Polymeric stationary phases based on poly(butylene terephthalate) and poly(4-vinylpirydine) in the analysis of polyphenols using supercritical fluid chromatography. Application to bee pollen

Laura Toribio*, Sandra Arranz, Ana M. Ares, José Bernal

I.U. CINQUIMA, Analytical Chemistry Group, University of Valladolid, Spain

* Corresponding author: Laura Toribio, I.U. CINQUIMA, Analytical Chemistry Group, University of Valladolid, C/ Paseo de Belén 5, E-47011 Valladolid, Spain.

Phone: 34-983185897, e-mail: ltoribio@qa.uva.es

Abstract

Two new polymer-based stationary phases; DCpak PBT (poly(butylene terephthalate)) and DCpak P4VP (poly(4-vinylpirydine)) were evaluated for the analysis of polyphenols using supercritical fluid chromatography (SFC). The compounds studied included phenolic acids and flavonoids. The different variables that influence the chromatographic separation, such as type and percentage of organic modifier, additive, pressure and temperature were examined. Using the DCpak P4VP column the retention was exceptionally high, obtaining better results with the DCpak PBT column. The separation of nine polyphenols was achieved using a gradient of modifier (methanol with 0.1% trifluoroacetic acid) from 5 to 50%, a pressure of 150 bar, a temperature of 35ºC and a flow-rate of 2 mL/min. The use of additives was necessary in order to obtain good peak shapes and efficiencies, achieving the best results with trifluoroacetic acid.. LODs and LOQs values were lower than 5 µg/mL in all the cases; meanwhile, the %RSD values for method repeatability and inter-day reproducibility were lower than 3% and 10% respectively. Finally, the proposed method was successfully applied to the analysis of polyphenols in commercial bee pollen; four compounds, namely cinnamic acid, p-coumaric acid, catechin and quercetin were identified and quantified.

Keywords: Bee pollen; Flavonoids; Phenolic acids; DCpak PBT; DCpak P4VP;Polymer based stationary phases; Polyphenols, SFC.

1. Introduction

The interest in supercritical fluid chromatography (SFC) continues growing because it offers a number of satisfactory benefits from a chromatographic point of view. These advantages include high efficiencies and resolutions, short analysis times and low consumption of organic solvents. In the past years, the development of SFC instrumentation was lower if compared with high performance liquid chromatography (HPLC); but recently there has been a significant advance, and the introduction of a new generation of instruments with improved robustness and performance has contributed to renew the interest in this technique. Taking into account that carbon dioxide is the main component of the mobile phase and it has a nonpolar nature, SFC has traditionally been used in the analysis of compounds with medium and low polarity. This limitation can be circumvented by using polar modifiers or additives, and in fact, at present, SFC covers a wider range of polarities being also applied to highly polar compounds [1–3]. Most of the SFC applications have been related to chiral separations, where SFC has shown all its potential and nowadays it is one of the preferred techniques [4,5]. Chiral stationary phases, based on polysaccharide derivatives, have demonstrated to have a high rate of success in the enantiomeric separation of a broad range of compounds, and can be considered of general use in SFC; which has favored the development of this sector. Achiral SFC is not as widely used as chiral SFC, because of the higher diversity of achiral compounds, and the fact that in most of the cases, there is not a single type of stationary phase that could provide the widespread applicability of C_{18} in HPLC High performance liquid chromatography (HPLC) is the preferred technique for achiral separations, because the selection of the stationary phase is simpler, since most of the separations are achieved using C_{18} stationary phases; while in achiral SFC there is not a stationary phase of general use [6]. Nevertheless, in the last few years there has been a growing interest in expanding the applicability of SFC into the achiral separation area. New achiral polar stationary phases have been commercialized [7], being most of them

based on low molecular compounds containing nitrogen heterocycles (principally pyridine) or hydrogen bonding groups such as the diol ones [8,9]. This has increased the number of published works related to the achiral SFC analysis of natural compounds [10-17], pharmaceuticals [18-22], or biological samples [23] y,. More recently, two new polymeric achiral columns, the DCpak PBT based on poly(butylene terephthalate) and the DCpak P4VP based on poly(4-vinylpirydine), have been introduced by Daicel. The possibilities that these stationary phases could offer, are currently being studied [24]. Good results have been obtained with the PBT based column in the planarity recognition of isomeric PHAs, as well as in the separation of structurally related compounds such as coumarin derivatives, phthalates and estrogenic hormones.

Among natural compounds polyphenols have attracted the attention not only of scientists but also of the public in general, due to their health benefits and their important properties such as the antioxidant, anticarcinogenic, cardioprotective, anti-inflammatory and antibacterial activities [25–29]. Polyphenols are secondary metabolites of plants and exhibit several important roles in the plants life [30, 31]. The term “polyphenols” include a large number of compounds which have a common structure: at least one aromatic ring in which one or more hydrogen atoms are substituted by hydroxyl groups. Phenolic acids and flavonoids are the most important groups of polyphenols, since they constitute around 30% and 70% of dietary polyphenols respectively [32, 33]. Supercritical fluid extraction (SFE) has been extensively used for the extraction of these compounds from foods or plants [34–38]. On the contrary, SFC has scarcely been used for their analysis, probably because polyphenols are polar compounds, with a high diversity of chemical structures, and, as previously mentioned, the nonpolar character of CO_2 make the separation of these kind of compounds more challenging. Ramirez et al. [39] developed specially designed stationary phases for the separation of phenolic compounds using pure CO_2. In other works, C_{18}

stationary phases have been employed at analytical [40, 41] and semi preparative scales [42], but usually polar stationary phases along with an organic modifier and acidic additive are used. In this way, different polar stationary phases such as silica [43], cyano [44, 45] or diol [46] have provided very good resolutions in short analysis times. However, most of the works published are focused on one family of polyphenols, usually phenolic acids or flavonoids, and different stationary phases are used for each group of compounds. The simultaneous separation of phenolic acids and flavonoids was studied in just one work [46] . In this case, the separation was achieved by coupling two diol columns. .

Nowadays the chromatographic separation of polyphenols is not a problematic issue, and it has been deeply studied using HPLC with satisfactory results [47–50], although the main drawbacks are the long analysis times and the consumption of organic solvents. Taking into account the renewed interest in SFC, and the effort to develop new stationary phases with better selectivities and specially designed to be used with this technique; it is important to explore the capabilities of these new polymer based columns, which have interaction mechanisms different from the conventional ones. In this way, it is interesting to study the analysis of polar compounds, such as polyphenols, using SFC and these columns, in order to check if it is possible to improve the results obtained previously. Moreover, the results obtained will contribute to a better knowledge of the type of compounds that could be resolved.. Thus, the aim of this work was to study the separation of nine polyphenols, including phenolic acids and flavonoids, using SFC and the two recently commercialized polymer-based columns DCpak PBT and DCpak P4VP; and to apply the proposed method to the analysis of a complex matrix as it is bee pollen.

