Separation and Purification Technology 170 (2016) 138–145

Contents lists available at ScienceDirect

Separation and Purification Technology

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

Development of a liquid-liquid extraction method of resveratrol from cell culture media using solubility parameters

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ARTICLE INFO

Article history:
Received 14 December 2015
Received in revised form 17 June 2016
Accepted 22 June 2016
Available online 23 June 2016

Keywords:
Benzyl benzoate
Liquid-liquid extraction
Partition coefficient
Plant cell culture
Resveratrol
Solubility parameters
Solvent consumption

ABSTRACT

The extraction of bioactive compounds, produced by plant cell cultures, directly from their culture medium, which contains other by-products, is a great challenge. Resveratrol extraction from its grapevine cell cultures is considered here as an example to improve the extraction processes from plant cell cultures using solubility parameters. Successive liquid-liquid extraction (LLE) processes were exploited to extract resveratrol from the culture medium with an extraction ratio approaching 100%, high selectivity and minimum amounts of solvents. The calculations of partition coefficients as a function of solubility parameters demonstrated that benzyl benzoate is the most suitable intermediate solvent to extract resveratrol from its aqueous medium. The calculations also illustrated the high ability of methanol and ethanol to extract resveratrol from benzyl benzoate. The physicochemical properties of benzyl benzoate and processing conditions were exploited to separate it from aqueous media and organic solvents. The agitation method, component ratios and extraction time were studied to maximize the extraction yield. Under the best studied conditions, the recovery of resveratrol from different culture media approached ~100% with a selectivity of ~92%. Ultimately, the improved extraction processes of resveratrol are markedly efficient, selective, rapid and economical.

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1. Introduction

Stilbenes are polyphenols identified in 33 taxonomically unrelated plant families and 72 plant species including grapevine (*Vitis vinifera*), peanut (*Arachis hypogaea*) and Japanese knotweed (*Polygonum cuspidatum*) [1–3]. Resveratrol is the major and most studied stilbene, and presents in a monomeric form as either the *trans*- or *cis*-isomer. *Trans*-resveratrol is the most bioactive form and has many pharmacological properties [4]. Cellular suspensions and hairy roots are plant cell culture systems used as bio-factories to produce bioactive compounds like resveratrol [5,6]. The efficient and economical separation of resveratrol from the culture medium is still a critical challenge. Liquid-liquid extraction (LLE) is a favorable method to recover bioactive compounds from their culture media with preservation of plant cell viability, especially when both production and extraction occur simultaneously [7]. Many researchers have used a continuous extraction process with an organic solvent directly contacting the aqueous phase, in which the bioconversion is carried out by the plant cells [8].

Extraction of resveratrol from plant parts is usually performed using solid-liquid extraction processes with ethanol [9], methanol [10], or ethyl acetate [11] as solvents. LLE is also used to extract resveratrol from different liquid media and has been shown to recover a large fraction of resveratrol using organic solvents, e.g., chloroform [12], methyl tert-butyl ether [3] and ethyl acetate. The latter is the most used solvent for LLE of resveratrol [5,6]. However, the organic solvent/aqueous medium ratio is usually 1:1 (v/v) thus large amounts of solvent are needed [3,5,6,12–16].

To reduce the volume of organic solvents, solvent supercritical fluid extraction and microwave-assisted extraction have been investigated as potential alternatives [17]. However, these need advanced and costly equipment. Furthermore, many different methodologies have been used to optimize extraction processes by maximizing the physical interactions between polyphenols and solvents, e.g., the response surface methodology [18], and

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http://dx.doi.org/10.1016/j.seppur.2016.06.037
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Hansen solubility parameters (HSPs) [19]; however, solvent volumes were not reduced.

Davis [20] and later Srebrenik [21] deduced a correlation between the partition coefficient of a solute between two solvents and the solubility parameters of the solute and the two solvents. Hiraga et al. [22] correlated the partition coefficients of benzene derivative compounds between ionic liquids (ILs) and carbon dioxide with their solubility parameters. Brookes and Livingston [23] found a good correlation between the membrane/aqueous phase partition coefficient and solubility parameters of organic compounds.

This work attempts to use HSPs as a helpful tool to select a solvent that is immiscible with water and to which resveratrol highly partitions from the aqueous medium, and then to determine another solvent that is separable from the first solvent and to which resveratrol highly partitions from the first solvent. Ideally, the chosen solvents should have physicochemical properties that preserve the aeration of the culture medium and enable the different phases to be easily separated. The parameters of the extraction processes will also be studied. Ultimately, the purpose of this work is to minimize the amount of organic solvents used during the extraction of resveratrol from hairy roots culture medium.

2. Materials and methods

2.1. Materials

Trans-resveratrol (99% purity) and the following solvents: benzyl benzoate (98% purity), methanol (99.8% purity), ethanol (100% purity), ether acetate (99.7% purity), and chloroform (99.9% purity) were purchased from VWR (France). Schenk and Hildebrandt (SH), Gamborg's (B5), Murashige and Skoog (MS) basal salts, vitamin mixtures, and sucrose (99.7% purity) were purchased from Duchefa (Haarlem, Netherlands). Water was deionized and double-distilled.

