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

Mohamad Houssam Al balkhi[a,b], Mohammad Amin Mohammad[b,c], Léo-Paul Tisserant[a,d], Michèle Boitel-Conti[a*]

[a] Biologie des Plantes et Innovation EA 3900, UFR des sciences, SFR Condorcet FR CNRS 3417, Université de Picardie Jules Verne, Amiens, France.
[b] Faculty of Pharmacy, University of Damascus, Damascus, Syria.
[c] School of Pharmacy, University of Bradford, Bradford, BD7 1DP, UK.
[d] Unité de Recherche Vignes et Vins de Champagne EA 4707, UFR Sciences Exactes et Naturelles, SFR Condorcet FR CNRS 3417, Université de Reims Champagne-Ardenne, Reims, France.
* Corresponding author

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 ~~optimize~~ 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 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.

Keywords

Benzyl benzoate; Liquid-liquid extraction; Partition coefficient; Plant cell culture; Resveratrol; Solubility parameters; solvent consumption

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 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 in this work. 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 salt and 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:

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

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

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

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:

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

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 (lnK_{S2S1}):

$$lnK_{S2S1} = \frac{V_m^D}{RT}[(\delta_t^{S1} - \delta_t^D)^2 - (\delta_t^{S2} - \delta_t^D)^2] + ln\frac{V_m^{S1}}{V_m^{S2}} \qquad (5)$$

where V_m is the molar volume, T is the temperature (in degrees Kelvin), R is the gas constant, and superscripts S1, S2, D indicate solvent one, solvent two, and the drug, respectively. Positive values of lnK_{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 x 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_3CN, 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 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 hours. HPLC was used to confirm the traces of resveratrol, thus ensuring that the transformed grapevine roots used in this study were non-resveratrol-accumulating hairy roots.

2.4.2. Preparation of cell culture media containing the hairy root extract and predetermined amounts of resveratrol

One gram of finely milled and dried non-resveratrol-containing grapevine hairy roots was extracted by a mixture of methanol/water (80:20, v/v) on a rotary shaker (100 rpm) for 24 h at 25°C in darkness. The extract was then dried using a rotary evaporator (BUCHI Rotavapor R-210/215) at 60°C. The dried residue was re-dissolved in 100 ml ½ SH culture medium [27], filtered (using 0.20 µm filter MILLEX) and then injected into HPLC. It was considered the blank of resveratrol-containing samples. 0.5 mg of resveratrol was spiked into 50 ml of the filtered medium to prepare a standard solution containing the original stilbenes and 1 mg/100 ml of resveratrol. The standard solution was also injected into HPLC. Benzyl benzoate was then used to extract resveratrol from this standard solution in order to evaluate

its selectivity for resveratrol over other compounds. Thus, 0.5 ml of benzyl benzoate solvent was mixed with 50 ml of the standard solution. Extraction was facilitated by centrifugation of the mixture for 20 min at 25°C (at 4900 rpm) using an Eppendorf 5810 R centrifuge. The benzyl benzoate phase was separated by micropipette then diluted 20 times with benzyl benzoate for injection into HPLC. The aqueous phase was filtered and then directly injected for analysis into HPLC.

The resveratrol selectivity coefficient percentage (*S*%) for a single-stage extraction process was considered the extracted amount of resveratrol (K_f) compared to the total amount of resveratrol with other unwanted by-products (K_i):

$$S\% = \frac{K_f}{K_i} \times 100 \qquad (6)$$

2.5. Preparation of cell culture media containing predetermined amounts of resveratrol

100 ml of a new autoclaved ½ SH culture medium containing 2% w/v sucrose as carbon source [29] was placed in a 250-ml Erlenmeyer flask, and then different amounts of resveratrol (0.1; 0.4; 1; 2; and 4 mg) were added and dissolved using a vortex for two minutes. The solubility of the highest amount (4 mg resveratrol in 100 ml of medium) was confirmed by HPLC. The effect of different mineral and vitamin compositions and sucrose concentrations on the recovery of resveratrol was tested, using the same procedure. Thus, 1 mg of resveratrol was dissolved in 100 ml of two other widely used media in plant tissue culture, Gamborg's (B5) [30] and Murashige and Skoog (MS) [31] both containing 3% w/v sucrose. This was to investigate the effect of different growth media on the extraction recovery of resveratrol. The pH of all culture media was adjusted to 5.7- 6, which is the pH of

plant cell growth culture media [29-31], with either dilute hydrochloric acid or sodium hydroxide and monitored with a pH meter (Thermo Scientific).