2. Material and methods

2.1. Reagents

All the organic solvents employed (methanol, ethanol, isopropanol, ethyl acetate) were HPLC grade and obtained from Lb Scan (Dublin, Ireland). The phenolic acids (cinnamic acid, ferulic acid, p-coumaric acid, sinapic acid, caffeic acid and gallic acid) and the flavonoids (naringenine, catechin and quercetin), were purchased from Sigma-Aldrich (Madrid, Spain). Their standard stock solutions were prepared in ethanol at the 300 µg/mL level. Triethylamine (TEA), formic acid (FA), trifluoroacetic acid (TFAA), acetic acid, ammonium sulfate and phosphoric acid were of analytical grade and obtained from Sigma-Aldrich (Madrid, Spain). Carbon dioxide was SFC grade and obtained from Carburos Metálicos (Barcelona, Spain).

2.2. Sample treatment

A commercial bee pollen sample was obtained from a local market (Valladolid, Spain). It was ground and sieved through 40 mesh, then it was dried overnight at 30ºC and three subsamples were submitted to analysis. The extraction of phenolic compounds was performed according to a previously published methodology [51] with minor modifications. Briefly, 5 g of ground pollen was dissolved in 25 mL of ethyl acetate; then 12.5 mL of 40% ammonium sulfate and 2.50 mL of 20% phosphoric acid were added. The flask was stirred for 20 min and centrifuged for 10 min (1000 rpm). The remaining solid residue was submitted to a second extraction process, and the supernatants were combined and transferred to a separation funnel. The organic phase was collected (top phase) and the aqueous phase was extracted again with 25 mL of ethyl acetate. All the organic phases were collected in a flask and concentrated to dryness in a vacuum rotary evaporator at 30ºC. Finally the residue was dissolved in 2 mL of ethanol and filtered through 0.45 µm pore size nylon filter. During all the process the extracts were protected from light using aluminum foil.

2.3. Instrumentation

The supercritical fluid chromatograph used was from Jasco (Tokyo, Japan). It was equipped with two PU-2080 pumps for supplying the carbon dioxide and the modifier (methanol with 0.1% TFAA), which was delivered using a gradient program from 5% to 50%. The autosampler was an AS-2059-SF model with a 20 µL loop injection volume. The column was thermostated in a CO-2065 oven and the detector employed was a MD-2015 diode-array model. Four wavelengths were monitored: 220 nm, 270 nm, 320 nm and 370 nm. The pressure was controlled by a BP-2080 pressure regulator. Two columns packed with polymer based stationary phases (see Figure 2) were employed: DCpak PBT (250 x 4.6 mm), initially DCpak SFC-A, and packed with poly(butylene terephthalate) (PBT) coated on a 5 µm silica gel support; the other column was DCpak P4VP (250 x 4.6 mm), initially DCpak SFC-B, and packed with poly(4-vinylpirydine) linked to a 5 µm silica gel support. Both of them were kindly donated by Daicel Corporation (Tokyo, Japan).

3. Results and discussion

3.1. Optimization of the SFC conditions

3.1.1. Selection of the stationary phase and organic modifier

As can be seen in Figure 2, the studied polyphenols possess several functional groups that can interact with the stationary phase through hydrogen bonding, as well as through π-π interaction with the aromatic rings. Therefore, the use of organic modifier was required to obtain reasonable retention times. The highest retentions were observed on the DCpak P4VP column which could be caused by the better accessibility of the functional groups on this stationary phase and the stronger hydrogen bonding interaction with the pyridine nitrogen. On this stationary phase, the compounds with two or more hydrogen-donor groups were strongly retained even using 40% of organic modifier and 0.5% of additive (see Table

1). This is the case of caffeic acid which has three hydrogen-donor groups and its retention time was higher than 40 minutes; while cinnamic acid, that only possesses one –OH group, showed the lowest retention. Other basic additives, such as isopropylamine or ammonia, did not improve the results, and acidic additives were not used with this column in order to not alter the stationary phase by protonation of the pyridine nitrogen, as it was stated by its manufacturer. Taking these results into account, the study was continued using the DCpak PBT column. On this column, the retention was lower and the elution order was similar to that observed on the DCpak P4VP (see Table 1). The first eluted compounds were the phenolic acids and the last ones the flavonoids; within each group the retention increased with the number of –OH groups. In order to achieve the baseline resolution of the nine compounds, a gradient of organic modifier was necessary. Firstly, several experiments were conducted with different organic modifiers (methanol, ethanol and isopropanol), and the results showed that the lowest retention times were obtained when using methanol; meanwhile, neither selectivity nor resolution were affected markedly by the nature of the organic modifier. Thus, methanol was the organic solvent selected as modifier. Afterwards, several gradients of modifier were tested to elute polyphenols without co-elutions (data not shown). The shortest analysis time was obtained by starting with 5% of organic modifier for 3 minutes, then it was increased to 20% at 1.25%/min, and finally increased at 15%/min to 50%, which was held for 7 minutes (see Table 1). Good resolutions were obtained in an analysis time of 22 minutes.

3.1.2. Effect of the additive

Additives are frequently used in SFC to improve the peak shape and the elution of highly retained compounds, by reducing unwanted interactions between the stationary phase and the analytes. As a rule, basic additives are used for the separation of basic compounds and

acidic additives for acidic compounds. Taking into account the acidic character of the compounds studied, several acidic additives (trifluoroacetic acid (TFAA), formic acid, citric acid and acetic acid) were checked, with the DCpak PBT column, in order to decrease the retention time and improve the peak shape of flavonoids, especially that of quercetin. The use of additives improved column efficiency especially for the last eluted compound (quercetin), as well as the separation between p-coumaric, sinapic and caffeic acids. The results obtained for the most retained compound, which also exhibited the worst peak shape, are shown in Table 2. As can be seen, additives did not decrease markedly the analysis time, but column efficiency and peak symmetry was clearly improved using trifluoroacetic, formic or citric acids. It should be noted that the effect of using acetic acid is negligible if compared with the results obtained using pure methanol, probably due to the weaker acid character. Among the different additives checked, TFAA provided the best results. Chromatograms obtained using different percentages of TFAA are shown in Figure 3. Changes on resolution and efficiency were negligible, it was just observed a slight increase of the retention when the percentage of TFAA decreased; thus the lowest percentage (0.1% TFAA) was selected to continue the work.

3.1.3. Effect of temperature

Temperature is another parameter that controls the chromatographic separations. Changes in the temperature affect retention and selectivity, and it can be used to improve the separation. In this study the temperature was varied between 20ºC and 35ºC, due to the fact that lower values were not achievable by the equipment and the maximum working temperature of the column was 40ºC. The results obtained (see Figure 4) showed that in most of the cases, the retention increased slightly when the temperature raised, but the opposite effect was observed for quercetin. At constant pressure, an increase in the temperature of a binary fluid causes a decrease in the density, but does not always signify

an increase of the retention; it depends on the column, the compound and the percentage of modifier. In fact there are numerous cases where a temperature rise can increase, decrease or have no effect on retention [52-54]. In the case of quercetin, the retention decrease could be justified by the high percentage of modifier needed for its elution (50%). In this case the mobile phase has a liquid-like behavior and increasing the temperature increases the solubility of the compound in the mobile phase. The effect observed would be similar to that observed in liquid chromatography. On the other hand, a significant influence was noticed on the selectivity and resolution of the pairs: ferulic–p-coumaric acids, caffeic--sinapic acids and naringenin-catechin. As can be seen (Figure 5), the separation of these compounds improved as the temperature increased, obtaining the best separation at 35ºC.

