Diastereoseparation of C-7 Pentacyclic Oxindole Alkaloids and Their Vibrational Circular Dichroism Characteristics

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Column chromatography of methanolic leaf extracts of *Uncaria longiflora* var. *pteropoda* has eluted fractions containing a mixture of diastereomeric C-7 pentacyclic oxindole alkaloids (POAs) known as the isopteropodine (**1**) and pteropodine (**2**). Previously, multiple developments of a preparative thin layer chromatography (PTLC) technique managed to separate the two diastereomers from the stem extract of the same plant. However, due to their similar retention times, the separation process was tedious and the purity of these compounds was unsatisfactory. The present work has successfully improved these unfavourable separation conditions within two complete cycles of a 74 minute recycling-High Performance Liquid Chromatography (r-HPLC) run. In addition, their respective C-7*S* and C-7*R* diastereomerism were unambiguously distinguished by their opposite vibrational circular dichroism (VCD) signals appearing at the regions *ca.* 1700 and 1100 cm^{-1}. The present work describes for the first time the r-HPLC separation technique and VCD spectral characteristics of these molecules. The findings revealed that the combination of r-HPLC and VCD are able to separate, characterize and distinguish the diastereomers.

Key words: *Uncaria longiflora*; diastereomeric alkaloids; pteropodine; isopteropodine; recycling HPLC; VCD characteristics

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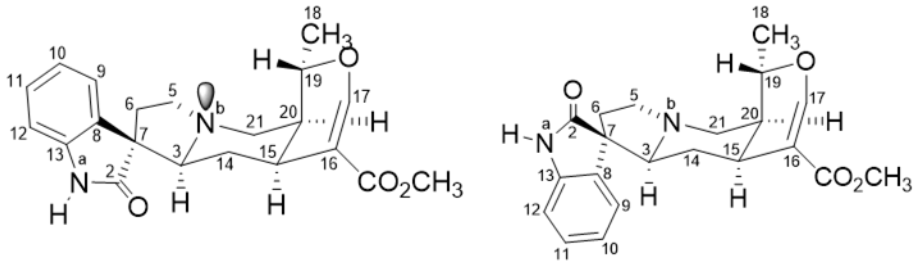
Isopteropodine (**1**) and pteropodine (**2**) are diastereomers at the *spiro* C-7 position in their molecular structures, whereby the former exhibits *S* while the latter possesses *R* configurations (Figure 1) [1, 2]. They belong to a group of pentacyclic oxindole alkaloids (POAs), which is the most prominent type of alkaloid that has been isolated from the genus *Uncaria* and some *Mitragyna* species (Rubiaceae) [3]. POAs **1** and **2** have been reported to possess several pharmacological properties including cytotoxic, muscarinic and neuroprotective activity, and are receptor modulators of glutamate and serotonin [3]. These findings warrant further exploration that may lead to new pharmacological outcomes. Our previous phytochemical work on the stem extract of *U. longiflora* var. *pteropoda* resulted in the successful purification of two alkaloids through repetitive preparative thin layer chromatography in a multiple developmental technique [1]. Due to their similar retention times, this purification process was tedious and time consuming, giving a low sample recovery and a product of unsatisfactory purity.



1-(3*S*, 7*S*, 15*S*, 19*S*, 20*S*) **2**-(3*S*, 7*R*, 15*S*, 19*S*, 20*S*)

Figure 1. Molecular structure of isopteropodine (**1**) and pteropodine (**2**)

Note. Reprinted from "Oxindole alkaloids of *Uncaria* (Rubiaceae, subfamily Cinchonoideae): A review on its structure, properties, and bioactivities," by Ahmad, R. and Salim, F, 2015, *Studies in Natural Products Chemistry*, 45. Copyright 2015 by Elsevier Inc.

