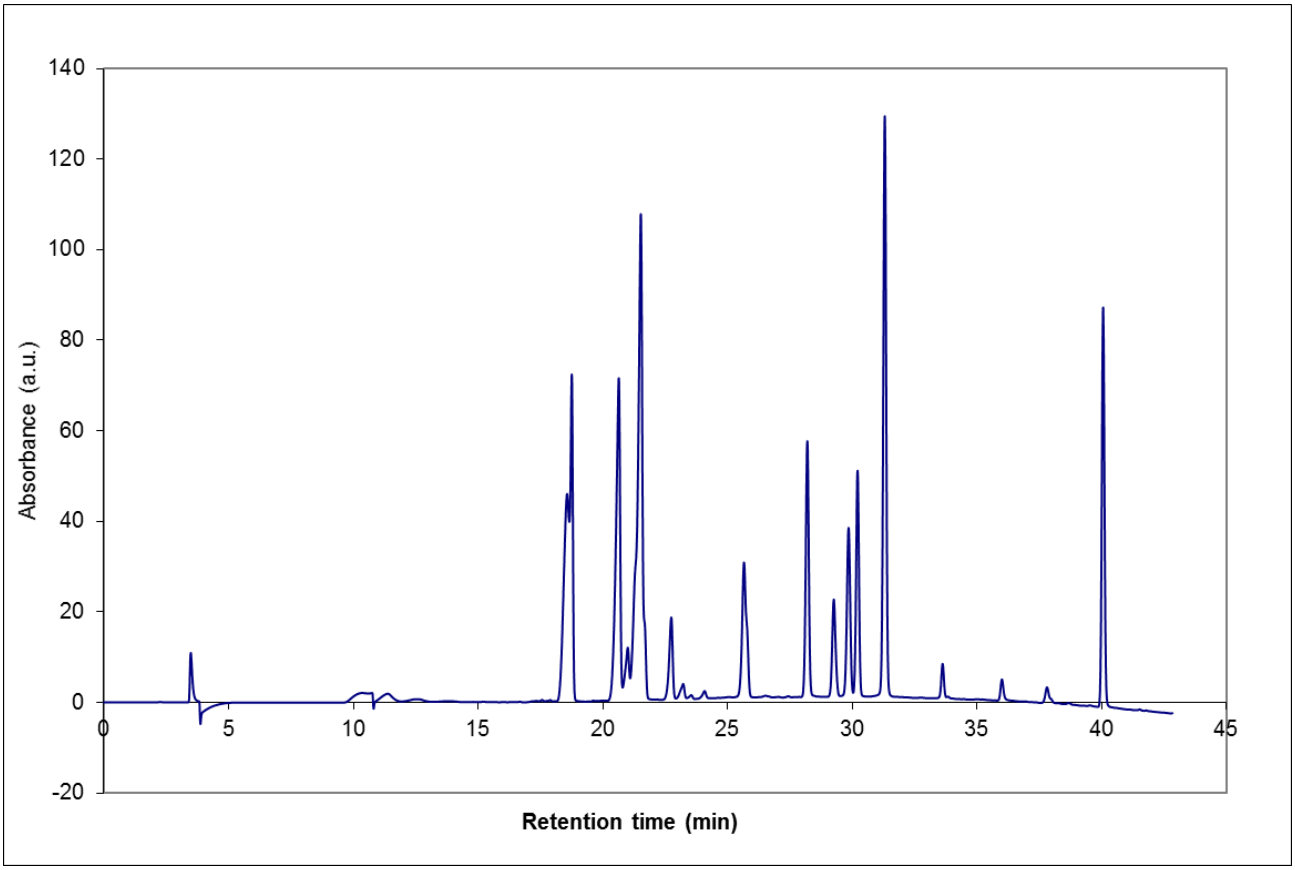
Appendix 1

HPLC methods:

1. Selected method and chromatogram of 17 phenolic compounds

Time (min)	% Aqueous formic acid (0.4 % v/v)	% Acetonitrile	Flow (mL/min)	Detector wavelength, λ (nm)
0	100	0	1	254
5	100	0		
50	0	100		
70	0	100		
75	100	0		
80	100	0		
Stationary phase		Phenomenex Column Gemini C18 (250 x 4.6 mm)		



Non selected methods:

Time (min)	% Aqueous formic acid (0.4 % v/v)	% Acetonitrile	Flow (mL/min)	Detector wavelength, λ (nm)
0	90	10	1	254
5	90	10		
25	10	90		
35	10	90		
36	0	100		
46	0	100		
48	90	10		
50	90	10		
Stationary phase	Phenomenex Column Gemini C18 (250 x 4.6 mm); Phenomenex Kinetex 5 u C18 (150 x 4.6 mm); Agilent Column Zorbax Eclipse XDB C6 (4,6 x 150 mm)			

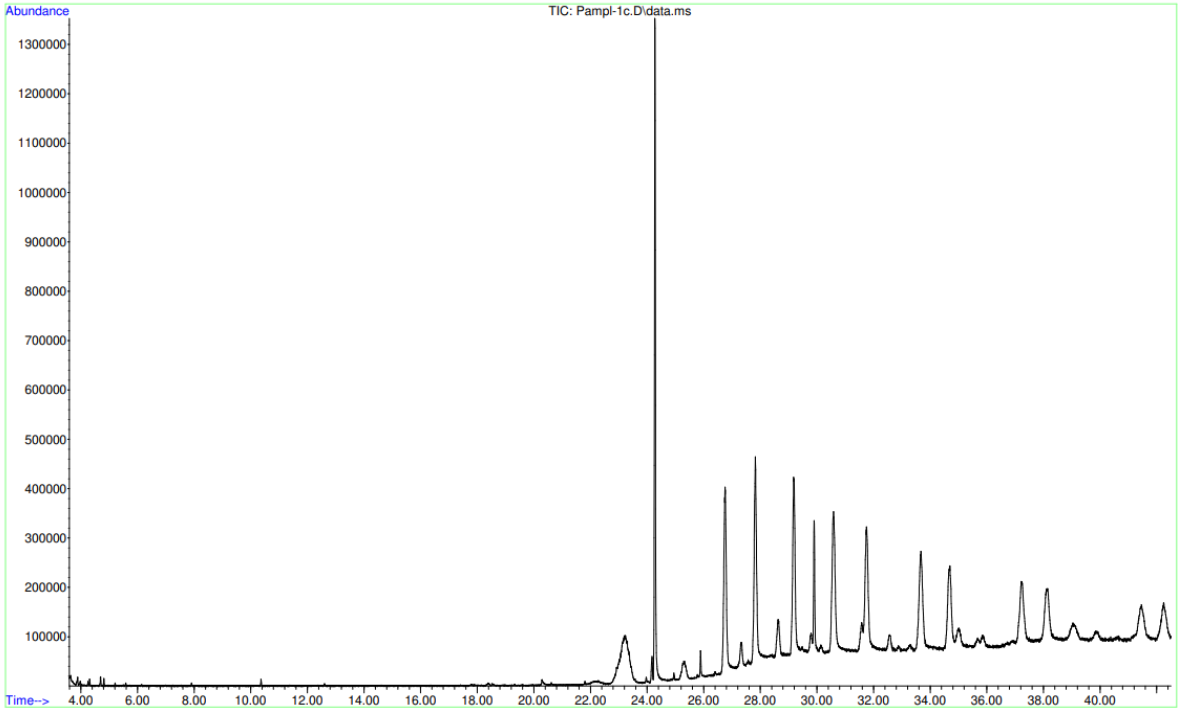
Time (min)	% Aqueous formic acid (0.4 % v/v)	% Acetonitrile	Flow (mL/min)	Detector wavelength, λ (nm)
0	90	10	1	254
40	0	100		
45	0	100		
46	90	10		
50	90	10		
Stationary phase	Phenomenex Column Gemini C18 (250 x 4.6 mm); Phenomenex Kinetex 5 u C18 (150 x 4.6 mm); Agilent Column Zorbax Eclipse XDB C6 (4,6 x 150 mm)			

