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Short communication

An approach to speed up the isolation of hydrophilic metabolites from natural sources at semipreparative level by using a hydrophilic–lipophilic balance/mixed-mode strong cation exchange–high-performance liquid chromatography/mass spectrometry system

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ABSTRACT

An approach to speed up the isolation of hydrophilic metabolites in complex natural matrixes by using a HLB/MCX–HPLC/MS system based on the retention properties of hydrophilic–lipophilic and cation exchange polymeric cartridges was developed. This methodology was successfully applied to the re-isolation of small water soluble compounds with completely different structures from two different natural extracts such as a dipeptide (vanchrobactin) from a bacterium culture broth and a pyrrolidine bearing a carboxylic acid moiety (clionapyrrolidine A) from a sponge. This method improved not only the efficiency of the isolation methodology but also the isolation time in relation to the existing methods.

1. Introduction

In recent decades considerable progress has been made in the search for bioactive secondary metabolites from microbial, plant and marine sources. This is a challenging task but is also rewarding because natural products have been the single most productive source of leads for the development of new drugs [1–4]. Advances in high-performance liquid chromatography (HPLC) and other chromatographic techniques for semi-preparative separation such as high-speed countercurrent chromatography, gel permeation chromatography and solid phase extraction (SPE), have enabled chemists to separate even the most complex mixtures of both non-volatile and volatile compounds into their individual components [5,6]. In addition, considerable progress has been made in detection and hyphenation in analytical techniques. The direct coupling of HPLC with either mass spectrometry (MS) or NMR and GC–MS now allows the quick and straightforward identification and quantification, with high sensitivity and selectivity, of individual components in complex mixtures [7–10].

Despite the remarkable advances in purification methods, the isolation of pure metabolites frequently remains not only highly labour-intensive and time-consuming but is also expensive and contaminating in terms of environmental conservation. Furthermore, as Shimizu and Li stated "the isolation of small water-soluble molecules still remains a mystery for natural-product researchers" [11]. Indeed, aqueous and other polar extracts, e.g. from the marine environment, are known to contain high levels of salts and such samples require extensive desalting and fractionation processes. The standard methods for desalting involve the use of reversed-phase columns (e.g. C-18 silica gel), organic polymer resins (e.g. XAD) or size-exclusion columns (e.g. Sephadex) while for fractionation implies in most cases the use of size exclusion, ion exchange chromatography or reversed phase silica gel based supports. But very often these methods lead to poor recoveries and, at worst, a loss of activity in the extract. Attempts to deal with these issues have included efforts not only to develop faster and more convenient extraction and fractionation approaches for purification of these water-soluble compounds, but also to hyphenate these steps with instrumental techniques to enhance the isolation of low

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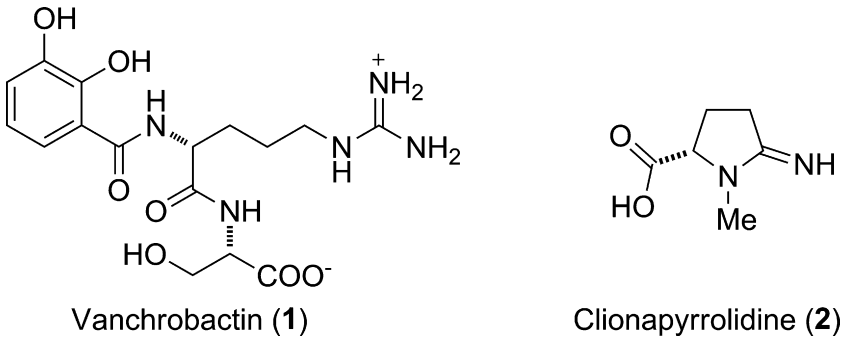

Fig. 1. Structure of the small water-soluble molecules isolated by this approach.

abundance secondary metabolites prior to their final purification by HPLC.