For the past decade, the isolation of isomeric compounds has been carried out using chiral columns mainly on a High Performance Liquid Chromatography (HPLC) system [4]. This technique is acceptable and gives good separation most of the time. However, optimization of the method is rather expensive and requires a high volume of organic solvents particularly for compounds that have similar retention times. These limitations were improvised in a number of ways including recycling the sample back into the column, now known as recycling HPLC (r-HPLC) [5, 6]. Separation efficiency is achieved by incorporating a recycle valve in the HPLC (preparative or semipreparative scale) system to recirculate the unresolved peaks into the column, hence no fresh solvent is required. Significant studies have proven that r-HPLC has successfully separated isomeric or structurally-related compounds having similar retention characteristics compared to conventional techniques [7-9].

Molecular chirality can be elucidated using an advanced yet widely utilised technique known as vibrational circular dichroism (VCD) [10]. VCD measures the differential in absorbance of left and right circularly polarized light of a molecular vibrational transition in the mid-IR spectral fingerprint region [11]. VCD is inferior to FTIR as it only analyses vibrational transitions in a narrow range that contains several signature bands of chiral compounds [11]. VCD is a highly sensitive technique in investigating conformation, configuration, solvation, and aggregation of a chiral molecule, particularly useful for molecules lacking chromophores but having a strong functional group [12]. Since there are several functional groups in the molecular structure of **1** and **2**, VCD would allow a clear interpretation of their diastereomeric chiroptical characteristics.

The present work describes for the first time the r-HPLC separation technique of diastereomers **1** and **2**. Their molecular structures were identified based on their NMR data and comparison with the literature values. For the first time too, this paper reports on the VCD spectral characteristics of **1** and **2** in distinguishing their C-7 antipodal structural configurations.

MATERIALS AND METHODS

1. Chemicals and Plant Materials

All the chemicals used in this work were analytical grade and the solvents were HPLC grade. A fraction containing a diastereomeric mixture of **1** and **2** (73 mg) was obtained from a column-chromatographed (Merck 7734 silica gel 60, 70-230 mesh ASTM) leaf extract of *U. longiflora* var. *pteropoda*. The mixture was eluted with dichloromethane (DCM) and methanol (MeOH) with an increasing solvent polarity. The leaves of *U. longiflora* var. *pteropoda* (voucher specimen HTBP 1336) were collected from Hutan Simpan Bangi, Selangor, Malaysia, and were identified by En. Ahmad Zainudin (botanist, Taman Botani Putrajaya, Malaysia).

2. Recycling-High Performance Liquid Chromatography (r-HPLC)

An optimised solvent system consisting of acetonitrile (MeCN) and ultra-pure water (UPW) in an 8:2 ratio was used on reversed-phase thin layer chromatography (RP-TLC) precoated with aluminum-backed supported silica gel 60 RP-18 F_{254} (0.2 mm thickness).

A mass of 70 mg diastereomeric mixture was dissolved in a 10 ml MeCN:UPW (8:2), then injected into an r-HPLC (JAI, model LC-9110 II NEXT) equipped with a reciprocating double plunger pump type P-9140B and a UV detector with four channel variable wavelengths. The separation was performed on a JAIGEL-ODS-C18 column, with an isocratic elution MeCN:UPW (8:2). The flow rate of the system was set at 10 ml/min and the absorbance was set to 210, 254, 270, and 360 nm. After two complete cycles (74 minutes), **1** (47 mg) and **2** (20 mg) were eluted at the 66^{th} and 50^{th} minutes, respectively. Prior to the analysis, the system was purged with MeCN to prevent the presence of air bubbles inside the tube, to condition the column and to monitor the baseline.

