Short communication

Secondary metabolites isolation in natural products chemistry: Comparison of two semipreparative chromatographic techniques (high pressure liquid chromatography and high performance thin-layer chromatography)

Thi Kieu Tien Do,a,b Francis Hadji-Minagloua, Sylvain Antoniottib, Xavier Fernandezb,*

a Botanicert, Espace Jacques-Louis Lions, 4 Traverse Dupont, 06130 Grasse, France
b Institut de Chimie de Nice, UMR 7272, Universite Nice Sophia Antipolis, CNRS, Parc Valrose, 06108 Nice Cedex 2, France

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ABSTRACT

Chemical investigations on secondary metabolites in natural products chemistry require efficient isolation techniques for characterization purposes as well as for the evaluation of their biological properties. In the case of phytochemical studies, the performance of the techniques is critical (resolution and yield) since the products generally present a narrow range of polarity and physicochemical properties. Several techniques are currently available, but HPLC (preparative and semipreparative) is the most widely used. To compare the performance of semi-preparative HPLC and HPTLC for the isolation of secondary metabolites in different types of extracts, we have chosen carvone from spearmint essential oil (Mentha spicata L.), resveratrol from Fallopia multiflora (Thunb.) Haraldson, and rosmarinic acid from rosemary (Rosmarinus officinalis L.) extracts. The comparison was based on the chromatographic separation, the purity and quantity of isolated compounds, the solvent consumption, the duration and the cost of the isolation operations. The results showed that semi-preparative HPTLC can in some cases offer some advantages over conventional semi-preparative HPLC.

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1. Introduction

The chemical diversity and the large variety of biological activities of secondary metabolites from natural products have attracted the attention of chemists, biochemists, pharmacists and biologists for a long time. Applications of these compounds in pharmaceuticals, cosmetics or flavors and fragrances are numerous, but are preceded by necessary chemical and biological studies [1]. To obtain pure samples of the compounds of interest in sufficient amounts from natural extracts and allow further characterization by spectral analysis such as 1H and 13C NMR, X-ray, or MS, efficient isolation techniques are required. The isolation of compounds from natural extracts is usually carried out in two steps. The first step is to enrich the extract by using various methods such as distillation, liquid–liquid partition, open-column chromatography (CC), and flash chromatography (FC), prior to the second step of isolation. These primary methods typically exhibit high loading capacity and low resolution. To improve the resolution, semipreparative techniques such as high pressure liquid chromatography (HPLC), counter current chromatography (CCC), overpressured layer chromatography (OPLC), and preparative thin-layer chromatography (TLC) (PTLC) can be used. OPLC has been used in many cases for the isolation of active compounds [2,3]. It has been reported to be best suited to the isolation of small amounts of partially purified samples but this technique requires a pressurized chamber [4]. HPLC however is the most frequently used [1,5,6]. The choice of an isolation procedure depends on the target product, the nature of the material source, and the concentration within the extract [7]. Most of the time, HPLC is chosen for the isolation of a wide variety of compounds such as higher terpenoids, alkaloids, saponins, polyphenols, etc. [8,9,10]. Conventional TLC has been used for the isolation of secondary metabolites such as flavonoids, phytosterols or terpenes, with preparative TLC plates, and quite often, in combination with a second isolation method [11,12]. TLC separation with TLC plates alone could also be performed on crude extracts without preliminary sample preparation [13,14] but no study has been done so far to our best knowledge to compare with high performance thin-layer chromatography (HPTLC). HPTLC is known to allow better separations than TLC [15]. HPTLC is simple and fast, uses disposable plates, which avoid cross-contamination from the stationary phase, and many derivatization reagents and phase pre-treatment procedures are available [16].
We described here in our results on the semi-preparative isolation of three compounds with different characteristics: volatile low-molecular weight terpenoid carvone (C<sub>10</sub>H<sub>14</sub>O, 150.22 g/mol), phenolic resveratrol (C<sub>14</sub>H<sub>22</sub>O<sub>2</sub>, 228.24 g/mol), and rosmarinic acid (C<sub>18</sub>H<sub>17</sub>O<sub>9</sub>, 360.31 g/mol), present in various concentrations in different untreated natural extracts. Carvone is a terpenoid naturally found in many essential oils such as spearmint (Mentha spicata L.) characterized by its high carvone content (60–70%) [17–19]. This monoterpenone exhibits antiseptic activity, and is usually analyzed by GC [18]. Resveratrol is a phenolic stilbene found in the skin of red grapes and in our case in Fallopia multiflora (Thunb.) Haraldson extract [20,21]. Rosmarinic acid is a phenolic compound known for its antioxidant activity [22]. The aim of the study was to assess the efficiency of HPTLC to isolate secondary metabolites compared to HPLC.

