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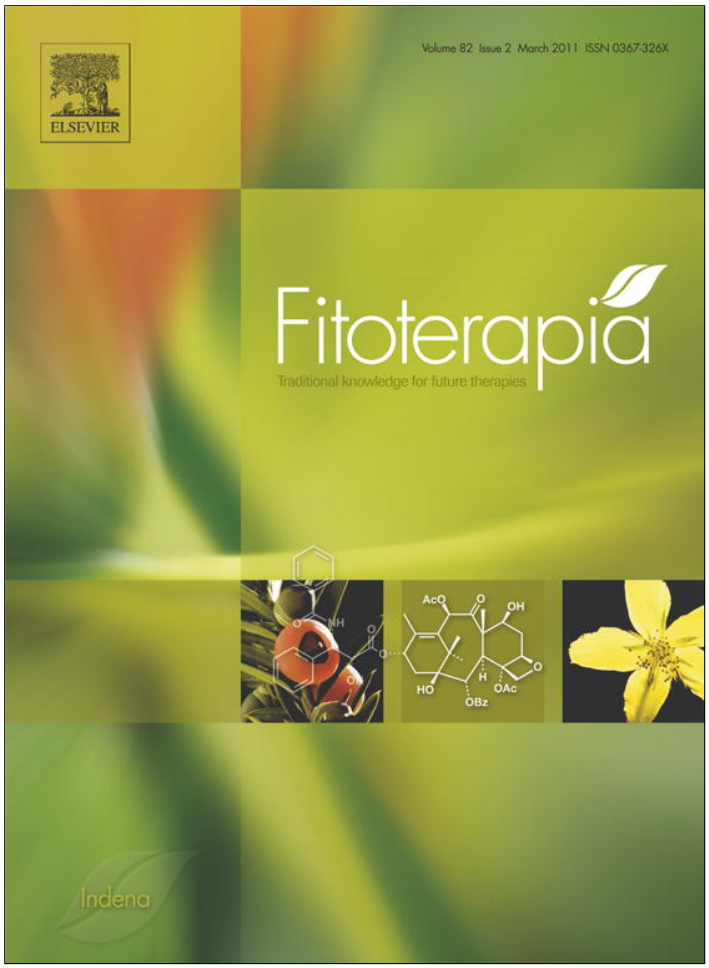

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Flash chromatography on cartridges for the separation of plant extracts: Rules for the selection of chromatographic conditions and comparison with medium pressure liquid chromatography

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ABSTRACT

Empirical rules for the selection of chromatographic conditions on flash chromatography cartridges were developed, with an emphasis on gradient mode. These rules were then tested with separation of extracts from important medicinal plants including *Curcuma xanthorrhiza*, *Piper nigrum* and *Salvia milthiorrhiza*. Sepacore® cartridges enabled a good separation of compounds with a broad range of polarity, as typically found in plant extracts. The chromatographic resolution remained, however, lower than that achieved on classical columns packed with material of smaller particle size. For poorly soluble extracts, solid introduction gave better results than liquid injection.

1. Introduction

During the last decade, several systems for rapid preparative chromatography with pre-packed cartridges have been commercialized. The most popular ones include Sepacore® (Büchi Labortechnik AG), ISCO combiflash®, Jones Flashmaster®, and various flash systems from Biotage. Pre-packed cartridges containing silica gel or RP-18 material ensure rapid separation cycles and ease of use. Some of these chromatography systems such as, for example, the Sepacore® system, are versatile and can accommodate cartridges or conventional glass columns. Depending on the applied pressure, one refers to flash chromatography (0.1–5(10) bars) or medium pressure liquid chromatography (MPLC) (5(10)–50 bars).

Flash chromatography systems were initially developed for rapid and easy purification of synthetic products, and numerous applications are documented in the experimental part of publications in synthetic chemistry (e.g. [1,2]), and in some patents (e.g. [3]). In contrast, application of such systems in the separation of complex natural product mixtures such as extracts has been neglected. Few examples can be found where pre-packed [4,5] or self-packed [6] cartridges, i.e. Sepacore® cartridges, have been used for the separation of natural product mixtures. To the best of our knowledge no chromatograms have been published, and guidelines for the selection of chromatographic conditions, in particular in gradient elution mode, are not available.

