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Optimisation of extraction of piceatannol from *Rhodomyrtus tomentosa* seeds using response surface methodology

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ABSTRACT

The extraction of piceatannol from the sim fruit (*Rhodomyrtus tomentosa*) was optimised. Firstly, the piceatannol content was determined in the different parts of the fruit (skin, pulp, and seed) and indicated that 94.20% of the piceatannol content was associated to the seeds, which were chosen as starting material to optimise the piceatannol extraction. A second-order polynomial model with three important variables (ethanol concentration, temperature and extraction time) was used. A rotatable central composite design consisting of 21 experimental runs with three replicates at the centre point was applied to describe the experimental data, *i.e.* the sim seed apparent piceatannol content. The experimental results fitted well to the model ($R^2 = 0.9647$). The optimised conditions were 78.8% ethanol, 85.3 °C and an extraction time of 78.8 min. Four extractions were performed in parallel at the optimal conditions to validate the model. The experimental value agreed with the predicted value ($p < 0.05$).

1. Introduction

Sim (*Rhodomyrtus tomentosa*) is a shrub of the Myrtaceae family, originating from South-East Asia. Parts of this plant (leaves, roots, buds and fruits) have been used in traditional Vietnamese, Chinese and Malay medicine for a long time. In particular, the fruits have been used to treat diarrhoea, dysentery, and to boost the immune system [1–3]. Sim has however been recently classified as one of 240 "Neglected and Underutilised Crop Species" of Vietnam, China, Thailand and Cambodia by the scientific project "Agrofolio" (www.Agrofolio.eu).

By means of high performance liquid chromatography coupled with a diode array detector and a high-resolution mass spectrometer (HPLC/DAD/HR-MS), we recently deciphered for the first time the phenolic profile of the sim fruit [4]. Nineteen phenolic compounds were putatively identified and quantified, with piceatannol as major component. Interestingly, the piceatannol content of the sim fruit was 1000–2000 times higher than that of red grapes, a major source of stilbenes in the human diet. As piceatannol has been shown to have potent biological activities, including antioxidant [5], anti-cancer [6], anti-inflammatory [7], and anti-obesity properties [8], sim is worth studying and exploiting as a new source of bioactive compounds.

Studying the distribution of phenolic compounds within the fruits is an important prerequisite for an efficient production of bioactive-rich products [9,10]. Indeed, the phenolic compounds and antioxidant capacity are not evenly distributed in fruits. Guo et al. [9] studied the antioxidant capacity of 28 different fruits and reported that most fruit peels and seeds present higher antioxidant activity than the pulps. More particularly, in some fruits such as red rose grapes and mangoes, the ferric reducing/antioxidant powers (FRAP values) of peel and seed parts were 22–113 times higher than those of pulp parts. In three cultivars of highbush blueberries named Legacy, Brigitta and Bluegold, the phenolic compounds were mostly located in the skins and included anthocyanins (80% of the whole fruit), chlorogenic acid (main phenolic acid) and rutin (major flavonol). In ripe fruits, the antioxidant capacities of skins were thereby around 192 times higher than those of pulps [11]. Dabai (*Canarium odontophyllum* Miq.) peel had the highest phenolic content (68 ± 1.2 mg of gallic acid equivalent (GAE) g^{-1}), while the lowest content was observed in the seed (10 ± 1.5 mg GAE g^{-1}) [10]. In contrast with blueberries and dabai fruits, raspberries and passion fruits had most phenolic

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compounds in the seeds [12,13]. Piceatannol was the major seed phenolic compound of passion fruit but was not detected in the rind or pulp [12]. The antioxidant capacity was far greater in raspberry seeds than in pulp or juice, while the opposite trend was found for total anthocyanin content [13].

Optimising the extraction of phenolic compounds from a plant material using solvents constitutes a further key step for industrial applications [14]. The use of this low-cost technology to obtain molecules to be used as food additives or nutraceutical products can be a reasonable strategy for the exploitation of plant materials in developing countries. Many factors, such as solvent composition, time of extraction, temperature, pH, solid-to-liquid ratio and particle size, may significantly influence the solid–liquid extraction [14–18]. Considering the diversity in composition of the natural sources of phenolic compounds, as well as the structure and physico-chemical properties of these compounds, a universal extraction protocol is not conceivable, and specific processes must be designed and optimised for each natural phenolic source [18].

Extraction studies can be done by using the one-factor-at-a-time approach [17,19] or a response surface methodology (RSM) [14,18,20–22]. In the one-factor-at-a-time approach, only one factor is variable at a time while all others are kept constant. This approach is time-consuming and expensive. In addition, possible interaction effects between variables cannot be evaluated [15,18]. RSM is a statistical method that uses data from appropriate experimental designs to determine and simultaneously solve multivariate equations. These equations can be graphically represented as response surfaces, which can be used in three ways: (i) to describe how individual test variables affect the response, (ii) to determine the relationships between the different test variables, and, (iii) to describe the combined effect of all test variables on the response [23]. This approach can overcome the drawbacks of the one-factor-at-a-time one [15] and has previously been used in the extraction of phenolic compounds from plant sources [18].

