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Novel methods to extract flavanones and xanthones from the root bark of *Maclura pomifera*

D. Martins Teixeira[a,b], C. Teixeira da Costa[a,c,*]

[a] *Departamento de Química da Universidade de Évora, CLAV Rua Romão Ramalho n° 59, 7000-617 Évora, Portugal*
[b] *Instituto de Ciências Agrárias e Mediterrânicas (ICAM), 7000-617 Évora, Portugal*
[c] *Centro de Química de Évora (CQE), 7000-617 Évora, Portugal*

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Abstract

A comparison between the extraction yields of xanthones and flavanones from the root bark of the *Maclura pomifera* by solid-liquid extraction (SLE), matrix solid phase dispersion (MSPD), and an alternative method using sea sand as a sample disruptor, is presented here. Two extraction solvents were used for all extraction techniques, dichloromethane and methanol:water, (9:1, v/v). The extraction procedures were reproducible as the R.S.D. values were less than 5% for almost all compounds. A recovery above 80% was obtained for macluraxanthone using the sea sand extraction procedure. Statistical treatment, ANOVA-single factor, was used to evaluate the different extraction procedures, and homogenization of plant material with sand followed by elution with dichloromethane provided the most efficient and rapid extraction method.
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Keywords: Sea sand; Matrix solid-phase dispersion; Flavanones; Xanthones; Phenolic compounds; *Maclura pomifera*

1. Introduction

The osange orange tree (*Maclura pomifera* Raf., Moraceae) is a very common tree in the Midwestern and Southwestern regions of the United States. Several compounds have been isolated and identified in various parts of this tree [1–5]. For example, in the root bark several phenolic compounds have been identified, namely four xanthones (osanjaxanthone, alvaxanthone, macluraxanthone, and 8-prenyltoxyloxanthone) and two flavanones (euchrestaflavanone B and euchrestaflavanone C) (see Fig. 1) [6–11].

Xanthones are a very interesting family of compounds not only because they occur in a few families of plants [12], but more important, because of their pharmacological properties. There are several reports of antimicrobial, antinflamatory, antitumoral and antidepressive properties of these compounds [13].

The extraction of phenolic compounds from plants has been traditionally performed using solvent extraction or steam distillation techniques. Traditional methods of extraction are labour-intensive, time consuming and require large volumes of solvents. In the last years, several new methods of extraction such as supercritical fluid extraction (SFE), pressurized fluid extraction (PFE) and matrix solid-phase dispersion (MSPD) have been developed. In 1999, C. da Costa et al. [14] compared SFE and PFE with conventional solid-liquid extraction (SLE) for their efficiency in extracting flavanones and xanthones from the root bark of the osange orange tree. They concluded that SFE and PFE remove those compounds from plant material, at similar or slightly higher yields than obtained with SLE, in a much shorter period of time and with small amount of solvent.

MSPD is a patented process [15] that permits simultaneous disruption and extraction of semi-solid and solid samples. This technique is based on the blending of a viscous, solid or semi-solid sample with an abrasive solid support material.

* Corresponding author.
E-mail address: cmtc@uevora.pt (C.T. da Costa).