2.6. Liquid-liquid extraction experiments

2.6.1. Medium-benzyl benzoate extraction

To determine the most suitable volume of benzyl benzoate, needed to extract resveratrol from mixtures of resveratrol-containing culture medium, each mixture was mixed with different amounts of benzyl benzoate to prepare medium-benzyl benzoate immiscible mixtures at different ratios (100:0.1, 100:0.25, 100:0.5, 100:1, 100:2 and 100:4 v/v). These immiscible mixtures were either - shaken, for 24 h, in a 250 ml-Erlenmeyer flask (containing 100 ml of mixture) on a rotary shaker at 100 rpm at 25°C in darkness (shaking method), - vortexed, for one minute, in a 50-ml tubes (containing 50 ml of mixture) at 2500 rpm (IKA MS2 Minishaker) (vortexing method) or – centrifuged, for 20 min, in a 50-ml tubes (containing 50 ml of mixture) using a centrifuge (Eppendorf 5810 at 4900 rpm at 25°C (centrifugation method).

Other experiments were also conducted to determine the best agitation time required to recover resveratrol from culture media, using one of these agitation methods. The three agitation processes were tested for time periods of (3, 7, 10, 14, 24, and 72 h) for the shaking method, and (1, 5, 10, 20, and 30 min) for the other two methods. The resveratrol-culture medium ratio was fixed at (1:100 w/v) with a medium-benzyl benzoate ratio of (100:1 v/v). For each extraction method, 1 ml samples of the aqueous phases were taken at the different mixing times mentioned then filtered (using 0.20 µm filter MILLEX) before analysis by HPLC. The benzyl benzoate always formed one drop within the medium and sank to the

bottom of the containers due to its high density compared to that of water and its complete immiscibility in water [32]. The drop of benzyl benzoate was easily removed using a micropipette (Fig. 1) and its volume was determined to calculate the volume loss (V_{loss}). This benzyl benzoate phase was then filtered and diluted 20 times with pure benzyl benzoate for injection into HPLC.

2.6.2. Benzyl benzoate-solvent extraction

After separating benzyl benzoate from the culture medium, resveratrol was extracted from benzyl benzoate by another liquid-liquid extraction process. Methanol, ethanol, and ethyl acetate, which are miscible with benzyl benzoate [33], were chosen depending on calculations to recover resveratrol from benzyl benzoate. These solvents were mixed separately with benzyl benzoate at a ratio of 1:1 v/v. The miscible mixtures were then agitated by centrifugation (4900 rpm, 10 min at 25ºC) followed by freezing at -20ºC (Proline freezer UFZ170) for one hour. In all these mixtures, the solvents stayed in their liquid state while benzyl benzoate was frozen due to its relatively high freezing point of 21°C [30] and sank to the bottom of the containers (Fig. 2). Then, the upper layer (solvent liquid phase) was rapidly separated from the frozen benzyl benzoate phase using a micropipette, in a cold place (<18°C). After dilution (20 times) with the same pure solvent, the solvent phase was injected for analysis into HPLC. The benzyl benzoate phase was thawed at ambient temperature in darkness, and then diluted 20 times with pure benzyl benzoate solvent for injection into HPLC.

3. Results and discussion

3.1. *Partition coefficient of resveratrol between different solvents:*

The HSPs calculations for resveratrol are given in Table 1. These were used to calculate the partition coefficients of resveratrol from water to 270 other different solvents (lnK_{SW}) and from benzyl benzoate to those same different solvents (lnK_{SB}) (Supplementary information, Table S1) using the same forms of Eq. 5:

$$lnK_{SW} = \frac{V_m^R}{RT}[(\delta_t^W - \delta_t^R)^2 - (\delta_t^S - \delta_t^R)^2] + ln\frac{V_m^W}{V_m^S} \qquad (7)$$

$$lnK_{SB} = \frac{V_m^R}{RT}[(\delta_t^B - \delta_t^R)^2 - (\delta_t^S - \delta_t^R)^2] + ln\frac{V_m^B}{V_m^S} \qquad (8)$$