The main purpose of this study was to optimise the extraction parameters of piceatannol from *R. tomentosa* fruit. In order to achieve this goal, we first determined and compared the phenolic composition and antioxidant capacity of different parts of the fruit (skin, pulp and seed). The highest piceatannol-containing tissue was then chosen as starting material to optimise the piceatannol extraction process, using RSM.

2. Materials and methods

2.1. Sample collection and preparation

The sim fruits (*R. tomentosa*) were harvested in the mountain of Thai Nguyen province (Vietnam) in August 2012. The mature fruits were hand-picked from five separate lots with about 50 plants per lot. For each lot, approximately 5 kg of fruits were collected. The fruits were placed in a plastic box, kept on ice and transported to the laboratory on the same day. All fruits were first washed by tap water and then rinsed in distilled water for three times. Skins, pulps and seeds were separated by hand from approximately 400 g of fresh fruits from each lot (Fig. 1). Firstly, the skins were separated from the fruits. Then, the pulps and the seeds were separated from each other by using a kitchen pored disc (about 1 mm pore size). The weight and the moisture of the three parts were determined. The different parts were then frozen, freeze-dried, ground and stored at −53 °C under nitrogen until analysis. For the experiment of optimisation of piceatannol extraction from sim seeds, a representative seed sample was prepared by mixing equal quantities of freeze-dried seed powder from each lot, which one being prepared from 200 g of fresh fruits.

2.2. Chemicals and reagents

Gallic acid, piceatannol, and quercetin dihydrate standards, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylicacid (Trolox), as well as the diphenyl-1-picrylhydrazyl (*DPPH*) radical were purchased from Sigma–Aldrich (St. Louis, MO). Resveratrol and cyanidin-3-glucoside were obtained from ExtraSynthese (Genay, France). Acetone and ethanol of analytical grade, acetonitrile and methanol of HPLC grade were obtained from VWR-Prolabo (Briare, France). Formic acid of LC-MS grade was supplied by Biosolve (Valkenswaard, The Netherlands).

2.3. Extraction of phenolic compounds from skin, pulp and seed parts

The extraction procedure was based on the one described in our previous work [4]. Briefly, approximately 0.4 g of powdered freeze-dried sample was mixed with 8 mL of acetone:water:acetic acid (50:49:1; v/v/v) and shaken for one hour at 37 °C. After centrifugation at 3642*g* for 10 min at 4 °C, the supernatant was collected and the residue was extracted two more times with the same quantity of the same solvent. Supernatants from the three extraction steps were combined and evaporated to dryness by rotary evaporating at 40 °C. The residue was then resuspended in 10 mL methanol 70%. The solution was filtered through a 0.22 μm syringe filter (Phenex™-NY, Utrecht, The Netherlands) before analysis by using HPLC-DAD. Each sample was extracted in triplicate.

2.4. Response surface procedure for piceatannol extraction from sim seeds

The RSM used a three-factor and central composite rotatable design (CCRD) consisting of 21 experimental runs with eight factorial points, six axial points (two axial points on the axis of each design variable at a distance of 1.68 from the design centre) and three replicates at the centre point and maximal and minimal factorial points. The CCRD was proceeded to obtain a quadratic model, consisting of factorial trails and axial points to estimate quadratic effects and central points to estimate the pure process variability with the response [20]. Design variables were the ethanol concentration (%v/v; X_1), the temperature (°C; X_2) and the time of contact (min; X_3). The selection of ranges within which each factor varied was based on our preliminary experimentation (data not shown). Each variable was coded at five levels −1.68, −1, 0, 1, and 1.68 (Table 1). The conversion of real values to coded values was as follows: $x_i = (X_i - X_0)/X_i$, where x_i and X_i are the dimensionless and the real values of the independent variable i (i = 1, 2, and 3), X_0 is the real value of the independent variable i at the central point, and ΔX_i is the step change of X_i corresponding to a unit variation of the dimensionless value. The variables liquid-to-solid ratio (20:1, v/m) and particle size (0.12 mm) were kept at constant values. The dependent variable (or response) was the apparent piceatannol content of the sim seeds.

For all runs, extractions were done in glass tubes with Teflon caps and in a shaken water bath (200 rpm). Extraction was terminated by centrifugation at 3642*g* for 10 min at 4 °C. The obtained extract was collected, filtered through a 0.22 μm syringe filter, and analysed by HPLC-DAD. Runs were done independently. Experimental data were fitted to the following second-order polynomial model.