Given the need for accelerating and streamlining not only the early compound identification but also the subsequent preparative isolation of compounds of interest, the aim of this work was to explore with a selection of examples the potential and limitations of cartridges for the purification of natural product extracts. In this context, empirical rules have been established for the determination of the separation conditions by preliminary TLC and HPLC analyses. The influence of solid introduction compared to liquid injection has been also investigated. The performance of the cartridges was compared

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to that of classical self-packed MPLC glass columns. Dichloromethane extracts of turmeric (*Curcuma xanthorrhiza*), danshen (*Salvia miltiorrhiza*) and black pepper (*Piper nigrum*) were chosen as representative examples of complex plant extracts of medicinal and commercial importance.

2. Experimental

2.1. Chemicals

Solvents were from Scharlau (Barcelona, Spain). Dichloromethane, ethyl acetate, *n*-hexane, petroleum ether and methanol used for MPLC were of technical quality and redistilled before use. Methanol used for HPLC was HPLC grade. Water was cleaned through an EasyPure R II water purification system (Barnstead; Dubuque IA, USA). Anethol, aesculetin, ferulic acid, herniarin and hesperetin were from Carl Roth (Karlsruhe, Germany). Coumarin and caffeic acid were from Fluka (Buchs, Switzerland). Cinnamic acid, p-coumaric acid, eugenol, piperonyl alcohol, and umbelliferone were from Sigma-Aldrich (Milwaukee, WI, USA). Naringenin was a gift of Induchem AG (Volketswil, Switzerland). Cryptotanshinone and tanshinone IIA were previously isolated from *S. milthiorriza* at the Institute of Pharmaceutical Biology, University of Jena, Germany [7]. Piperin and further piperamides were purified in our labs from an ethyl acetate extracts of *P. nigrum* [8].

2.2. Plant extracts

The rhizomes of *C. xanthorrhiza* and the fruits of *P. nigrum* were purchased from Dixa AG (St. Gallen, Switzerland); the roots of *Salvia miltiorrhiza* were obtained from Yong Quan GmbH (Ennepetal, Germany). 500 g of powder from each dried plant were extracted with 2 L dichloromethane for 24 h under stirring. After filtration, the extracts were evaporated to dryness under reduced pressure.

2.3. Instrumentation

Preparative separations were performed on a Sepacore® chromatography system (Büchi Labortechnik, Flawil, Switzerland) consisting of two C 605 pump modules, a C 620 control unit, a C 635 UV detector and a C 660 fraction collector. The system was controlled by the software SepacoreControl 1.0. Flash chromatography separations were performed on pre-packed silica gel (40–63 μm) and RP18ec (40–63 μm) polypropylene cartridges (12×150 or 40×150 mm, Büchi) at a flow rate of 10 ml (12×150 mm cartridges) and 30 mL/min (40×150 mm cartridges), respectively. Medium pressure liquid chromatography (MPLC) separations were carried out on a glass column (26×460 mm) packed with silica gel Si60 (15–40 μm, Merck) or LiChroprep RP18 (25–40 μm, Merck; Darmstadt, Germany), at a flow rate of 10 mL/min. Liquid injection was carried out through a 6-way valve with a 20 ml loop. Solid introduction was performed by means of a PrepElut cartridge (flash chromatography) or a glass precolumn (MPLC) connected to the top of the cartridge or the column, respectively. The samples were adsorbed to silica gel Si60 or LiChroprep RP-18, respectively, prior to introduction.

HPLC analyses were performed on a Waters Alliance 2695 Separations Module equipped with a 996 PDA detector and controlled with Empower Pro 2 software (Milford, MA, USA). Separations were made on a Nucleodur C18 (5 μm, 4×150 mm i.d.) column equipped with a ChromCart C18 (5 μm, 4×8 mm, i.d.) precolumn (both from Macherey Nagel; Düren, Germany); the flow rate was 1 mL/min.

TLC was performed on silica gel 60 F254 plates (Merck) in twin trough developing chamber (10×10 cm) with stainless steel lid (Camag, Muttenz, Switzerland). Samples were applied with a Linomat IV sample applicator (Camag).