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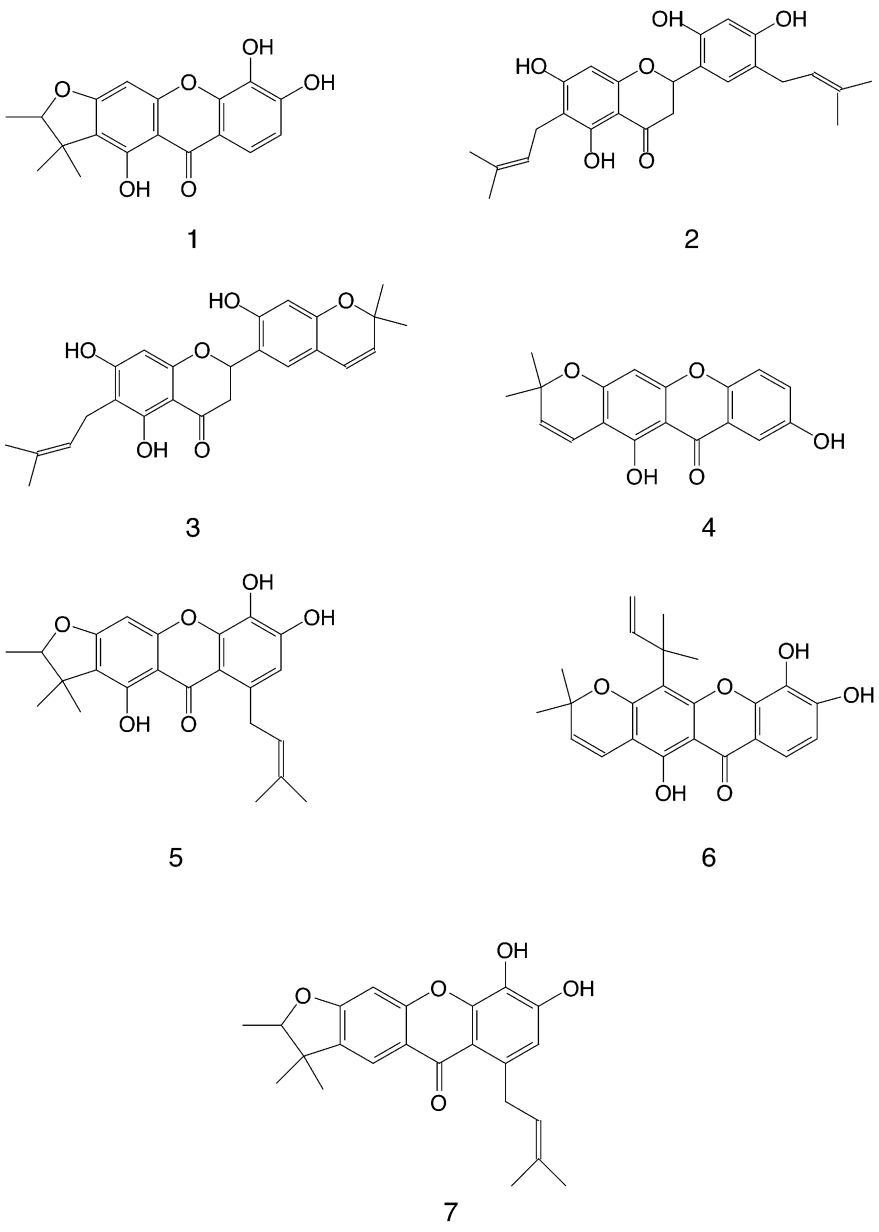

Fig. 1. Structure of flavanones and xanthones identified in the root bark of osange orange tree, *M. pomifera* [6–11]. Toxyloxanthone C (1); euchrestaflavanone B (2); euchrestaflavanone C (3); osajaxanthone (4); alvaxanthone (5); macluraxanthone (6); 8-prenyltoxyloxanthone (7).

This method has been applied mainly to the analysis of herbicides, pesticides and pollutants from animal tissues, fruits, vegetables and also from other matrices [16–21].

Only a few reports have been published using MSPD technique for the extraction of phenolic compounds from plant material. In 2003, A. Ziakova et al. [22] used MSPD for the extraction of phenolic acids in *Melissa officinalis*, and in 2004, H.B. Xiao et al. [23] removed isoflavonoids from *Radix astragali* using a MSPD procedure. The most common solid support material used in MSPD is modified silica, usually C_{18}, and Ziakova et al. [22] compared different C_{18} bulk materials but found no significant differences in phenolic acid yields with the different sorbents.

The aim of this work is to compare SLE, a MSPD procedure with a C_{18} silica bonded phase, and a new extraction method using sea sand for the extraction of flavanones and xanthones from the root bark of osange orange tree (*M. pomifera*). Sand has been used as a sample-disrupting medium for decades, especially in the isolation of drugs and natural products from tissues and semi-solid samples [16]. Sand was used here to extract the plant phenolics with a procedure similar to the one applied for MSPD but, instead of a bonded phase, the sample was mixed with sea sand. Two different elution media were tested for all extraction procedures, dichloromethane and a commonly used solvent for total plant phenolic extraction, methanol:water (9:1, v/v) [24]. The

extracts obtained were compared by LC analysis using diode array detection.