where superscripts B, R and W indicate benzyl benzoate, resveratrol and water, respectively. The calculations showed that the highest lnK_{SW} is 14.3 with methanol, revealing that methanol has the highest ability to extract resveratrol from water (cell culture medium). Ethanol also has a high ability to extract resveratrol from water with lnK_{SW} equal to 12.7 (Table 2). However, methanol and ethanol are miscible with water and so cannot be used to extract resveratrol directly from its aqueous cultures. Benzyl benzoate is a dense liquid [32], immiscible with water [32], with a relatively high lnK_{SW} of 7.0. Therefore, a small amount of benzyl benzoate can extract a high percentage of resveratrol from cell culture media and separate out at the bottom of the aqueous media making it the most suitable liquid to extract resveratrol from its aqueous cultures. For the recovery of resveratrol from benzyl benzoate in a second-step extraction process, lnK_{SB} values were calculated also for 270 solvents (Table S1), then were employed to select the best solvent for ~~optimize~~ the extraction of resveratrol from benzyl benzoate. They demonstrated that methanol was the best solvent for this extraction because it has the highest calculated lnK_{SB} value of 7.3 (Table 2). To test this hypothesis, resveratrol was extracted from benzyl benzoate by not only methanol but also two

other evaporating solvents, ethanol and ethyl acetate with lnK_{SB} rangeing from -3.6 to 7.3 (Table 2).

3.2. *Choice of solvents*

The choice of potential solvents for this process is a crucial step in performing an effective extraction. It was generally determined following a compromise between technical considerations and physicochemical properties [34, 35]. To achieve the goal of efficient extraction with a minimum volume of solvent, this solvent must satisfy certain requirements, including a great affinity towards the solute. Another critical property is the ease of recovery of the desired solute from the solvent. Thus, a relatively low boiling point, i.e., high vapor pressure point, is desirable to avoid the large energy consumption by solvent volatilization. The miscibility of ethanol and methanol in water makes them impossible for direct liquid-liquid extraction. Water-immiscible solvents that have a lower density than that of water are also not suitable for direct liquid-liquid extraction of resveratrol in hairy root culture conditions. This is because they will float on the surface of the culture medium and thus prevent air exchange [36].

The physicochemical properties of benzyl benzoate are suitable for this extraction. It is immiscible with water and has a higher density (ρ = 1.1 kg dm^{-3}) [30]. Moreover, the octanol/water partition coefficients ($logP_{oct}$) of resveratrol and benzyl benzoate are 3.1 and 3.97, respectively [37, 33], so they have similar hydrophobicity and a high affinity for each other. This corresponds to the high calculated value (7.0) of lnK_{SW} for benzyl benzoate. Therefore, a small volume of benzyl benzoate extracted resveratrol from the aqueous medium and sank to the bottom of the flask (Fig. 1). However, the boiling point of benzyl benzoate is relatively high (324°C) with a very low vapor pressure of 0.000224 mmHg at 25°C [32],

which makes the recovery of resveratrol from benzyl benzoate impossible by solvent evaporation. Thus, a second-step liquid-liquid extraction using an evaporating solvent at lower temperature (< 80 °C) is needed to recover resveratrol from benzyl benzoate. Methanol, ethanol and ethyl acetate are miscible with benzyl benzoate and melt at -97.8, -144, and -83.8°C [38], respectively. However, benzyl benzoate has a relatively high melting point of 21°C [33] compared to those of the other solvents used. This difference was exploited to separate benzyl benzoate from its mixtures by freezing as described above and shown in Fig. 2. Thus, these solvents were used to recover resveratrol from the benzyl benzoate phase in a second-step extraction process. Their ability to extract resveratrol from benzyl benzoate depends on their $\ln K_{SB}$ values, which were calculated and are tabulated in Table 2.

According to the calculations and the physicochemical solvent properties discussed above, benzyl benzoate was selected as the best candidate solvent for the medium-solvent extraction process, and methanol as the best, ethanol as an excellent and ethyl acetate as a poor evaporating solvent to extract resveratrol from benzyl benzoate.

3.3. High-performance liquid chromatography (HPLC)

The linearity of the calibration for resveratrol in both benzyl benzoate and ½ SH medium was acceptable with $R^2 = 0.9983$ and $R^2 = 0.9989$, respectively. The very low concentration of resveratrol of 0.1 mg/100 ml was measurable in the aqueous phase. The highest studied concentrations of 4 mg/100 ml and 4 mg/ml were also within the linearity range in both aqueous and benzyl benzoate phases, respectively. This indicates that these concentrations are still under the saturation point in both phases.