3. Results and discussion

Guiding principles for selecting chromatographic conditions for both normal and reversed phase cartridges were derived from preliminary experiments with reference compounds. For practical reasons, we selected commercially available natural products with strong UV absorption. Given that constituents in extracts usually cover a wide polarity range, emphasis was put on gradient separations. The empirical rules derived from these experiments were then applied to the separation of complex plant extracts. The performance of pre-packed cartridges was compared to MPLC. Finally, we compared the separation performance with solid sample introduction and conventional liquid injection.

3.1. Correlation between HPLC and reversed phase flash chromatography

For reversed phase separations, selection of mobile phase and optimization of gradient profiles is most conveniently performed by HPLC, as RP TLC is of limited use due to the poor wettability of the plates. In a series of preliminary experiments, we established some empirical rules for translating HPLC separation into preparative separations on Sepacore cartridges. Phenolic acids (cinnamic, p-coumaric, ferulic and caffeic acids) and flavanones (naringenin and hesperitin) were selected as reference mixtures, and separated using different isocratic conditions on a Nucleodur C18 HPLC column and Sepacore® 12×150 mm cartridges. A good correlation was found between the capacity factors k' on the HPLC column and Sepacore cartridges ($k'_{\text{Sepacore}} = 1.15 \times k'_{\text{HPLC}}$; $R^2 = 0.97$) (See Supporting Information, Fig. 1). However, it should be mentioned here that compounds such as the methylxanthines caffeine and theobromine showed an unexpected behavior on Sepacore cartridges. No chromatographic retention was obtained even with organic modifier (methanol) content as low as 2%, despite significant retention on HPLC. This must be due to differences in the phase chemistry of the HPLC and flash column packing materials.

The influence of the gradient profile was evaluated with a mixture of phenolic acids. Using a gradient of 5–50% methanol in water, the resolution of caffeic acid and p-coumaric acid on a 10×150 mm Sepacore® cartridge improved from $R = 0.79$ to $R = 0.91$ if the gradient time was increased twofold as compared to HPLC (60 vs 30 min). Increasing the gradient time by a factor of 4 (120 min) provided little additional improvement to $R = 0.96$. Extending the gradient time with respect to HPLC is also necessary to

ensure complete elution of late eluting compounds on Sepacore® cartridges.

Based on these preliminary findings, we evaluated the potential of Sepacore® cartridges for the separation of complex plant extracts. We selected dichloromethane extracts of *P. nigrum* and *C. xanthorrhiza* as examples, since they both contain complex mixtures of strongly UV absorbing compounds. Separations by analytical HPLC, flash chromatography on a Sepacore (40×150 mm) cartridge, and MPLC on a glass column (26×420 mm; LiChroprep RP18 25–40 μm) were compared. The roots of *C. xanthorrhiza* (turmeric) are known to contain curcuminoids and sesquiterpene derivatives as main constituents [9]. The separation of 500 mg of the dichloromethane extract of *C. xanthorrhiza* is shown in Fig. 1. Good preliminary fractionation was achieved for extract constituents, which were well separated on HPLC due to significant differences of polarity. On the other hand, separation efficiency on the flash cartridge was not sufficient for full resolution of closely eluting HPLC peaks. As expected, the glass column with smaller particle size and increased length had a higher resolution. Baseline separation of most peaks including the major constituent xanthorrhizol could be achieved. However, the separation time increased 5-fold compared to Sepacore® cartridges, due to the length of the column and a lower flow rate which had to be used because of high back-pressure. HPLC analysis of the MPLC fractions is shown in Supporting Information, Fig. 3.

Typical constituents of black pepper (*P. nigrum*) fruits are pungent principles referred to as piperamides. These amides occur as complex mixtures of closely related compounds, and separation is challenging even on HPLC [8]. Fig. 2 shows the separation of a lipophilic pepper extract by analytical HPLC, by flash chromatography on a Sepacore® cartridge, and by MPLC. Due to the complexity of the extract, a 60 min HPLC gradient was necessary for chromatographic resolution of all constituents. As for the turmeric extract, a good fractionation was achieved on Sepacore® cartridges by increasing the gradient time by a factor 2 or 4, the latter giving slightly higher resolution. Nevertheless, the resolution remained in both cases inferior to that achieved on the MPLC column.

