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Ultra high efficiency/low pressure supercritical fluid chromatography with superficially porous particles for triglyceride separation☆

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ABSTRACT

This paper reports the development of the separation of vegetable oil triglycerides (TG) in supercritical chromatography (SFC), using superficially porous particles (SPPs). The SPP, having a small diameter (2–3 μm), provide a higher theoretical plate number (*N*), which allows to improve separation of critical pairs of compounds. However, compared to fully porous particles of larger diameter (5 μm), the pressure drop is also increased. Fortunately, supercritical fluids have a low viscosity, which allows coupling several columns to achieve high *N* values, while maintaining flow rate above 1 ml/min, ensuring a ultra high efficiency (UHE) at low pressure (LP) (below 40 MPa), with regards to the one reached with liquid and sub-two micron particles (around 100 MPa). The use of two detector systems (UV and ELSD) connected in series to the UHE–LP-SFC system provides complementary responses, due to their specific detection principles. Working in a first part with three coupled Kinetex C18 columns (45 cm total length), the effect of modifier nature and percentage were studied with two reference oils, argan and rapeseed, chosen for their different and well-known TG composition. The analytical method was developed from previous studies performed with fully porous particles (FPP). Optimized conditions with three Kinetex were as follows: 17 °C, 12% of ACN/MeOH (90/10; v/v). With these conditions, and by using an increased length of Kinetex C18 column (60 cm), another additional column was selected from ten different commercial SPP C18 bonded phases, by applying a Derringer function on varied parameters: theoretical plate number (TPN), separation index (SI) for critical pairs of peaks (the peaks of compounds difficult to separate due to subtle structural differences), the analysis duration, and the total peak number. This function normalizes the values of any parameters, between 0 and 1, from the worst value to the better, allowing to take account of various parameters in the final choice. Finally, by using four Kinetex C18 plus one Accucore C18 (75 cm total column length), a high-performance separation of triglycerides was achieved, with reasonable analysis duration and isocratic conditions. These conditions can be applied to varied vegetable oils. Identification of the numerous separated peaks of rapeseed oil was achieved by using published data and chromatographic retention behaviour.

1. Introduction

Triglycerides (TG) are the main constituents of vegetable oils. They are composed by three fatty acid chains, having varied length, from 14 to 24 carbons, and double bonds, from 0 to 4 (Table 1). Due to their low volatility, they are generally not analyzed in gaseous phase chromatography (GC), but either by high-performance liquid chromatography (HPLC) or by supercritical fluid chromatography (SFC).

Two HPLC methods can be used, reverse phase liquid chromatography (RPLC) [1–5], or silver-ion mode [6–8]. With the former, using non-polar octadecylsiloxane-bonded silica (ODS) stationary phases, the retention of TG increases with the partition number (PN). PN is calculated from the total carbon number of the fatty acid chain, minus twice the total double bond number. For instance, this partition number is equal to 36 for LnLnLn (Ln = C18:3), this value is obtained from this calculation: $PN = 3 \times 18 - 2 \times 9 = 36$; 18 being the carbon number of each Ln chain and 9 the total double bond number. Moreover, due to the low polarity of the studied compounds ($\log P > 5$), the use of water is generally avoided in the mobile phase, leading to a non-aqueous mode, called NARP. Elution gradients are also often used [1,3–5] to

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Table 1
List of name, abbreviation, carbon number (CN), double-bond number (DB) and partition number (PN) for main fatty acids of vegetable oils.

Trivial name	Abbreviation	CN	DB	PN
Myristic	M	14	0	14
Palmitic	P	16	0	16
Palmitoleic	Po	16	1	14
Margaric	Ma	17	0	17
Margaroleic	Mo	17	1	15
Stearic	S	18	0	18
Oleic	O	18	1	16
Linoleic	L	18	2	14
Linolenic	Ln	18	3	12
Arachidic	A	20	0	20
Gadoleic	G	20	1	18
Behenic	B	22	0	22
Lignoceric	Li	24	0	24

reduce the retention time which could be equal to 300 min in isocratic mode with two coupled columns [2] against 100–140 min in gradient mode [3,5]. However, the separation of several critical pairs remains difficult by NARP-LC [5]. First, separation of compounds composed by three C18 chains, having the same total carbon and double-bond number (identical PN), but with a different repartition onto the three chains, for instance: LLLn and OLnLn, LLL and OLLn, OLL and OOLn or SLL and SOLn [9,10].

These couples can be separated by HPLC using two-coupled C18 column, and an elution gradient with water in the first part, thereby increasing the analysis duration from 100 to 140 min [3]. Another separation seems impossible whatever the system used: the separation between couples SLL/POL, SLLn/POLn and SOL/POO having the same partition number with one saturated chain, either S (C18) or P (C16) [3,11]. The same difficulties of separation are encountered for the couples P_0OO/OOL or P_0PO/POL [5].

