Journal of Chromatography A, 1216 (2009) 7088–7095

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Improved separation of furocoumarins of essential oils by supercritical fluid chromatography

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ARTICLE INFO

Article history:
Received 8 June 2009
Received in revised form 20 August 2009
Accepted 28 August 2009
Available online 1 September 2009

Keywords:
Supercritical fluid chromatography
Furocoumarin
Pentofluorophenyl (PFP)
Lemon residue

ABSTRACT

Separation of furocoumarins has become of a great interest for cosmetic industry and human health, since the recent directive of the European Union. Furocoumarins are a class of compounds presenting varied substituents linked mainly in two positions to an identical skeleton made by a furan ring bonded to a coumarin nucleus (Psoralen). The substituents are mainly methoxy, or alkyl chains, which can contain double bonds, hydroxyl or epoxy groups. Due to the variety of compounds, and their subtle structure differences, their separation requires high-performance methods. Multi-gradient high-performance liquid chromatography (HPLC) and two-dimensional chromatography are usually applied. This paper describes a new approach, by using super/subcritical fluid chromatography (SFC), with a green mobile phase: CO_2–ethanol. The choice of the stationary phase from varied types of phases, and the effects of numerous analytical parameters (flow rate, modifier percentage, temperature and outlet pressure) are studied, described and discussed, on the basis of the separation of a complex sample: lemon residue. From these studies, isocratic conditions are determined to obtain a satisfactory separation in 10 min. A two-dimensional analysis was also investigated, by performing first a class fractionation of compounds on an ethylpyridine (EP) phase, then by separating each class on a pentafluorophenyl phase (Discovery HS F5) with the selected isocratic mobile phase. A gradient elution is also studied to improve separation of some minor compounds. Structure of the eluted compounds was determined by comparison with standards, HPLC-DAD, HPLC–MS analysis, and NMR analysis of collected fractions. All these approaches allow relating structure of compounds to retention behaviour, which is unusual due to the selected pentafluorophenyl stationary phase.

1. Introduction

Furocoumarins are natural plant metabolites, mainly found in the Umbelliferae (celery, parsnip, parsley) and Rutaceae (citrus plants). Because these compounds (psoralen, bergapten) have UV-light absorbing properties, essential oil, such as Citrus oil, were used in many cosmetic and fragrance products, especially in sun protection and bronzing products. However, these compounds also display phototoxic properties, and they are unfortunately believed to cause mutagenesis, carcinogenesis and photodermatitis [1]. Consequently, the European Union modified the European Cosmetic Directive to limit the final concentration of furocoumarins to 1 mg/kg in cosmetics [2], thus furocoumarins should not be used in cosmetic products anymore because of their adverse effects. In fact, all products used by manufacturers of fragrance ingredients and containing furocoumarins are concerned, and require accurate quantifications. Among these products one can notice the lemon residue, obtained from Sicilian lemon oil hydrodistillation, is especially rich in varied furocoumarins.

On the other hand, when ingested, furocoumarins can interfere with the oral bioavailability of certain drugs, by inhibition of CYP 3A4 enzymes, which are involved in the oxidative biotransformation of these drugs [3–7]. Thus there are varied reasons why the determination of furocoumarins is of interest.

Few studies were performed on furocoumarin separation. These compounds are psoralen derivatives in position 5 or 8 or both (Fig. 1), with hydroxyl, methoxy, alkyl, and some other groups with double bonds or epoxy functions. Structure differences are varied, explaining that normal-phase (NPLC) [7–12] and reversed-phase liquid chromatography (RPLC) were used [4–6,13–16].

Most of the recent RPLC methods use multistep elution gradient [13,16], allowing separation of 15 different compounds, with 6 steps and analysis duration equal to 55 min [13]. Most compounds were baseline separated, except for bergapten/heraclenin and isoimperatorin/epoxybergamottin. Bergapten (5-methoxypsoralen) is often one of the major furo-

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doi:10.1016/j.chroma.2009.08.080