Up to now, only a few examples of HPLC–MPLC transposition have been presented and discussed in some detail [11–13]. Our results demonstrate that good preliminary fractionation can be obtained on pre-packed cartridges, and excellent MPLC separations can be achieved by HPLC-driven optimization of the chromatographic conditions.

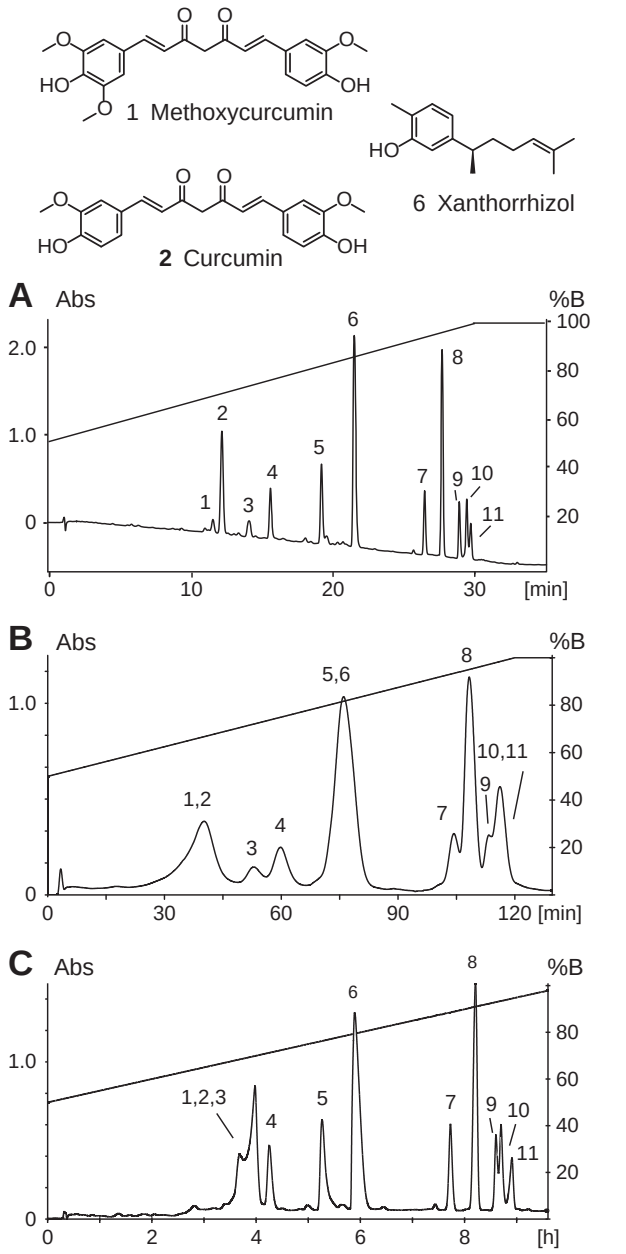

Fig. 1. Separation of a dichloromethane extract of *C. xanthorrhiza* on RP-18. A) HPLC column, 4×150 mm, B) Sepacore® cartridge 40×150 mm, C) glass column 26×240 mm. Mobile phase: water (A), methanol (B) both with 0.1% formic acid; gradient: 50–100% B in A) 30 min, B) 120 min, C) 600 min; flow rate: A) 1 mL/min, B/C) 30 mL/min, D) 10 mL/min. Samples: A) 100 μg in 10 μL, B/C) 500 mg in 5 mL. Detection at 220 nm. Identification of curcumin and xanthorrhizol from ESI-MS and NMR data of the isolated peaks and by comparison with reference compounds.