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii}x_i^2 + \sum_{\substack{i=1\\ i<j}}^{2}\sum_{j=2}^{3}\beta_{ij}x_i x_j$$

where Y is the measured response, β_0, β_i, β_{ii}, β_{ij} are regression coefficients for intercept, linear, quadratic and interactions terms,

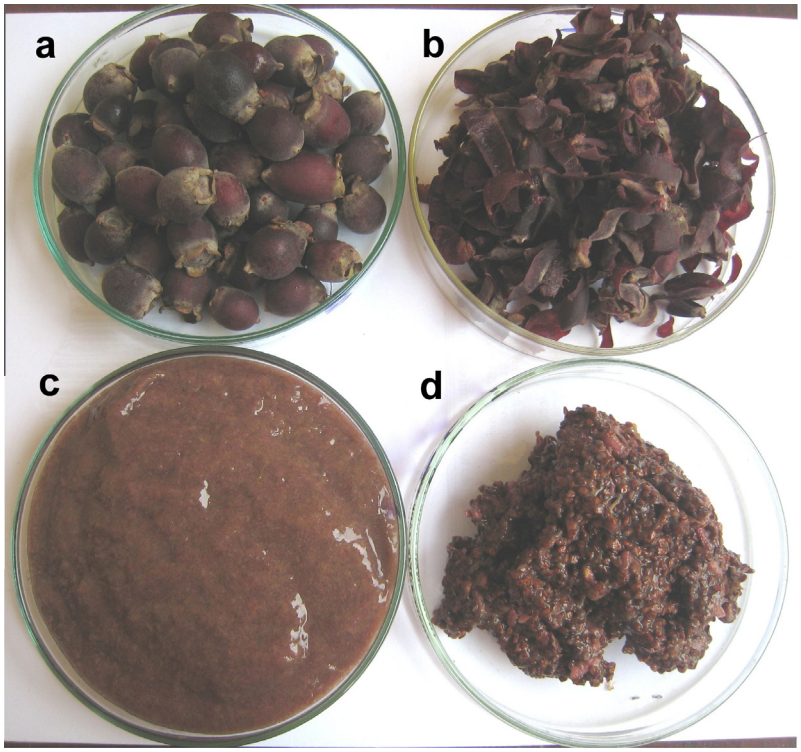

Fig. 1. Whole fruit (a), skin (b), pulp (c) and seed (d) parts of the sim fruit (*Rhodomyrtus tomentosa*).

Table 1
Rotatable central composite design setting in the coded form (x_1, x_2 and x_3) and real values of the independent variables (X_1, X_2 and X_3) and experimental results for the response variable (apparent piceatannol content of sim seeds).

Runs	Coded forms			Real values			Piceatannol (mg g^{-1} DW)
	x_1	x_2	x_3	X_1 EtOH (%)	X_2 T (°C)	X_3 Time (min)	
1	1	1	1	80	85	85	7.30
2	1	1	1	80	85	85	6.92
3	1	1	1	80	85	85	6.96
4	−1	1	1	40	85	85	5.62
5	1	−1	1	80	45	85	5.85
6	−1	−1	1	40	45	85	3.47
7	1	1	−1	80	85	25	6.74
8	−1	1	−1	40	85	25	5.90
9	1	−1	−1	80	45	25	5.37
10	−1	−1	−1	40	45	25	3.99
11	−1	−1	−1	40	45	25	3.91
12	−1	−1	−1	40	45	25	3.91
13	1.68	0	0	93.6	65	55	6.34
14	−1.68	0	0	26.4	65	55	3.33
15	0	1.68	0	60	98.6	55	6.18
16	0	−1.68	0	60	31.4	55	4.33
17	0	0	1.68	60	65	105.4	5.58
18	0	0	−1.68	60	65	4.6	5.82
19	0	0	0	60	65	55	5.82
20	0	0	0	60	65	55	6.42
21	0	0	0	60	65	55	6.56

respectively, and x_i and x_j are the coded values of the independent variables.

The optimum conditions of piceatannol extraction were determined using the JMP 10 software. The software was set to search the optimum desirability of the response variable being maximum apparent piceatannol content of the sim seeds. The verification of the validity and adequacy of the predictive extraction model was realised in these optimum conditions of ethanol concentration, temperature and time of contact. Four experimental replicates were performed at the optimised conditions and the experimental and predicted values were compared.

2.5. HPLC-DAD analysis and quantification of phenolic compounds

Quantification of the phenolic compounds was performed by HPLC-DAD as previously described [4] using a Thermo Finnigan system (San Jose, CA) equipped with a P1000XR pump, an AS3000 autosampler, a SN4000 interface, and a UV6000LP DAD. Simultaneous monitoring was set at 280 nm, 320 nm, and 520 nm for quantification of ellagitannins, stilbenes and flavonols, and anthocyanins, respectively. Phenolic compounds were identified by their retention times and spectral data as compared to authentic standards and were quantified using five-point

calibration curves. Gallic acid, quercetin, piceatannol and resveratrol, and cyanidin-3-O-glucoside were used as standards to quantify ellagitannins, flavonols, stilbenes and anthocyanins, respectively. The phenolic concentrations were first expressed in mg per 100 g DW in order to allow a comparison of concentrations between the three parts of the fruits. The values were then converted to mg per 100 g of fresh fruits in order to express the contribution of each fruit part to the fruit phenolic content. The phenolic content of the whole fruit was calculated as the sum of those of each of the three parts.