2. Experimental

2.1. Materials and reagents

Acetonitrile (HPLC gradient grade) and methanol (analytical reagent) were purchased from SDS (Peypin, France). Dichloromethane and *n*-hexane (analytical reagents) were obtained from Labscan (Dublin, Ireland). Formic acid (HPLC gradient grade) was obtained from Merck (Darmstadt, Germany). Water from an EASY pure® (BARNSTEAD Thermolyne Corporation, USA) system was used for sample preparation and LC analysis.

The solid support material used for MSPD was Polygoprep C_{18}, 40 μm, non-end-capped 14% C, (Macherey-Nagel, Germany). Sea sand was collected in Faro Beach, Portugal.

The roots of the *M. pomifera* tree were collected on the grounds of the National Arboretum in Washington, DC. The bright orange, paper-thin root bark was peeled off, air-dried for 3 days and stored at 4 °C. A food processor was used to grind the bark into fine particles. The same batch of ground bark was used with the different extraction techniques.

Standards of the xanthones (osajaxanthone, alvaxanthone, macluraxanthone, 8-prenyltoxyloxanthone) and flavanones (euchrestaflavanone B and C) were kindly provided by Dr. Derek Horton (American University, Washington, DC).

2.2. Preparation of standards

A 1.70 mg amount of macluraxanthone was weighed, dissolved and transferred to a 5 mL volumetric flask with a mixture of methanol:acetonitrile (4:1, v/v) to yield a stock solution (340 μg/mL). By serial dilution of this solution with acetonitrile, calibration standards at levels of 10.20, 8.16, 6.12, 4.08 and 2.04 μg/mL of macluraxanthone were obtained. All the stock solutions and working solutions were stored at 4 °C, and brought to room temperature before use.

A small amount of all other available standards were dissolved in 5 mL methanol:acetonitrile (4:1, v/v).

2.3. Extraction procedures

2.3.1. Solid-liquid extraction (SLE)

One hundred fifty milligrams of samples of dry root bark were soaked in 20 mL of dichloromethane or methanol:water (9:1, v/v) for 24 h. All extracts were dried under vacuum, redissolved in 5 mL of a mixture acetonitrile:water (4:1, v/v), and filtered through a 0.45 μm PTFE filter (Macherey-Nagel, Germany).

2.3.2. Matrix solid phase dispersion (MSPD) and sea sand extraction method

Both C_{18} solid support material and sand were cleaned before use: C_{18} was washed three times with methanol and the sea sand was washed several times with deionised water, and three times with methanol. Both materials were air dried before use.

Aliquots of 150 mg of dried plant were placed in a glass mortar with 600 mg of the previously cleaned C_{18} or sea sand, and 2 mL of *n*-hexane. The materials were mixed in the glass mortar using a glass pestle to obtain a homogenous material suitable for column packing. The blend was then quantitatively transferred into a 5 mL syringe with three circles of filter paper on the bottom. The packing material was covered with another circle of filter paper and compressed using the syringe plunger. The filled syringe was then dried under vacuum. The flavanones and xanthones were eluted with two different elution media: 20 mL of dichloromethane or methanol:water (9:1, v/v). All extracts were dried under vacuum, redissolved in 5 mL of a mixture acetonitrile:water (4:1, v/v), and filtered through a 0.45 μm PTFE filter (Macherey-Nagel, Germany).

2.3.2.1. Optimal elution volume determination. The determination of optimal elution volume was done using sea sand as solid support and dichloromethane as elution media. Five different elution volumes of dichloromethane were tested: 1.5, 2.5, 5.0, 10.0 and 20.0 mL. All extracts were dried under vacuum, redissolved in 5 mL of a mixture acetonitrile:water (4:1, v/v), and filtered through a 0.45 μm PTFE filter (Macherey-Nagel, Germany).

2.4. Reproducibility and recovery

The reproducibility of the analytical methods and the repeatability of the extraction procedures were assessed by evaluating the peak area variation of the seven major compounds present in the extracts. Five replicates were performed for each extraction assay and three replicate LC–DAD analyses were performed on each filtrate.

The recovery of the sea sand extraction method was assessed by measuring the recovery of 60 μL of the macluraxanthone stock solution (equivalent to 20.4 μg of macluraxanthone) after it was added to the mortar with 150 mg of plant and 600 mg of sand. The extraction was performed with 20 mL dichloromethane. The extract was dried, recovered in 5 mL of a mixture of acetonitrile:water (4:1, v/v), and filtered through a 0.45 μm PTFE filter (Macherey-Nagel, Germany). This assay was repeated five times and three replica analyses were performed on each extract.