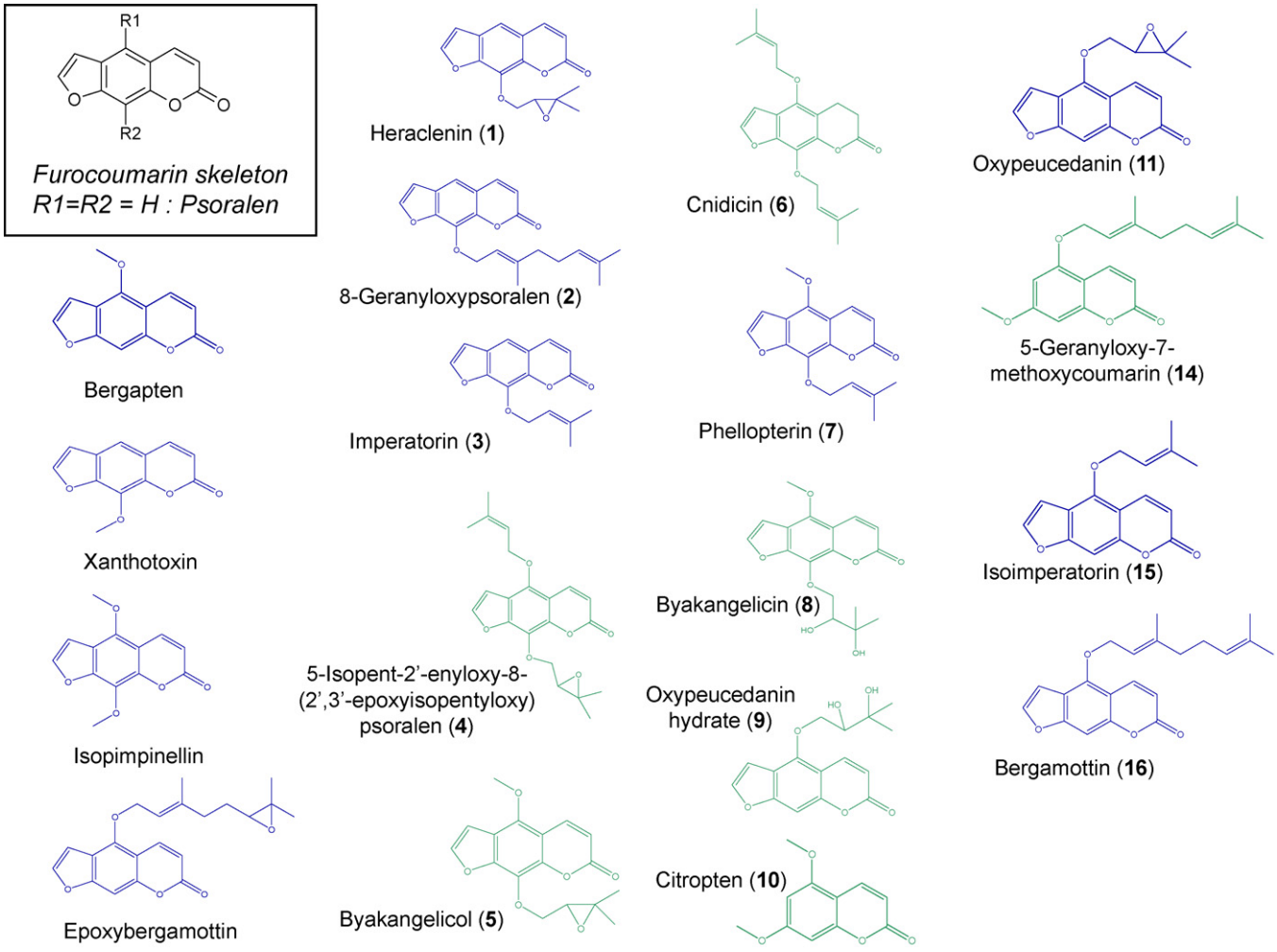

Fig. 1. Structure of furocoumarins investigated in this study.

coumarin encountered. However it is not present in some lemon oils.

Two-dimensional methods were also developed, either NPLC–RPLC [17,18], or SFC–RPLC [19]. Whatever the method and the stationary phases used, the total analysis duration ranged from 45 to 80 min. Whatever the mobile phase nature, liquid of supercritical fluid, the first dimension was performed with a polar stationary phase (diol, cyano, or silica) and the second one, on an octadecylsiloxane-bonded silica (ODS). The low fluid viscosity of supercritical mobile phase allowed the use of four coupled columns in the first dimension, increasing peak capacity [19].

The use of supercritical fluids often allows achieving high separation quality, due to the properties of these fluids: low viscosity and high eluotropic strength, which lead to rapid separations. Thanks to the supercritical fluids nature all column types can be used in SFC with the same mobile phase, from the most polar (silica) to the least polar (ODS), going through varied aromatic and/or polymeric phases [20,21]. Moreover, due to the absence of water in the mobile phase, the stationary phase/solutes interactions take a greater part in separations, and the choice of the well-suited stationary phase becomes more important [20,21]. By using systematic studies applying a LSER approach [22–26], we developed a system map to classify varied phase types in relation to five main interactions encountered in chromatographic systems: dispersion, charge transfer, acido-basic (hydrogen bond donor and acceptor) and dipole–dipole [27–29].

From these studies, we selected a set of columns for furocoumarin separation, to retain the one best suited to achieve the required separation. Then, an optimisation based on the understanding of the chromatographic parameter effects was performed, and relationships between chromatographic behaviours and chemical structures were observed.

2. Materials and methods

2.1. Column selection for SFC analysis and effect of analytical parameters

The system used for checking the column properties and to study the retention behaviour of furocourins was described elsewhere [29].

The following columns were tested: Uptisphere ODB (ODS) (Interchim, Montluçon, France); 2-Ethylpyridine (EP) (Princeton Chromatography, Princeton, USA); Kromasil Silica (Eka Nobel, Sweden); Princeton CN (cyanopropyl) (Princeton, USA); Synergi Polar RP (OPHE) (propyloxyphenyl) (Phenomenex, Le Pecq, France); Synergi Fusion RP (Phenomenex, Le Pecq, France); Gemini Phenylhexyl (Phenomenex, Le Pecq, France); Pursuit Diphenyl XRs (Varian, Les Ulis, France); Alltima HP AQ (Discovery Science, Deerfield, IL, USA); Acclaim Polar Advantage II (Dionex, Voisins-le-Bretonneux, France); Discovery HS F5 (Supelco/Sigma–Aldrich, l'Ile d'Abeau, France); Hypersil Gold PFP (Thermo, Les Ulis, France); Pursuit

PFP (Varian); Cogent C18 Bidentate (MicrosolvTech, USA); Cosmosil Cholester (Nacalai Tesque, Kyoto, Japan). All columns were packed with 5 μm particles, apart from EP and CN (6 μm), and were 250 mm × 4.6 mm, except the Acclaim Polar Advantage II (150 mm × 4.6 mm).