2.6. DPPH assay and determination of antioxidant capacity

The antioxidant capacity of the extracts of the different fruit parts was measured by the DPPH radical scavenging test described by Duan et al. [24] with minor modifications. Briefly, 100 μL of extract were mixed with 2900 μL of 0.1 mM DPPH radical in methanol solution. The solution was incubated for 30 min at 25 °C in the dark and the decrease in absorbance at 517 nm was measured. The control contained methanol instead of the antioxidant solution. The inhibition of DPPH radicals by the sample was calculated according to the following equation: DPPH-scavenging activity (%) = 100 ∗ (absorbance of control − absorbance of sample)/absorbance of control. Trolox was used as standard. The antioxidant capacity was calculated by the use of a calibration curve describing the relation between Trolox concentration and DPPH-scavenging activity. The antioxidant capacity was expressed in μmol Trolox equivalent per gram dry weight (μmol TE g^{-1} DW).

2.7. Statistical analysis

The contents of the different sim fruit parts in each phenolic compound under investigation, as well as their antioxidant capacity were analysed by the SAS 9.3 software (SAS Institute, Cary, NC) and expressed as mean ± standard deviation. One way analysis of variance (ANOVA) and Duncan test were used to determine the differences amongst the means. p-values <0.05 were considered to be significantly different. In the RSM experiment, multiple linear regression analysis was performed by the software JMP 10 (SAS Institute, Cary, NC).

3. Results and discussion

3.1. Distribution of the phenolic compounds in the sim fruit

Five samples of *R. tomentosa* fresh fruit were taken and about 400 g of each sample were separated into three parts corresponding to the skin, pulp and seed, as described in Section 2. The pulp part contributed to 47.99 ± 2.75% of the bulk of the fruit weight while the skin and the seed parts constituted 25.78 ± 2.38% and 26.33 ± 0.76%, respectively. The dry matter content of the seed part was the highest (43.70 ± 0.36%) while that of the pulp part was the lowest (18.73 ± 0.36%). The skin fraction had a dry matter content of 28.90 ± 0.23%. The phenolic concentrations of the three fruit parts as well as the contribution of each fruit part to the fruit phenolic content are presented in Table 2.

The phenolic content and antioxidant capacity significantly varied amongst the sim fruit parts ($p < 0.0001$). Amongst the three fractions of sim fruit, the seed part showed the highest total phenolic content, which was 1.75 and 9.72 times higher than that of the skin and pulp parts, respectively (Table 2). Similarly, the antioxidant capacity of the seed fraction was the highest (603.95 ± 18.41 μmol TE g^{-1} DW), while that of the skin fraction was twice lower (265.31 ± 13.15 μmol TE g^{-1} DW) and that of the pulp fraction was the lowest (48.98 ± 6.36 μmol TE g^{-1} DW). With regard to the concentrations of individual phenolic classes (Table 2), the seed part presented the highest contents in ellagitannins, stilbenes and gallic acid, whereas it did not contain any anthocyanins. Anthocyanin concentrations were 12 times higher in the skin than in the pulp. Besides, the skin part was the richest fraction in flavonols and contained 14.00 and 2.38 times more flavonols than the pulp and seed fractions, respectively. Piceatannol has recently been identified by our group as the major phenolic compound in the sim fruit [4]. Interestingly, in the present study, we identified high levels of piceatannol in the seeds. Skin and pulp parts contained 22.85 and 24.08 times less piceatannol than the seed part, respectively (Table 2). In addition, piceatannol contributed to 36.59% of the total phenolic content in the seeds, while only to 2.81% and 14.77% for the skin and pulp fractions, respectively.

The contribution of each fruit part to the total phenolic content of the whole fruit and to its content in each individual phenolic compound is presented in Table 2. The seed fraction was the main contributor to the total phenolic content (68.78%) and antioxidant capacity (74.14%) of the whole fruit, followed by the skin part (25.69% and 21.11%, respectively). Most phenolic compounds of the sim fruit, including total stilbenes (91.17%), piceatannol (94.20%), phenolic acids (59.51%) and ellagitannins (82.28%), concentrated in the seed part, while 90.87% of anthocyanins and 58.13% of flavonols of the whole fruit occurred in the skin fraction. Besides, most individual phenolic compounds within each family, distributed similarly in the sim fruit, except for isorhamnetine/rhamnetine-deoxyhexoside, which predominated in the seed part while two other flavonols concentrated in the skin fraction. As people eat the whole sim fruit, including the seeds and peel, they also ingest the phenolic compounds mainly located in the seeds (*e.g.* piceatannol) and in the skin (*e.g.* anthocyanins and flavonols).