3.1.4. Effect of pressure and flow-rate

In order to decrease the analysis time, an increase of the pressure and flow-rate were also checked. Increasing the pressure up to 200 bar led to a slight decrease of the analysis time from 22 to 20 min, but only a partial resolution (Rs= 0.8) was obtained between ferulic acid and p-coumaric acid. The same effect was observed when increasing the flow-rate to 3 mL/min, some compounds were partially resolved and the analysis time decreased to 17 min.

3.1.5. Selection of the injection volume

The injection volumes assayed were 5 µL, 10 µL and 20 µL. The chromatograms obtained are showed in Figure 6. As it was expected, when the injection volume was lower than 20 µL, peak efficiency increased specially for the first four eluted compounds, and narrower peaks were obtained. Nevertheless, in the cases of caffeic acid, gallic acid, catechin and quercetin the improvement in the efficiency was negligible, moreover the detection limits obtained with 5 µL or 10 µL were higher than those obtained with 20 µL. Taking into account that using 20 µl all the peaks were baseline resolved and the detection limits were the lowest,

this injection volume was selected to continue the work. **3.1.6. Comparison with previous SFC publications**

Taking into account all the above-mentioned experiments, the chromatographic conditions finally selected were: 35ºC, 2mL/min, 150 bar and a gradient of modifier (methanol with 0.1% TFAA) from 5% to 50%. Comparing these conditions with those obtained in the previous publication in which SFC was employed to determine a mixture of phenolic acids and flavonoids s by using two diol columns (250 x 4.6 mm, 5µm) [46], it could be said that, although the gradient of modifier employed was different, the behavior was very similar as far as the elution order was concerned. On the other hand, it should be noted that the analysis time was similar in both cases, but in our work only one column was used. Tang et al [44] employed SFC and a Platisil CN column (250 mm × 4.6 mm, 5 µm) to resolve 12 phenolic acids. In this case the retention (considering the same compounds) was higher than the obtained in our work with the PBT column. In the work of Tang et al, the elution of the phenolic acids was achieved with a higher percentage of organic modifier; moreover the addition of two polar additives (water and formic acid) was needed to elute gallic acid in a reasonable time and with better peak shape. It should be also commented that, in this work, the detection limits obtained with the DAD detector were higher than those obtained in our work. As far as elution order is concerned, it was very similar to that obtained in our work, just a slight difference was observed; on the CN column sinapic acid eluted before than p-coumaric acid.

Taking into account all the above mentioned reasons, it could be said that the DCpak PBT column, provided good results, and in some aspects more advantageous than those obtained in previous works with other columns.

3.2. Method Performance

The limits of detection (LOD) and quantification (LOQ), were determined at the wavelength of the maxima absorption for each compound, and they were calculated as 3 and 10 times the signal to noise ratio (S/N) respectively. As can be seen in Table 3, the LOD values ranged from 0.12 to 1.42 μg/mL; meanwhile, LOQ values varied between 0.38 to 4.73 μg/mL. The instrumental repeatability was evaluated at three different levels (1.0, 5.0, and 15.0 μg/mL) by injecting each standard solution six times during the same day. The relative standard deviation (%RSD) of peak areas and retention times were in all the cases below 3% and 1.5% respectively (data not shown). The inter-day reproducibility was determined by injecting three times each standard solution during three consecutive days. The %RSD values were close to 10% and 5% for the peak areas and retention times, respectively. Finally, standard calibration curves were prepared for each compound from the LOQ to 100 μg/mL. Calibration curves (n=3, at six concentration levels) were constructed by plotting the signal on the *y*-axis (analyte peak area) against the analyte concentration on the *x*-axis. The graphs obtained in all the calibration curves were straight lines, and the coefficient of the determination values (R^2) was above 0.99 in all cases (data not shown). Absence of bias was confirmed by a *t* test and by studying the distribution of residuals (data not shown). Method accuracy was determined at three concentration levels (1.0, 5.0 and 15.0 μg/mL), by injecting three replicates of each solution. The mean recovery ranged from of 98.4% to 102.5% and the %RSD from 0.7% to 0.5%.

3.3. Application to bee pollen analysis

In order to check the feasibility of the proposed method in the analysis of complex samples, it was applied to the analysis of commercial bee pollen. Peak identification was based on peak spectra, peak purity and retention time. As can be seen in Figure 6, four polyphenols (cinnamic acid, p-coumaric acid, catechin and quercetin) were identified. Other compounds

eluted in the region of phenolic acids and flavonoids, could be also polyphenols. Although the purpose of this work was not to determine the polyphenolic content of bee pollen, the identified compounds were also quantified and the results are summarized in Table 4. The highest concentrations were found for the flavonoids catechin and quercetin.

4. Conclusions

The separation of nine polyphenolic compounds using SFC was favorably achieved by using a recently commercialized polymer-based column (DCpak PBT). As far as elution order is concerned the compounds showed a similar behavior on the DCpak PBT and DCpak P4VP columns, nevertheless the retention for gallic acid and flavonoids was extremely high on the DCpak P4VP column. Therefore, the PBT stationary phase was finally selected, and it was observed that the retention increased with the number of –OH groups and aromatic rings, eluting firstly phenolic acids and then flavonoids. A gradient of modifier (methanol with 0.1% of TFAA) from 5% to 50% was necessary in order to achieve the baseline separation of the nine compounds and to elute the flavonoids in a reasonable time. The use of 0.1% TFAA as additive was required to improve peak shape and column efficiency, especially for the last eluted compound. Moreover, it was also found that the temperature affected the resolution between some pairs of compounds, obtaining the best results at 35ºC. Comparing these results with those obtained in a previous SFC work in which two diol columns were required to perform the separation of a mixture of phenolic acids and flavonoids, it should be said that as far as elution order is concerned the DCpak PBT column behaved in a similar way but the separation was obtained by using just one column. On the other hand, it should be noted that the use of the PBT stationary phase in the analysis of flavonoids would have limitations due to the high retention obtained for these compounds. Finally, the proposed

method was successfully applied to the determination of polyphenolic compounds in bee pollen.

Acknowledgments

Authors thank Chiral Technology Europe (Illkirch France) and specially Dra. Pilar Franco, for the generous donation of the columns.

References

[1] M.A. Patel, F. Riley, M. Ashraf-Khorassani, L.T. Taylor, Supercritical fluid chromatographic resolution of water soluble isomeric carboxyl/amine terminated peptides facilitated via mobile phase water and ion pair formation, J. Chromatogr. A. 1233 (2012) 85–90.