The mobile phase used was CO_2–EtOH 90:10 (v/v); temperature: 25 °C; outlet pressure: 15 MPa; flow rate: 3 ml/min; UV detection at 310 nm. Prior to injection, the samples were diluted with ethanol and centrifuged, then only the supernatant was analysed.

2.2. Final SFC separations

The system used for chromatographic identification and gradient separation in SFC was a Berger Minigram (Thar Technologies, a Waters company, Pittsburgh, PA, USA). For the gradient, the temperature was set at 35 °C, the outlet pressure at 10 MPa, the flow rate at 3 ml/min. Step gradient as follows: 7% EtOH during 4 min, 8% EtOH during 3.2 min and 9% EtOH during 4 min.

Isocratic analyses were also performed at varied EtOH percentages (from 7 to 10) with optimal pressure, temperature and flow rate, as well as a linear gradient from 7 to 9% of EtOH. Despite satisfactory separation, none of these experiments allowed to reach the separation performance of the step gradient, especially in regards of the last eluted minor compounds.

2.3. Compounds and samples

A sample of lemon residue was obtained as the residue of an Italian lemon oil distillate. It was selected as a complex sample containing a large fraction of furocoumarin species.

The following coumarins and furocoumarins (Fig. 1) were purchased from varied suppliers (structures in blue in Fig. 1): bergamottin (**16**), xanthotoxin, bergapten (Fluka); heraclenin (**1**), epoxybergamottin, 8-geranyloxypsoralen (**2**), oxypeucedanin (**11**), imperatorin (**3**), isoimperatorin (**15**), isopimpinellin, phellopterin (**7**) (Phytolab).

Byakangelicol (**5**), oxypeucedanin hydrate (**9**), 5-isopentyl-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy)-psoralen (**4**), cnidicin (**6**), 5-geranyloxy-7-methoxycoumarin (**14**), citropten (**10**) and byakangelicin (**8**) (structures in green in Fig. 1) were identified by preparative NPLC and semi-preparative SFC of a lemon residue sample, followed by HPLC-DAD and HPLC–MS using the method described by Frérot and Decorzant [13], UV–vis, FTIR and NMR analyses.

The HPLC apparatus was an Agilent Series 1100 (Agilent, Palo Alto, USA) equipped with a G1312A binary pump, a G1330A autosampler, a column thermostat G1316A oven, a G1315A diode-array detector. The column effluent was directly introduced into a single quadrupole G1946A mass spectrometer with APPI ionisation source. The stationary phase was Nucleodur Sphinx (250 mm × 2.0 mm, 5 μm, Macherey-Nagel). Flow rate was set at 0.5 ml/min, temperature 40 °C, injection volume 5 μl. Mobile phase gradient (methanol-buffer) was as described by Frérot and Decorzant [13].

The APPI-MS was operated in the positive ion mode with the following parameters: source temperature: 350 °C; quad. temperature: 100 °C; threshold 10; voltage 70 V; *m*/*z* range 50–1000 amu; gas: nitrogen; gas flow: 2; fragmenter voltage: 60 V; capillary voltage: 3500 V. Excitation was carried out with a Krypton lamp at energies of 10.0 and 10.6 eV. Toluene had been tested as a dopant at different flow rates but results were better with no dopant addition.

UV–vis absorption spectra were recorded with a diode-array spectrophotometer (Agilent, model 8453). Spectra were acquired in the 190–500 nm range. Cell thickness was 1 cm. Ethanol was used as sample and blank solvent.

Infra-red spectra were recorded with a Bruker model Vector 22 FT-IR spectrometer (Bruker, Ettlingen, Germany), equipped with a Specac's Golden Gate ATR/diamond crystal device. Measurement range was 4000–600 cm^{-1} (mid-IR region). Automatic signals for the neat samples were collected in 16 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean diamond ATR objective.

NMR spectra were recorded on a Bruker Avance DPX-400 or on a Bruker Avance 500 at 400 MHz and 500 MHz (^1H), 100 MHz and 125 MHz (^{13}C). The NMR pulse techniques HSQC, HMBC, COSYDFQ (COSY double quantum filtered), NOESY and gradient accelerated spectroscopy were used to correlate ^1H and ^{13}C NMR signals. Experiments were recorded in $CDCl_3$ (Bruker Avance DPX-400) or C_6D_6 (Bruker Avance 500).

3. Results and discussion

3.1. Initial column screening

Amongst the numerous tested columns, Fig. 2 displays separations of lemon residue obtained on four columns allowing varied separation with 10% ethanol, 15 MPa (outlet pressure), and 25 °C. Some compounds classically encountered in citrus oil were also analysed (flavanone, xanthotoxin and bergapten). On the two polar stationary phases, 2-Ethylpyridine (EP) and silica (SIL) the retention profile is close, but retention and separation factors are lower on SIL. On the other hand, bergapten and xanthotoxin are not well resolved, and coelute with some compounds. These two compounds only differ by the position of the substituent on the skeleton (5 or 8-methoxy group).

On the propyloxyphenyl phase (OPHE), more peaks appear in a narrow range of retention. The profile seems in part reversed in regards to the one obtained on SIL and EP. This remark is in accordance with the different polarity of the stationary phases, the Synergi Polar RP, despite its name, is a less polar phase than the other two. Two other phases (profiles not shown) display retention profiles somewhat close to the one obtained on OPHE: Pursuit Diphenyl XRs and Gemini Phenylhexyl, underlying the effect of the aromatic ring of the stationary phase on furocoumarin separation.