In an effort to address the challenges associated with the isolation and purification of hydrophilic metabolites from natural sources, we introduce here an approach to speed up the isolation of this type of natural products using a HLB/MCX–HPLC/MS system (hydrophilic–lipophilic balance/mixed-mode strong cation exchange–liquid chromatography/mass spectrometry) based on the retention properties of HLB and MCX polymeric cartridges. This approach involves a quick and straightforward fractionation step on solid phase extraction (SPE) polymeric cartridges with the optimal combination of two highly orthogonal and complementary retention mechanisms (reversed-phase and/or mixed-mode cation exchange-reversed-phase and/or hydrophilic interaction chromatography HILIC) followed by purification on a RP-HPLC column. The entire process is monitored by HPLC/MS. The nature and the high specific surface area of these SPE polymeric sorbents allow the loadability of crude extracts to be enhanced to obtain an optimum yield of the hydrophilic metabolite with high purity using the minimum elution and washing steps. We applied this methodology to speed up the isolation of two hydrophilic bioactive marine natural products, the siderophore vanchrobactin (**1**), as shown in Fig. 1, responsible for the iron uptake of the pathogen bacteria *Vibrio anguillarum* serotypes O2 and O3 [12], and the allelopathic clionapyrrolidine A (**2**) (Fig. 1) from the excavating sponge *Cliona tenius* that kills coral tissue upon contact [13]. Although SPE polymeric hydrophilic–lipophilic balance (HLB) and mixed-mode strong cation exchange (MCX) Oasis® cartridges have been widely used in pre-concentration (enrichments) processes or clean-up procedures at the analytical level [14–19], this is the first time that they have been applied to natural crude extracts for the isolation of hydrophilic compounds at a semipreparative level.

2. Experimental

2.1. Apparatus

Chromatographic separation was carried out on an Agilent 1100 liquid chromatography system equipped with a solvent degasser, quaternary pump, auto sampler, column compartment and a diode array detector (Agilent Technologies, Waldbronn, Germany). The UV wavelength was set up at 214 nm, band width 8 nm. Electrospray mass spectrometry measurements were performed on an MSD ion trap mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) interfaced to the HPLC system. MS measurements were acquired in positive ionization mode over the mass range of 100–700 amu. NMR spectra were measured on a Bruker Avance DRX500 MHz in D_2O. Spectra were referenced to the residual solvent peak.

SPE cartridges were Oasis® HLB and MCX from Waters (Milford, MA, USA) and Strata silica from Phenomenex (Torrance, CA, USA). SPE steps were conducted on an IST VacMaster SPE manifold of 10 positions. The HPLC columns were Atlantis dC18 100 mm × 4.6 mm, 5 μm and Atlantis Hilic 4.6 mm × 50 mm 3 μm from Waters (Milford, MA, USA), Chromolith SpeedRod 50 mm × 4.6 mm from Merck (Darmstadt, Germany), Discovery F5 50 mm × 4.6 mm, 3 μm and 100 mm × 10 mm, 5 μm from Supelco (Bellefonte, PA, USA). The HPLC mobile phases were water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid (FA) or 0.05% triflouroacetic acid (TFA). The gradient programs employed are described in each chromatogram.

2.2. Reagents

Acetonitrile and methanol HPLC grade were obtained from Lab Scand (Dublin, Ireland), formic acid and trifluoroacetic acid, were from Sigma–Aldrich (Steinheim, Germany), ammonia 7 N solution in methanol (CH_3OH:7 N NH_3) was from Acros (Geel, Belgium) and water was purified in house with a Milli-Q plus system from Millipore (Bedford, MA, USA).