Time (min)	% Aqueous formic acid (0.4 % v/v)	% Acetonitrile	Flow (mL/min)	Detector wavelength, λ (nm)
0	90	10	1	254
50	0	100		
55	0	100		
56	90	10		
60	90	10		
Stationary phase	Phenomenex Column Gemini C18 (250 x 4.6 mm); Phenomenex Kinetex 5 u C18 (150 x 4.6 mm); Agilent Column Zorbax Eclipse XDB C6 (4,6 x 150 mm)			

Appendix 2

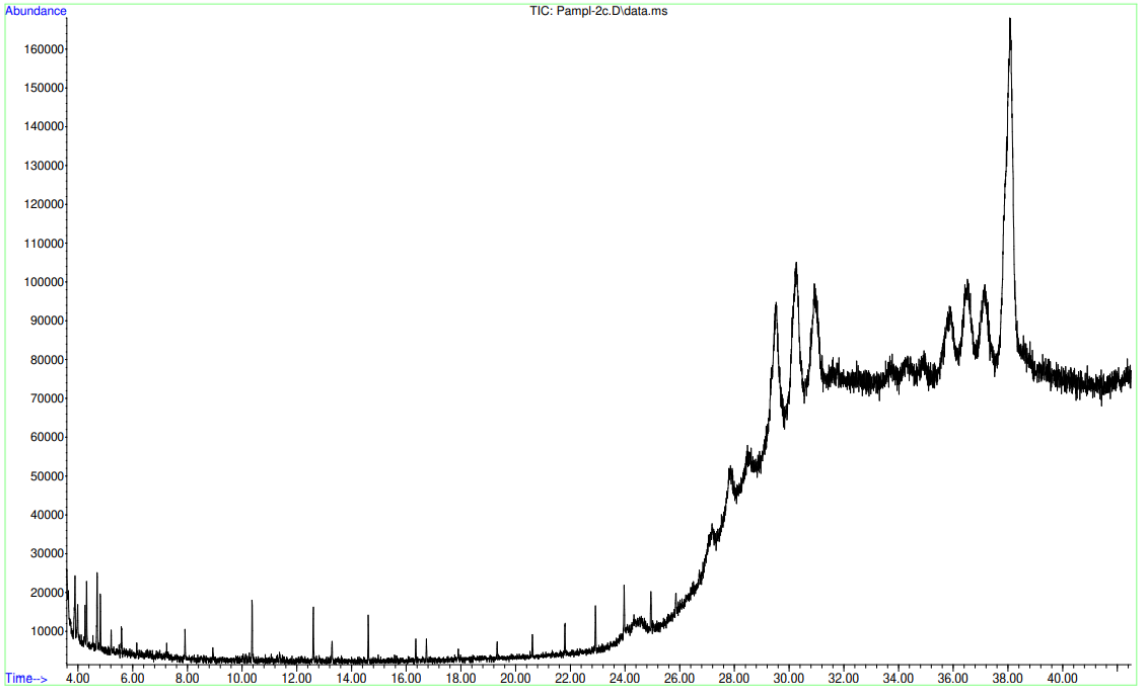
1. GC-MS of hexane Soxhlet extract. No peaks were identified

```
File       :C:\msdchem\1\data\Pampl-1c.D
Operator   :
Acquired   : 21 Jan 2021  10:43     using AcqMethod EXTRAC-PAMPLONA.M
Instrument :   GCMSD
Sample Name: Pampl-1
Misc Info  :
Vial Number: 1
```



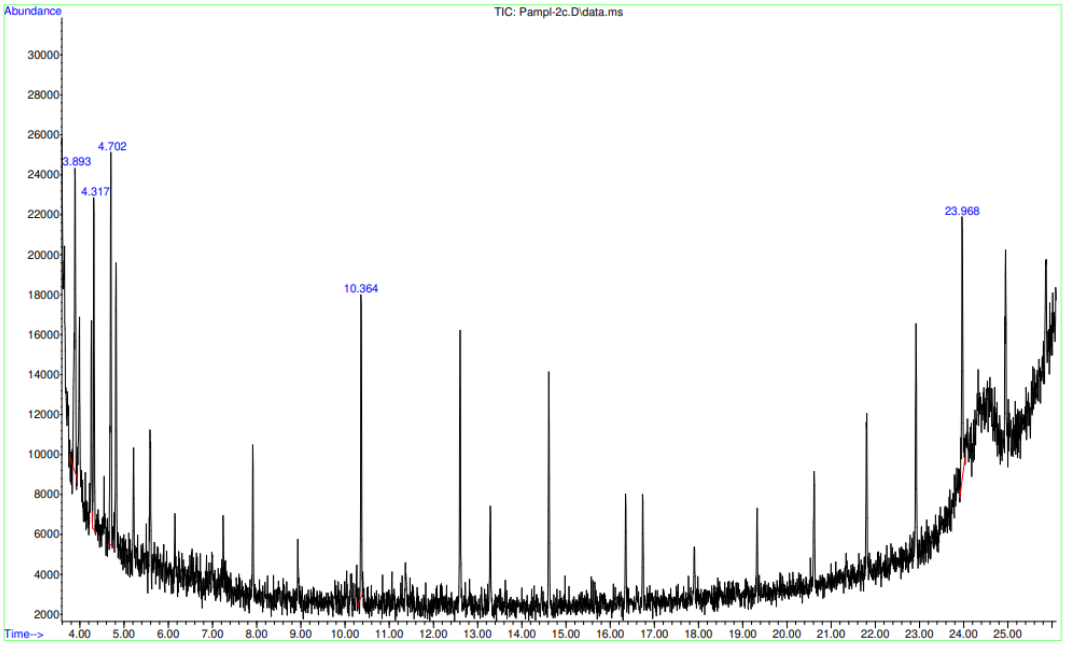
2. GC-MS of DCM Soxhlet extract:

```
File       :C:\msdchem\1\data\Pampl-2c.D
Operator   :
Acquired   : 21 Jan 2021  11:34     using AcqMethod EXTRAC-PAMPLONA.M
Instrument :   GCMSD
Sample Name: Pampl-2
Misc Info  :
Vial Number: 2
```