It could in part explain the rather good separation obtained on a pentafluorophenyl (PFP) phase (Discovery HS F5), based on an aromatic ring substituted by five fluorine atoms. Moreover, amongst the standard compounds, bergapten is well separated being eluted after the other furocoumarins in the lemon residue sample. In previous works, this phase was shown to display unique retention behaviour, wholly based on charge transfer and dipole–dipole interactions [27–29]. Some other works underlined the suitability of PFP phases to separate tocopherol isomers [30].

We also investigated the separation on two other PFP phases: Hypersil Gold PFP and Pursuit PFP. The profiles obtained on Discovery HS F5 and Hypersil Gold PFP are close, showing very similar elution order, but the retention is lower on Hypersil Gold and separation quality is poorer. These results are in accordance with the available data on surface area of the two silica gels and on their carbon content (300 and 220 m^2/g; 12 and 8% carbon, respectively).

On the other hand, the separation profile achieved with the Pursuit PFP was dramatically different, and less performing (in terms of peak number).

Thus the Discovery HS F5 column was retained for further developments.

3.2. Study of chromatographic behaviour on PFP

The first parameter studied was the modifier percentage. Ethanol was selected in order to work with a green mobile phase, without methanol or acetonitrile.

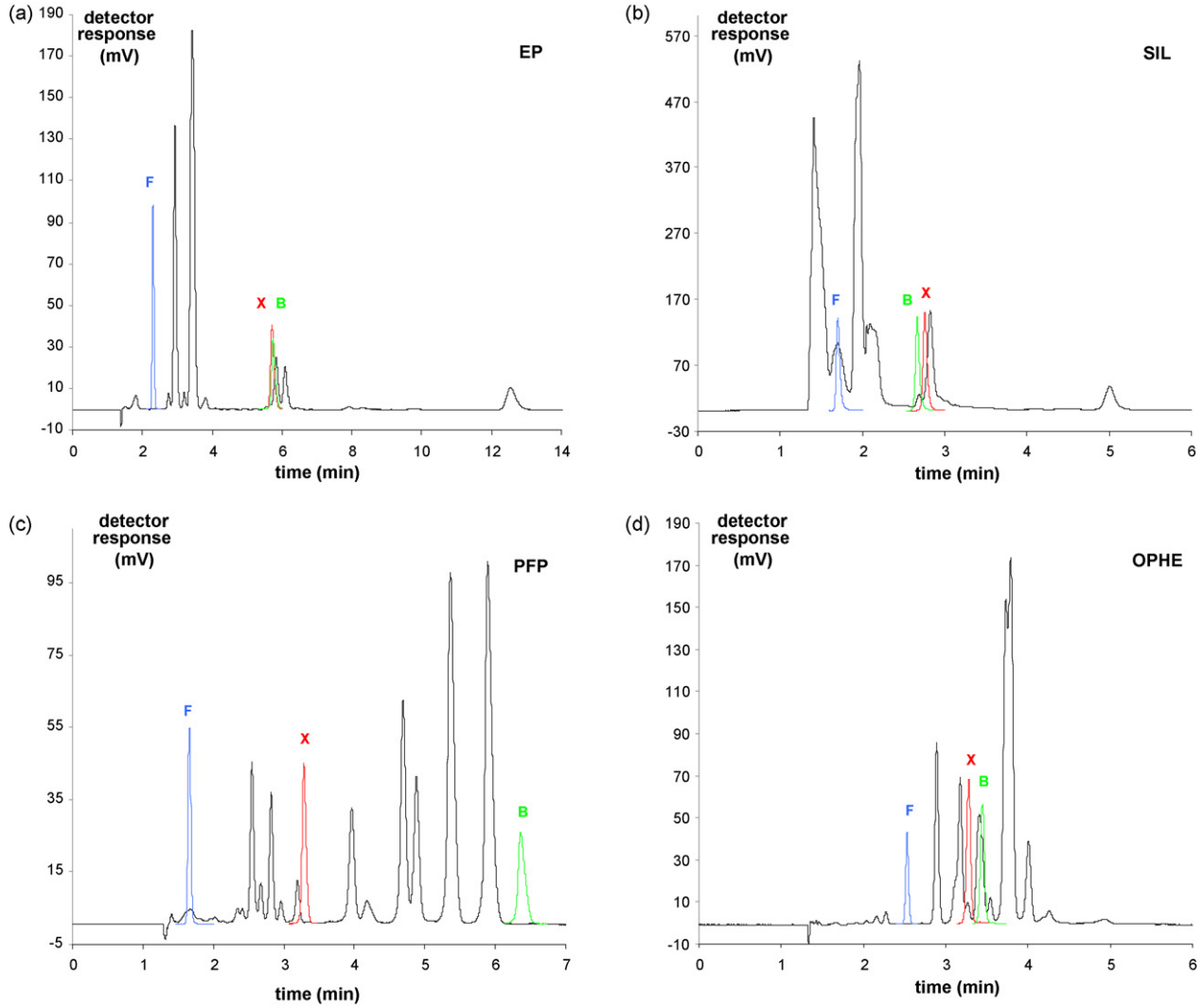

Fig. 2. Separation of lemon residue (black curves) and standards (flavanone F, xanthotoxin X and bergapten B, respectively blue, red and green curves) on four stationary phases. (a) Ethylpyridine (EP); (b) Kromasil Silica (SIL); (c) Discovery HS F5 (PFP); (d) Synergi Polar RP (OPHE). *Conditions*: mobile phase CO_2–EtOH 90:10 (v/v); 25 °C; backpressure 15 MPa; 3 ml/min; λ = 310 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Fig. 3a shows that the increase in modifier percentage reduces retention, as often observed in SFC. The curves are not all parallel. One can remark that for compounds **1**, **4** and **5** (heraclenin, 5-isopent-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy)-psoralen and byakangelicol), the retention decrease is stronger than for most other compounds, which show similar retention behaviours. Compounds **1**, **4** and **5** all have an epoxy function on the alkyl chain, which is located in position 8 of the psoralen skeleton. The greater retention change indicates stronger interaction modifications when ethanol percentage increases. Another compound possessing an epoxy function on an alkyl chain in position 5 (oxypeucedanin, **11**) does not show such a retention variation. These behaviours could appear surprising because it is unclear why the epoxy group in position 5 would be involved in lowest interactions variation with ethanol, whereas for the epoxy group in position 8, despite the proximity of the ether and ester groups on this side of the ring, these variations would be greater. We can suppose that, when the epoxy group is in position 8, there are intramolecular interactions, which are reduced by ethanol addition.

A larger retention variation is also noticed for two compounds: no. **8** (byakangelicin) and no. **9** (oxypeucedanin hydrate). Both have two hydroxyl groups on the side chain, leading to greater interaction modification due to the ethanol addition. As for the compounds having an epoxy group, the variation of retention is greater for compound **8**, where the chain is in position 8, than for compound **9**, where the chain is in position 5.

These retention variations are related to the chemical structure of the compounds, for those having epoxy or hydroxyl group, i.e. to the position of the chain. Thus studying the chromatographic behaviours, a first identification of the chemical structure can be done, helping in the final structure determination. Such relationships were used in the past for the identification of triglycerides in SFC [31,32].

Nevertheless, to improve the separation of compounds **7**, **8**, **9** and **10**, Fig. 3a suggests that the final composition should be comprised between 8 and 10% modifier.

Temperature effect was studied in the range 25–40 °C (Fig. 3b). The increase in temperature induces an increase in retention of furocoumarins. This type of variation is not encountered in HPLC, but is often reported in SFC because of the fluid density variation [31]. With compressible fluids, the temperature increase reduces the density, i.e. the eluotropic strength. Thus the interactions between the mobile phase and the compounds decrease. Of course, this behaviour depends on the modifier percentage and the average pressure in the column. The greater these two parameters