2.5. Liquid chromatography

An Agillent 1100 system (Agilent Technologies, Germany) with a diode-array detector (DAD) and an HP ChemStation (Agilent Technologies, Germany) was used for LC analyses. The analytical column was a reversed-phase Zorbax Eclipse XDB-C_{18}, 250 mm × 4.6 mm (length × i.d.) and 5 μm particle size (Agilent Technologies, Germany). The analytical guard column was a Zorbax Eclipse XDB-C_{18},

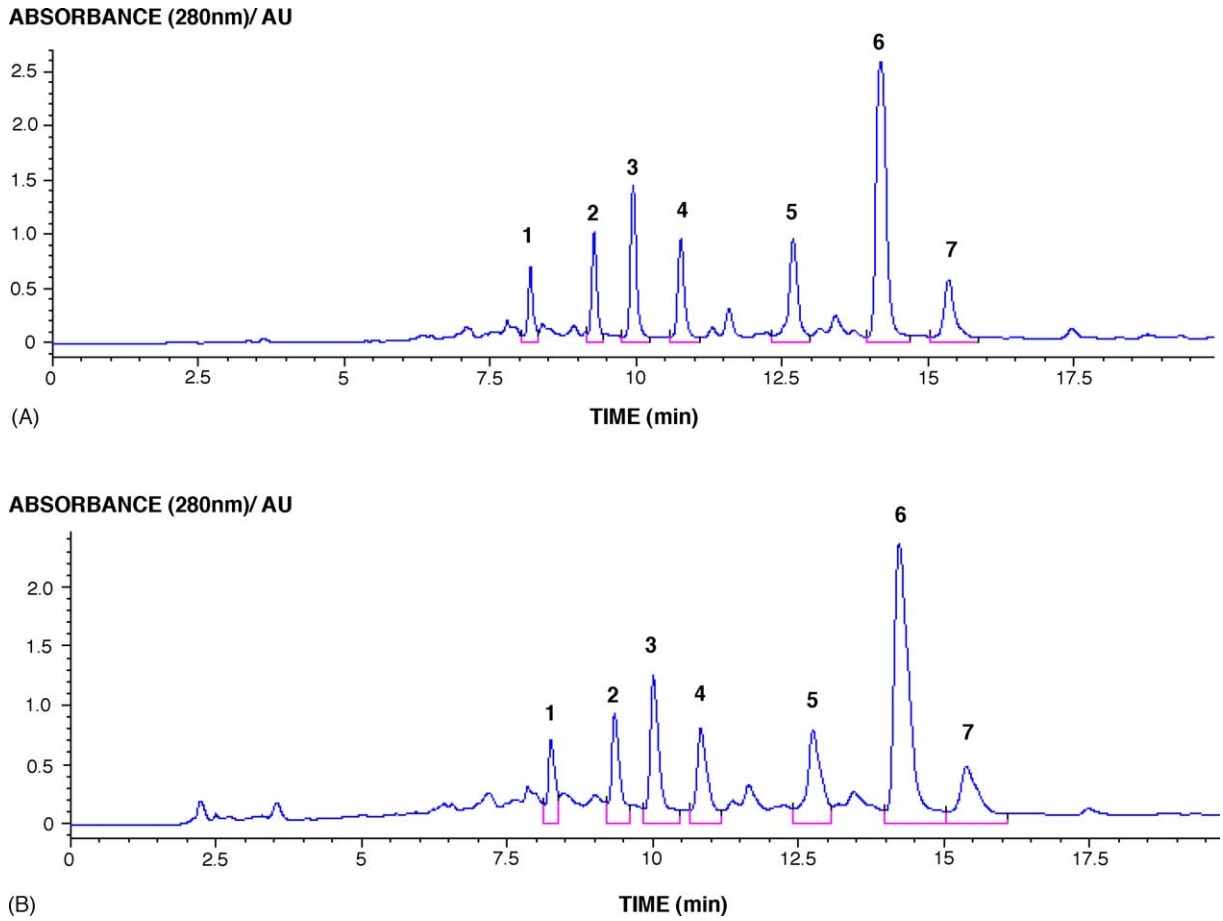

Fig. 2. LC–DAD chromatogram of dichloromethane (A) and methanol:water (9:1, v/v) (B) extracts of *Maclura pomifera* root bark samples, using sea sand extraction method. Column: Zorbax eclipse XBD-C_{18}. Elution conditions: solvent A, acetonitrile; solvent B water with acetonitrile (2.5%) and formic acid (0.5%). Gradient program: linear from 40 to 80% of solvent A (0–5 min) and from 80 to 92% of solvent A (5–20 min). Peak identification: toxyloxanthone C (1); euchrestaflavanone B (2); euchrestaflavanone C (3); osajaxanthone (4); alvaxanthone (5); macluraxanthone (6); 8-prenyltoxyloxanthone (7).

12.5 × 4.6 mm (length × i.d.) and 5 μm particle size (Agilent Technologies, Germany). The mobile phase was: solvent A: acetonitrile; solvent B: water with acetonitrile (2.5%) and formic acid (0.5%). Gradient program was adopted as follows: linear from 40 to 80% of solvent A (0–5 min) and from 80 to 92% of solvent A (5–20 min). LC analyses were performed at room temperature; the injection volume was 20 μL and the flow-rate was 1.0 mL/min; the DAD detector was scanned from 200 to 500 nm and the chromatographic profile was recorded at 280 nm (Fig. 2).

3. Results and discussion

3.1. Comparison of extraction procedures

3.1.1. Solid-liquid extraction

Solid-liquid extraction (SLE) is the most commonly used method for extraction of phenolic compounds from plant material. The xanthones and flavanones present in the root bark of the *M. pomifera* have been extracted by SLE using dichloromethane and diethyl ether [14], and the authors concluded that dichloromethane was the most effective extraction solvent. Different solvents can be used depending on the compounds to be extracted., but the most widely used solvent for extracting phenolic substances is methanol and methanol/water mixtures [24]. Here we compare the extraction yields obtained by SLE extraction of the root bark with dichloromethane and methanol:water (9:1, v/v). Slightly higher yields seamed to have been obtained with the methanol: water solvent (see Fig. 3 and Table 1). However, careful examination of the chromatograms (data not shown) reveals that the dichloromethane extracts present a much better chromatographic separation. This is likely due to the fact that the methanol:water solvent is a much stronger solvent and likely extracts unwanted matrix compounds. The difference in the peak areas shown in Table 1 are probably not due to a poorer extraction of the xanthones and flavanones by the dichloromethane, but to the co-elution in the LC system used of unwanted compounds extracted with the methanol:water solvent. A similar effect was observed with the two other extraction procedures used.