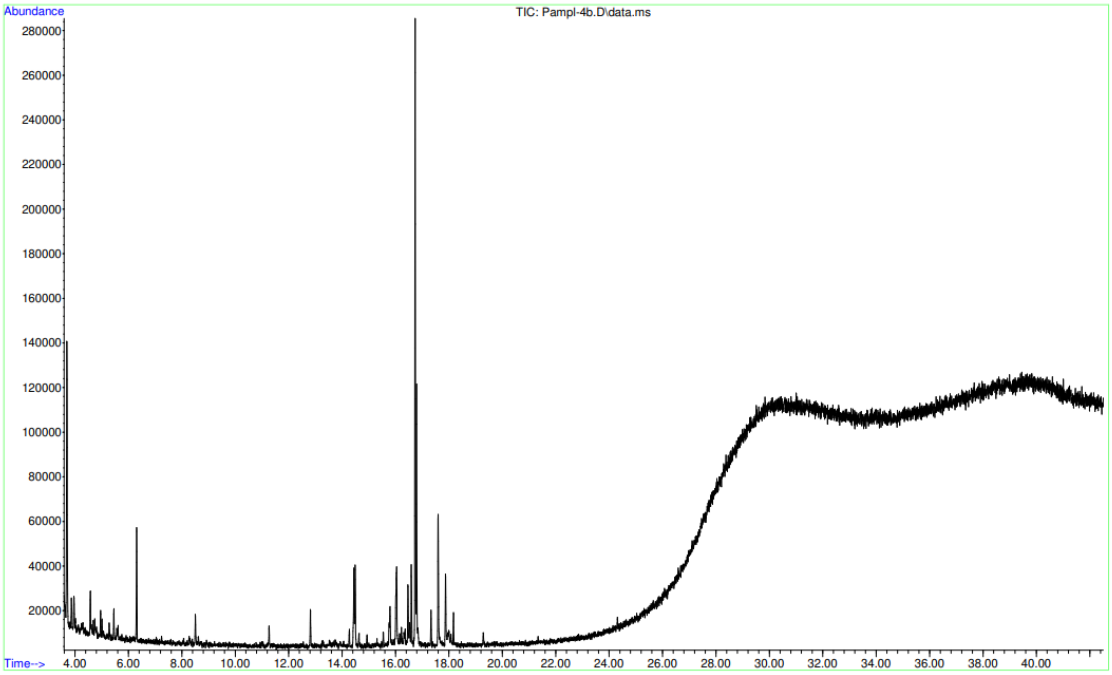
Enhancement of the GC-MS from minute 4 to 25:

```
File       :C:\msdchem\1\data\Pampl-2c.D
Operator   :
Acquired   : 21 Jan 2021  11:34     using AcqMethod EXTRAC-PAMPLONA.M
Instrument :   GCMSD
Sample Name: Pampl-2
Misc Info  :
Vial Number: 2
```



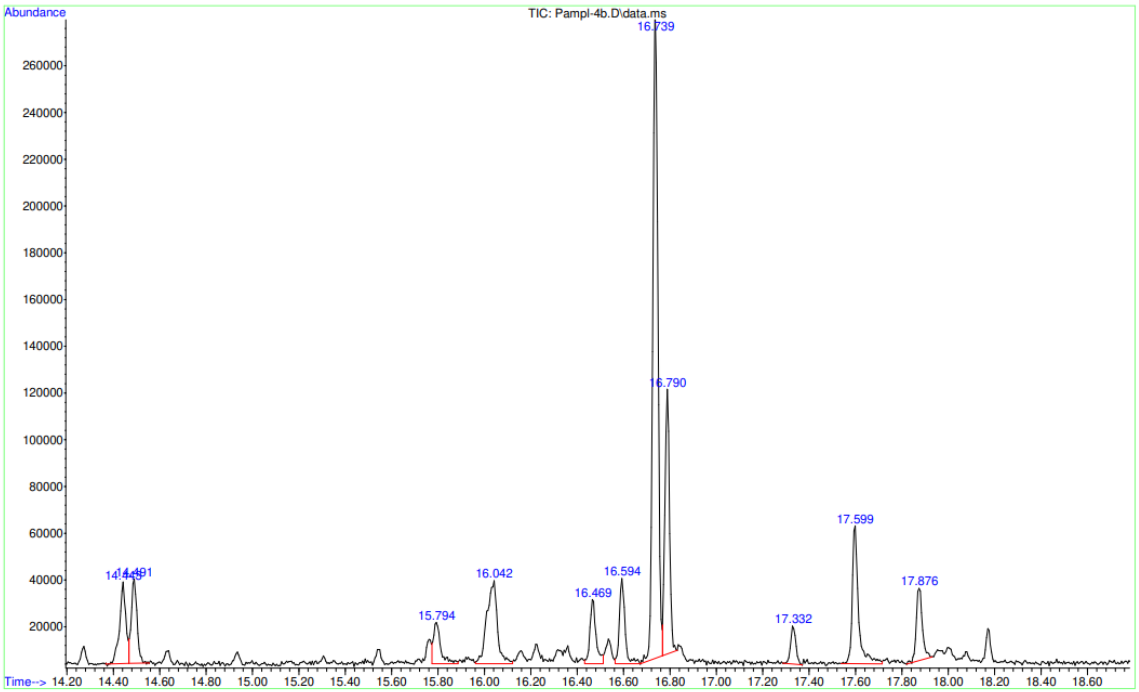
3. GC-MS of ethyl acetate Soxhlet extract:

```
File       :C:\msdchem\1\data\Pampl-4b.D
Operator   :
Acquired   : 19 Jan 2021  12:11     using AcqMethod EXTRAC-PAMPLONA.M
Instrument :   GCMSD
Sample Name: Pampl-4
Misc Info  :
Vial Number: 4
```