3.2. Transposition from TLC to flash chromatography on silica gel

TLC is the most straightforward approach for mobile phase selection in view of flash chromatography separations on silica gel. We investigated the correlation between chromatographic behavior of reference compounds on TLC plates and on Sepacore® cartridges. Experiments were performed with 12×150 mm cartridges using petrol ether–ethyl acetate mixtures, and coumarins (coumarin and herniarin) and phenolic derivatives (anethol, eugenol and piperonyl alcohol) as reference mixtures. A satisfactory correlation was found between $1/R_f$ and k' values ($k' = 1.1 \times 1/R_f$; $R^2 = 0.90$) (See Supporting Information, Fig. 2). Based on these findings, the mobile phase composition for starting and end point of a gradient should be selected such as to obtain R_f values of 0.2 for the most lipophilic component and the most hydrophilic constituent, respectively. We applied these principles to the separation of a dichloromethane extract of the traditional Chinese herbal drug *S. milthiorrhiza* roots (Danshen). Danshen contains a series of highly colored diterpene quinones which can be easily detected on TLC [10]. Separations on a Sepacore® silica gel cartridge (40×150 mm) and a MPLC column (26×460 mm, silica gel 15–40 μm), respectively, are shown

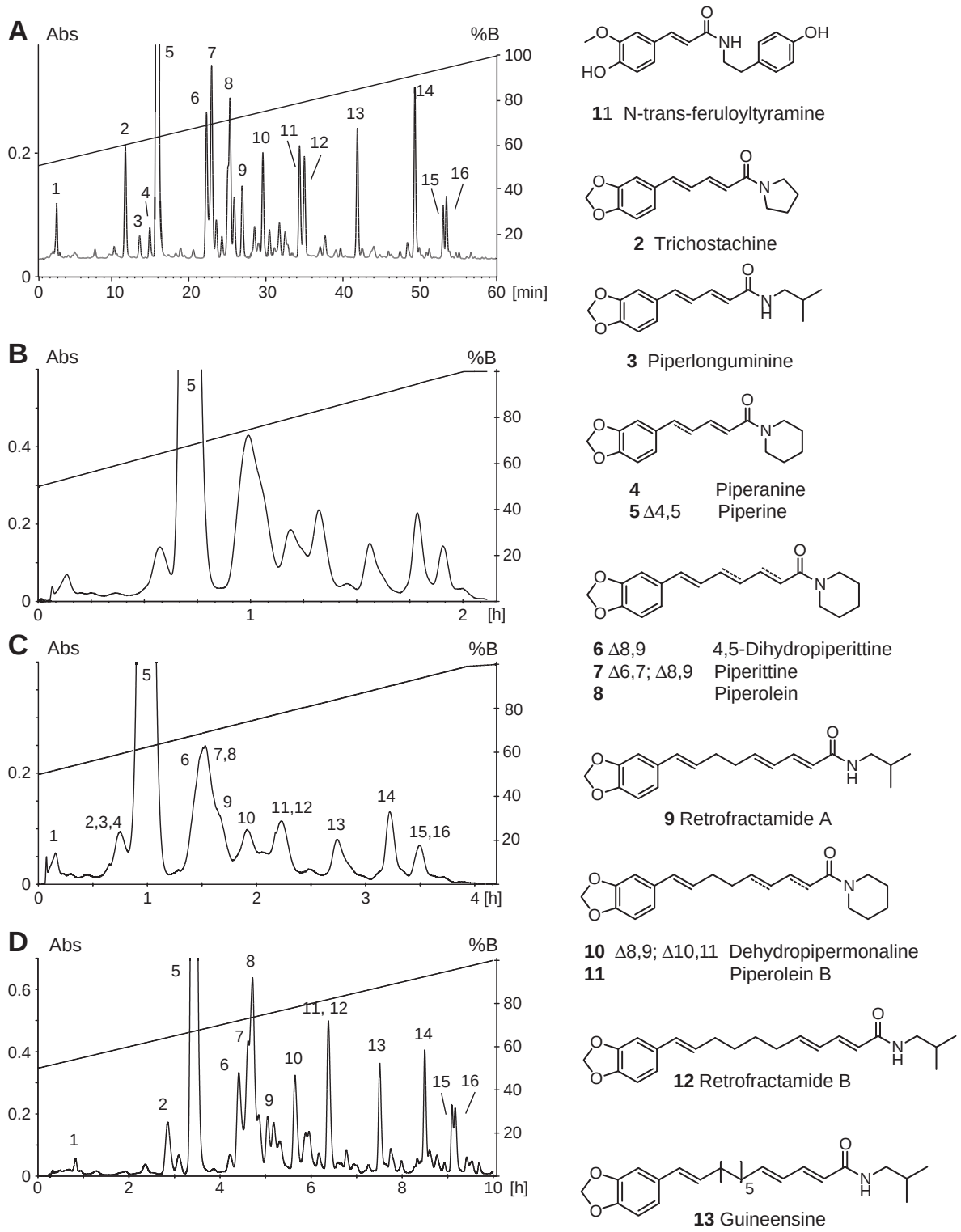

Fig. 2. Separation of a dichloromethane extract of *P. nigrum* on RP-18. A) HPLC column (4×150 mm). B/C) Sepacore® cartridge 40×150 mm, D) Glass column 26×240 mm. Mobile phase: water (A), methanol (B) both with 0.1% formic acid; gradient: 50–100% B in A) 60 min, B) 120 min, C), 420 min, D) 600 min; flow rate: A) 1 mL/min, B/C) 30 mL/min, D) 10 mL/min. Samples: A) 100 μg in 10 μL, B/C/D) 500 mg in 5 mL. Detection at 270 nm. Peak assignments by comparison with reference compounds previously isolated from *P. nigrum* [8].

on Fig. 3. A separation of the major diterpene quinones could be achieved in both cases. As expected, the separation efficiency of the MPLC column was slightly superior, but the separation time was increased by a factor of 5.

3.3. Influence of the mode of sample introduction

Sample dissolution is a major issue in preparative chromatography, and in particular for crude extracts and fractions.