2.2. Calculation of solubility parameters

2.2.1. HSPs calculations

The Hansen solubility parameters, i.e. dispersion (δ_d), polar (δ_p) and hydrogen bonding (δ_h) of 270 solvents were taken from the literature [24] and are tabulated in the supplementary data (Table S1). The HSPs for resveratrol were calculated using the combined group contribution methods of Van Krevelen–Hoftyzer and Fedors [25,26] as follows:

$$\delta_d = \frac{\sum_i F_{d_i}}{\sum_i V_i} \quad (1)$$

$$\delta_p = \frac{\left(\sum_i F_{p_i}^2\right)^{0.5}}{\sum_i V_i} \quad (2)$$

$$\delta_h = \left(\frac{\sum_i E_{h_i}}{\sum_i V_i}\right)^{0.5} \quad (3)$$

where i is the structural group within the molecule, F_{d_i} is the group contribution to the dispersion forces, F_{p_i} is the group contribution to the polar forces, E_{h_i} is the group contribution to the hydrogen-bonding energy, and V_i is the group contribution to the molar volume.

The total solubility parameter (δ_t) [24] is calculated from the partial solubility parameters as follows:

$$\delta_t = \left(\delta_d^2 + \delta_p^2 + \delta_h^2\right)^{0.5} \quad (4)$$

2.2.2. Partition coefficient calculations using solubility parameters

Srebrenik and Cohen [21] used the following equation to predict the partition coefficient of a drug between two solvents ($\ln K_{S2S1}$):

$$\ln K_{S2S1} = \frac{V_m^D}{RT}\left[\left(\delta_t^{S1} - \delta_t^D\right)^2 - \left(\delta_t^{S2} - \delta_t^D\right)^2\right] + \ln\frac{V_m^{S1}}{V_m^{S2}} \quad (5)$$

where V_m is the molar volume, T is the temperature (in Kelvin), R is the gas constant, and superscripts S1, S2, D indicate solvent one, solvent two, and the drug, respectively. Positive values of $\ln K_{S2S1}$ mean that the concentration of the drug in solvent two is higher than in solvent one; the higher the value, the higher the concentration of the drug in solvent two compared to solvent one.

The paucity of data on partial molar volumes is the major restriction to the use of Srebrenik equation (5). An important source is the published results of Hildebrand and coworkers [27]. Among them, Srebrenik and Cohen have studied two systems [21]. These are the solutions of iodine and bromine in CC14 and CS2, for which the experimental partition coefficients have also been given in the 1iterature [28]. Their results confirmed very good agreement between the theoretical and experimental values.

2.3. High-performance liquid chromatography (HPLC)

The chemical stability and content of resveratrol in both aqueous and organic phases were determined by HPLC (a pre-column, a LC20AD pump, a SPDM 10 A diode array detector and a SIL20AC automatic injector, Shimadzu, France). Resveratrol was separated on a C18 Shim-pack column (250 mm × 4.6 mm, 5 μm). The HPLC analysis was conducted at 30 °C, and the temperature was controlled using a CTO20AC system column heater. UV detection at 305 nm was used and the mobile phase was (A) H_2O and (B) CH_3-CN, both with 0.1% formic acid. The flow rate of the mobile phase was 0.4 ml/min using a gradient program of 45 min as follows: initial 0–5 min A:B (95:5); 5–40 min linear change to A:B (50:50); and 40–45 min linear change to A:B (95:5). The injection volume was 5 μL. Under these conditions, the retention time of *trans*-resveratrol was 18 min. The chromatographic peak of resveratrol was confirmed by comparing the retention time with that of the reference compound. The linearity of resveratrol concentration versus the measured integration areas was validated using a blank and ten solutions of pure resveratrol in 100% benzyl benzoate with different concentrations (0.01, 0.0125, 0.02, 0.025, 0.1, 0.125, 0.2, 0.25, 0.4, and 0.5 mg/ml), and using another blank and seven solutions of pure resveratrol in half strength Shenck and Hildebrandt (½ SH) medium culture [29] used as a nutrition medium for grapevine hairy roots with different concentrations (0.001, 0.002, 0.004, 0.01, 0.02, 0.03, 0.04 mg/ml). Each concentration was injected three times. The linearity of the calibration for resveratrol in both benzyl benzoate and ½ SH media was validated.

2.4. Resveratrol-selectivity of benzyl benzoate

Benzyl benzoate was evaluated for the selectivity of resveratrol over other stilbenes assumed to be synthetized by grapevine hairy roots and secreted into the culture medium (Tisserant L.P., personal communication).

2.4.1. Non- resveratrol-accumulating hairy roots

Grapevine hairy roots were recently established in our laboratory. These roots, without stress conditions, produce several stilbenes with traces of resveratrol. The best rooting was recorded on half strength Shenck and Hildebrandt medium (½ SH) [29] with sucrose 2% w/v as carbon source for 21 days. The grown hairy roots were removed and dried in an oven at 40 °C for 24 h. HPLC was used to confirm the traces amount of resveratrol, thus ensuring

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