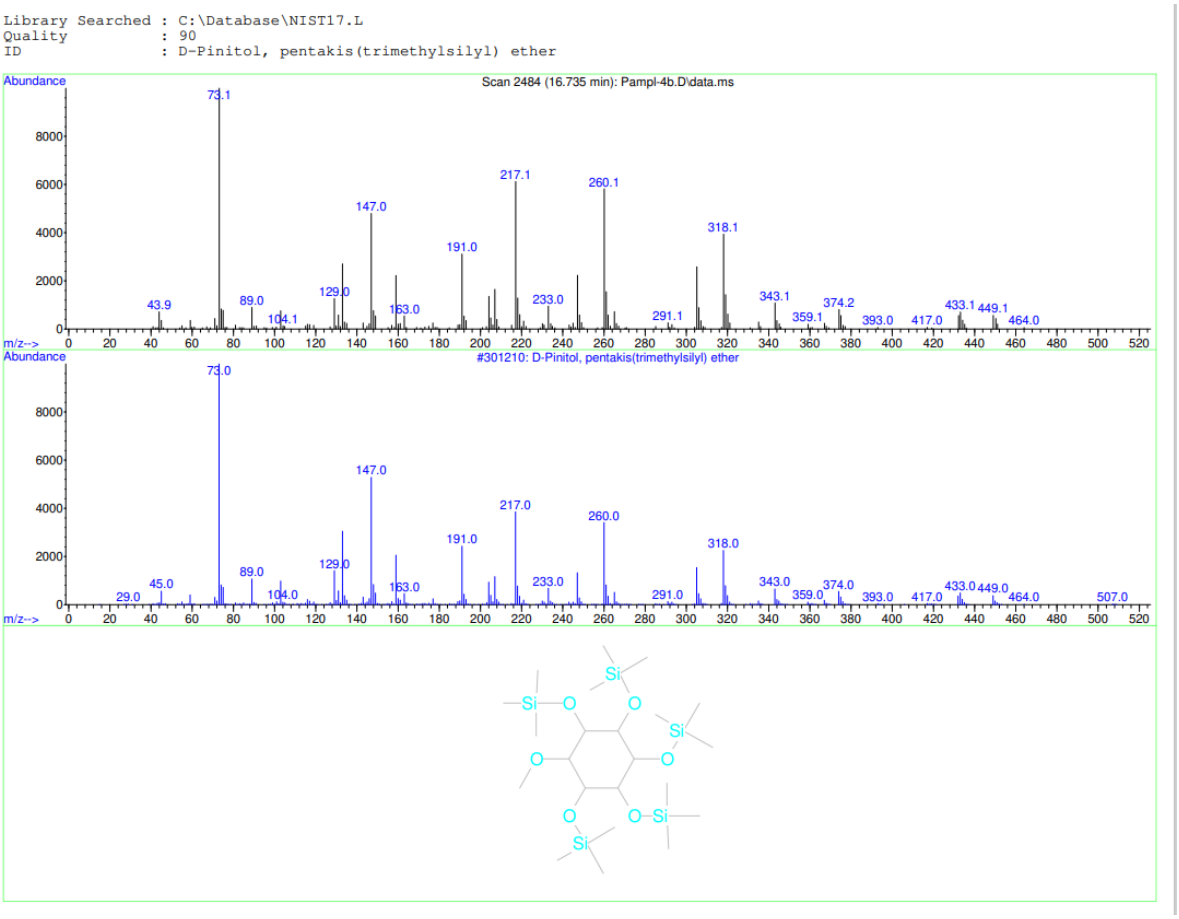
Enhancement of the GC-MS from minute 14 to 19:

```
File       :C:\msdchem\1\data\Pampl-4b.D
Operator   :
Acquired   : 19 Jan 2021  12:11     using AcqMethod EXTRAC-PAMPLONA.M
Instrument :   GCMSD
Sample Name: Pampl-4
Misc Info  :
Vial Number: 4
```

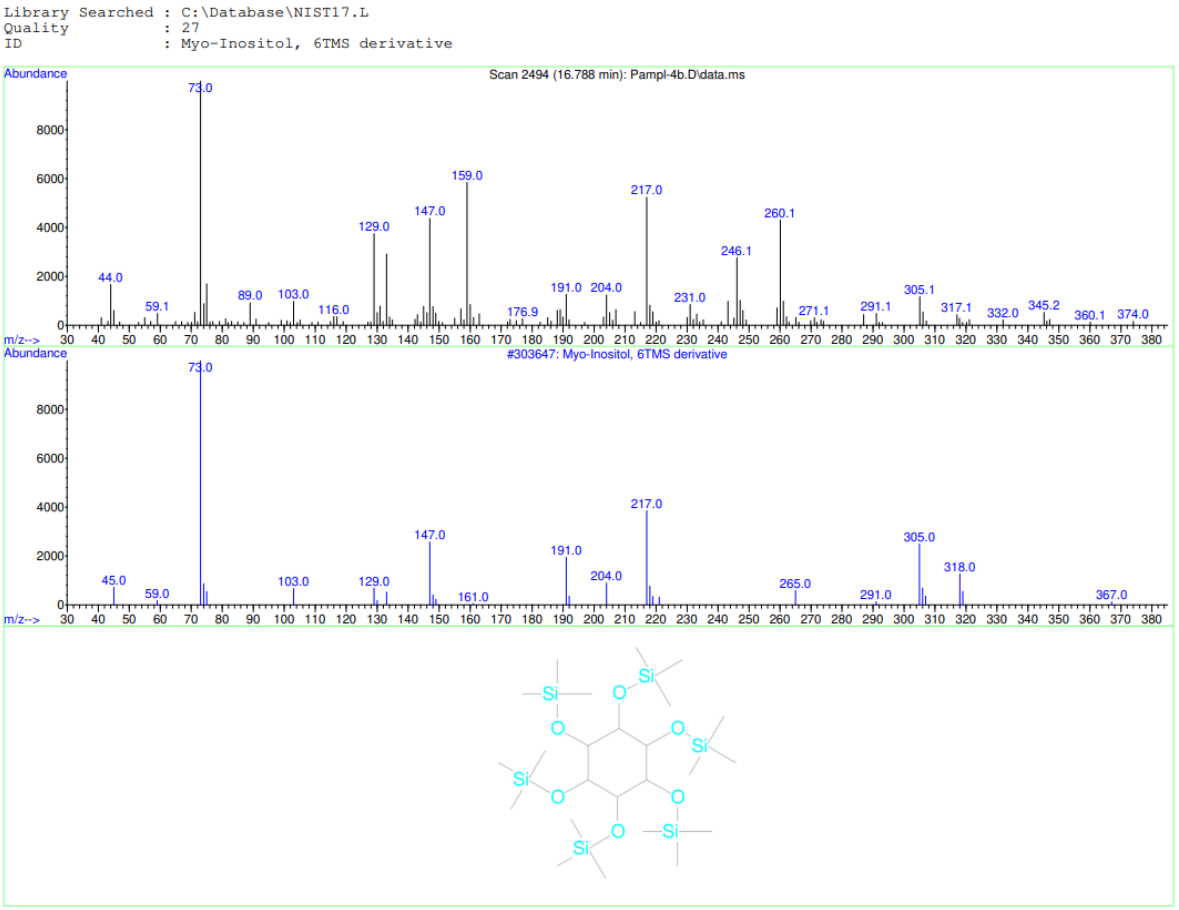


a. Most important identified peaks (Ethyl acetate Soxhlet extract):