[2] L.T. Taylor, Packed column supercritical fluid chromatography of hydrophilic analytes via water-rich modifiers, J. Chromatogr. A. 1250 (2012) 196–204.

[3] L.T. Taylor, Separation of Ionic Analytes Using Supercritical Fluid Chromatography, LCGC North Am. 27 (2009) 490–502.

[4] E. Lemasson, S. Bertin, C. West, Use and practice of achiral and chiral supercritical fluid chromatography in pharmaceutical analysis and purification, J. Sep. Sci. 39 (2016) 212–233.

[5] J.M. Płotka, M. Biziuk, C. Morrison, J. Namieśnik, Pharmaceutical and forensic drug applications of chiral supercritical fluid chromatography, TrAC - Trends Anal. Chem. 56 (2014) 74–89.

[6] R. McClain, M. Przybyciel, A Systematic Study of Achiral Stationary Phases Using Analytes Selected with a Molecular Diversity Model, LC-GC North Am. 29 (2011) 894–906.

[7] T. Berger, B. Berger, R. E. Majors A Review of Column Developments for Supercritical Fluid Chromatography, LC-GC North Am. 28 (2010) 344-357.

[8] C.F. Poole, Stationary phases for packed-column supercritical fluid chromatography, J. Chromatogr. A. 1250 (2012) 157–171.

[9] A. Grand-Guillaume Perrenoud, J. Boccard, J.L. Veuthey, D. Guillarme, Analysis of

basic compounds by supercritical fluid chromatography: Attempts to improve peak shape and maintain mass spectrometry compatibility, J. Chromatogr. A. 1262 (2012) 205–213.

[10] B. Li, H. Zhao, J. Liu, W. Liu, S. Fan, G. Wu, R. Zhao, Application of ultra-high performance supercritical fluid chromatography for the determination of carotenoids in dietary supplements, J. Chromatogr. A 1425 (2015) 287-292.

[11] D. N. Rathi, C.Y. Liew, M.N. Mohd Fairulnizal, D. Isameyah, G. Barknowitz, Fat-Soluble Vitamin and Carotenoid Analysis in Cooking Oils by Ultra-Performance Convergence Chromatography, Food Anal. Methods 10 (2017) 1087–1096.

[12] M. Ashraf-Khorassani , G. Isaac, P. Rainville, K. Fountain, L.T Taylor, Study of UltraHigh Performance Supercritical Fluid Chromatography to Measure Free Fatty Acids With Out Fatty Acid Ester Preparation, J. Chromatogr. B, 997 (2015) 45-55.

[13] V. Pauk, T. Pluhàček, V. Havlíček, K. Lemr, Ultra-high performance supercritical fluid chromatography-mass spectrometry procedure for analysis of monosaccharides from plant gum binders, Anal. Chim. Acta 989 (2017) 112-120.

[14] O. Cicleta, D. Barron, S. Bajic, J.L. Veuthey, D. Guillarme, A. Grand-Guillaume Perrenoud, Natural compounds analysis using liquid and supercritical fluid chromatography hyphenated to mass spectrometry: Evaluation of a new design of atmospheric pressure ionization source, J. Chromatogr. B 1083 (2018) 1–11.

[15] B. Andria, P. Lebrun, A. Dispas, R. Klinkenberg, B. Streel, E. Ziemons, R.D. Marini,Ph. Huberta, Optimization and validation of a fast supercritical fluidchromatography method for the quantitative determination of vitamin D3 and its related impurities, J. Chromatogr. A 1491 (2017) 171–181.

[16] J. Duval, C. Colas, V. Pecher, M. Poujol, J. F. Tranchant, E. Lesellier, Hyphenation of ultra high performance supercritical fluid chromatography with atmospheric pressure chemical ionisation high resolution mass spectrometry: Part 1. Study of the coupling parameters for the analysis of natural non-polar compounds, J. Chromatogr. A 1509 (2017) 132-140.

[17] K. Taguchi, E. Fukusaki, T. Bamba, Simultaneous Analysis for water- and fat-soluble vitamins by a novel single chromatography technique unifying supercritical fluid chromatography and liquid chromatography. J. Chromatogr. A, 1362 (2014) 270– 277.

[18] C. West, E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, Interest of achiral-achiral tandem columns for impurity profiling ofsynthetic drugs with supercritical fluid chromatography, J. Chromatogr. A 1534 (2018) 161–169.

[19] A.J. Alexander, L. Zhang, T.F. Hooker, F.P. Tomasella, Comparison of supercritical fluid chromatography and reverse phase liquid chromatography for the impurity profiling of the antiretroviral drugs lamivudine/BMS-986001/efavirenz in a combination tablet, J. Pharm. and Biomed. Anal. 78– 79 (2013) 243– 251

[20] E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West, Comparison of ultra-high performance methods in liquid andsupercritical fluid chromatography coupled to electrospray ionization– mass spectrometry for impurity profiling of drug candidates, J. Chromatogr. A 1472 (2016) 117–128.

[21] M.L. de la Puente, P. López Soto-Yarritu, J. Burnett, Supercritical fluid chromatography in research laboratories: Design, development and implementation of an efficient generic screening for exploiting this technique in the achiral environment, J. Chromatogr. A 1218 (2011) 8551– 8560.

[22] L. Herpin, E. Bichon, L. Rambaud, F. Monteau, B. Le Bizec, Comparison between

liquid chromatography and supercritical fluid chromatography coupled to mass spectrometry for beta-agonists screening in feeding stuff, J.of Chromatogr. B 1086 (2018) 130–137.

[23] A. Dispas, H. Jambo, S. André, E. Tyteca, P. Hubert, Supercritical fluid chromatography: apromising alternative to current bioanalytical techniques, Bioanalysis 10 (2017) 107-124.

[24] K. Nagai, T. Shibata, S. Shinkura, A. Ohnishi, Poly(butylene terephthalate) based novel achiral stationary phase investigated under supercritical fluid chromatography conditions, J. Chromatogr. A 1549 (2018) 85–92.

[25] L. Bravo, Polyphenols : Chemistry , Dietary Sources , Metabolism , and Nutritional Significance, Nutr. Rev. 56 (1998) 317–333.

[26] E. Coppo, A. Marchese, Antibacterial activity of polyphenols., Curr. Pharm. Biotechnol. 15 (2014) 380–90.

[27] F.A. Tomás-Barberán, C. Andrés-Lacueva, Polyphenols and health: Current state and progress, J. Agric. Food Chem. 60 (2012) 8773–8775..

[28] S. V. Joseph, I. Edirisinghe, B.M. Burton-Freeman, Fruit Polyphenols: A Review of Anti-inflammatory Effects in Humans, Crit. Rev. Food Sci. Nutr. 56 (2016) 419–444.

[29] E. Middleton, C. Kandaswami, T.C. Theoharides, The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer., Pharmacol. Rev. 52 (2000) 673–751.