The distribution of the phenolic compounds and antioxidant capacity within the sim fruit appears to be similar to the one described for other fruits. The richest fraction is made of the seeds, and is followed by the skin and the pulp. Indeed, in lychee, pear, and persimmon, antioxidant capacities (FRAP values) of the seed fraction were 2.39–7.74 and 10.57–113.36 times higher than those of the skin and the pulp fractions, respectively [9]. Accordingly, the raspberry seeds had the highest oxygen radical absorbance capacity (ORAC) value (273.27 ± 11.15 μmol TE g^{-1}), followed by the pulp (24.45 ± 0.43 μmol TE g^{-1}) and the juice (18.40 ± 0.39 μmol TE g^{-1}) [13]. In avocado, jackfruit, longan, and tamarind, the seeds showed a much higher antioxidant activity and phenolic content than the pulp. In most of these fruits, the contribution of the fruit seed fraction to the total antioxidant activity and phenolic content was more than 95%, except for the jackfruit for which it was about 70% [25]. Similarly, the average total phenolics were 2178.8, 374.6, and 23.8 mg GAE per 100 g in the seed, skin, and pulp of grapes, respectively, contributing to 87.1%, 11.3%, and 1.6% of the phenolic compounds of the whole fruit [26,27]. The antioxidant capacity was distributed as follows: 93.9% in seeds, 5.6% in skin, and 0.5% in pulp [27]. Besides, the distribution of different phenolic groups including hydrolyzable tannins, stilbenes, anthocyanins, flavonols, and phenolic acids within the sim fruit seems to be the same one as in other fruits. Anthocyanins and flavonols such as myricetin, quercetin, and kaempferol glycosides predominated in the phenolic profile of grape skin while the major phenolic compounds of the seeds were hydrolyzable tannins, flavan-3-ols ((+)-catechin and epicatechin, and condensed tannins), and phenolic acids [26,27]. In the blueberry fruit, the anthocyanins as well as the major flavonol of the fruit, *i.e.* rutin, concentrated in the skin [11].

The distribution of the different phenolic compounds in the sim fruit as well as in other fruits could be explained by their role in the plant, including protection against adverse environmental conditions and reproduction. The plants need birds and other animals

Table 2
Concentration of phenolic compounds in different parts of the sim fruit.

Phenolic compounds	Concentration (mg per 100 g DW)[x]			Contribution to the whole fruit phenolic content (mg per 100 g fresh fruit)[x]		
	Skin	Pulp	Seed	Skin	Pulp	Seed
Ellagitannins (quantified as gallic acid equivalents at 280 nm)						
DiHHDP-galloyl-glucose	nd	6.7 ± 0.8 b	49.8 ± 2.7 a	nd	0.5 ± 0.1	5.1 ± 0.4
HHDP-galloyl-glucose	nd	4.8 ± 0.3 b	57.7 ± 5.2 a	nd	0.4 ± 0.0	5.9 ± 0.7
HHDP-digalloyl-glucose	19.8 ± 3.0 b	9.3 ± 0.7 c	77.6 ± 3.4 a	1.3 ± 0.2	0.8 ± 0.1	7.9 ± 0.5
Furosin	58.8 ± 3.4 b	8.1 ± 0.8 c	81.3 ± 7.9 a	3.8 ± 0.2	0.7 ± 0.0	8.7 ± 0.5
HHDP-trigalloyl-glucose	15.6 ± 6.4 b	8.6 ± 1.0 b	149.9 ± 5.8 a	1.0 ± 0.4	0.7 ± 0.1	15.6 ± 0.6
Total ellagitannins	88.0 ± 7.7 b	37.5 ± 2.4 c	416.3 ± 23.2 a	6.3 ± 0.6	3.0 ± 0.3	43.1 ± 2.3
Stilbenes (quantified at 320 nm)						
Astringin[y]	20.0 ± 0.8 b	7.8 ± 0.3 c	85.1 ± 1.0 a	1.3 ± 0.2	0.6 ± 0.1	8.7 ± 0.2
Piceatannol	21.4 ± 1.5 b	20.3 ± 2.5 b	488.9 ± 12.3 a	1.4 ± 0.2	1.6 ± 0.3	49.8 ± 1.3
Piceatannol-galloyl-hexoside[y]	18.92[z]	7.7 ± 0.4 b	77.6 ± 2.0 a	1.4 [z]	0.6 ± 0.1	7.9 ± 0.3
Resveratrol	15.3 ± 0.9 b	7.1 ± 0.3 c	87.4 ± 0.8 a	1.0 ± 0.1	0.6 ± 0.1	8.9 ± 0.2
Total stilbenes	56.2 ± 15.7 b	42.9 ± 3.3 b	739.2 ± 12.3 a	3.8 ± 1.3	3.4 ± 0.4	74.43 ± 3.0
Flavonols (quantified as quercetin equivalents at 320 nm)						
Myricetine-pentoside	187.2 ± 4.1 a	9.0 ± 0.9 c	68.0 ± 6.3 b	12.4 ± 1.3	0.7 ± 0.1	6.9 ± 0.6
Myricetin-deoxyhexoside (Miricitrin)	109.8 ± 6.2 a	9.1 ± 0.5 c	49.1 ± 20.0 b	7.3 ± 0.7	0.7 ± 0.1	4.5 ± 2.8
Isorhamnetine/rhamnetin-deoxyhexoside	18.1 ± 4.7 b	4.4 ± 0.2 c	35.0 ± 10.4 a	1.2 ± 0.3	0.4 ± 0.0	3.6 ± 1.0
Total flavonols	315.1 ± 12.6 a	22.5 ± 1.0 c	132.5 ± 34.1 b	20.9 ± 2.0	1.8 ± 0.2	13.2 ± 3.5
Phenolic acids (quantified at 280 nm)						
Gallic acid	35.9 ± 5.3 b	12.0 ± 0.7 c	48.4 ± 0.8 a	2.4 ± 0.5	1.0 ± 0.1	4.9 ± 0.2
Anthocyanins (quantified as cyanidin-3-O-glucoside equivalents at 520 nm)						
Delphinidin-3-glucoside	26.4 ± 1.1 a	1.3 ± 0.1 b	nd	1.8 ± 0.2	0.1 ± 0.0	nd
Cyanidin-3-O-glucoside	106.3 ± 2.9 a	9.8 ± 0.3 b	nd	7.1 ± 0.7	0.8 ± 0.1	nd
Pelargonidin-3-hexoside + Petunidin-3-glucoside [t]	27 .7 ± 0.9 a	2.0 ± 0.2 b	nd	1.8 ± 0.2	0.2 ± 0.0	nd
Peonidin-3-glucoside + Malvidin-3-glucoside [t]	106.6 ± 1.1 a	9.2 ± 0.4 b	nd	7.1 ± 0.7	0.7 ± 0.1	nd
Total anthocyanins	267.0 ± 5.0 a	22.2 ± 0.9 b		17.7 ± 1.8	1.8 ± 0.2	
Total phenolics	762.2 ± 23.3 b	137.4 ± 5.6 c	1336.1 ± 53.4 a	51.0 ± 5.9	11.0 ± 1.0	136.6 ± 6.3