Retention time: 16.7 min

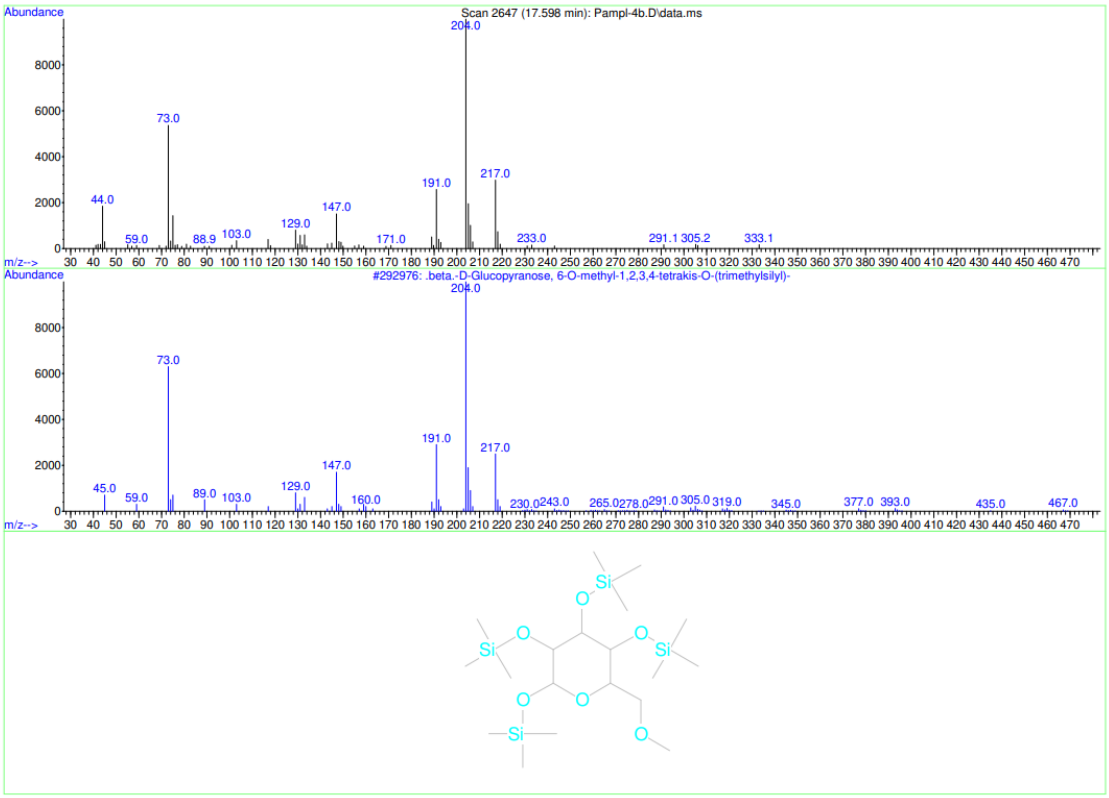


Retention time: 16.8 min



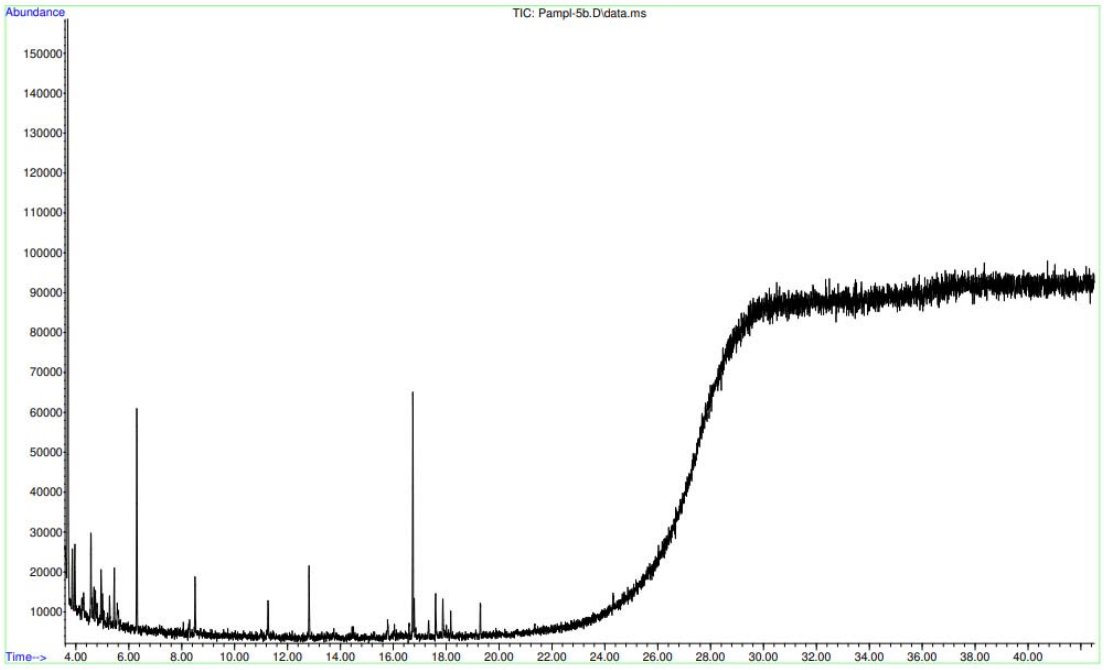
Retention time: 17.6 min

```
Library Searched : C:\Database\NIST17.L
Quality          : 91
ID               : .beta.-D-Glucopyranose, 6-O-methyl-1,2,3,4-tetrakis-O-(trimethylsilyl)-
```



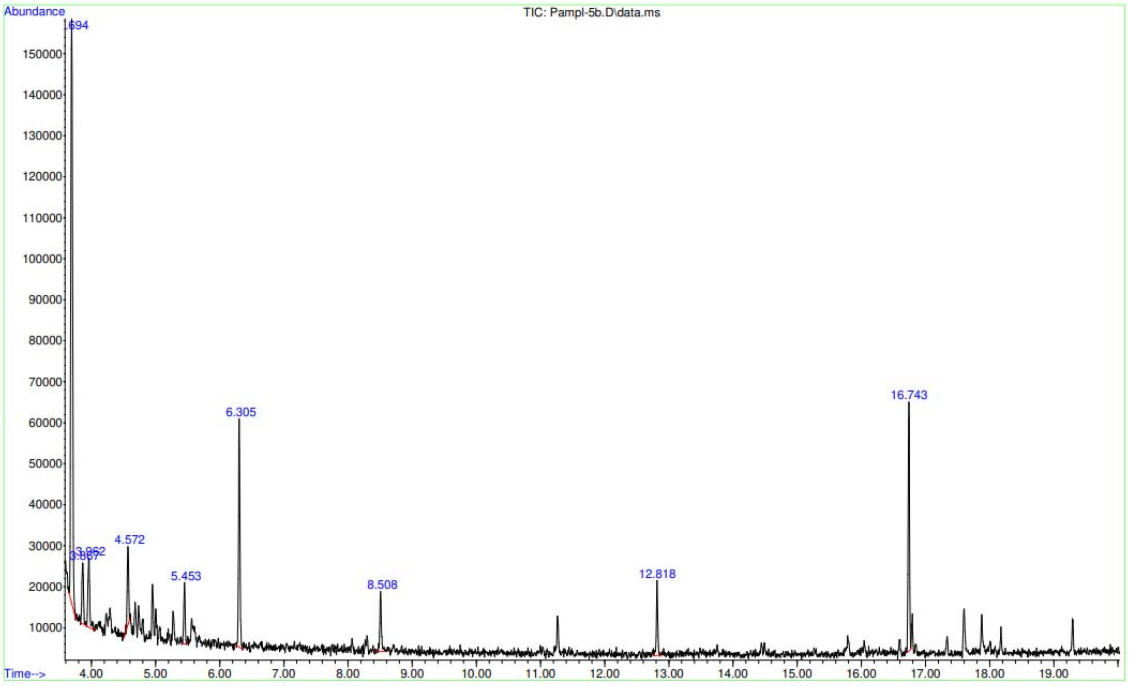
4. GC-MS of methanol Soxhlet extract:

```
File       :C:\msdchem\1\data\Pampl-5b.D
Operator   :
Acquired   : 19 Jan 2021  13:37     using AcqMethod EXTRAC-PAMPLONA.M
Instrument :   GCMSD
Sample Name: Pampl-5
Misc Info  :
Vial Number: 5
```