2.3. Separation procedure

2.3.1. V. anguillarum serotype O2

Two litres of cell-free culture broth was lyophilized to give 3 g of material, which was dissolved in water (4.5 mL) and distributed in three HLB cartridges (35 cm^3, 6 g), loading 1.5 mL in each one. The mobile phase employed with the HLB cartridges was water (solvent A) and acetonitrile (solvent B), each containing 0.1% TFA (v/v). The cartridges were conditioned and equilibrated in parallel with 60 mL of solvent B and 60 mL of solvent A. After washing the cartridges with 60 mL of solvent A, the siderophore was eluted with 30 mL of a mixture of solvents A:B 1:1 and was detected by RP-HPLC/(+)-ESI-MS at m/z 398 ($[M+H]^+$). Each of the three fractions without drying was directly loaded into three MCX cartridges in parallel (20 cm^3, 1 g, conditioned with 10 mL of CH_3OH:TFA 0.1%, v/v) and then washed with 10 mL of solvent A, 10 mL of CH_3OH, and 20 mL of CH_3OH:7 N NH_3. RP-HPLC/(+)-ESI-MS showed that the fraction eluted with the latter mixture (2.4 mg after evaporation to dryness) contained siderophore **1** as the major component with a relative UV purity 76%. Final purification by HPLC using an Atlantis dC18 column with a mobile phase consisting of a 6 min gradient from 0 to 50% CH_3CN/TFA 0.05% (v/v) at a flow rate of 1 mL/min gave 1.2 mg of vanchrobactin (**1**) with purity greater than 95%.

2.3.2. V. anguillarum serotype O3

The lyophilized material (2.15 g) obtained from 2 L of cell-free culture of this organism was also subjected to the above mentioned protocol. Fractionation steps by HLB and MCX were monitored by RP-HPLC/(+)-ESI-MS. Thus, 17 mg of the MCX fraction containing siderophore **1** were finally purified by HPLC to yield 0.7 mg of vanchrobactin (**1**) with purity greater than 90%.

2.3.3. C. tenius

Two hundred milligram of the final aqueous fraction, obtained by the modified Kupcham protocol from the methanol crude extract as described previously, [13] was dissolved in 1 mL H_2O:CH_3OH:TFA (2:1:0.1, v/v/v) and then loaded into an MCX cartridge (20 cm^3, 1 g per cartridge, conditioned with 10 mL of CH_3OH/TFA 0.1%, v/v). After washing with 10 mL of H_2O:TFA 0.1% (v/v) followed with 10 mL of CH_3OH, clionapyrrolidine A (**2**) was eluted with 10 mL H_2O/CH_3OH:7 N NH_3 (2/1, v/v) and was detected by RP-HPLC/(+)-ESI-MS at *m/z* 143 ($[M+H]^+$). This fraction was dried under nitrogen stream, resolubilised in 500 μL of H_2O/CH_3CN/FA (2:1:0.1, v/v/v) and loaded into a Strata silica cartridge (6 cm^3, 1 g per cartridge conditioned with 10 mL of 1:9 H_2O:FA 0.1% (v/v) as solvent A and CH_3CN:FA 0.1% (v/v) as solvent B and eluted in HILIC mode with mixtures of solvents A and B 1:9, 2:8, 3:7, 4:6 and 6:4 (5 mL each). Clionapyrrolidine A (**2**) was identified by RP-HPLC/(+)-ESI-MS in the fraction eluted with 70% of solvent B and it was then purified by HPLC on a Discovery F5 semipreparative column using a mixture of H_2O:FA 0.1% and CH_3CN:FA 0.1% (85:15, v/v) as the mobile phase at a flow rate of 3 mL/min. The HPLC fraction containing clionapyrrolidine A (**2**) was subsequently trapped in an MCX cartridge (20 cm^3, 1 g per cartridge, conditioned with 6 mL H_2O). This cartridge was first eluted with 6 mL of H_2O and 6 mL of CH_3CN followed by 6 mL of CH_3CN:NH_4OH 10% (1:4, v/v). The latter fraction was evaporated to dryness under nitrogen stream to yield 1.2 mg of clionapyrrolidine (**2**).

3. Results and discussion

3.1. Vanchrobactin (*1*) from V. anguillarum serotype O2

We initially demonstrated the use of this methodology with the re-isolation and purification of the siderophore vanchrobactin (**1**), from the culture of the marine bacterium *V. anguillarum* serotype O2 in hours. This bacterium is the causative agent of vibriosis, a fatal hemorrhagic septicemia, in marine and freshwater fish species and results in considerable economic losses in aquaculture farming worldwide [20].

