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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)

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ABSTRACT

Chemical investigations on secondary metabolites in natural products chemistry require efficient isolation techniques for characterization purpose 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 semipreparative 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 semipreparative HPTLC can in some case offer some advantages over conventional semipreparative HPLC.

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 ^{1}H and ^{13}C 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 time, HPLC is chosen for the isolation of a wide variety of compounds such as higher terpenoids, alkaloids, saponins, polyphenols, etc. [6,8–10]. 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].

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We described here in our results on the semi-preparative isolation of three compounds with different characteristics: volatile low-molecular weight terpenoid carvone ($C_{10}H_{14}O$, 150.22 g/mol), phenolic resveratrol ($C_{14}H_{12}O_3$, 228.24 g/mol), and rosmarinic acid ($C_{18}H_{16}O_8$, 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 monoterpene 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^2 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_2 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; 5/95/0–0/50/50; 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_{254} (20 cm × 10 cm) glass HPTLC analytical plate, and Merck (0.20 mm) silica gel 60 F_{254} (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 238, 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.