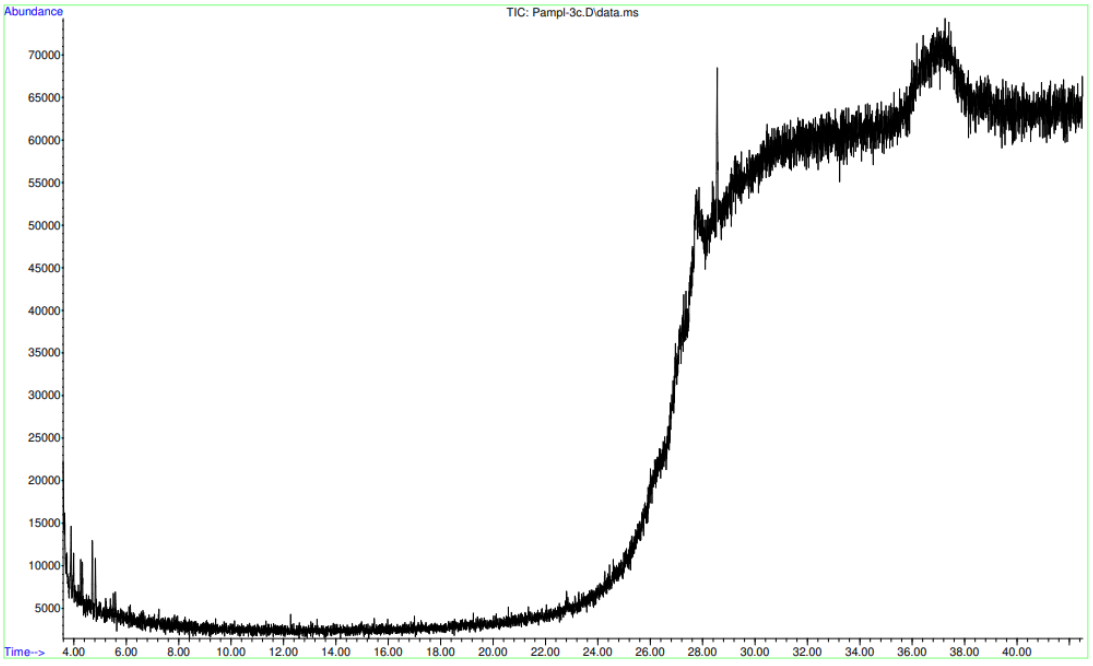
Enhancement of the GC-MS from minute 4 to 20:

```
File       :C:\msdchem\1\data\Pampl-5b.D
Operator   :
Acquired   : 19 Jan 2021  13:37     using AcqMethod EXTRAC-PAMPLONA.M
Instrument :   GCMSD
Sample Name: Pampl-5
Misc Info  :
Vial Number: 5
```



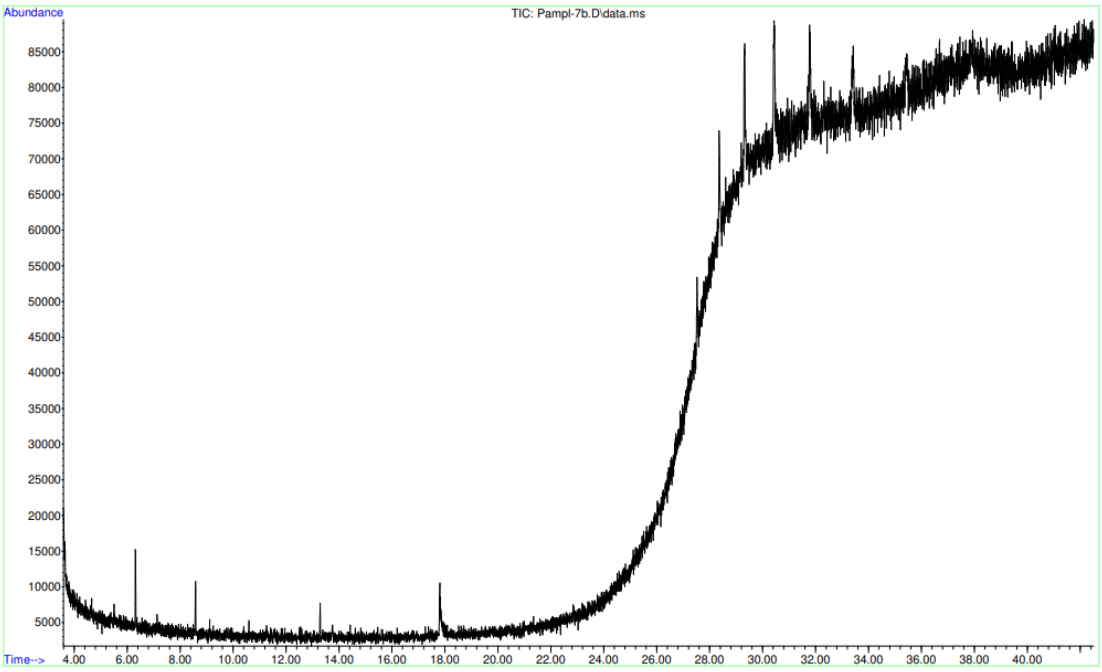
5. GC-MS of hexane column:

```
File       :C:\msdchem\1\data\Pampl-3c.D
Operator   :
Acquired   : 21 Jan 2021  12:25     using AcqMethod EXTRAC-PAMPLONA.M
Instrument :   GCMSD
Sample Name: Pampl-3
Misc Info  :
Vial Number: 3
```