nd: Not detected
[x] Mean ± SD (n = 5). Values within a line with different letters (a, b or c) are significantly different ($p < 0.05$).
[y] Quantified as piceatannol equivalents.
[z] Detected in one amongst 5 samples. LOD = 0.012 μg mL^{-1}.
[t] Peaks were co-eluting.

to eat their fruits and then to disperse the seeds that lie within the fruit [28]. A high content in anthocyanin pigments [29] together with flavones and flavonols as co-pigments [30] in the skin contributes to the colour of the fruit, which is important for attracting birds and other animals [31]. By contrast, the seed and the seed coat, usually contain "effective deterrents" such as phenolic acids and tannins, which reduce the use of seeds in insect and other animal feed. This is to ensure that the seed is not consumed along with the fruit and is intact at the end of the digestion [28,31]. In addition, as both UV-B and UV-A lights induce the accumulation of anthocyanins by stimulating the expression of genes encoding enzymes in the anthocyanin biosynthetic pathway [31,32], anthocyanins concentrate in the skin part. Thanks to their antioxidant property [29], they protect, in turn, the internal tissues against DNA-damaging UV lights [31].

Unlike anthocyanins and flavonols, which concentrated in the skin, piceatannol and other stilbenes were predominantly in the seed part. This opposite behaviour may be caused by a competition for substrates between chalcone synthase, the enzyme catalysing the flavonoid synthesis, and stilbene synthase within a certain tissue. Indeed, both enzymes use *p*-coumaroyl-CoA and malonyl-CoA and catalyse the same condensing-type of enzymatic reaction. Nevertheless, they form two different products, directing the flavonoid route on one hand, and stilbene synthesis on the other one. This competition has been well described in grapes by Jeandet et al. [33].

As 94.20% of the sim total piceatannol content occur in the seeds, they were chosen as starting material to optimise the piceatannol extraction process. Preparation of a representative seed sample which was used in the optimisation experiment is described in Section 2.

3.2. Optimisation of the piceatannol extraction process from sim seeds through RSM

3.2.1. Fitting the model

In our study, the dependent variable was apparent piceatannol content of the sim seeds while the three independent variables were ethanol concentration, temperature and time of contact. The selection of ethanol as extraction solvent was justified by the fact that ethanol is a food grade solvent, is less toxic, and is more abundant as compared to acetone, methanol and other organic solvents [15,22]. The use of ethanol at different concentration in water was chosen because binary-solvent systems demonstrated higher yield of phenolic compounds as compared to mono-solvent systems [14,17–19,34]. The experimental design of five-level, three-variable CCRD and the experimental results of extraction are shown in Table 1. By applying multiple a regression analysis, the relation between the tested independent variables and the response was explained in Eq. (1) in which x_i were standardised or coded variables.

$$Y = 6.2466 + 0.8250 \times x_1 + 0.7261 \times x_2 - 0.0175 \times x_3 - 0.4370 \times x_1^2 - 0.2885 \times x_2^2 - 0.1312 \times x_3^2 - 0.1781 \times x_1 \times x_2 + 0.2140 \times x_1 \times x_3 + 0.0240 \times x_2 \times x_3 \quad (1)$$

By converting the coded values into real values, the equation of the extraction process was as follows (Eq. (2)):

Table 3
Analysis of variance for the response surface quadratic model of apparent piceatannol content of sim seeds.

Source of variance	DF[x]	Sum of square	Mean square	F ratio
Model	9	29.03	3.23	33.42
Error	11	1.06	0.10	$p < 0.0001$
Lack of fit	6	0.66	0.13	1.98
Pure error	5	0.40	0.07	$p = 0.2148$
Total	20	30.09		

[x] Degree of freedom.

Table 4
Parameter estimates[x] of the predicted second-order model for the response variable (apparent piceatannol content of sim seeds).