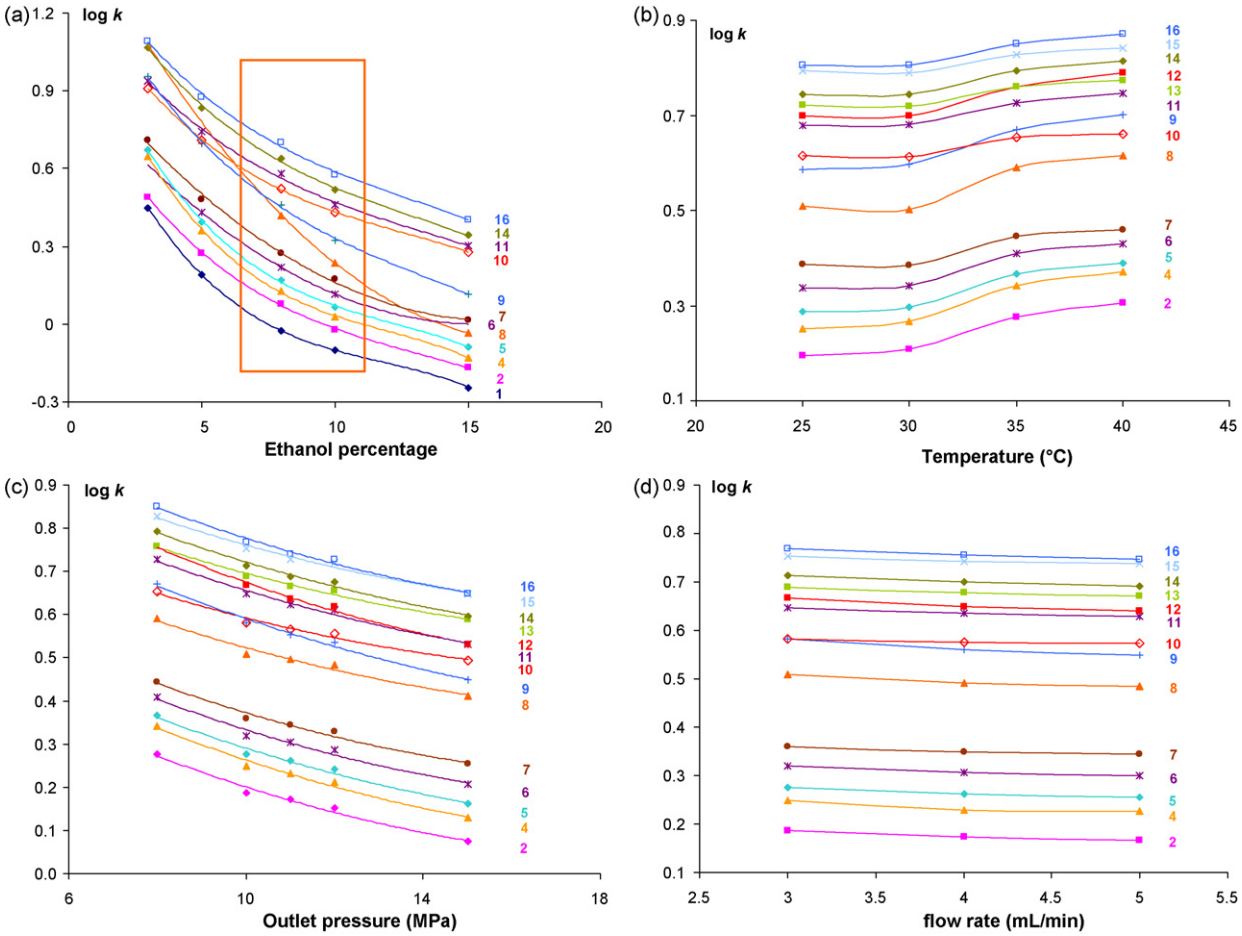

Fig. 3. Log *k* of compounds *vs.* (a) ethanol percentage (other conditions as Fig. 2), (b) temperature (CO_2–EtOH 91:9 (v/v); 8 MPa; 3 ml/min), (c) outlet pressure (CO_2–EtOH 91:9 (v/v); 35 °C; 3 ml/min), (d) flow rate (CO_2–EtOH 91:9 (v/v); 35 °C; 10 MPa) on Discovery HS F5.

are, the lower is this effect. With higher modifier percentages and average pressure, temperature acts in a manner similar to what is observed in HPLC.

The retention variations are greater for three compounds: **8**, **9** and **12**. Compound **12** was not identified, and was not detected in conditions of Fig. 3a thus was probably co-eluted with some other compound. Because of the chemical nature of compounds **8** and **9**, one can suspect that compound **12** also possesses two hydroxyl groups on the side chain. As explained previously, identical chromatographic behaviours are related to similar structural composition. However, the effect of temperature does not depend on the position of the side chain, because byakangelicin (**8**) and oxypeucedanin hydrate (**9**) do not have the chain on the same side of the central ring. Comparing the retentions between phellopterin (**7**) and byakangelicin (**8**) (both compounds have the chain in position 8), and between isoimperatorin (**15**) and oxypeucedanin hydrate (**9**) (both compounds have the chain in position 5), the addition of two hydroxyl group on the chain double bond favours retention when the chain is in position 8, but causes a decrease in retention when the chain is in position 5. By considering the retention differences between these two couples, and the compounds having a double bond which could by hydroxylated, compound **12** could be the 6′,7′-dihydroxybergamottin.

Moreover, the temperature change induces a reversal in the elution order, which could dramatically modify the separation, mainly between 30 and 40 °C.

The effect of outlet pressure appears in Fig. 3c. The increase of outlet pressure favours retention decrease, because of the increase of the fluid density.