[30] V. I. Popa, M. Dumitru, I. Volf, N. Anghel, Lignin and polyphenols as allelochemicals, Ind. Crops Prod. 27 (2008) 144–149

[31] M. Naczk, F. Shahidi, Phenolics in cereals, fruits and vegetables: Occurrence,

extraction and analysis. J. Pharm.l Biomed. Anal., 41 (2006) 1523–1542

[32] A. Larrauri, O. Núñez, S. Hernández-Cassou, J. Saurina, Determination of Polyphenols in White Wines by Liquid Chromatography: Application to the Characterization of Alella (Catalonia, Spain) Wines Using Chemometric Methods, J. AOAC Int. 100 (2017) 323-329

[33] C. Manach, A. Scalbert, C. Morand, C. Rémésy, L. Jiménez, Polyphenols: food sources and bioavailability, Am. J. Clin. Nutr. 79 (2004) 727–747

[34] S. Yammine, S. Brianceau, S. Manteau, M. Turk, R. Ghidossi, E. Vorobiev, M. Mietton-Peuchot, Extraction and purification of high added value compounds from by-products of the winemaking chain using alternative/nonconventional processes/technologies, Crit. Rev. Food Sci. Nutr. 58 (2018) 1375–1390.

[35] L.F. Guido, M.M. Moreira, Techniques for Extraction of Brewer's Spent Grain Polyphenols: a Review, Food Bioprocess Technol. 10 (2017) 1192–1209.

[36] C. Da Porto, A. Natolino, Supercritical fluid extraction of polyphenols from grape seed (Vitis vinifera): Study on process variables and kinetics, J. Supercrit. Fluids. 130 (2017) 239–245.

[37] C.G. Pereira, M.A.A. Meireles, Supercritical fluid extraction of bioactive compounds: Fundamentals, applications and economic perspectives, Food Bioprocess Technol. 3 (2010) 340–372.

[38] R.P.F.F. da Silva, T.A.P. Rocha-Santos, A.C. Duarte, Supercritical fluid extraction of bioactive compounds, TrAC - Trends Anal. Chem. 76 (2016) 40–51.

[39] P. Ramírez, T. Fornari, F. J. Señooráns, E. Ibañez, G. Reglero, Isolation of phenolic antioxidant compounds by SFC, J. of Supercrit. Fluids 35 (2005) 128–132

[40] M. Ganzera, Supercritical fluid chromatography for the separation of isoflavones, J. Pharm. Biomed. Anal. 107 (2015) 364–369

[41] M. Sun, G. Lidén, M. Sandahl, C. Turner, Ultra-high performance supercritical fluid chromatography of lignin-derived phenols from alkaline cupric oxide oxidation, J. Sep. Sci., 39 (2016) 3123–3129

[42] M.T..Fernández-Ponce, L..Casas, C..Mantell,.E. Martínez de la Ossa, Fractionation of Mangifera indica Linn polyphenols by reverse phase supercritical fluid chromatography (RP-SFC) at pilot plant scale, J. of Supercrit. Fluids 95 (2014) 444–456

[43] Y. Huang, Y. Feng, G. Tang, M. Li, T. Zhang, M. Fillet, J.Crommen, Z. Jiang, Development and validation of a fast SFC method for the analysis of flavonoids in plant extracts, J. Pharm. Biomed. Anal. 140 (2017) 384–391

[44] G. Tang, Y. Huang, T. Zhang, Q. Wang, J. Crommen, M.Fillet, Z. Jiang, Determination of phenolic acids in extra virgin olive oil usingsupercritical fluid chromatography coupled with single quadrupole mass spectrometry, J. Pharm. Biomed. Anal. 157 (2018) 217–225

[45] W. Gao, X. Dong, R. Wang, X. G. Liu, P. Li, H. Yang, The use of ionic liquid as a mobile phase modifier in analytical supercritical fluid chromatography for the separation of flavonoids, RSC Adv., 6 (2016) 61418-61422.

[46] A. Karnangerpour, M Ashraf-Khorassani, L T Taylor, L Chorida, Supercritical Fluid Chromatography of Polyphenolic Compounds in Grape Seed Extract, Chromatographia. 55 (2002) 417–421.

[47] K.M. Kalili, A. De Villiers, Recent developments in the HPLC separation of phenolic

compounds, J. Sep. Sci. 34 (2011) 854–876.

[48] R. García-Villalba, J.C. Espín, F.A. Tomás-Barberán, N.E. Rocha-Guzmán, Comprehensive characterization by LC-DAD-MS/MS of the phenolic composition of seven Quercus leaf teas, J. Food Compos. Anal. 63 (2017) 38–46.

[49] P. Lucci, J. Saurina, O. Núñez, Trends in LC-MS and LC-HRMS analysis and characterization of polyphenols in food, TrAC - Trends Anal. Chem. 88 (2017) 1–24.

[50] M.J. Motilva, A. Serra, A. Macià, Analysis of food polyphenols by ultra high-performance liquid chromatography coupled to mass spectrometry: An overview, J. Chromatogr. A. 1292 (2013) 66–82.

[51] J. Serra Bonvehi, M. Soliva Torrentó, E. Centelles Lorente, Evaluation of polyphenolic and flavonoid compounds in honeybee-collected pollen produced in Spain, J. Agric. Food Chem. 49 (2001) 1848–1853.

[52] K. Anton, C. Berger (Eds.), Supercritical Fluid Chromatography with Packed Columns-Techniques and Applications, Marcel Dekker, New York, 1997

[53] C. West, A. Bouet, S. Routier, E. Lesellier, Effects of mobile phase composition and temperature on the supercritical fluid chromatography enantioseparation of chiral fluoro-oxoindole-type compounds with chlorinated polysaccharide stationary phases, J. Chromatogr. A 1269 (2012) 325– 335

[54] L. Toribio, J. L. Bernal, M. T. Martín, J. Bernal and M. J. del Nozal, Effects of organic modifier and temperature on the enantiomeric separation of several azole drugs using supercritical fluid chromatography and the Chiralpak AD column, Biomed. Chromatogr. 28 (2014) 152–158

Figure Captions

Figure 1. Names and structures of the compounds studied

Figure 2. Structures of the stationary phases employed

Figure 3. Effect of the percentage of trifluoroacetic acid (TFAA) on the separation using the DCpak PBT column. Chromatographic conditions are described in subsections 2.3 and 3.1. Peak identification: **1** = cinnamic acid, **2** = ferulic acid, **3** = p-coumaric acid, **4** = sinapic acid, **5** = caffeic acid, **6** =gallic acid, **7** = naringenin, **8** = catechin, **9** = quercetin

Figure 4. Effect of temperature on the retention using the DCpak PBT column. Chromatographic conditions are described in subsections 2.3 and 3.1.

Figure 5. Effect of temperature on the resolution of the pairs ferulic–p-coumaric acids, caffeic--sinapic acids and naringenin-catechin using the DCpak PBT column. Chromatographic conditions are described in subsections 2.3 and 3.1.