Comparison between a conventional protocol used previously in the isolation of vanchrobactin and the present approach is illustrated in Fig. 2. A total of 18 L of cell-free culture broth was required to yield 0.8 mg of vanchrobactin using a conventional process that took several weeks [21]. In contrast, only 2 L of cell-free culture broth was required to obtain 1.2 mg of highly pure vanchrobactin within a few hours using our approach. Moreover, this approach can be considered as a green methodology due to the low volume of solvent and the small amount of stationary phase used in our procedure compared to those employed in the traditional one (see Fig. 2).

The coupling of two different retention mechanisms in this separation system allows the high degree of orthogonality and selectivity in the straightforward isolation of the siderophore (see Fig. S1 in the Supporting Information). Firstly, vanchrobactin (pKa_1 2.7, pKa_2 11.8) [22] was selectively trapped and strongly retained by electrostatic interactions in the hydrophilic–lipophilic balance (HLB) cartridge. Salts and other components of the culture medium were rapidly removed as a result of washing the cartridge with aqueous acidic modifier and the siderophore was desorbed with an acidic pH solution containing organic solvent (H_2O:CH_3CN:TFA 50:50:0.1). This acidic sample matrix was directly loaded without evaporation into the mixed-mode cation exchange-reversed-phase (MCX) cartridge. In this second step, the protonated siderophore was captured and retained by ion exchange mechanism because of the strong interaction of the basic functionality (nitrogen atom of the arginine moiety) and the $-SO_3^-$ groups in the MCX stationary phase. This second step facilitated the removal of neutral and acidic impurities after washing the cartridge with acidic and neutral aqueous solvents. Vanchrobactin (**1**) was desorbed almost pure with a non-aqueous basic solvent (CH_3OH:7 N NH_3) and finally purified by RP-HPLC.

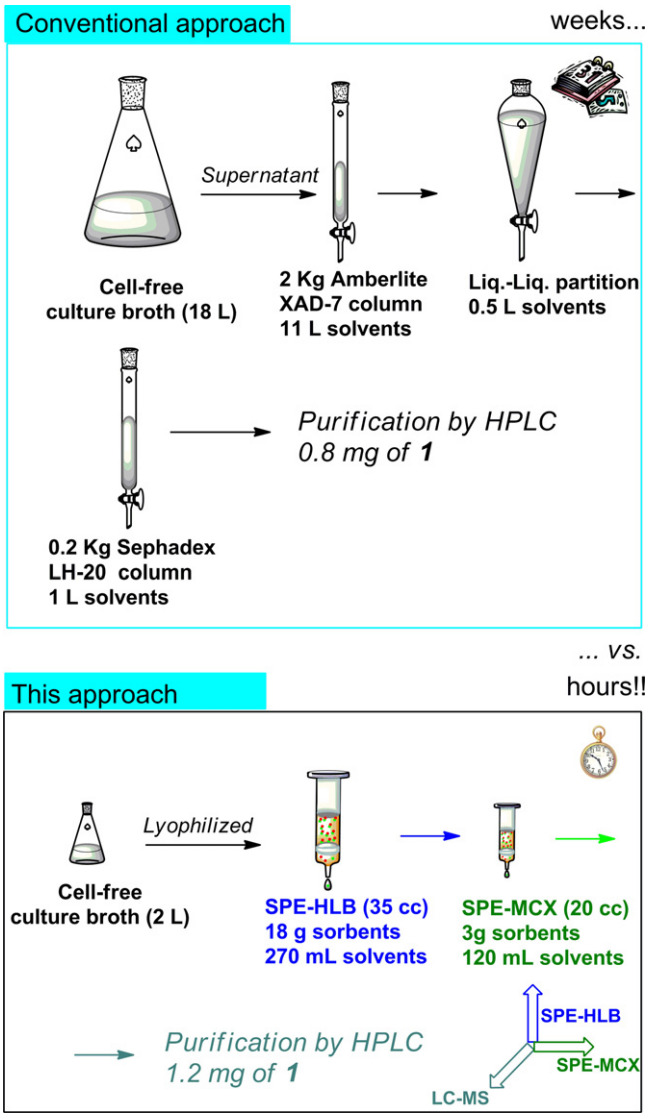

Fig. 2. Flow charts for the experimental design used in the isolation of vancrobactin (**1**) from *Vibrio anguillarum* serotype O2.