3. Ultrahigh-Performance Liquid Chromatography (HPLC)

The purity check on isolated POAs **1** and **2** followed the normalization procedure whereby the percentage of their peak areas in the chromatogram was calculated by assuming the extinction coefficient at the given wavelength is the same for peaks and the detector signal is linear. The sample was dissolved in MeCN to the concentration of 1000 ppm by weight and filtered through a 0.45 µm membrane filter into an HPLC vial. The UHPLC analysis was performed on a DIONEX Ultimate 3000 HPLC system (ThermoFisher, USA), equipped with a vacuum degasser, a quaternary pump, an auto-sampler injector, and a photodiode array detector (PDA). The separation was achieved with a Hypersil GOLD C18 column (Thermo Scientific, Malaysia) with a pore size of 175 A, and dimensions 250 mm x 4.6 mm, i.d. 5 µm. The chromatographic conditions were a gradient of MeCN:UPW (5:95 to 95:5 for 10 minutes) with a flow rate of 1 mL/min, and peaks were detected at 210, 254, 270 and 360 nm. The control of the instrument and the data analyses were conducted using Chromeleon software version 7.2 provided by the supplier.

4. Physical and Spectroscopic Characterisation

Melting points of **1** and **2** were determined using a X-4 melting-point apparatus equipped with a microscope JM628 digital thermometer. Their ^{1}H- and ^{13}C-NMR were analysed in chloroform-*D* on a Bruker 500 Ultrashield NMR spectrometer measured at 500 and 125 MHz, respectively. Their mass spectra were recorded on an Agilent Technologies 6890N GC equipped with an Agilent Technologies 5973 inert mass selective detector. Their optical rotations were measured on a JASCO P1020 digital polarimeter at 589 nm. Their UV and IR spectra were obtained using a JASCO UV/Vis Spectrophotometer V-730 and a Bruker FT-IR Spectrometer TENSOR II model, respectively. The physical and spectroscopic data for **1** and **2** matched with those previously reported [13].

4.1. Isopteropodine (1)

Colourless needles, 47 mg, mp 216 – 220 ºC (dec.). $[\alpha]_D^{20}$ –126.01 (MeOH, *c* 0.03); MS *m/z* = 368 $[M]^+$, $C_{21}H_{24}N_2O_4$; UV (MeCN) λ_{max} nm: 207, 248, 286; IR (KBr) υ_{max} cm^{-1}: 3202 (NH), 2946 (C-H aromatic), 1714 (C=O amide), 1631 (C=O conjugated ester), 1469 (C=C olefinic), 1229 (C=C aromatic), 1079 (C-O cyclic ether); ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) spectroscopic data: refer **Table 1**; VCD ($CDCl_3$) υ_{max} cm^{-1}: refer **Table 2**.

4.2. Pteropodine (2)

Whitish amorphous solid, 20 mg, mp 206 – 210 ºC (dec.). $[\alpha]_D^{20}$ –151.99 (MeOH, *c* 0.03); MS *m/z* = 368 $[M]^+$,$C_{21}H_{24}N_2O_4$; UV (MeCN) λ_{max} nm: 207, 248, 286; IR (KBr) υ_{max} cm^{-1}: 3206 (NH), 3151 (C-H aromatic), 1688 (C=O amide), 1622 (C=O conjugated ester), 1629 (C=C aromatic), 1471 (C=C olefinic), 1083 (C-O cyclic ether); ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) spectroscopic data: refer **Table 1**; VCD ($CDCl_3$) υ_{max} cm^{-1}: refer **Table 3**.

5. Vibrational Circular Dichroism Analysis

The vibrational absorbance (VA) and vibrational circular dichroism (VCD) spectra of **1** and **2** were obtained in chloroform-*D* with a concentration of 0.98 M on a JASCO FVS-6000 spectrometer using a 50 μm quartz cell. The analysis was acquired in the wavelength reciprocal (1/λ) range of 900 – 1800 cm^{-1} with a band resolution set to 4.0 cm^{-1} and the temperature controlled at 25 ºC. The VCD spectra were obtained over 48000 scans numbers which is equivalent to eight hours. The spectra were then solvent-subtracted and smoothed using JASCO Spectra Analysis software.