3.4. Extraction study

The study of the liquid-liquid extraction process of resveratrol from the culture medium was conducted in order to determine the best conditions leading to a recovery approaching 100% with high selectivity. Several factors, including extraction time and agitation methods, benzyl benzoate/medium ratio, resveratrol/medium ratio, and the nature of the culture medium, were studied to improve resveratrol extraction.

3.4.1. Extraction time and agitation methods

The effect of extraction time and agitation methods on the recovery yield of resveratrol from ½ SH culture medium was determined at a benzyl benzoate-medium ratio of 1:100 v/v and a resveratrol concentration of 1 mg/100 ml in the medium. The results demonstrated that both parameters influenced the extraction efficiency. For example, 24 h was required to reach the maximum recovery yield (69.7±5%) by the shaking method but an increase in the extraction time to 72 h did not significantly change the recovery yield (t-Test: $P < 0.05$) (Fig. 3). A similar extraction yields of 69±5 and 71.6±6 (t-Test: $P < 0.05$) were obtained by vortexing for one minute and centrifugation for 20 minutes, respectively. No significant increase in the recovery yields was noticed by increasing the agitation time for vortexing or centrifugation. (Fig. 4).

In the tested conditions, vortexing method was more efficient in reducing the extraction time of resveratrol. One minute of vortexing achieved the maximum recovery yield of resveratrol in a single-stage extraction process. This could be due to the different mixing mechanisms and rates applied during the different extraction methods. Shaking uses a slower mixing rate compared to the high speed mixing of a vortexer, while a centrifuge uses a

relatively strong centrifugal force to separate phases [35]. This suggests that intensive mixing is required to increase the mass transfer in the system, and also leads to equilibrium being reached at shorter time scales. The extraction was carried out using the agitation conditions usually applied during the normal culture process of hairy roots, and other stronger agitation methods were tested to explore the possibility of minimizing the extraction time. The shaking method using rotary shakers is usually applied during the growth of hairy root cultures [5, 15], therefore shaking for 24 h using a rotary shaker at a speed of 100 rpm at 25°C in darkness was selected as the extraction method for subsequent studies.

3.4.2. Benzyl benzoate-medium ratio

The benzyl benzoate-medium ratio was changed to evaluate the effect of its volume on the recovery process further. The results showed that the ratio affected the amount of resveratrol extracted from the culture medium (Fig. 5). The recovery ratio of resveratrol increased as the volume of benzyl benzoate increased, reaching the maximum (~ 80%) at a benzyl benzoate-medium ratio of 4:100 v/v. With a benzyl benzoate-medium ratio of 1:100 v/v, the recovery of resveratrol (~ 70%) was similar to that of 2:100 v/v (73±4 %). and significantly (t-Test: $P < 0.05$) higher to that of 0.5:100 v/v (60±4 %). and also comparatively close to that of 4:100 v/v. The higher interfacial area between the two phases when the volume of benzyl benzoate was increased explains the increase in the recovery. Three subsequent extraction processes with the ~~best~~ ratio (1:100 v/v) were enough to recover the total amount of resveratrol (~ 100%). Benzyl benzoate-medium ratio of 4:100 v/v was the most efficient for the extraction of resveratrol. Otherwise, the goal of this work is to reduce markedly the volume of solvent. The ratio of 1:100 v/v is the best, 3 ml are sufficient to recover the total amount of resveratrol from 100 ml of medium culture compared to 4 ml

needed to recover 80 % with ratio of 4:100 v/v. Based on these results, a benzyl benzoate-medium ratio of 1:100 v/v was selected to perform further experiments

The separation of benzyl benzoate from the culture medium was easily performed using a micropipette. However, a slight loss of benzyl benzoate volume (V_{loss}) was noticed, which was relatively independent of the initial volume. This means that, for a solvent volume of 1 ml to 4 ml, the solvent loss was on average about 0.07 ml of the total volume. However, for a solvent volume of less than 1 ml, the loss was significantly lower ($V_{loss=}$ 0.02 ml). The total amounts of resveratrol added to the culture medium were found in the calculations by adding the amount extracted into the organic phase to that remaining in the aqueous phase (Fig. 3S).