3.2. Vanchrobactin (*1*) from V. anguillarum serotype O3

In order to demonstrate the applicability of the current method for isolation water-soluble compounds in another complex natural matrix, we applied this approach to cell-free culture broth of *V. anguillarum* serotype O3 to isolate the siderophore responsible for its iron uptake mechanism [23].

Of the more than 20 recognized *V. anguillarum* serotypes, O1, O2 and O3 are the main ones implicated in vibriosis outbreaks [12]. Although the siderophores from *V. anguillarum* serotypes O1 and O2 have been identified, the corresponding to serotype O3 remained to be characterized. Thus, 0.7 mg of pure vanchrobactin (**1**) was obtained from 2 L of cell-free culture broth in just 5 h on using this methodology (see Figs. S2–S4 in the Supporting Information).

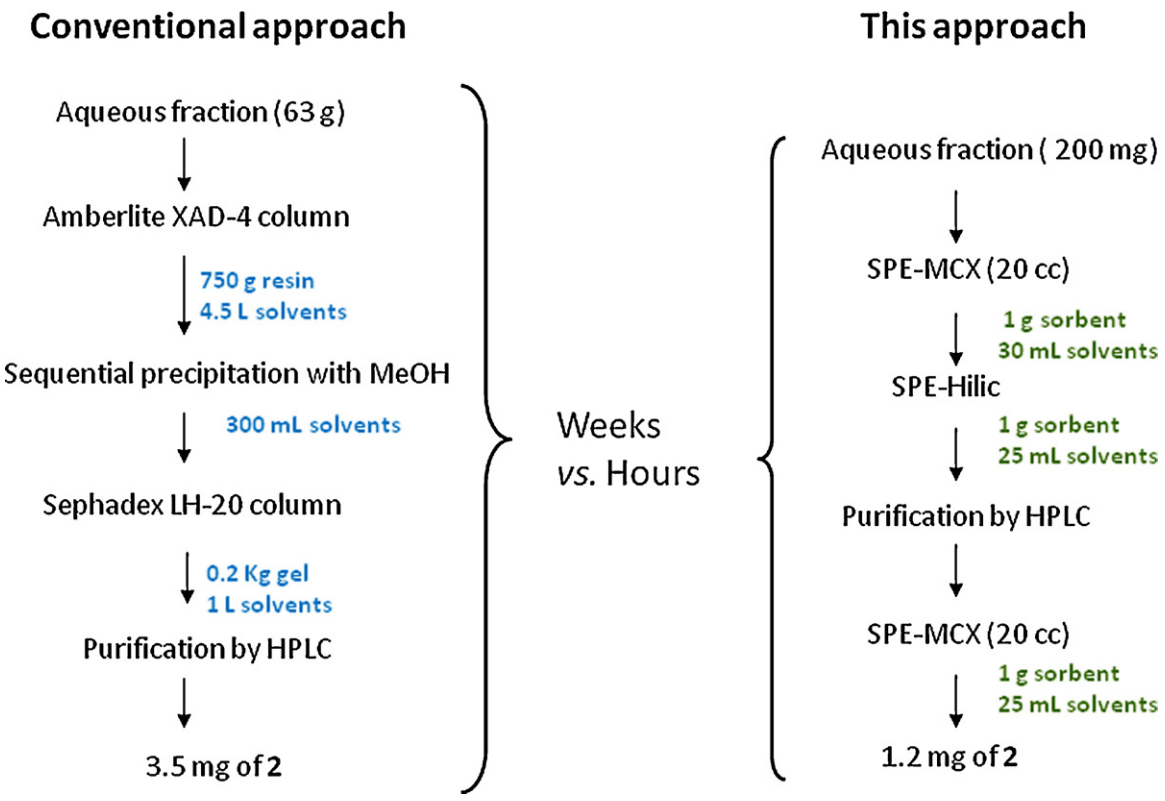

Fig. 3. Flow charts for the experimental design used in the isolation of clionapyrrolidine A (**2**) from *Cliona tenius*.