6. GC-MS of DCM column:

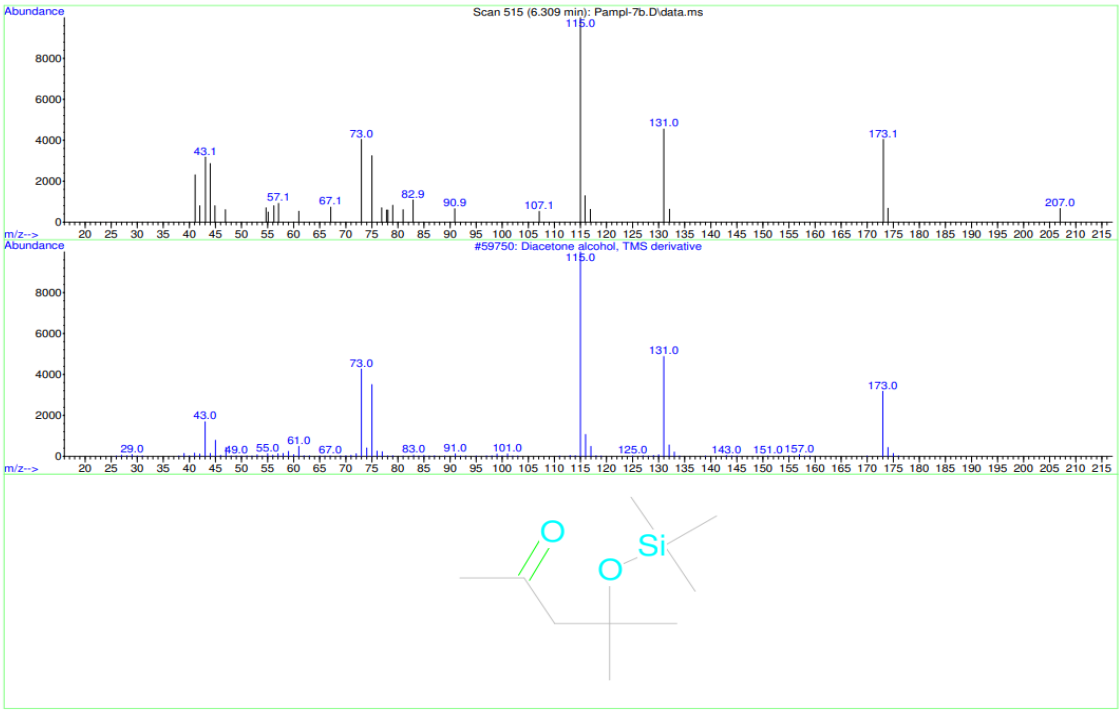
```
File       :C:\msdchem\1\data\Pampl-7b.D
Operator   :
Acquired   : 19 Jan 2021  15:19     using AcqMethod EXTRAC-PAMPLONA.M
Instrument :   GCMSD
Sample Name: Pampl-7
Misc Info  :
Vial Number: 7
```



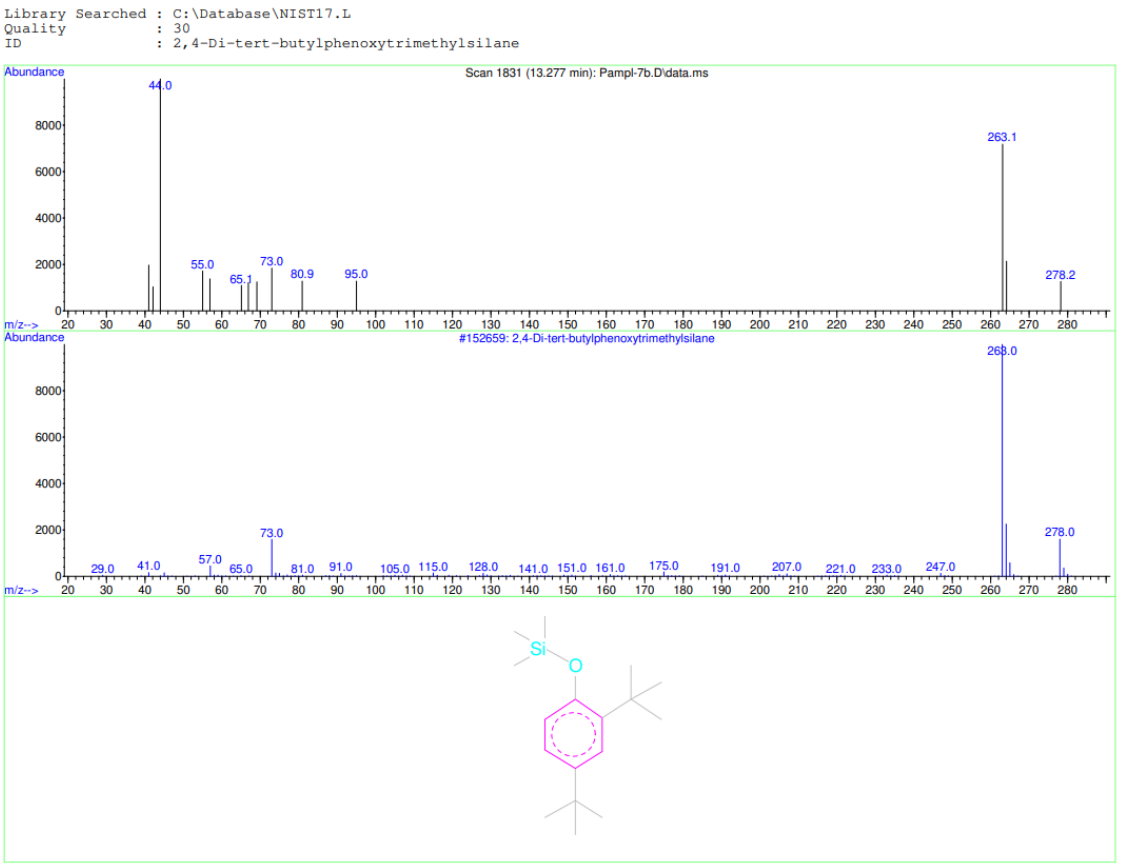
a. Most important identified peaks (DCM column):

Retention time: 6.3 min

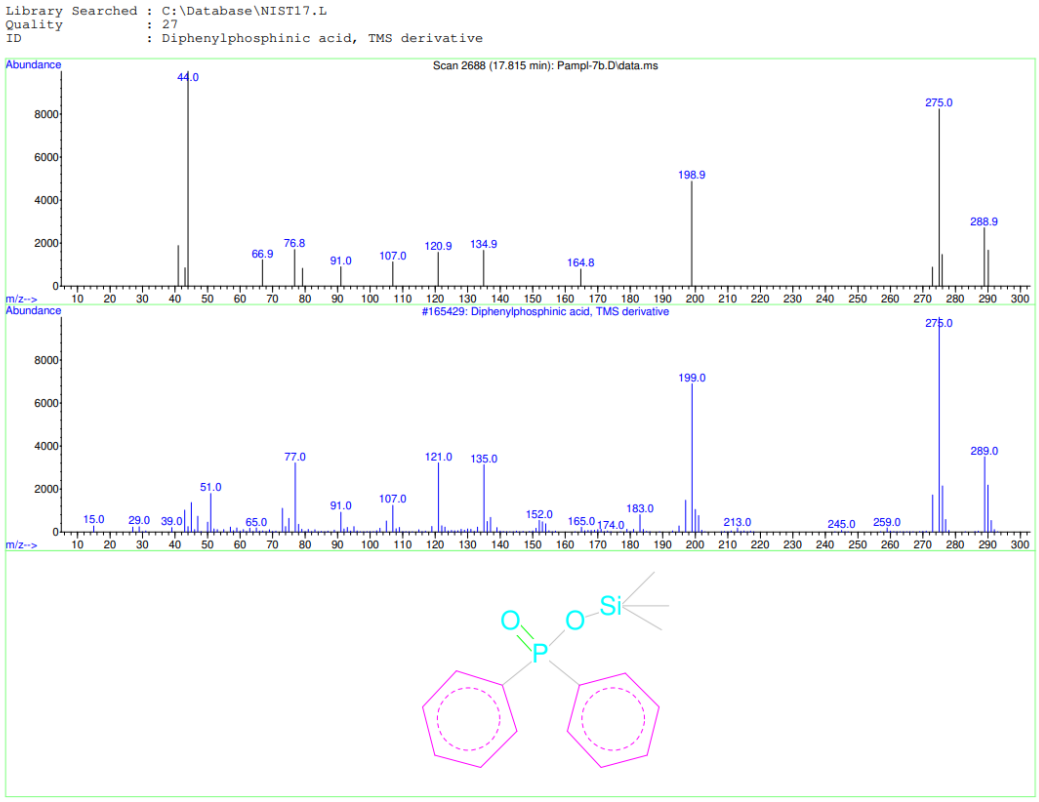
```
Library Searched : C:\Database\NIST17.L
Quality          : 86
ID               : Diacetone alcohol, TMS derivative
```



