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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

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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 35 °C 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.

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]. More

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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 Corporation. 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 (Fig. 1), 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 30 °C 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 30 °C. 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 Fig. 2) were employed: DCpak PBT (250 × 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 × 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 Fig. 1, 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

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