This variation of fluid density causes an increase in the apparent void volume, i.e. the volume pumped to fill the void volume into the column, and an increase in the eluotropic strength too, i.e. the interactions between solutes and mobile phase.

Just as it was observed for other parameters, the variation of retention is greater for compounds **9** (oxypeudecanin hydrate) and **12**. However, the variation of byakangelicin (**8**), with two hydroxyl groups on the chain in position 8 does not vary to the same extent. This is consistent with the previous hypothesis that compound **12** would have two hydroxyl groups on a chain in position 5. No clear difference can be noticed between epoxy compounds.

Finally, the flow rate variation is reported in Fig. 3d. The increase in the flow rate from 3 to 5 ml/min reduces the retention factor. For compressible fluids, and such as it occurs for outlet pressure changes, the flow rate modification induces density changes, which modify the eluotropic strength. The retention variations are greater for compounds **9** and **12**, in accordance with the pressure change (Fig. 3c), modifying the selectivity for some couples of compounds. The flow rate is a parameter acting on separation in SFC, and could be used to fine tune the selectivity factor.

Several isocratic conditions provide satisfactory, but not optimal separation. Fig. 4 shows a separation obtained with 9% ethanol, 3 ml/min, 25 °C, 8 MPa. The separation is achieved in less than 10 min.

3.3. Fraction separation

For natural extracts used in cosmetics or pharmaceuticals, fractionation can be useful to purify specific compound families,

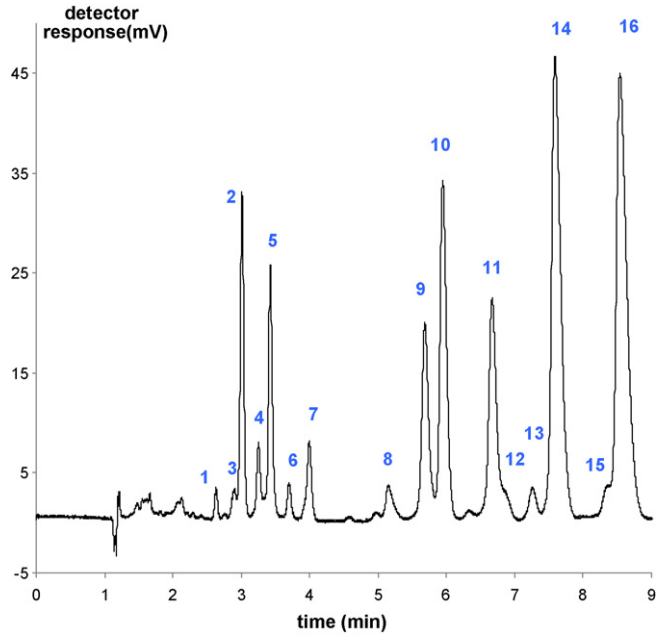

Fig. 4. Analysis of lemon residue with optimized isocratic conditions on Discovery HS F5. CO_2–EtOH 91:9 (v/v); 25 °C; 8 MPa; 3 ml/min. For compound identification, see Fig. 1.

avoiding contamination of samples used for skin products. Moreover, in the goal to improve the knowledge on the composition of a complex mixture, fractionation can be helpful in evidencing coelutions of minor compounds.