Term	Estimate	Standard error	*t* Ratio	Prob > |*t*|
Intercept	6.2466	0.1790	34.90	<.0001
EtOH	0.8250	0.0772	10.68	<.0001
Temperature	0.7261	0.0772	9.40	<.0001
Time	−0.0175	0.0772	−0.23	0.8253
EtOH * Temperature	−0.1710	0.0995	−1.72	0.1139
EtOH * Time	0.2140	0.0995	2.15	0.0547
Temperature * Time	0.0240	0.0995	0.24	0.8137
EtOH * EtOH	−0.4370	0.0919	−4.75	0.0006
Temperature * Temperature	−0.2885	0.0919	−3.14	0.0094
Time * Time	−0.1312	0.0919	−1.43	0.1814

[x] Parameter estimates are coefficients of the model with coded variables.

$$Y = -6.32 + 181.88 \times 10^{-3} \times X_1 + 156.25 \times 10^{-3} \times X_2 - 7.85 \times 10^{-3} \times X_3 - 1.10 \times 10^{-3} \times X_1^2 - 0.73 \times 10^{-3} \times X_2^2 - 0.14 \times 10^{-3} \times X_3^2 - 0.43 \times 10^{-3} \times X_1 \times X_2 + 0.35 \times 10^{-3} \times X_1 \times X_3 + 0.03 \times 10^{-3} \times X_2 \times X_3 \quad (2)$$

To fit the response function and experimental data, the linear and quadratic effects of the independent variables, as well as their interactions on the response variable were evaluated by analysis of variance (ANOVA) and regression coefficients were determined (Tables 3 and 4). The ANOVA of the regression model showed that the model was highly significant or useful due to a very low probability value ($p < 0.0001$) (Table 3). The fitness and adequacy of the model was judged by the coefficient of determination (R^2) and the significance of lack-of-fit, respectively [21]. R^2, which was defined as the ratio of the variation explained by the model (Estimate Sum of Square – ESS) to the total variation (Total Sum of Square – TSS), was used as a measure of the degree of fit. In this study, R^2 value for the regression model of piceatannol content of sim seeds was 0.9647, which was close to 1 suggesting that the predicted second order polynomial model defined well the piceatannol extraction process from sim seeds and that 96.47% of variation for the apparent piceatannol content was attributed to the three studied factors [20]. Besides, the lack of fit test is used to verify the adequacy of the model. In this test, the null hypothesis is that the model for $E(Y)$ is correct at locations of X with replicated observations. The sum of squared errors (SSE) is split into two components called pure error (variation between observed and average values at X) and lack of fit (variation between average and predicted values at X). In our study, the absence of lack of fit ($p = 0.2148$) meant that the total error of the model was due to the pure error. This strengthened the reliability of the model (Table 3).

The effects of ethanol concentration, temperature and time of extraction on piceatannol content are presented in Table 4 and Fig. 2. Based on Eq. (2), the ethanol concentration appears to be the most affecting factor of the piceatannol extraction process from the sim seeds since its coefficient has the highest value. The ethanol concentration showed significant linear ($p < 0.0001$) and quadratic ($p = 0.0006$) effects for apparent piceatannol content. The negative quadratic effect of x_1 indicated that there was a maximum apparent piceatannol content at a certain ethanol concentration. Indeed, the apparent piceatannol content mounted up with an increase in ethanol concentration, reached its highest value at about 80% ethanol and then began to decrease (Fig. 2). This result is in accordance with the one of Matsui et al. [35], who reported that the extraction with 80% aqueous ethanol provided the highest efficiency for piceatannol extraction from the passion fruit seeds.