3.4.3. *Resveratrol-medium ratio*

The effectiveness of the above parameters of the extraction process was investigated when the amount of resveratrol was changed (0.1; 0.4; 1; 2; and 4 mg) in 100 ml of medium. The resveratrol/medium ratio (mg/ml) had a minor effect on the resveratrol extraction (Fig. 6); although a small significant increase was noticed when the amount of resveratrol was increased from 0.1 to 0.4 mg in 100 ml of medium, no significant change occurred when the amount increased further (t-Test: $P < 0.05$). These results agree with previous reported findings. Wang et al. [3] showed that the extraction recovery of resveratrol from its aqueous medium by ethanol (95%) increased when the ratio of the resveratrol increased to reach the maximum at which no significant change occurred.

3.4.4. *Effect of the nature of the culture medium on resveratrol recovery*

The results showed that changing the culture medium had no significant effect on the recovery of resveratrol, for neither agitation nor centrifugation single-stage extraction methods. The recovery percentages were ~70% irrespective of the medium used (½ SH, MS or B5) using the same previous defined conditions, i.e., a benzyl benzoate-medium ratio of 1:100 v/v and a resveratrol-medium ratio of 1:100 mg/ml (Fig. 7).

3.4.5 *Benzyl benzoate-solvent extraction*

Recovery of resveratrol from benzyl benzoate, by one stage benzyl benzoate/solvent extraction, was 97±3%, 86±4% and 5±2% using methanol, ethanol and ethyl acetate, respectively, i.e., methanol was the best extractor, ethanol was an excellent extractor and ethyl acetate was not effective. Sun et al. [39] studied the solubility of resveratrol in several aliphatic alcohols including methanol and ethanol and found that it decreased as the carbon number of alcohol solvents increased.

These experimental results verified that the theoretical data obtained from calculations of partition coefficients of resveratrol from benzyl benzoate to other different solvents (lnK_{SB}) are an excellent screening tool to find suitable solvents for LLE. Calculated data in Table 2 show that methanol is the best solvent with the highest lnK_{SB} value of 7.3, ethanol is an excellent solvent and ethyl acetate is a poor solvent for extracting resveratrol from benzyl benzoate.

3.5. *Resveratrol selectivity*

In this extraction procedure, the capacity of benzyl benzoate to extract resveratrol efficiently is essential, while the selectivity for resveratrol over structurally similar stilbenes

has the potential to reduce the recovery costs significantly in plant cell culture systems [40]. For this reason, benzyl benzoate was evaluated for the selectivity of resveratrol over other stilbenes assumed to be secreted into the plant cell culture medium. First, the chromatogram from the extract-containing medium spiked with resveratrol was compared with the chromatogram after extraction by benzyl benzoate (Fig. 4S). The results showed that the selectivity coefficient *S*% for resveratrol over the total extracted compounds was 92±3.5%. Therefore, using benzyl benzoate as the organic solvent in an LLE process markedly increases the ratio of resveratrol to other products in the extract stream. One possible explanation for this is the difference in structure between resveratrol and other monomeric stilbenes of *Vitis vinifera*, which are present in glycosylated forms or in oligomeric and polymeric forms [41]. This increases the hydrophilic properties (more soluble in water) of these compounds compared to resveratrol.

4. Conclusion

Calculations based on solubility parameters were used to predict the partitioning of resveratrol between different liquids. The calculations and the physicochemical properties indicated that benzyl benzoate would be the best liquid to extract resveratrol from its culture medium, and methanol or ethanol would be the best liquid to recover the extracted resveratrol from benzyl benzoate. The growth process conditions of hairy roots in their cultures were mimicked, such as the shaking method, pH, temperature and composition. The ratio of the extracting liquids and the amount of spiked resveratrol in the medium were also manipulated. Thus, the best extraction parameters to extract resveratrol (~100%) from its medium were a three-stage extraction process using benzyl benzoate at a benzyl benzoate/medium ratio of 1:100 v/v and an extraction time of 24 h by shaking. The best parameters to recover resveratrol (~97%) from benzyl benzoate were a single-stage extraction process using

methanol at a methanol/ benzyl benzoate ratio of 1:1 v/v and freezing at -20°C to separate the phases. These methods were not only efficient and selective in the recovery of resveratrol but they also reduced organic solvent consumption to the lowest volume ever reported for a conventional liquid–liquid extraction of resveratrol, thus less negative effects resulting usually by the use of high volume of solvents. Cost and time of the extraction are also reduced. Although extraction by vigorous vortexing and centrifugation was more rapid in the tested conditions, extraction by shaking may be used for a direct recovery of resveratrol from the culture medium of hairy roots with preservation of plant cell viability. Further studies are needed on benzyl benzoate solvent biocompatibility and toxicity assays.