RESULTS AND DISCUSSION

1. Recycling-HPLC Separation and Structural Identification

Prior to r-HPLC analysis, solvent system optimisation was carried out to secure a retention factor (R_f) value close to 0.5 for the spot. This practice is important to ensure that the sample contains only the compounds of interest, and that the interactions of the compounds with the stationary and mobile phases are balanced, besides minimising the chromatographic cycles in r-HPLC analysis. For the diastereomer mixture, a solvent system of MeCN and UPW in an 8:2 ratio was sufficient to achieve the abovementioned R_f value.

Figure 2 shows the chromatographic cycles in the separation process of the diastereomers detected through four UV wavelengths of 210, 254, 270, and 360 nm. Of these wavelengths, 210 nm showed strong π-π* and n-π* electronic transition absorptions for POAs compounds [14]. Upon sample injection, the eluent was drained before the 15th minute to remove impurities. When the desired peaks appeared at the 23th minute, the valve was turned to channel the eluent into the column. After the desired peaks were completely eluted, the eluent was drained again to remove the remaining impurities. This practice ensures that the second cycle only contains the compounds of interest. As shown in the figure, two cycles were adequate to achieve a baseline resolution between the desired peaks, where each cycle took 37 minutes to complete. There was no further intention to recycle the POAs as there may be a possibility for compounds to be mixed up after further recycling processes. This is possible due to the tailing part of the first peak being mixed up with the leading part of the later peak and thus can result in a decrease in peak resolution [15].

Consequently, the POAs were collected manually at the 50th and 66th minutes and labelled as **Ca** and **Cb**, respectively (Figure 2). The peak-shaving technique was applied in this process to ensure the maximum purity of the compounds obtained [16]. The purities of the **Ca** and **Cb** were 97.52% and 96.44%, respectively, as determined through ultrahigh-performance liquid chromatography. The solvent was evaporated using a vacuum evaporator and the residue was dissolved in chloroform and left to dry, yielding 20 mg of a whitish amorphous solid (**Ca**) and 47 mg of a colourless crystalline solid of (**Cb**). The structures of the two compounds were identified by comparing their ^{1}H and ^{13}C NMR data to those of the published values [2, 13]. The data for **Ca** and **Cb** are in accordance with those reported for pteropodine (**2**) and isopteropodine (**1**), respectively (Table 1).