Retention time: 13.2 min

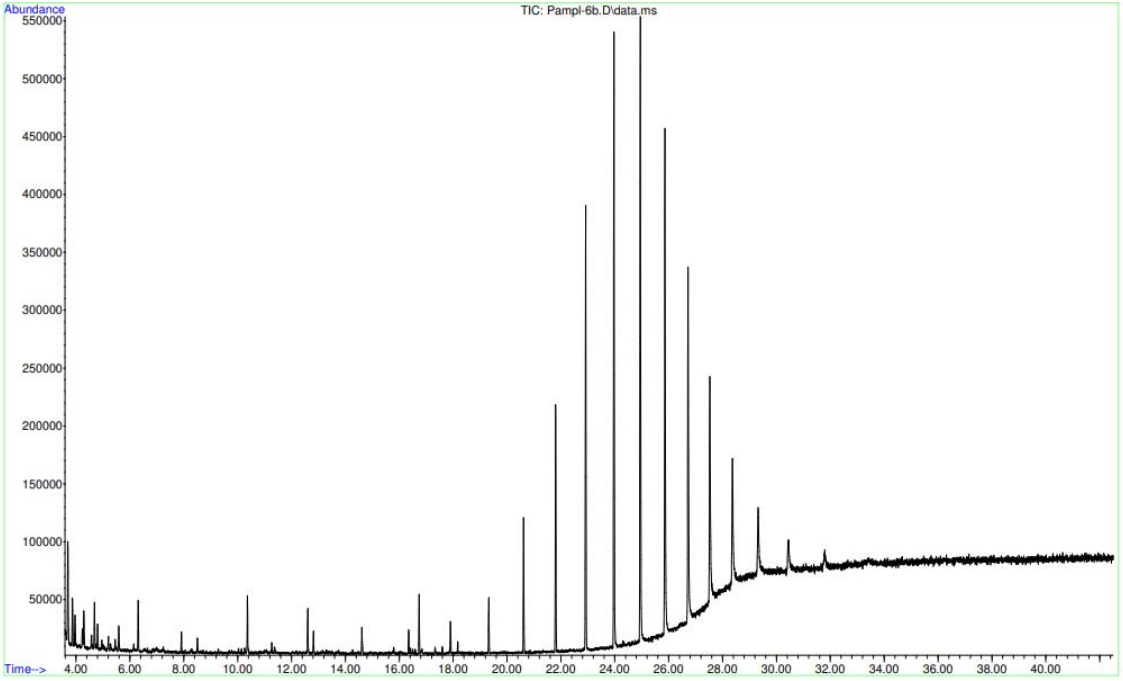


Retention time: 17.8 min



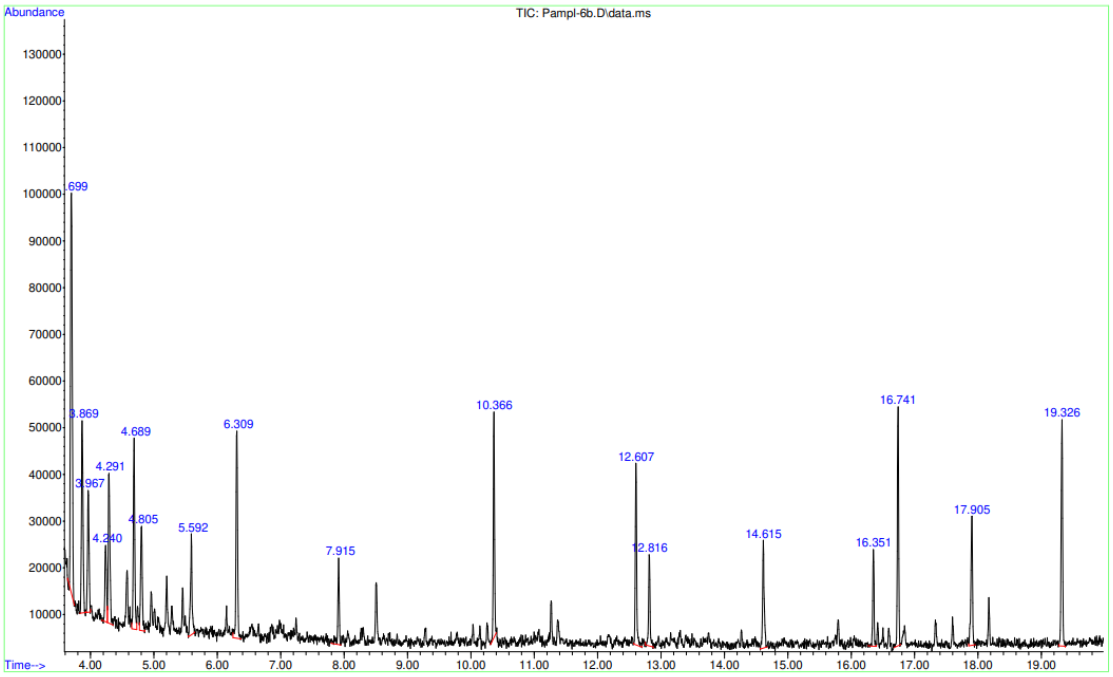
7. GC-MS of Methanol column

```
File       :C:\msdchem\1\data\Pampl-6b.D
Operator   :
Acquired   : 19 Jan 2021  14:28     using AcqMethod EXTRAC-PAMPLONA.M
Instrument :   GCMSD
Sample Name: Pampl-6
Misc Info  :
Vial Number: 6
```



Enhancement of GC-MS from minute 4 to 20

```
File       :C:\msdchem\1\data\Pampl-6b.D
Operator   :
Acquired   : 19 Jan 2021  14:28     using AcqMethod EXTRAC-PAMPLONA.M
Instrument :   GCMSD
Sample Name: Pampl-6
Misc Info  :
Vial Number: 6
```



a. Identified peak (methanol column):

Retention time: 16.7 min