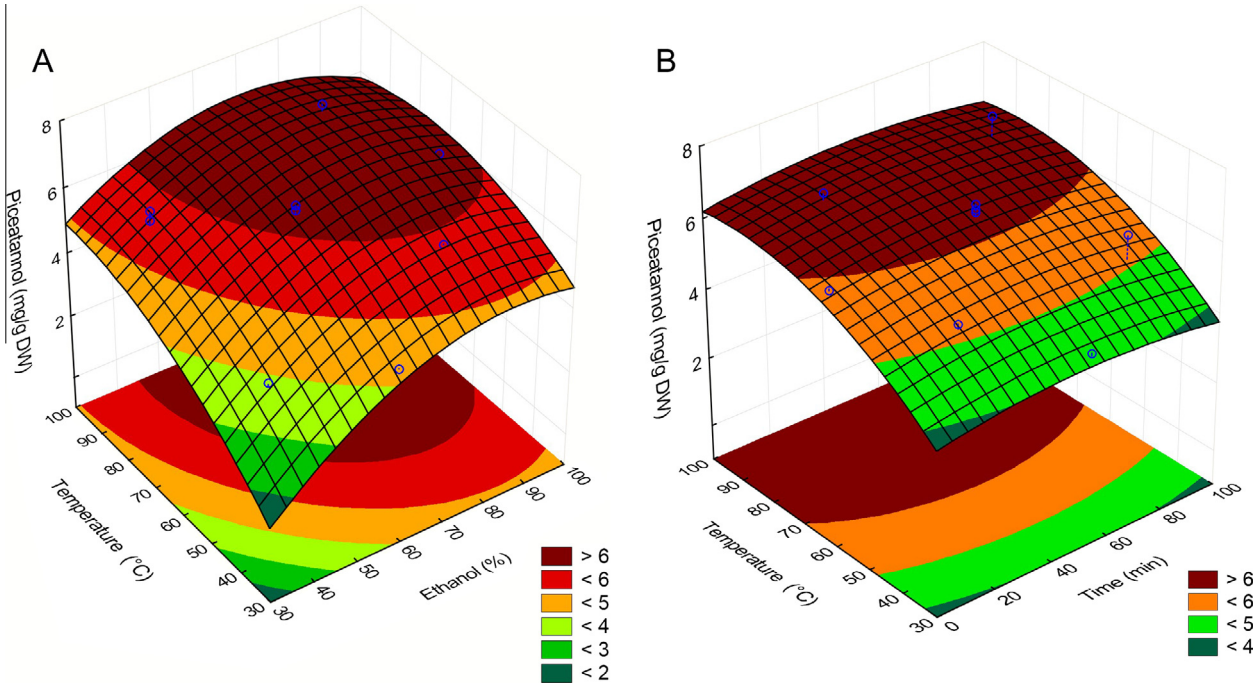

Fig. 2. Response surface for apparent piceatannol content in function of ethanol concentration, temperature and time of extraction.

An effect of the ethanol concentration in the extraction medium on the phenolic compound yield has been observed in various studies. Chan et al. [34] found that the ethanol concentration had the most critical role in the extraction of phenolic compounds from limau purut peel with 52.9% as optimal value. Chew et al. [15] reported that the highest apparent total phenolic content of centella was achieved at 60% ethanol concentration. The optimised ethanol concentrations for the extraction of phenolic compounds from ice-cream-bean (*Inga edulis*) leaves and from açai fruits were 86.8% and 70–80%, respectively [14,18]. The impact of the ethanol concentration is due to its effect on the polarity of the extraction solvent and the resulting solubility of the phenolic compounds. The general principle is "like dissolve like", which means that solvents only extract those phytochemicals, which have a similar polarity to that of the solvent [15,17].

The temperature had significant linear ($p < 0.0001$) and quadratic ($p = 0.0094$) effects on the piceatannol extraction (Table 4). The negative quadratic coefficient of temperature evidences the existence of an optimal temperature leading to a maximal piceatannol extraction. Fig. 2 shows that the apparent piceatannol content increased when the temperature increased, and reached a maximum value at about 80–90 °C and then decreased. This effect of temperature is in accordance with studies on phenolic extraction from limau purut peel [34], from mangrove leaves [20], and from neem leaves [21]. An increase in the extraction temperature may increase the diffusion coefficient and hence improve the rate of diffusion of phenolic compounds [14,21]. However, increasing the temperature beyond certain values may promote a possible concurrent decomposition of phenolic compounds, which were already mobilised at a lower temperature or even the break down of phenolics that still remain in the plant matrix [34]. Our study shows that the apparent piceatannol content decreased only when the temperature was above 85 °C (Fig. 2) suggesting that piceatannol is a quite thermo-resistant compound that could resist to various food production processes.

According to Table 4, the time of contact had neither a linear nor a quadratic effect on the apparent piceatannol content ($p = 0.8253$ and 0.1814, respectively). In run 18 (Table 1), a high concentration of piceatannol (5.82 mg g^{-1} DW) was observed when the time of extraction was only 4.6 min. When the extraction time increased from 4.6 to 55 min, the apparent piceatannol content increased very slightly (from 5.82 mg g^{-1} DW for run 18 to 6.27 mg g^{-1} DW, as average value for runs 19, 20, and 21). This would mean that an important quantity of piceatannol is extracted during the first minutes of extraction. Accordingly, the maximal rates of extraction of phenolic compounds from agrimony, sage, and savoury leaves were found to take place during the first minutes [16]. In our study, long times of extraction (for example 105.4 min for run 17) gave smaller apparent piceatannol contents (5.58 mg g^{-1} DW for run 17). Silva et al. [18] have also shown that longer extraction times do not necessarily lead to a more thorough extraction of phenolic compounds. Moreover, prolonged extraction processes might lead to phenolic oxidation or degradation due to light, oxygen or high temperature exposure [21,34].

3.2.2. Determination of the optimal conditions and validation of the model

The optimum conditions of piceatannol extraction from sim seeds acquired by using JMP 10 were as follows: ethanol concentration, 78.8%, temperature, 85.3 °C, and time of contact, 78.8 min. In order to examine the validity of the model, an extraction was done with four replicates under these conditions. The measured values (7.18 ± 0.11 mg g^{-1} DW) lay within a 95% mean confidence interval of the predicted value (6.67–7.32 mg g^{-1} DW). These results confirmed the predictability of the model. The second-order polynomial model can thus be effectively applied to predict piceatannol content of the extract from sim seeds.

4. Conclusion

We reported for the first time on the phenolic distribution between the different parts of the sim fruit. High ellagitannin and stilbene contents were found in the seed part whereas anthocyanins and flavonols concentrated in the skin. Interestingly, nearly 95% of the fruit total piceatannol content was localised in the seeds. The RSM was successfully employed to describe and to optimise the piceatannol extraction process from sim seeds. Overall, this study should be considered as a first step for the production of piceatannol-rich products to be used as nutraceuticals from this under-utilised plant species from South-East Asia.

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