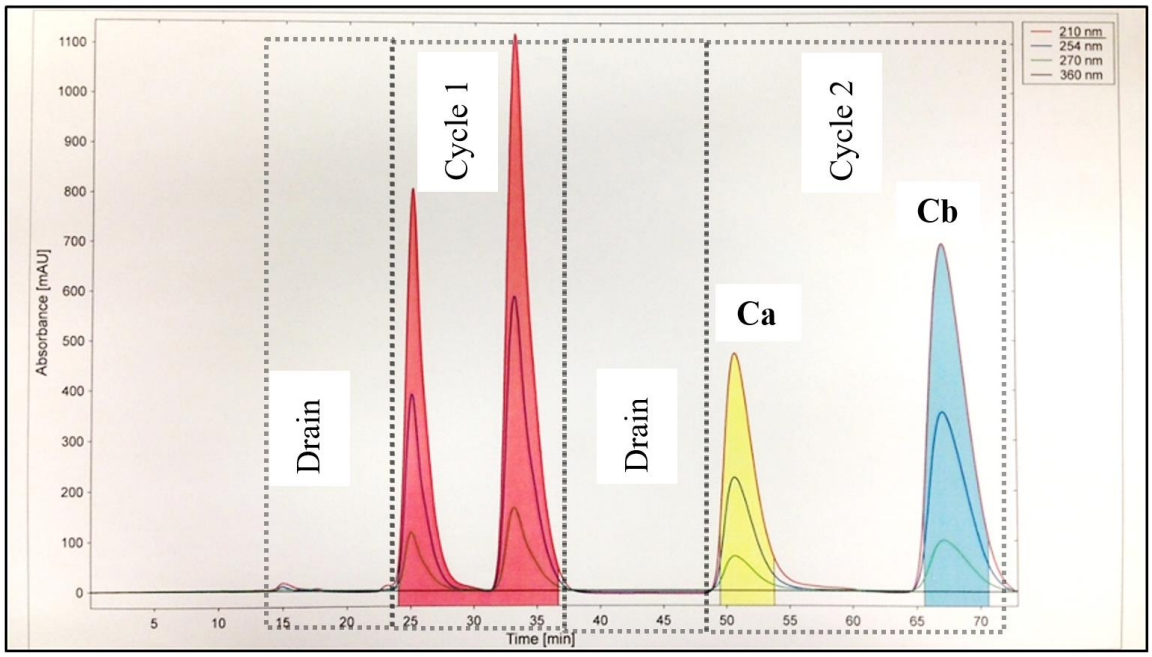


Figure 2. Recyling-HPLC chromatogram of **1** and **2** recorded at 210, 254, 270, and 360 nm.

Table 1. ^{1}H and ^{13}C NMR chemical shifts acquired in chloroform-*D* for **1** and **2** in comparison with the reported values for isopteropodine and pteropodine.

Position	Isopteropodine (**1**)						Pteropodine (**2**)					
	δH (ppm)			δC (ppm)			δH (ppm)			δC (ppm)		
	exp.	[a]ref.	[b]ref	exp.	[a]ref	[b]ref	exp.	[a]ref	[b]ref	exp.	[a]ref	[b]ref
3	2.41	2.40	2.36	74.44	74.42	74.43	2.50	2.48	2.57	71.33	71.34	71.24
5a	2.41	2.40	2.36	55.19	55.17	55.18	2.50	2.48	2.46	54.15	54.15	54.10
5b	3.31	3.30	3.30				3.24	3.27	3.22			
6a	2.03	2.03	1.99	34.62	34.59	34.67	2.01	2.01	2.00	34.86	34.86	34.83
6b	2.41	2.40	2.40				2.50	2.48	2.39			
7	-	-	-	56.11	56.08	56.13	-	-	-	56.87	56.86	56.92
8	-	-	-	133.43	133.41	133.47	-	-	-	133.76	133.75	133.75
9	7.23	7.22	7.20	123.22	123.27	123.05	7.29	7.29	7.27	124.63	124.64	124.54
10	7.07	7.07	7.04	122.73	122.74	122.60	7.06	7.04	7.02	122.56	122.58	122.49
11	7.21	7.19	7.18	127.99	127.98	127.92	7.23	7.21	7.19	127.69	127.69	127.66
12	6.84	6.83	6.87	109.34	109.25	109.56	6.88	6.88	6.89	109.48	109.46	109.62
13	-	-	-	140.56	140.48	140.77	-	-	-	140.09	140.04	140.21
14a	1.74	1.74	1.72	29.60	29.59	29.59	1.62	1.61	1.62	30.20	30.19	30.16

14b	1.51	1.51	1.51				0.87	0.88	0.88			
15	2.41	2.40	2.44	31.00	30.98	31.00	2.50	2.48	2.51	30.49	30.49	30.47
16	-	-	-	109.22	109.22	109.18	-	-	-	109.85	109.85	109.94
17	7.51	7.49	7.41	155.31	155.29	155.25	7.44	7.43	7.41	155.02	155.00	154.94
19	4.57	4.57	4.55	72.23	72.23	72.18	4.36	4.36	4.36	72.17	72.16	72.13
20	1.54	1.61	1.59	37.90	37.88	37.87	1.61	1.61	1.59	37.91	37.90	37.89
21a	2.41	2.40	2.32	53.66	53.63	53.68	2.50	2.48	2.42	53.53	53.53	53.50
21b	3.35	3.36	3.32				3.29	3.27	3.29			
CH3	1.43	1.43	1.40	18.99	19.00	18.97	1.43	1.43	1.41	18.65	18.65	18.62
O-CH3	3.63	3.63	3.60	50.94	50.93	50.89	3.63	3.62	3.60	50.99	50.99	50.95
O-C=O	-	-	-	167.67	167.77	167.72	-	-	-	167.66	167.64	167.59
N-C=O	-	-	-	180.42	181.00	181.23	-	-	-	180.91	180.88	181.25
N-H	7.73	8.66	8.52	-	-	-	7.95	7.83	8.42	-	-	-