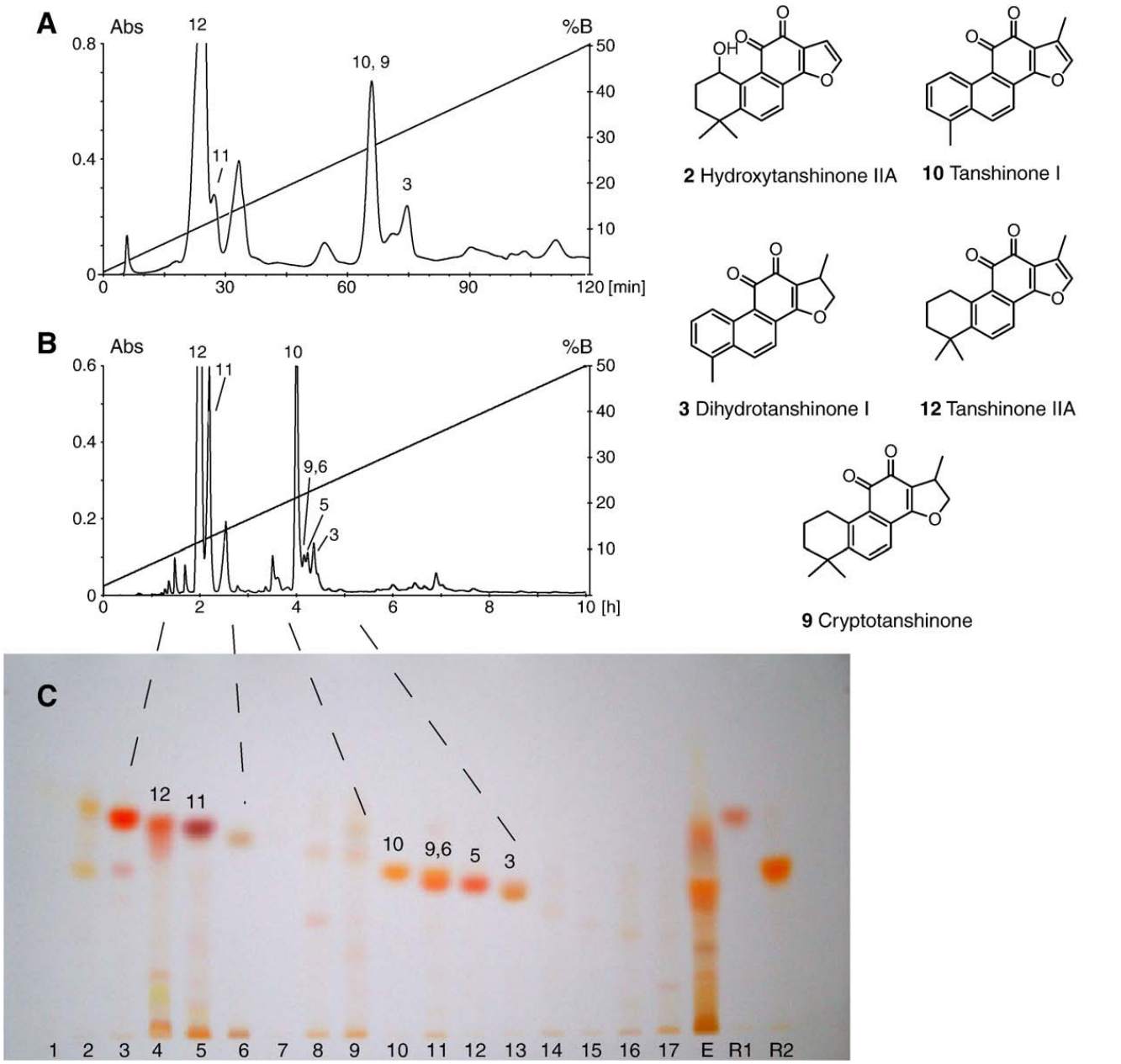

Fig. 3. Separation of a dichloromethane extract of *S. miltiorrhiza* on silica gel. A) Sepacore® cartridge 40×150 mm, B) glass column 26×240 mm. Mobile phase: A/B) n-hexane (A), ethyl acetate (B); gradient: A) 2–50% B in 120 min, B) 2–50% B in 600 min; flow rate: A) 30 mL/min, B) 10 mL/min. Sample: A) 500 mg in 10 mL *n*-hexane-ethyl acetate (6:4 v/v), B) solid introduction using a precolumn. Detection at 265 nm. C) TLC analysis of the extract and the fractions obtained from B with *n*-hexane-ethyl acetate-methanol (25:25:1 v/v); R1: tanshinone IIA, R2: cryptotanshinone (reference compounds). Further peaks tentatively assigned from HPLC-ESI-MS data.

Dissolution of the sample in the mobile phase is often not possible. To circumvent the solubility problem, samples are then dissolved in the mobile phase component of high elution strength. However, this may considerably disturb the separation and affect the chromatographic resolution. Solid phase introduction has been developed as an alternative, where samples are adsorbed onto stationary phase and packed into a precolumn device, which is then connected in-line for elution. We compared liquid and solid phase introduction with the example of *S. miltiorrhiza*. Dichloromethane extract of *S. milthiorrhiza* (500 mg) was either injected in a solvent of high elution strength (8 ml methanol) or introduced as dry material adsorbed on LiChroprep RP-18 material and subsequently packed in a PrepElut cartridge. Migration of compounds through the cartridge could be easily followed thanks to their intrinsic colours (Fig. 4). In liquid introduction, the solvent (methanol) led to strong perturbation of the column equilibrium and resulted in asymmetric bands. In contrast, solid introduction led to regular migration and consequently higher resolution.

4. Conclusion

Despite their relative short length and large particle size, pre-packed cartridges provide good preliminary separation of complex natural product extracts. Under gradient elution, they are well suited for the first fractionation of compounds covering a broad polarity range, which is typically the case in plant extracts. For the fine separation of close peaks,