3.3. Clionapyrrolidine A (**2**) from C. tenius

To validate this methodology and to study the scope of this approach, we applied it to the isolation of another extremely polar bioactive metabolite with a very different structure, clionapyrrolidine A (**2**). This compound was initially isolated by a long bioassay-guided fractionation protocol on an aqueous fraction obtained from the methanol extract of the excavating sponge *C. tenius* [24]. This metabolite was finally identified as the compound that kills coral tissue upon contact [13]. The rapid and successful isolation of 1.2 mg of clionapyrrolidine A (**2**) from just 200 mg of that aqueous fraction using our methodology confirmed the broad scope this approach.

As depicted in Fig. 3, the 2D-SPE orthogonal retention mechanism employed here was mixed mode (MCX cartridge) and HILIC (SiO_2 cartridge). Quantification of clionapyrrolidine A (**2**) in the aqueous fraction by the electronic reference to access in vivo concentration (ERETIC) method in NMR calibration revealed a purity of 2% [25]. This extremely polar small molecular weight metabolite (pKa_1 2.8, pKa_2 10.1) was non retained in the Oasis HLB cartridge (data not shown), therefore the sample matrix was dissolved in aqueous acidic solvent and fractionated on a MCX cartridge. Clionapyrrolidine A (**2**) was selectively retained by ion exchange mechanism and desorbed by flushing the MCX cartridge using alkalinized methanol that was then dried, resolublized and loaded into normal phase silica cartridge. The elution of the silica cartridge was carried out in HILIC mode where the elution is promoted by the use of polar mobile phases as it has been successfully reported in the separation of highly polar natural products [26]. Thus, a clean clionapyrrolidine A (**2**) was obtained after flushing the silica cartridge with a 10–40% aqueous gradient with formic acid as modifier (see Fig. S5 in the Supporting Information). Taking into account that this type of metabolites elutes too close to the void volume on conventional C18 alkyl phases, a highly retentive column Discovery F5 (pentafluorophenylpropyl phase) that exhibits sufficient retention of small polar compounds [15] was selected to monitor the isolation of clionapyrrolidine A (**2**) by LC/MS. Final purification by HPLC and using a fraction trapping step to simplify solvent removal and to avoid salt contamination allowed obtaining highly pure clionapyrrolidine A (**2**). It is worthy to note that in previous studies clionapyrrolidine A (**2**) was isolated with high concentrations of formate salt after its final purification by HPLC [24].

4. Conclusions

In summary, we have developed a SPE-HPLC/MS strategy based on the retention properties of hydrophilic–lipophilic and cation exchange polymeric sorbents as well as silica (HILIC mode) cartridges followed by semipreparative HPLC combined with hyphenated analytical LC/DAD/MS techniques for detection. This approach is very useful for the isolation of small water-soluble bioactive molecules. The higher capacity and interaction with organic polar compounds suggest an attractive alternative to traditional C18-SPE cartridges and XAD resins. Furthermore, we found that the cartridges can be reused several times and retain their hydrophobic retention properties, which is an additional cost and resource benefit (unpublished results). This approach was successfully applied to the re-isolation of hydrophilic compounds with completely different structures such as a dipeptide (vanchrobactin) and a pyrrolidine bearing a carboxylic acid moiety (clionapyrrolidine A). It was also useful for the characterization – for the first time – of the siderophore responsible for the iron uptake of *V. anguillarum* serotype O3. This method improved not only the efficiency of the isolation methodology but also the isolation time in relation to the existing methods, and so we can speed up the isolation of hydrophilic secondary metabolites in complex environmental matrixes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.01.072.

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