2. Materials and methods

2.1. Chemical and material

Carvone (98%), resveratrol (≥99%) and rosmarinic acid (≥98%) standards, methanol, acetonitrile, water, toluene and chloroform (HPLC grade), formic acid (≥98%) and ethyl acetate (≥99.5%) were purchased from Sigma–Aldrich. M. spicata L. essential oil, F. multiflora (Thunb.) Haraldson and rosemary (Rosmarinus officinalis L.) dry extracts were purchased from Naturex (Avignon, France).

2.2. Samples and standards preparation

Standard stock solutions were obtained by dissolution of carvone (1.7 mg), resveratrol (11.7 mg), and rosmarinic acid (3.0 mg) in 10 mL MeOH. For each standard, a calibration curve was realized with a range of 5 concentrations from 0.02 mg/mL to 0.17 mg/mL for carvone using GC, from 0.13 mg/mL to 1.17 mg/mL for resveratrol using HPLC/DAD, and from 0.01 mg/mL to 0.3 mg/mL for rosmarinic acid using HPLC/DAD. Response factors were determined by linear regression for each standard with R<sup>2</sup> coefficients all deemed acceptable above 0.99.

2.3. GC/FID analysis

The quantitative analysis of carvone samples was performed by GC-FID using an Agilent 6890N system equipped with a HP1 column polydimethylsiloxane (50 m × 0.2 mm i.d. and 0.33 μm phase thickness) and operated using the following conditions: carrier gas: helium; constant flow: 1 mL/min; injected volume: 1 μL and split ratio: 1:100. GC oven temperature was set to 100 °C and increased to 250 °C with a rate of 10 °C/min and remained at 250 °C for 10 min. The conditions for FID were: detector temperature: 250 °C; hydrogen flow: 40 mL/min; air flow: 450 mL/min and make up flow N<sub>2</sub> 45 mL/min. The characterization of carvone was performed by comparison with a standard. The quantifications were obtained using FID signal integrations according to the calibration curve. GC/FID analyses were carried out in duplicates.

2.4. HPLC analysis and isolation

HPLC analyses were performed on a HPLC Agilent 1200 series equipped with evaporative light-scattering detector (ELSD) and diode array detector (DAD) using a Phenomenex Luna C18 column (4.6 mm × 150 mm, 5 μm). The column is equipped with a Phenomenex guard C18 (4.0 mm × 3.0 mm). The ELSD was used under the following conditions: temperature: 40 °C; nebulizing gas pressure: 3.6 bar; Gain: 3; sampling time: 100–10 Hz; Filter: 3 s. The DAD provided 3 characteristic UV wavelengths 238 nm (carvone), 307 nm (resveratrol), and 330 nm (rosmarinic acid). For quantitative analyses, a standard HPLC method was set up with water containing 0.1% formic acid (A), methanol (B) and isopropanol (C). The gradient was set as follows (with A%/B%/C%): 0–15 min, 95/5/0; 15–45 min, 95/5/0–5/95/0; 45–50 min, 5/95/0; 50–51 min; 5/95/0–0/100/0; 51–61 min, and back to the initial conditions (95/5/0) in 10 min. The flow rate was constant at 1 mL/min with an injection volume of 20 μL.