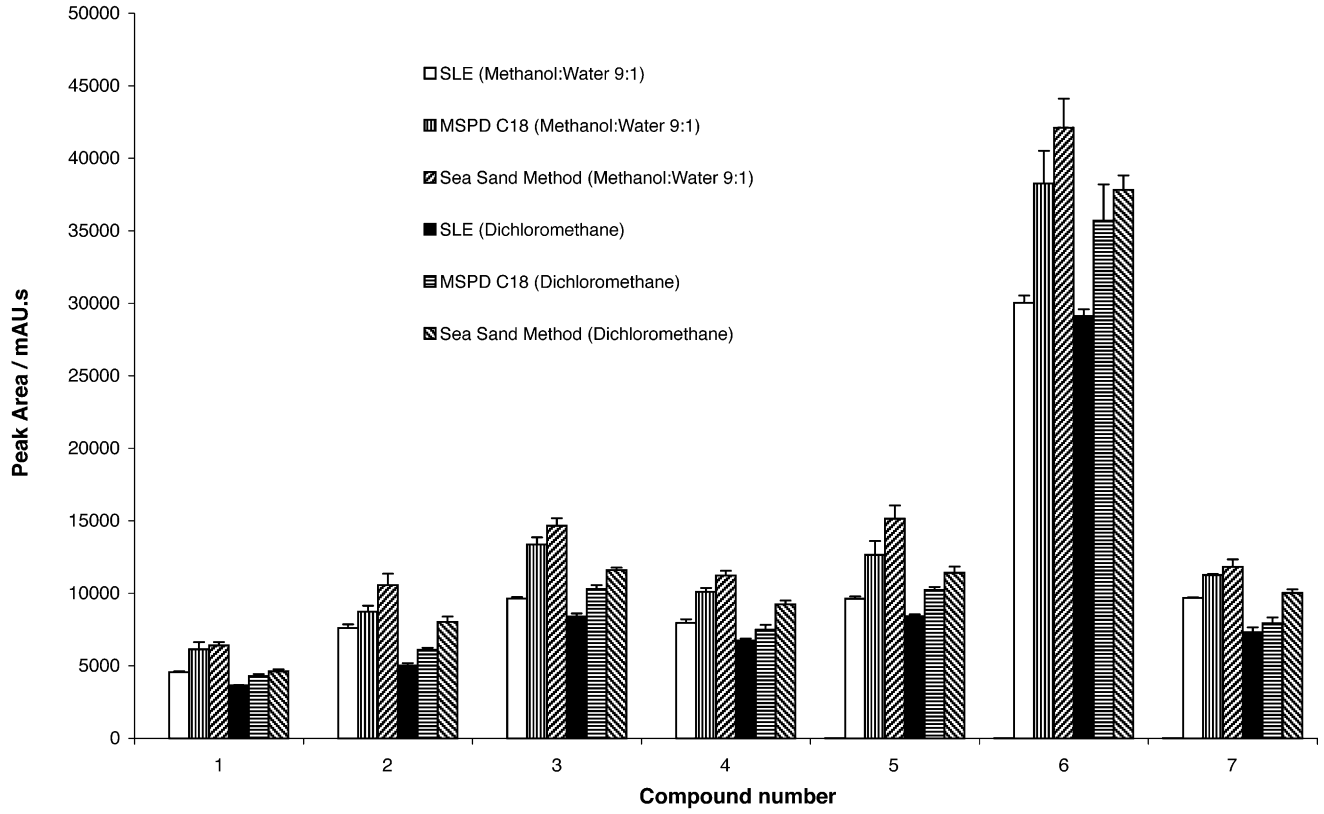

Fig. 3. Comparison between SLE, MSPD (C18) and sea sand extraction of *M. pomifera* root bark samples with dichloromethane and methanol:water (9:1, v/v). Conditions: 150 mg of plant; agitation at room temperature with 20 mL of solvent for 24 h in SLE; 600 mg of C_{18} or sea sand eluted with 20 mL of solvent in MSPD and sea sand extraction method; all extracts dried, redissolved in 5 mL of acetonitrile:water (4:1, v/v) and analyzed by LC–DAD. Compound identification: see Fig. 1.

Table 1
Evaluation of the precision on the SLE, MSPD and sea sand method extraction and LC analysis of plant phenolic compounds from the root bark of *M. pomifera*

Compound number	SLE: peak area[a] (mAUs)		MSPD C_{18}: peak area[a] (mAUs)		Sea sand extraction method: peak area[a] (mAUs)			
	Mean[b] (S.D.)[c]	%R.S.D.[d]	Mean[b] (S.D.)[c]	%R.S.D.[d]	Mean[b] (S.D.)[c]	%R.S.D.[d]	Recovery (%)	LOD[e] (μg/g)
Elution with dichlorometane								
1	3648.96[1] (20.47)	0.56	4284.44[2] (147.35)	3.44	4612.41[3] (145.33)	3.15		