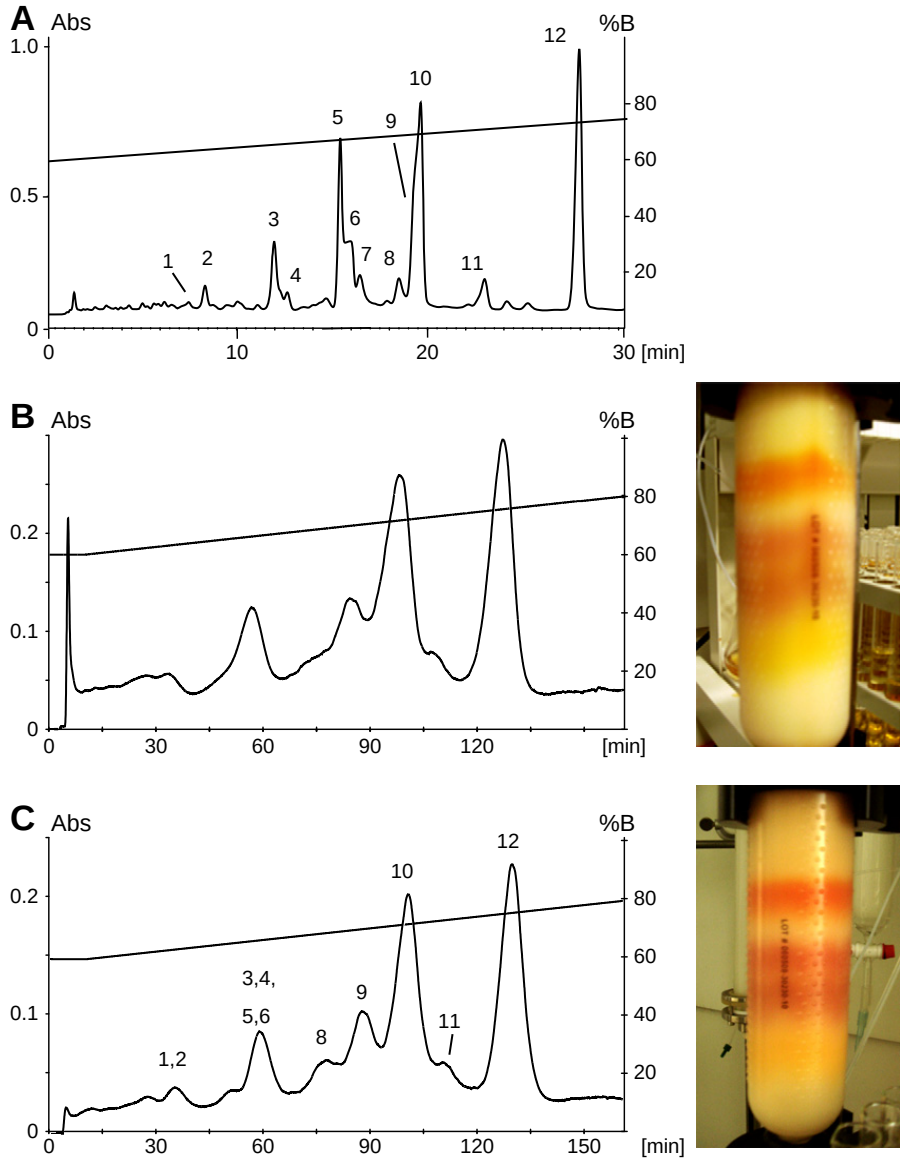

Fig. 4. Influence of the sample introduction mode on the separation of a dichloromethane extract of *S. milthiorrhiza*. A) HPLC column (4×150 mm). B/C) Sepacore® cartridge 40×150 mm. Mobile phase: water (A), methanol (B) both with 0.1% formic acid; gradient: A) 60–75% B in 30 min, B/C) 60% B for 10 min, 60–80% B in 110 min; flow rate: A) 1 mL/min, B/C) 30 mL/min. Detection at 250 nm. Sample: A) 100 μg in 10 μL, B) liquid introduction, 500 mg in 8 mL methanol, C) solid introduction, 500 mg. Peak assignment to tanshinone IIA and cryptotanshinone based on comparison with reference compounds; further peaks tentatively assigned from HPLC-ESI-MS, and literature data [10]. Compound structures: see Fig. 3.

however, their efficiency remains clearly inferior to classical MPLC columns. In the reversed phase mode, gradients developed by HPLC can be directly translated into flash chromatography separations, if the time for the gradient is increased by a factor of 2 to 4. For flash separations on silica gel, gradient end points can be determined by selecting R_f values around 0.2 for the most lipophilic and hydrophilic constituents, respectively. Solid introduction appears to be the best approach when a sample to be separated is poorly soluble in the mobile phase, even though sample preparation is more time consuming and requires some experience. The versatility of sample introduction is an asset for flash chromatography and MPLC as compared to preparative HPLC. While better separation performance can be expected for classical MPLC, pre-packed cartridges are an attractive alternative for the purification of extracts and crude fractions, due to their ease of use and speed of separation. Due to differences in the chemistry of cartridges and columns from diverse manufacturers the precise correlation between HPLC and MPLC conditions will have to be determined for each particular system. However, this should be relatively straightforward using the approach and the selection of reference compounds described above.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.fitote.2010.08.013.

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