The compound isolations were carried out by semipreparative HPLC/DAD on the same HPLC with a Phenomenex Luna C18 column (10 mm × 250 mm, 5 μm) operating at 20 °C with a flow rate of 2.5 mL/min. The column is equipped with a Phenomenex semiprep guard C18 (10 mm × 10 mm). For semipreparative isolation a standard method was set up with water containing 0.1% formic acid (A), acetonitrile containing 0.1% formic acid (B) and isopropanol (C). The gradient profile for carvone isolation was set as follows (with A%/B%/C%): 0–3 min, 30/70/0; 3–13 min, 5/95/0–15/85/0; 13–15 min, 15/85/0–0/50/50; 15–25 min, 0/50/50 and back to the initial conditions (30/70/0) in 10 min with an injection volume of 40 μL for a concentration of the solution of 20.3 mg/mL. The gradient profile for resveratrol isolation was (with A%/B%/C%): 0–10 min, 55/45/0; 10–15 min, 55/45/0–0/100/0; 15–16 min, 0/100/0–0/50/50; 16–26 min, 0/50/50 and back to the initial conditions (55/45/0) in 10 min with an injection volume of 50 μL for a concentration of the solution of 35.1 mg/mL. The gradient profile for rosmarinic acid isolation was: (with A%/B%/C%): 0–10 min, 70/30/0–55/45/0; 10–15 min, 55/45/0–0/100/0; 15–17 min, 0/100/0–0/50/50; 17–27 min, 0/50/50 and back to the initial conditions (70/30/0) in 10 min with an injection volume of 40 μL for a concentration of the solution of 75.7 mg/mL. The solvent of fraction obtained was evaporated under vacuum and analyzed by GC/FID and HPLC/DAD after solubilization with methanol.

2.5. HPTLC analysis and isolation

HPTLC analyses were performed using Merck (0.20 mm) silica gel 60 F<sub>254</sub> (20 cm × 10 cm) glass HPTLC analytical plate, and Merck (0.20 mm) silica gel 60 F<sub>254</sub> (20 cm × 10 cm) glass HPTLC plate with concentrating zone, using a Camag (Muttenz, Switzerland) HPTLC system equipped with an automatic TLC sampler (ATS 4), an automatic developing chamber ADC2 with humidity control, a visualizer and a TLC scanner 4 controlled with WinCATS software. All the plates were pre-washed by developing (80 mm) using 10 mL methanol, and then dried in oven at 120 °C for 30 min. All HPTLC analyses were developed from the lower edge of the plate until 70 mm, humidity control (33–38%), with 20 min saturation. Visual inspection and documentation of the chromatograms were carried out under 254 nm and 366 nm. Plates were scanned under the following conditions: scanning mode, reflectance mode at 328, 307, and 330 nm, D2 and W lamp, slit dimension 8.00 mm × 0.40 mm, scanning speed 20 mm/s, data resolution 100 μm/step. Pre-washing was realized simultaneously during the application of the next plate. For analytical purposes, standard and sample solutions (15 tracks per plates) were applied bandwise (band length 8 mm, 50 Nl/s delivery speed, track distance 11.4 mm, distance from the left and right edges 20 mm) and for semipreparative separation, sample solutions were applied bandwise (19 tracks per plates) (band length 8 mm, 50 Nl/s delivery speed, track distance 8.8 mm, distance from the edge 20 mm). Carvone isolation (23.5 mg) was carried out with 14 analytical HPTLC plates with toluene:ethyl acetate (95:5, v/v), for a processing time of 9 h. Resveratrol isolation (8.5 mg) was carried out with 12 analytical HPTLC plates with chloroform:methanol (9:1, v/v), for a processing time of 8 h. Rosmarinic acid isolation (4.1 mg) was carried out with 15 analytical HPTLC plates with toluene:ethyl acetate:formic acid (5:4:1, v/v/v), for a processing time of 9 h.
3. Results and discussion

Two semipreparative isolation chromatographic techniques (HPLC and HPTLC) have been applied and compared for the cases of carvone, resveratrol and rosmarinic acid from mint essential oil (M. spicata L.), F. multiflora (Thunb.) Haraldson and rosemary (R. officinalis L.) extracts, respectively. Carvone which is present at 65.2% in the essential oil of mint (measured by GC/FID) and resveratrol present at 50.6% in the extract (measured by HPLC/DAD) are well separated from the other analytes by analytical HPLC/DAD (resolution (Rs) = 7.9; Rs = 4.1). By analytical HPTLC, with detection at 238 and 307 nm, carvone (Ratio of flow (Rf) = 0.37) and resveratrol (Rf = 0.48) were well separated from the other analytes as well (Rs = 1.7; Rs = 2.9). Concerning rosmarinic acid, present at 22.6% in the extract, two times longer method development times was required, to optimize the separation from other compounds using both HPLC and HPTLC (Rf = 0.30) instruments (Rs = 2.2; Rs = 0.8) (Fig. 1).