Among the varied columns tested, 2-Ethylpyridine (EP) displays an interesting separation, which could serve to fractionate the lemon residue, which is a complex mixture, as shown in Fig. 4. Several analyses were performed on EP (Fig. 5), and five fractions were manually collected, then analysed on Discovery HS F5 (PFP) in the same conditions as in Fig. 4. Obviously, due to the polar nature of the EP phase, one expects that the most polar compounds are eluted the latest.

Fig. 6 displays the chromatographic profile of the crude samples (lemon residue) and four of the five collected fractions on the PFP stationary phase. No peaks were detected in fraction 1.

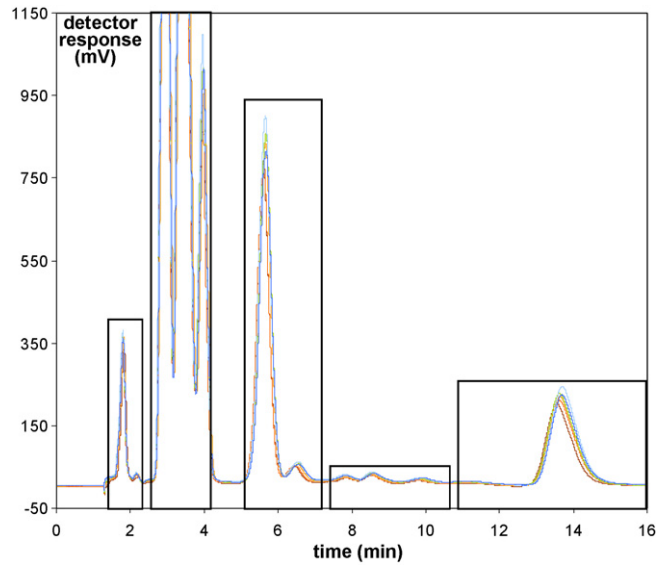

Fig. 5. Analysis of lemon residue on 2-Ethylpyridine for fraction collection. Conditions as in Fig. 2a.

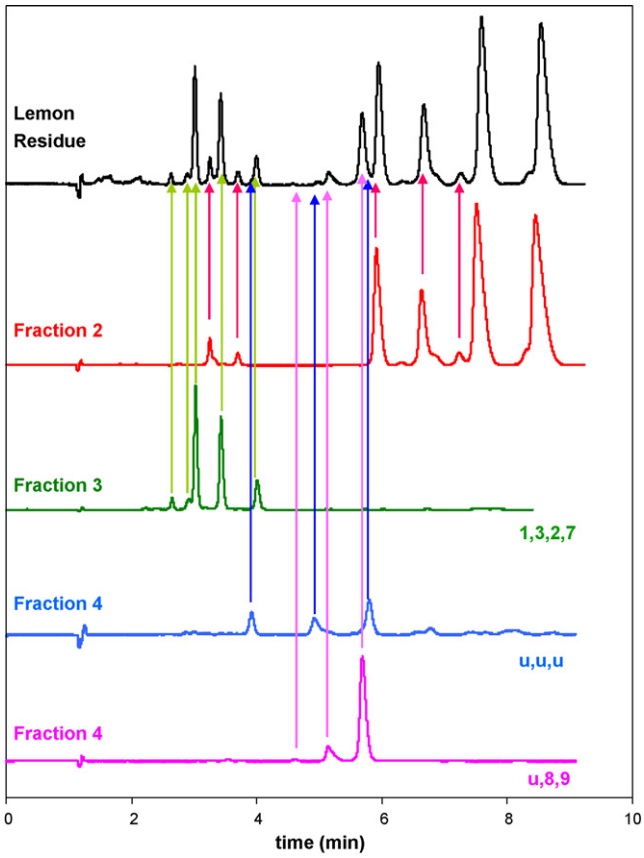

Fig. 6. Chromatograms of collected fractions (F2–F5) and of original lemon residue. Conditions as in Fig. 4.

Fraction 2 contains many compounds, having varied retention times. The first two (**4** and **6**) are substituted both on 5 and 8 positions, and just differ by the epoxydation of the double bond in the alkyl chain. The most retained (**10**, **11**, **14**, **15**, **16**) are not substituted in position 8. We can conclude that all compounds in this fraction do have a substitution in position 5.

Fraction 3 contains peaks located at the beginning of the chromatogram (**1**, **2**, **3**, **5** and **7**) which all have a chain in position 8 and either no substituent or a methoxy group in position 5.

Fraction 4 contains few amounts of compounds (the profile intensity was greatly enhanced). These compounds are mainly eluted with other peaks and are not well resolved by this analysis. They have not been identified.

Fraction 5 is supposed to contain the most polar compounds. Compounds **8** and **9**, which are both dihydroxyl compounds, are included in this fraction. Other minor compounds appear in this fraction, but were not identified.