Figure 6. Chromatograms obtained with different injection volumes using the DCpak PBT column. Chromatographic conditions are described in subsections 2.3 and 3.1. Peak identification: 1 = cinnamic acid, 2 = ferulic acid, 3 = p-coumaric acid, 4 = sinapic acid, 5 = caffeic acid, 6 =gallic acid, 7 = naringenin, 8 = catechin, 9 = quercetin

Figure 7. Chromatogram of a bee pollen sample. Chromatographic conditions are described in subsection 3.1.6. Peak identification: **1** = cinnamic acid, **3** = p-coumaric acid, **8** = catechin, **9** = quercetin.

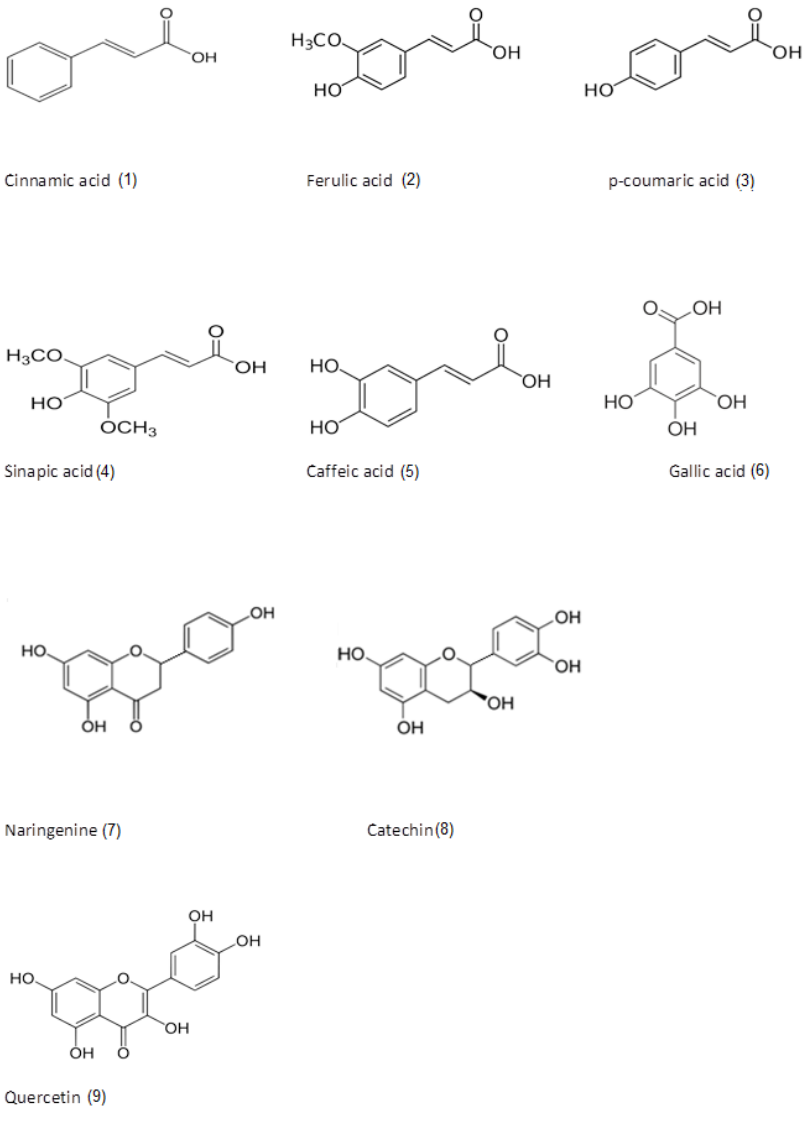
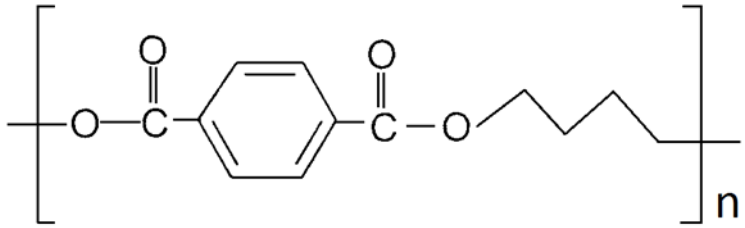


Figure 1. Names and structures of the compounds studied

DCpak PBT column: Poly(butylen terephtalate)



DCpak P4VP column: Poly(4-vinylpyridine)

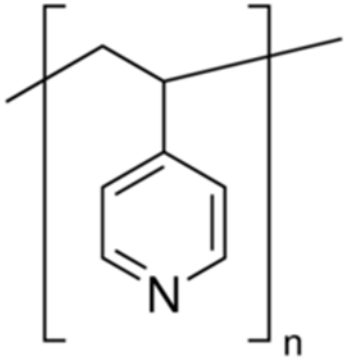


Figure 2. Structures of the stationary phases employed

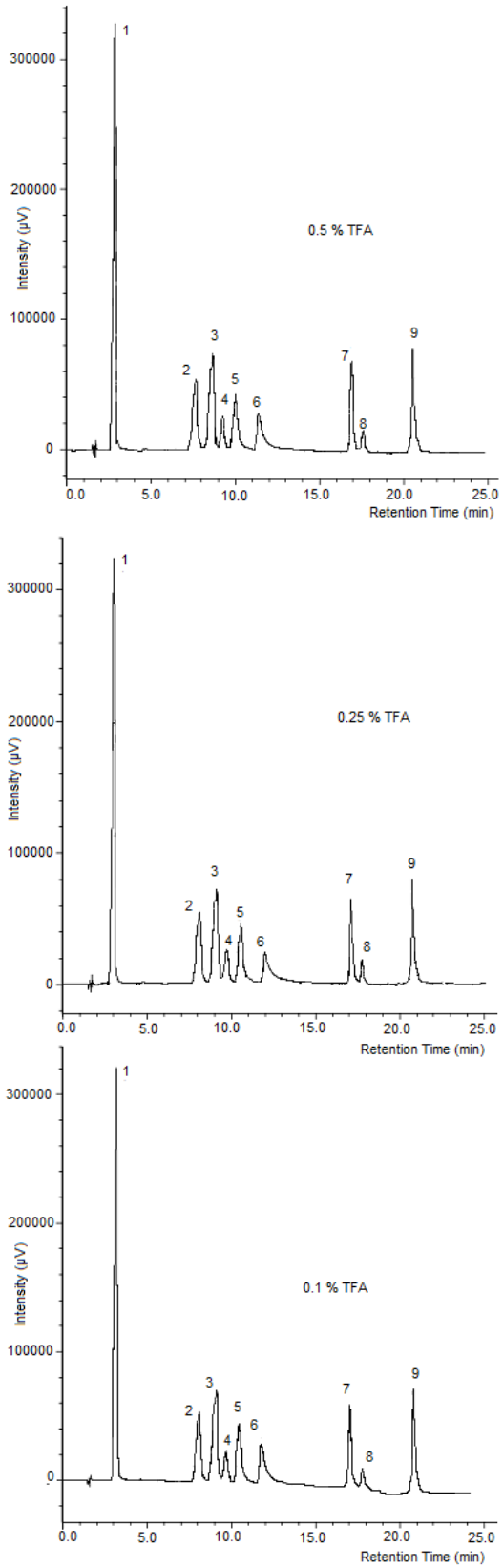


Figure 3. Effect of the percentage of trifluoroacetic acid (TFAA) on the separation using the DCpak PBT column. Chromatographic conditions are described in subsections 2.3 and 3.1. Peak identification: **1** = cinnamic acid, **2** = ferulic acid, **3** = p-coumaric acid, **4** = sinapic acid, **5** = caffeic acid, **6** =gallic acid, **7** = naringenin, **8** = catechin, **9** = quercetin