2	5032.23[1] (156.74)	3.11	6115.98[2] (131.41)	2.15	8021.39[3] (368.73)	4.60		
3	8413.69[1] (200.36)	2.38	10285.23[2] (278.09)	2.70	11596.11[3] (174.29)	1.50		
4	6750.89[1] (127.77)	1.89	7482.81[2] (348.50)	4.66	9239.62[3] (261.47)	2.83		
5	8415.90[1] (133.83)	1.59	10235.29[2] (205.58)	2.01	11411.11[3] (423.44)	3.71		
6	29120.00[1] (466.05)	1.60	35711.11[2] (2493.62)	6.98	37820.00[3] (1004.79)	2.66	83.33	0.29
7	7312.69[1] (344.16)	4.71	7926.32[2] (411.70)	5.19	10022.86[3] (254.56)	2.54		
Elution with methanol:water (9:1, v/v)								
1	4568.16[4] (56.54)	1.24	6133.28[5] (502.76)	8.20	6408.06[6] (230.83)	3.60		
2	7615.98[4] (249.53)	3.28	8730.88[5] (414.76)	4.75	10567.61[6] (780.52)	7.39		
3	9638.06[4] (101.59)	1.05	13366.67[5] (491.03)	3.67	14655.56[6] (516.76)	3.55		
4	7961.24[4] (252.28)	3.17	10094.92[5] (272.94)	2.70	11229.10[6] (322.03)	2.87		
5	9622.33[4] (164.16)	1.71	12655.56[5] (945.36)	7.47	15143.16[6] (918.69)	6.06		
6	30040.00[1] (492.74)	1.64	38266.67[4] (2251.91)	5.88	42100.00[5] (2011.91)	4.77		
7	9676.72[4] (39.52)	0.41	11266.67[5] (66.67)	0.59	11822.22[6] (510.27)	4.32		