The method development in HPTLC involved different steps. The first attempts were performed on HPTLC plates with concentrating zone that allow larger volume application. In this case the crude extract separation was not sufficient for isolation purpose. A second series of attempts was performed with regular HPTLC plates and showed better result. For each HPTLC development method, one single type of plate was used. On each plate, one track of analytical standard and a range of 5 concentrations of the extracts

were applied to select in each case the maximum concentration for the application (carvone: 69.0 mg/mL; resveratrol: 41.1 mg/mL; rosmarinic acid: 79.8 mg/mL). The methods were already known in the literature [15,23,24]. Regarding HPLC, from the processing time point of view, the method development took an average of half a day per extract. For the isolation step, the number of samples is limited by the necessary elution time for each one, since the samples are injected one at a time. In general, HPLC instrument requires a more trained user than HPTLC. Concerning the cost of the isolation procedures, in our analysis conditions, up to 400 injections of natural extract can be performed by HPLC on the same semipreparative column, with a change of precolumn every 200 analyses. Concerning HPTLC, in our analysis conditions, up to 19 tracks can be applied on a plate. By considering all this information, HPLC is 10 times more expensive than HPTLC without taking into account the solvent consumption.

The results for the isolation of carvone, which is present at 65.2% in essential oil of mint, showed that for a processing time shorter by using HPTLC (9 h) than HPLC (10 h), 2.6 times more amount of compounds was isolated by using HPTLC. Regarding the yields of the isolation, the results are similar with both methods (HPLC: 98.9 ± 0.1% ; HPTLC: 98.1 ± 0.1%). Concerning the purity, the compound isolated was of higher purity by using HPLC than HPTLC (HPLC: 97.1 ± 0.1% ; HPTLC: 94.3 ± 0.1%). From the solvent consumption point of view, differences were much more significant. For those range of mass of isolated compound, average of solvent consumption by using HPTLC was more than 5 times lower than using semipreparative HPLC (153 mL/mg compared to 27 mL/mg) (Table 1).

In the case of resveratrol, titrated at 50.6% in the current extract and well separated, the mass of isolated compound results were 12.7 ± 0.1 mg using HPLC and 8.5 ± 0.0 mg using HPTLC. The processing time was shorter by using HPTLC (8 h for 8.5 mg) than HPLC (9 h for 12.7 mg). The yield was better using HPLC (95.8 ± 0.1%) compared to HPTLC (89.9 ± 0.1%). Regarding the purity, analyses reveal no significant difference (98.1 ± 0.1% for both methods). For this range of mass of isolated compound, average of solvent consumption by using HPTLC was ca. 30% lower than using semipreparative HPLC (94 mL/mg compared to 61 mL/mg).

In the case of rosemary extract, rosmarinic acid was present at 22.6% and not well separated by HPLC and HPTLC, and a processing time of 6 h and 9 h using HPLC and HPTLC, respectively, was needed. The solvent consumption was 38% lower in the case of HPTLC (116 mL/mg vs 72 mL/mg), for an isolated mass of 5.8 ± 0.1 mg using HPLC and 9.4 ± 0.1 mg using HPTLC. With HPTLC, the compound isolated was less pure (86.8 ± 0.1%) than using HPLC (93.2 ± 0.1%) but the yield is better using HPTLC (91.3 ± 0.1%) than HPLC (84.0 ± 0.1%). With this case, it seems that for challenging extracts, with target compounds hardly separated from the other metabolites, HPTLC is not enough efficient, and preference should be given to the use of HPLC. Alternatively, an additional enrichment step should be implemented with HPTLC in the separation procedure (Table 1).

### 4. Conclusion

The two approaches (semipreparative HPLC and HPTLC) allowed successful and rapid isolation of the target metabolites in the three cases studied. For a high content of the target in the natural extract, considering a green and sustainable chemistry approach aiming at reducing the amount of waste, of material, and of energy costs, HPTLC appeared as the method of choice (case of carvone and resveratrol). For challenging cases, the choice of the technique depends on the priority between purity and yield: for a high purity regardless of the amount isolated, semipreparative HPLC remains the most efficient, while for a higher yield, HPLC should be preferred.

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