All these data reinforce the previous observations on the relationships between chemical structure and chromatographic behaviours of furocoumarins on the PFP stationary phase. However, it is surprising that, on the EP stationary phase, the compounds in fraction 3, which have a substituent in position 8 are more retained than compounds in fraction 2, which do not have substitution in position 8 (for the most retained ones). Indeed, a chain in position 8 would be expected to reduce the interactions between the ester group included in the skeleton and the polar stationary phase, leading to a decrease in the retention time.

Nonetheless, this study shows the potential of SFC to achieve two-dimensional and orthogonal separations, by using varied and complementary stationary phases without changing the mobile phase as it often occurs in HPLC [17,18]. Moreover, at least 25 com-

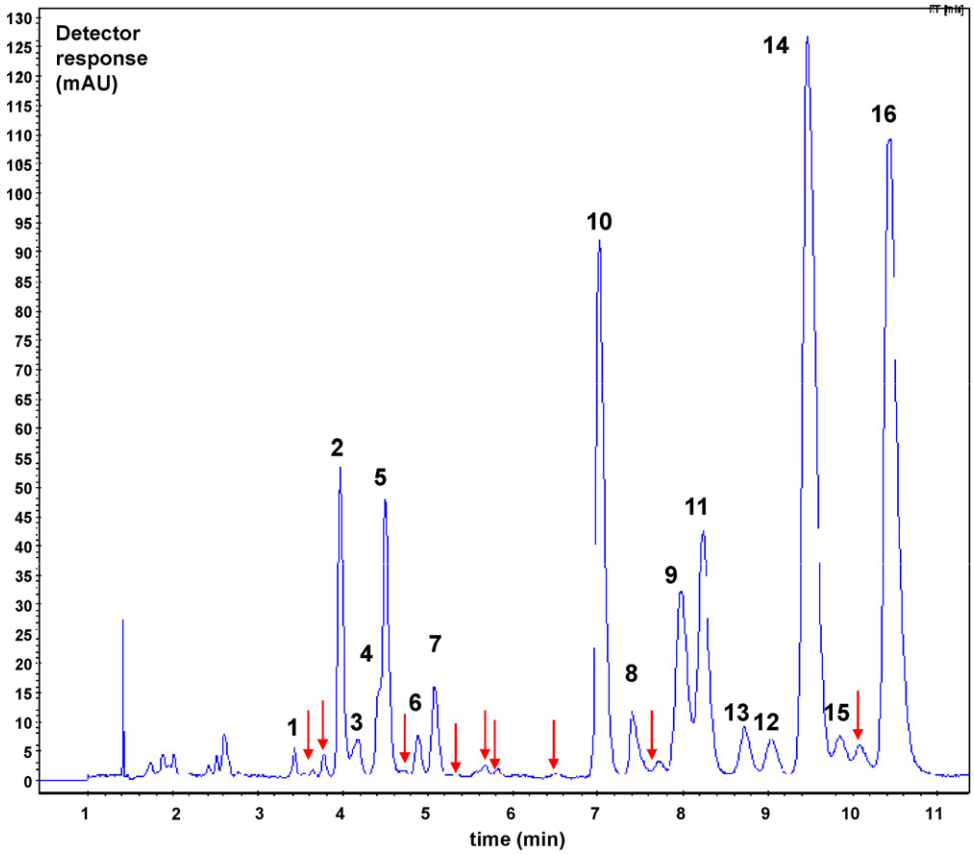

Fig. 7. Chromatogram of lemon residue on Discovery HS F5 with elution gradient. Mobile phase CO_2 with step gradient of EtOH as follows: 7% during 4 min, 8% during 3.2 min, 9% during 4 min. 35 °C; 10 MPa; 3 ml/min. For compound identification, see Fig. 1.

pounds are separated by using the two selected columns, showing the high separative ability of SFC in regards of LC–LC [17–19].

3.4. Elution gradient

Finally, the essential oil of lemon residue was analysed by using an elution gradient (Fig. 7). This gradient is a two-step one, going to 7–9% of ethanol. A linear gradient between 7 and 9%, run on the same duration, was not able to reach separation of the unknown compound eluted between compounds **15** and **16**. Numerous other minor peaks appear (at least 8, marked by red arrows).

4. Conclusion

The analysis of furocoumarin of essential oils is of a prime importance for human health. The separation of such compounds represents a challenge because of the great variety of compounds, and because of minor structural differences between the compounds.

The results obtained show that SFC is a perfectly suited method to investigate the essential oil composition, because of the great number of compounds separated in a reduced analysis time (around 10 min), and with a very short time for re-equilibration of the system at the end of the gradient analysis. Several ways can be followed to achieve such separations, either isocratic or with a two-step gradient, reducing the complexity of the analyses performed by HPLC. A two-dimensional approach can also be suggested, with two orthogonal stationary phases.

Because of the absence of water in the mobile phase in SFC, the stationary phase can establish more varied interactions than in HPLC, making the stationary phase choice very important. Due to the variety of compounds, a choice based on predictive calculation such as linear solvation energy relationships seems difficult. A screening of well-chosen stationary phases remains the simplest way to achieve a complex separation. However, as shown, nominally identical stationary phases, such as pentafluorophenyl phases, can provide different separations.

Acknowledgment

Dr. Gerhard Brunner is greatly acknowledged for his help with NMR analyses.

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