[a] Normalized to 150 mg of root bark extracted, sample dried and redissolved in 5 mL of acetonitrile:water (4:1, v/v); 20 μL injection.
[b] The values represent the mean of three replicate measurements on the five different extracts. For each compound means with different index numbers are significantly different (ANOVA: single factor Microsoft® Excel 2000, $P < 0.001$).
[c] Standard deviation of a single measurement.
[d] Relative standard deviation.
[e] Limit of detection.

3.1.2. Matrix solid phase dispersion (MSPD) and sea sand extraction method

Some families of phenolic compounds have been extracted from plant sources by MSPD [22,23] but, as far as we know, this technique has never been used to extract flavanones and xanthones. As mentioned before, the more commonly used solid support for MSPD is C_{18} derivatized silica which not only serves as a disruptor of the sample architecture, but also can act as a solvent dispersing the sample components [28]. The resulting mixture presents a greatly enhanced surface area, enabling higher extraction yields [28]. A major drawback of these substituted silica materials is that, they are very expensive and suitable alternatives are needed. Sea sand, although lacking the solvent effect of the silica bound phase, has the potential to be a very effective sample architecture disruptor, likely resulting in good extraction yields.

Both C_{18} (a MSPD procedure) and sand (an alternative extraction method) were used here to extract the root bark of the *M. pomifera* and the data is presented in Table 1 and Fig. 3. In order to compare SLE with these other two extraction methods, both dichloromethane and methanol: water (9:1, v/v) were used as eluting solvents. Independently of the solvent used, higher yields are obtained with the sea sand extraction procedure. The increase in extraction yields is also accompanied with a significant decrease in sample preparation time: MSPD and sea sand extraction procedures take about 1 h, while SLE extraction takes 24 h.

The higher yields achieved with the sand are likely due to a more effective disruption of the plant cells when this material is used. The sand sharp edges and rough surface serve to provide shearing during mechanical blending of a sample, exposing the cell components to solvent extraction. However, confirmation of sample disruption enhancement can only be done by scanning-electron microscopy (SEM) [16].

As observed in the SLE method, the methanol: water solvent seems to be the more effective extraction solvent. However, examination of the chromatograms (see Fig. 2) revealed, as it had already been observed for the SLE extracts (data not shown), that the dichloromethane extracts are cleaner. As stated before, this is likely due to the fact that the stronger methanol based solvent co-extracts unwanted matrix components.

3.2. Determination of optimum elution volume for MSPD extraction

The assays performed for the determination of the optimum elution volume were done using the optimized conditions for sea sand extraction using dichloromethane as elution solvent.

The peak areas of all analyzed compounds were evaluated for various volumes of elution solvent. The data presented in Table 2 show that, for compounds number 1–6, the amounts extracted with 5 mL of solvent are not significantly different from those extracted with larger volumes. Only for compound number 7 is the peak area maximized when 10 mL of solvent

Table 2
Evaluation of the precision on the optimal volume determination in the sea sand extraction with dichloromethane, and LC analysis of plant phenolics from the root bark of *M. pomifera*