Acknowledgements

Mohammad Amin Mohammad gratefully acknowledges CARA (The Council for At-Risk Academics, Stephen Wordsworth and Ryan Mundy) for providing the financial support for an academic fellowship.

References

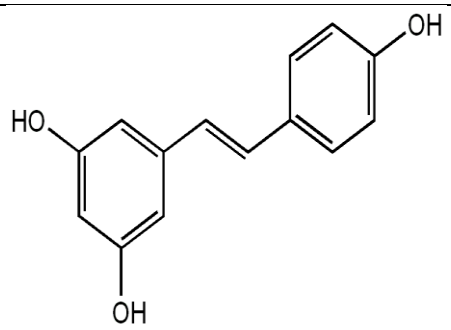
[1] J.A. Baur, D.A. Sinclair, Therapeutic potential of resveratrol: the in vivo evidence, Nat. Rev. Drug Discov. 5 (2006) 493–506.
[2] T. Shen, X.-N. Wang, H.-X. Lou, Natural stilbenes: an overview, Nat. Prod. Rep. 26 (2009) 916–35.
[3] D.-G. Wang, W.-Y. Liu, G.-T. Chen, A simple method for the isolation and purification of resveratrol from *Polygonum cuspidatum*, J. Pharm. Anal. 3 (2013) 241–247.
[4] K.A. Roupe, C.M. Remsberg, J.A. Yáñez, N.M. Davies, Pharmacometrics of stilbenes: seguing towards the clinic, Curr. Clin. Pharmacol. 1 (2006) 81–101.
[5] F. Medina-Bolivar, J. Condori, A.M. Rimando, J. Hubstenberger, K. Shelton, S.F. O'Keefe, et al., Production and secretion of resveratrol in hairy root cultures of peanut, Phytochemistry. 68 (2007) 1992–2003.
[6] D. Donnez, K.-H. Kim, S. Antoine, A. Conreux, V. De Luca, P. Jeandet, et al., Bioproduction of resveratrol and viniferins by an elicited grapevine cell culture in a 2L stirred bioreactor, Process Biochem. 46 (2011) 1056–1062.