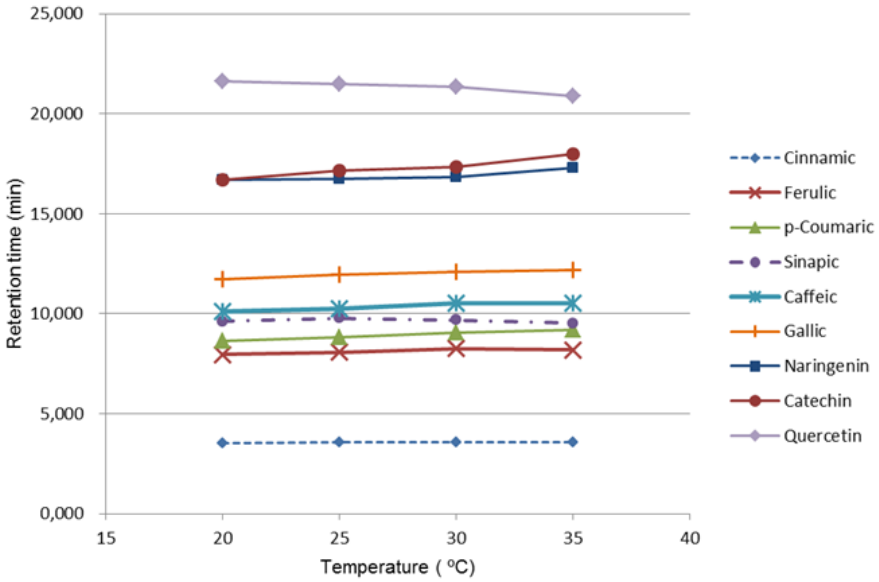


Figure 4. Effect of temperature on the retention using the DCpak PBT column. Chromatographic conditions are described in subsections 2.3 and 3.1.

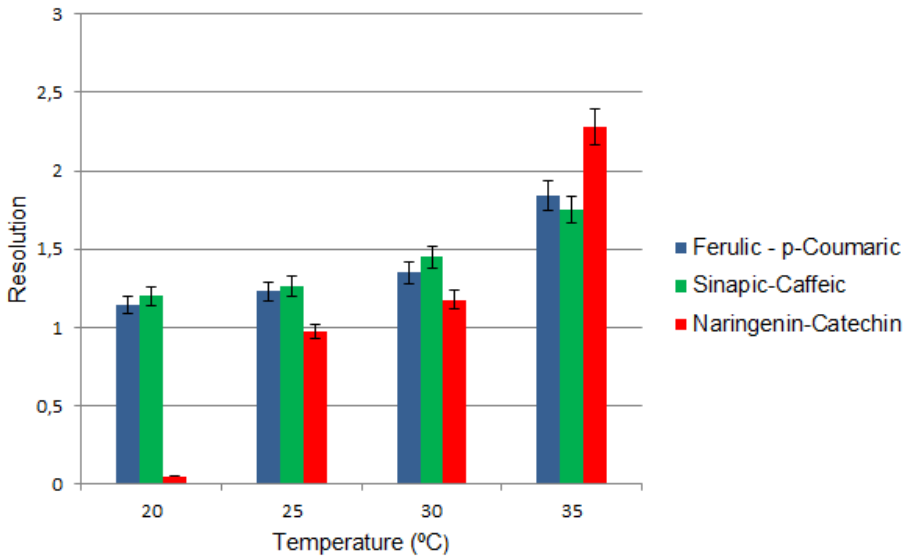


Figure 5. Effect of temperature on the resolution of the pairs ferulic–p-coumaric acids, caffeic--sinapic acids and naringenin-catechin using the DCpak PBT column. Chromatographic conditions are described in subsections 2.3 and 3.1.

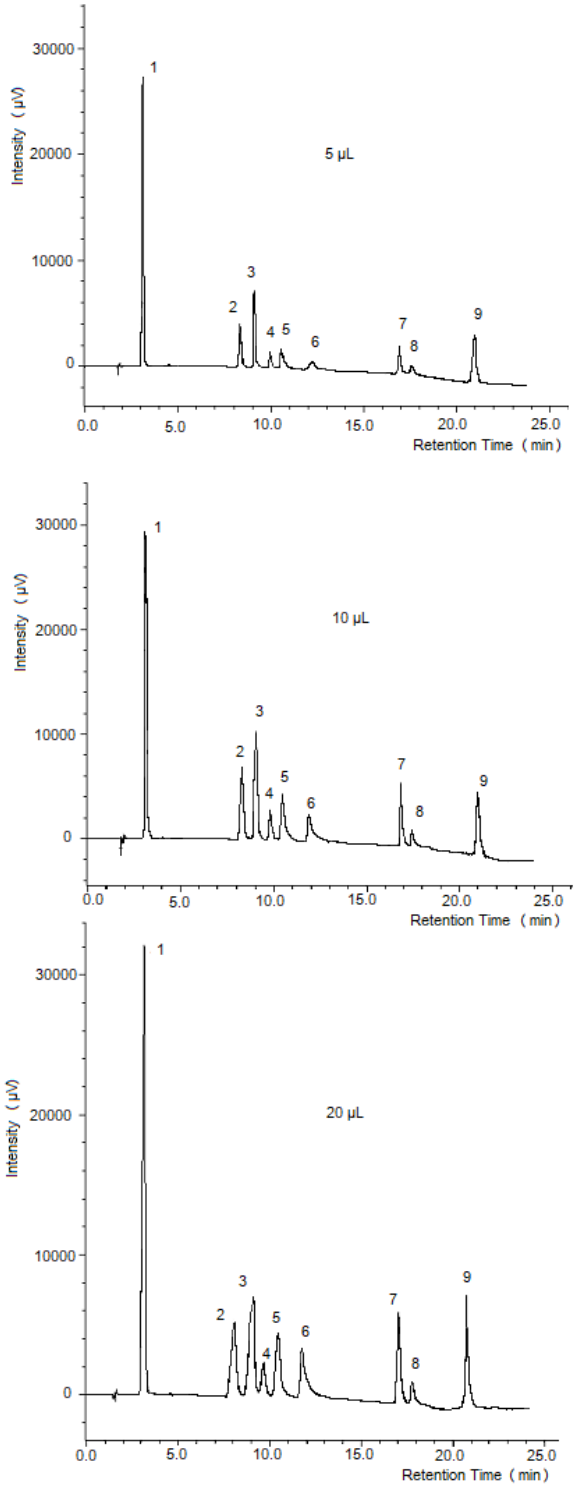


Figure 6. Chromatograms obtained with different injection volumes using the DCpak PBT column. Chromatographic conditions are described in subsections 2.3 and 3.1. Peak identification: 1 = cinnamic acid, 2 = ferulic acid, 3 = p-coumaric acid, 4 = sinapic acid, 5 = caffeic acid, 6 =gallic acid, 7 = naringenin, 8 = catechin, 9 = quercetin