Separations of regio-isomers (*sn*-2; *sn*-3) are achieved by NARP-LC, when using polymeric bonded ODS phases at low temperature [12], whereas LC–MS/MS systems allow to quantify these isomers on the basis of ion abundance [9]. The use of low temperature in NARP-LC was also reported in other studies but the retention times were prohibitive [13,14]. For this kind of separation, temperatures of 25–40 °C are preferred for silver-ion chromatography to favour good peak shapes [15].

Silver-ion chromatography displays a different retention order in regards of the one in NARP-LC, as larger double-bond number induces longer retention. It also allows the separation of regio-isomers, having the same chains but with a different position onto the glycerol moiety (as OLL and LOL) [8]. Unfortunately, silver-ion chromatography suffers from poor reproducibility and peak shapes, requires long equilibration times and freshly prepared mobile phases [16]. However, reproducibility seems improved by the addition of 2-propanol to the hexane–acetonitrile mobile phase, favouring the miscibility between the two previous solvents [15].

Increase in theoretical plate number can improve overall separation performance. This can be achieved by using superficially porous particles (SPPs) increasing the total column length. Despite the lower viscosities of non-aqueous mobile phases in regards to aqueous ones, the column length, and/or the flow rate, is limited when using pumps allowing for classical inlet pressure conditions [17,18]. Recently the use of SPP, also called core-shell or fused-core particles, has allowed to couple columns in HPLC, for the analysis of a very complex oil sample [197]. Four columns were serially coupled, and the inlet pressure was around 900 bars at the end of the elution gradient. 137 compounds from menhaden (fish) oil were identified with mass spectrometry, with total analysis duration equal to 190 min.

Two-dimensional studies using both liquid modes were achieved [19,20]. In this case, silver-ion mode is performed first, then NARP-LC.

However, despite the short column used in the second dimension, the flow rate in the first one should be in the 10–20 μl/min range, to ensure the separation of all peaks in the second dimension. This low flow rate dramatically increases the analysis duration.

SFC has also been used for the separation of TG, with ODS stationary phases [21–26]. Due to the low viscosity of the CO_2-based mobile phase, high flow rates and long columns together provide shorter retention time and greater separation efficiencies in regards to HPLC.

By using C18-bonded phases, the general retention rules are identical to those observed in RPLC, *i.e.* they are based on the partition number. Besides, due to the nature of the mobile phase, selectivity between TG having P chains are somewhat modified due to the increase in the P chain solubility in SFC, leading to improved separation. For instance the retention order in NARP-LC is LLL/OLL/PLL, whereas in SFC it is LLL/PLL/OLL. The improved solubility of the P chains in regards to the O one provides a retention inversion of the TG differing only by one P or one O chain. Moreover, the selectivity between the three (LLL, PLL, OLL) is strongly improved in SFC due, in part to the greater peak capacity and to the larger retention difference between PLL and OLL.

Recently, SPP were also used in SFC to achieve 120,000 theoretical plates, by coupling four 15 cm columns [27]. Due to their reduced particle size, and to their great particle size homogeneity, these SPP phases provide higher efficiencies, with a reduced inlet pressure, compared to the ones observed using sub-2 μm fully porous particles. This property, in addition of the low viscosity of super(sub)critical fluids would allow the use of very long columns to improve the separation efficiency for TG, without the need for ultra-high pressure chromatographic systems. The use of such high column length leads to get ultra high efficiency/low pressure SFC (UHE/LP-SFC).

This paper will describe the method development used in UHE/LP-SFC, based on a previous separation obtained by SFC using fully porous particles (FPP) (Hypersil ODS). The composition of the mobile phase, the flow rate, temperature, backpressure and the choice of the stationary phases will be studied to ensure the best TG separation. Finally, the coupling of five columns filled with superficially porous particles, allows improving the separation performance of TG of vegetable oils with regard to previous works performed in the last century [23].

2. Materials and methods

Chromatographic separations were carried out using equipment manufactured by Jasco (Tokyo, Japan). One pump model 2080-CO_2 *Plus* was used for carbon dioxide and a second one model 2080 *Plus* for the modifier. Control of the mobile phase composition was performed by the CO_2 pump. When the two solvents (modifier and CO_2) were mixed, the fluid was introduced into a dynamic mixing chamber PU 4046 (Pye Unicam, Cambridge, UK) connected to a pulsation damper (Sedere, Orleans, France). The injector valve was supplied with a 20 μL loop (model 7125 Rheodyne, Cotati, CA).

The columns were thermostated by an oven (Jetstream 2 Plus, Hewlett-Packard, Palo Alto, CA). The detector was a Gilson UV 151 detector (provided by Waters, Milford, MA) equipped with a pressure-resistant cell. The detection wavelength was 210 nm. After the detector, the outlet column pressure was controlled by a Jasco BP-2080 *Plus* back pressure regulator (BPR). The outlet regulator tube (internal diameter 0.25 mm) was heated to 60 °C to avoid ice formation during the CO_2 depressurization. The ELSD model