[7] M. Mojaat, A. Foucault, J. Pruvost, J. Legrand, Optimal selection of organic solvents for biocompatible extraction of beta-carotene from *Dunaliella salina*, J. Biotechnol. 133 (2008) 433–41.
[8] M.A. Hejazi, C. de Lamarliere, J.M.S. Rocha, M. Vermuë, J. Tramper, R.H. Wijffels, Selective extraction of carotenoids from the microalga *Dunaliella salina* with retention of viability, Biotechnol. Bioeng. 79 (2002) 29–36.
[9] A. Berna, A. Cháfer, J. Montón, High-pressure solubility data of the system resveratrol (3)+ethanol (2)+CO2 (1), J. Supercrit. Fluids. 19 (2001) 133–139.
[10] F. Larronde, J.P. Gaudillère, S. Krisa, A. Decendit, G. Deffieux, J.M. Mérillon, Airborne methyl jasmonate induces stilbene accumulation in leaves and berries of grapevine plants, Am. J. Enol. Vitic. 54 (2014).
[11] N. Landrault, F. Larronde, J.-M. Mérillon, P.-L. Teissedre, Etude de stilbenes-trans (astringine, resvératrol, picéïde) par CLHP-fluorimétrie au cours de la transformation du raisin en vin, Ann. des Falsif. l'expertise Chim. Toxicol. 92 (1999) 443–453.
[12] E. Kübra İnal, S. Öz, A. Atakol, M. Abdülkadir Akay, O. Atakol, Liquid-Liquid Extraction Based Simple *Trans*-Resveratrol Analysis In Wine Samples, Suleyman Demirel University Journal of Science 8 (2013) 114.
[13] V.M. Burin, N.E. Ferreira-Lima, C.P. Panceri, M.T. Bordignon-Luiz, Bioactive compounds and antioxidant activity of *Vitis vinifera* and *Vitis labrusca* grapes: Evaluation of different extraction methods, Microchem. J. 114 (2014) 155–163.
[14] F. Nave, M.J. Cabrita, C.T. da Costa, Use of solid-supported liquid–liquid extraction in the analysis of polyphenols in wine, J. Chromatogr. A. 1169 (2007) 23–30.
[15] J. Condori, G. Sivakumar, J. Hubstenberger, M.C. Dolan, V.S. Sobolev, F. Medina-Bolivar, Induced biosynthesis of resveratrol and the prenylated stilbenoids arachidin-1 and arachidin-3 in hairy root cultures of peanut: Effects of culture medium and growth stage, Plant Physiol. Biochem. 48 (2010) 310–8.
[16] Z. Cai, D. Knorr, I. Smetanska, Enhanced anthocyanins and resveratrol production in *Vitis vinifera* cell suspension culture by indanoyl-isoleucine, N-linolenoyl-l-glutamine and insect saliva, Enzyme Microb. Technol. 50 (2012) 29–34.
[17] L. Wang, C.L. Weller, Recent advances in extraction of nutraceuticals from plants, Trends Food Sci. Technol. 17 (2006) 300–312.
[18] M. Rabesiaka, L. Rakotondramasy-Rabesiaka, I. Mabille, C. Porte, J.-L. Havet, Extraction of *trans*-resveratrol from red wine and optimization by response surface methodology, Sep. Purif. Technol. 81 (2011) 56–61.
[19] M. Savova, T. Kolusheva, A. Stourza, I. Seikova, The use of group contribution method for predicting the solubility of seed polyphenols of *Vitis vinifera* L. within a wide polarity range in solvent mixtures, J. Univ. Chem. Technol. Metallurgy. 42 (2007) 295–300.
[20] S.S. Davis, Molar attraction constants applied to structure activity relationships, Experientia. 26 (1970) 671-672.
[21] S. Srebrenik, S. Cohen, Theoretical derivation of partition coefficient from solubility parameters, J. Phys. Chem. 80 (1976) 996–999.
[22] Y. Hiraga, W. Endo, H. Machida, Y. Sato, T.M. Aida, M. Watanabe, R.L. Smith Jr, Infinite dilution partition coefficients of benzene derivative compounds in supercritical carbon dioxide + ionic liquid systems: 1-butyl-3-methylimidazolium chloride [bmim][Cl], 1-butyl-3-methylimidazolium acetate [bmim][Ac] and 1-butyl-3-methylimidazolium octylsulfate [bmim][OcSO4], J. Supercrit. Fluids. 66 (2012) 49-58.
[23] P.R. Brookes, A.G. Livingston, Aqueous-aqueous extraction of organic pollutants through tubular silicone rubber membranes, J. Membr. Sci. 104 (1995) 119-137.
[24] C. Hansen, Hansen Solubility Parameters: A User's Handbook, CRC Press, Boca Raton, FL, USA. (2007).