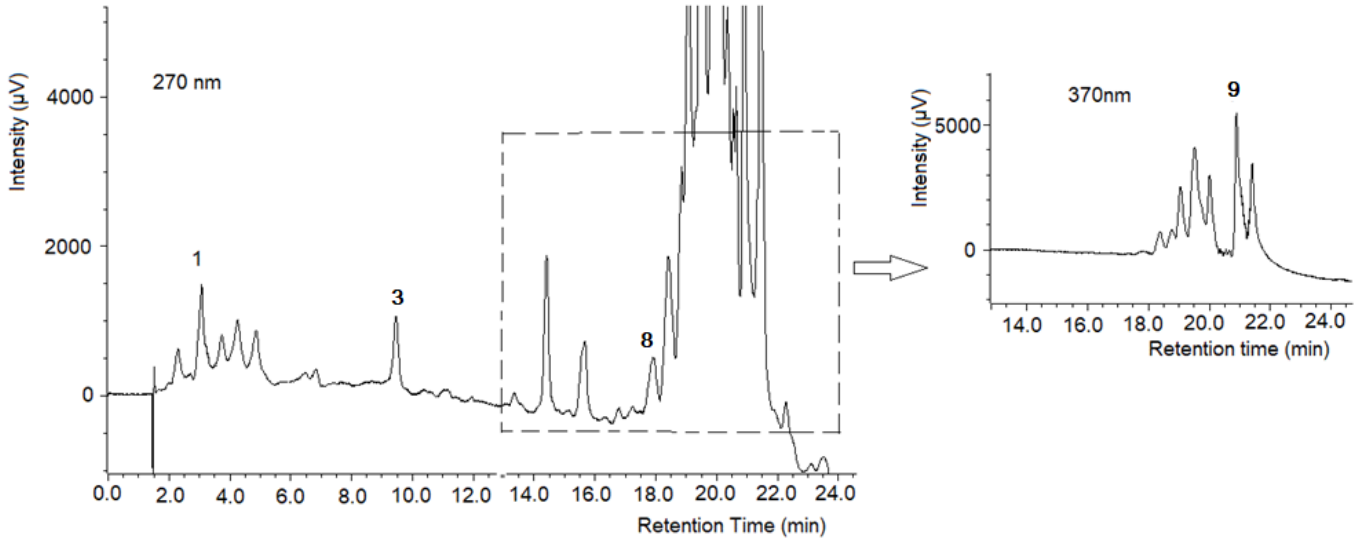


Figure 7. Chromatogram of a bee pollen sample. Chromatographic conditions are described in subsection 3.1.5. Peak identification: **1** = cinnamic acid, **3** = p-coumaric acid, **8** = catechin, **9** = quercetin

Table 1- Retention time (min) of the compounds studied with the two stationary phases assayed. Chromatographic conditions: 150 bar, 35ºC, 2mL/min, percentage of organic modifier as indicated in the table headings.

Compound	Stationary phase				
	P4VP	PBT 10% organic modifier with 0.5% TFAA			PBT
	40% Methanol with 0.5% TEA	Methanol	Ethanol	Isopropanol	Final gradient[1] methanol with 0.1% TFAA
Cinnamic acid	3.42 ± 0.04	1.82 ± 0.04	3.02 ± 0.03	4.13 ± 0.04	3.16 ± 0.03
Ferulic acid	9.91 ± 0.06	4.41 ± 0.06	6.38 ± 0.05	7.52 ± 0.06	8.08 ± 0.05
p-Coumaric acid	10.24 ± 0.06	5.25 ± 0.05	6.63 ± 0.05	8.31 ±0.04	9.09 ± 0.05
Sinapic acid	11.51 ± 0.07	5.43 ± 0.06	6.92 ± 0.06	8.52 ± 0.07	9.68 ± 0.05
Caffeic acid	> 40	5.61 ± 0.09	7.02 ± 0.10	12.35 ± 0.09	10.47 ± 0.09
Gallic acid	---	6.32 ± 0.10	10.21 ±0.10	20.31 ± 0.08	11.91 ± 0.10
Naringenin	---	25.61 ± 0.08	35.52 ± 0.07	> 40	17.01 ± 0.02
Catechin	---	25.58 ± 0.07	> 40	---	17.77 ± 0.06
Quercetin	---	>40	---	---	20.91 ± 0.09

TEA: triethylamine, TFAA: trifluoroacetic acid, --- not eluted

[1] 5% of organic modifier for 3 minutes, then it was increased to 20% at 1.25%/min, and finally increased at 15%/min to 50%, which was held for 7 minutes

Table 2. Effect of the additive on the retention times (t_r), column efficiency (N) and symmetry factor of the quercetin peak. Chromatographic conditions are described in subsections 2.3 and 3.1.

	t_r (min)	N	Symmetry factor
without additives	23.63 ± 0.12	2837 ± 12	6.33 ± 0.05
0.5% Trifluoroacetic acid	21.14 ± 0.06	10533 ± 51	1.74 ± 0.01
0.5% Citric acid	21.31 ± 0.06	6802 ± 30	2.30 ± 0.02
0.5% Formic acid	21.72 ± 0.05	5702 ± 18	3.88 ± 0.02
0.5% Acetic acid	22.14± 0.08	3691 ± 11	6.01 ± 0.03

Table 3. Detection (LOD) and quantification limits (LOQ) of the studied compounds obtained at the corresponding optimal wavelengths. Chromatographic conditions are described in subsections 2.3 and 3.1.

Compound	LOD (µg/mL)	LOQ (µg/mL)	Wavelength (nm)
Cinnamic acid	0.12	0.38	270
Ferulic acid	0.21	0.71	320
p-Coumaric acid	0.34	1.14	320
Sinapic acid	0.28	0.92	320
Caffeic acid	0.86	2.87	320
Gallic acid	1.35	4.49	220
Naringenin	0.78	2.58	220
Catechin	0.85	2.80	220
Quercetin	1.42	4.73	370

Table 4. Results of the investigation of a bee pollen sample in which polyphenols were detected[A] (means of triplicate analyses (mg/kg).

Compound	Concentration
Cinnamic acid	3.70 ± 0.22
p-Coumaric acid	11.62 ± 0.23
Catechin	22.15 ± 0.57
Quercetin	22.02 ± 0.60

[A]Other polyphenols were <LOD.