Compound number	Volume of elution media (mL): peak area[a] (mAUs)									
	1.5		2.5		5.0		10.0		20.0	
	Mean[b] (S.D.)[c]	%R.S.D.[d]	Mean[b] (S.D.)[c]	%R.S.D.[d]	Mean[b] (S.D.)[c]	%R.S.D.[d]	Mean[b] (S.D.)[c]	%R.S.D.[d]	Mean[b] (S.D.)[c]	%R.S.D.[d]
1	2464.30^{1} (60.31)	2.45	3716.62^{2} (156.49)	4.21	4651.74^{3} (173.80)	3.74	4770.11^{3} (100.32)	2.10	4612.41^{3} (145.34)	3.15
2	4157.53^{1} (225.97)	5.44	6204.34^{2} (56.12)	0.90	7339.19^{3} (375.43)	5.12	7700.38^{3} (207.65)	2.70	8021.39^{3} (368.73	4.60
3	7753.02^{1} (980.12)	12.64	10020.60^{2} (408.11)	4.07	11104.24^{3} (468.72)	4.22	11777.78^{3} (390.63)	3.32	11417.78^{3} (370.25)	3.24
4	5020.32^{1} (663.52)	13.22	7631.81^{2} (266.25)	3.49	8521.86^{3} (257.26)	3.02	8581.26^{3} (191.21)	2.23	8964.59^{3} (218.03)	2.43
5	6571.98^{1} (952.38)	14.49	10545.26^{2} (103.03)	0.98	11149.68^{3} (411.01)	3.69	10908.37^{3} (62.96)	0.58	11013.33^{3} (477.21)	4.33
6	32533.33^{1} (360.56)	1.11	35322.22^{2} (1368.83)	3.88	37922.22^{3} (1500.49)	3.96	39610.12^{3} (2268.03)	5.94	37820.00^{3} (1004.79)	2.66
7	6430.14^{1} (461.07)	7.17	8165.54^{2} (609.20)	7.46	8388.61^{3} (359.98)	4.29	9105.14^{4} (202.53)	2.22	9632.67^{4} (471.83)	4.90

[a] Normalized to 150 mg of root bark extracted, sample dried and redissolved in 5 mL of acetonitrile:water (4:1, v/v); 20 μL injection.
[b] The values represent the mean of three replicate measurements on the five different extracts. For each compound means with different index numbers are significantly different (ANOVA: single factor Microsoft® Excel 2000, $P < 0.001$).
[c] Standard deviation of a single measurement.
[d] Relative standard deviation.

is used. However the difference in peak areas for compound 7 is very small, and could only be recognized when the data were statistically analyzed.

Sea sand and MSPD extraction require an elution volume smaller than that required for SLE extraction of the same amount of plant material. This is a very important issue and these procedures, when compared to the traditionally extraction methods, are much more environmental friendly techniques.

3.3. Validation: reproducibility and recovery

The reproducibility of the analytical methods and the repeatability of the extraction procedures were assessed by evaluating the peak area variation of the seven major compounds. Five replicates were performed for each extraction assay and three replicate LC–DAD analyses were performed on each filtrate. The data presented in Table 1 demonstrate that the extraction procedures are reproducible as R.S.D. values were less than 5% for almost all compounds, for all the elution media and extraction procedures used. Additionally, statistical treatment (ANOVA-single factor, Microsoft Excel® 2000) was performed to the data to determine significant differences when they occurred.

To evaluate the recovery, spiking experiments were done with macluraxanthone (compound number 6) the major compound in the extracts. The mean peak area of the spiked macluraxanthone was calculated by subtracting the total peak area of macluraxanthone after spiking from the mean peak area in the extract of the plant before spiking. Calibration curve for this compound was constructed using the standard solutions prepared, $y = 112.54x + 21.99$, $r = 0.9999$ $(n = 5)$, and the recovery of macluraxanthone was calculated as 83.3% as shown in Table 1. The limit of detection (LOD) for macluraxanthone was estimated as 2.88 μg/g of plant, corresponding to the analyte concentration giving a signal equal to the blank signal plus three standard deviations of the blank [29].

4. Conclusions

MSPD using C_{18}, and a similar extraction procedure using sand were compared with SLE for extraction of flavanones and xanthones from plant material. The results presented here prove that extraction using sea sand as sample disrupting media and dichloromethane as eluent is the optimum extraction procedure to obtain flavanones and xanthones from the *M. pomifera* root bark. Higher yields were obtained using smaller amounts of solvents and less sample preparation time when compared with SLE. When compared with the MSPD method, the use of sea sand avoids the cost of the expensive C_{18} solid support materials.

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