[25] D.W. Van Krevelen, P.J. Hoftyzer, Properties of polymers: Their estimation and correlation with chemical structure, Elsevier Scientific Publ., Amsterdam. 2nd ed. (1976).
[26] R. Fedors, A method for estimating both the solubility parameters and molar volumes of liquids, Polym. Eng. Sci. 14 (1974) 147–154.
[27] J. H. Hildebrand, J. M. Prausnitz, and R. L. Scott, Regular and Related Solutions, Van Nostrand-Reinhold, New York. N.Y, (1970).
[28] A. Leo, C. Hansch, D. Elkins, Partition coefficients and their uses, Chem. Rev. 71 (1971).
[29] R.U. Schenk, A.C. Hildebrandt, Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures, Can. J. Bot. 50 (1972) 199–204.
[30] O.L. Gamborg, R.A. Miller, K. Ojima, Nutrient requirements of suspension cultures of soybean root cells, Exp. Cell Res. 50 (1968) 151–158.
[31] T. Murashige, F. Skoog, A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures, Physiol. Plant. 15 (1962) 473–497.
[32] S. Budavari, The Merck Index - Encyclopedia of Chemicals, Drugs and Biologicals. Rahway, NJ: Merck and Co., Inc. (1989) 176.
[33] M.J. O'Neil, The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. Whitehouse Station, NJ: Merck and Co. Inc. (2006) 645.
[34] J.D. Seader, E.J. Henley, Separation process principles. New York: Wiley. (1998).
[35] P.F. Leal, T.S. Almeida, G.H.C. Prado, J.M. Prado, M.A.A. Meireles, Extraction Kinetics and Anethole Content of Fennel (*Foeniculum vulgare*) and Anise Seed (*Pimpinella anisum*) Extracts Obtained by Soxhlet, Ultrasound, Percolation, Centrifugation, and Steam Distillation, Sep. Sci. Technol. 46 (2011) 1848–1856.
[36] K. Kanokwaree, P.M. Doran, The extent to which external oxygen transfer limits growth in shake flask culture of hairy roots, Biotechnol. Bioeng. 55 (1997) 520–6.
[37] M.E. Herbig, D.-H. Evers, Correlation of hydrotropic solubilization by urea with logD of drug molecules and utilization of this effect for topical formulations, Eur. J. Pharm. Biopharm. 85 (2013) 158–160.
[38] W.M. Haynes, (ed.), CRC Handbook of Chemistry and Physics, CRC Press LLC, Boca Raton. FL. 94th ed. (2014) 3-250.
[39] X. Sun, B. Peng, W. Yan, Measurement and correlation of solubility of *trans*-resveratrol in 11 solvents at T=(278.2, 288.2, 298.2, 308.2, and 318.2)K, J. Chem. Thermodyn. 40 (2008) 735–738.
[40] D. Donnez, P. Jeandet, C. Clément, E. Courot, Bioproduction of resveratrol and stilbene derivatives by plant cells and microorganisms, Trends Biotechnol. 27 (2009) 706–13.
[41] P. Jeandet, A.-C. Douillet-Breuil, R. Bessis, S. Debord, M. Sbaghi, M. Adrian, Phytoalexins from the Vitaceae: Biosynthesis, Phytoalexin Gene Expression in Transgenic Plants, Antifungal Activity, and Metabolism, J. Agric. Food Chem. 50 (2002) 2731–2741.

Tables:

Table 1
Calculation of HSPs and molar volume for resveratrol according to the Hoftyzer-Van Krevelen method.



Group	Frequency	F_{d_i} ($J^{1/2}cm^{3/2}mol^{-1}$)	F_{p_i} ($J^{1/2}cm^{3/2}mol^{-1}$)	E_{h_i} (J/mol)	V_m(cm^3/mol)
=CH-	5	1000	0	0	67.5
>C=	3	210	0	0	-16.5
Phenylene (o, m, p)	1	1270	12100	0	52.4
-OH	3	630	750000	60000	30
Ring closure 5 or more atoms	1	190	0	0	16
Conjugation in ring for each double bond	3	0	0	0	-6.6
Σ		3300	762100	60000	142.8

$\delta_d = \frac{\sum_i F_{d_i}}{\sum_i V_i}$	$23.1\, MP_a^{0.5}$
$\delta_p = \frac{\left(\sum_i F_{p_i}^2\right)^{0.5}}{\sum_i V_i}$	$6.1\, MP_a^{0.5}$
$\delta_h = \left(\frac{\sum_i E_{h_i}}{\sum_i V_i}\right)^{0.5}$	$20.5\, MP_a^{0.5}$
$\delta_t = \left(\delta_d^2 + \delta_p^2 + \delta_h^2\right)^{0.5}$	$31.5\, MP_a^{0.5}$

* Molar Volume is calculated according to (Fedors, 1974)

Table 2

The partition coefficients of resveratrol from water to different solvents (lnK_{SW}) and from benzyl benzoate to other different solvents (lnK_{SB}).

	δ_d* ($MP_a^{0.5}$)	δ_p* ($MP_a^{0.5}$)	δ_h* ($MP_a^{0.5}$)	δ_t ($MP_a^{0.5}$)	V_m* (cm^3/mol)	lnK_{SW}	lnK_{SB}
Benzyl benzoate	20.0	5.1	5.2	21.3	191.2	7.0	
Ethanol	15.8	8.8	19.4	26.5	58.6	12.7	5.8
Ethyl acetate	15.8	5.3	7.2	18.2	98.6	3.4	-3.6
Methanol	14.7	12.3	22.3	29.4	40.6	14.3	7.3

* Data are taken from Hansen (2007). Hansen Solubility Parameters: A User's Handbook. CRC Press, Boca Raton, FL, USA.