[a]Reprinted from "Isopteropodic acid from Malaysian *Uncaria longiflora* var. *pteropoda*," by Salim, F. and Ahmad, R., 2010, *World Applied Science Journal,* 10(11), 1334-1337.
[b]Reprinted from "Nuclear magnetic resonance study on the eleven stereoisomers of heteroyohimbine-type oxindole alkaloids," by Seki, H., Takayama, H., Aimi, N., Sakai, S., Ponglux, D, 1993, *Chemical and Pharmaceutical Bulletin*, 41, 2077–2086.

2. Vibrational Absorbance and Vibrational Circular Dichroism (VCD) Characteristics

The initial vibrational absorbance (VA) analyses of **1** and **2** measured using the KBr pellet method, showed typical absorption bands for skeletal POA at ν_{max} *ca.* 3200, 3000, 1630, 1470, and 1100 cm^{-1} attributable to the amino (N-H), aromatic (C-H), conjugated ester carbonyl (C=C-C=O), olefinic (C=C), and cyclic ether (C-O-C) groups, respectively [13]. In addition, the ν_{max} absorptions for the amide (N-C=O), and aromatic (C=C) functional groups for **1** and **2** were clearly observed at 1714 and 1229 cm^{-1}, and 1688 and 1629 cm^{-1}, respectively. This might be due to changes in the functional group's electronic environment due to the changing structural configuration at the *spiro* C-7 of **1** (7*S*) and **2** (7*R*) [14].

In vibrational circular dichroism (VCD) analysis, functional group absorptions are monitored through the VA spectrum which is acquired in a deuterated solvent that reduces the effects of solvent absorption. For the case of **1** and **2**, deuterated chloroform (chloroform-*D*) was used to match their solubility. Even though the molecules exhibited a few strong and sharp absorption peaks above the region 2800 cm^{-1}, it is not suitable for VCD due to the unstable and broad vibrational absorptions [17]. Therefore, to obtain strong VCD signals by the diastereomers, the scanning was carried out in the 1800 – 900 cm^{-1} range.

As shown in Figure 3, there are plenty of ν_{max} that could be observed in the VA spectrum of **1** and **2** (Top panel). Although some of the absorptions were slightly shifted compared to the KBr pellet method, these deviations are acceptable, since they are due to solute-solvent and solute-solute interactions in the respective methods [18]. On the other hand, their VCD spectra (Lower panel) showed several Cotton effects (CEs) in the observed region. However, CEs of interest were restricted to the functional groups' absorption near the C-7 *spiro* centre where the diastereomers differed (see Figure 1). The most distinctive CEs were observed at *ca.* 1700 (labelled as 1), 1600 (labelled as 2), 1200 - 1300 (labelled as 6), and 1100 cm^{-1} (labelled as 10) which were mainly due to the stretching of the amide carbonyl, stretching and bending of the aromatic group, and stretching of the amino groups, respectively.

The VCD spectrum of **2** presented a bisignate CE while **1** revealed a monosignate CE at the amide carbonyl (labelled 1) and aromatic regions (labelled 2).

The exciton couplet from the interaction between the amide carbonyl and the aromatic in the skeleton of **2** showed an intense and distinctly positive CE, implying an *R* configuration at its *spiro* C-7 centre. While an opposite CE sign (negative) was observed for the respective functional groups in the spectrum of **1**, evidence of an S-configuration at its *spiro* C-7 centre. This assignment is further supported by the opposite CE sign (labelled as 10) which is due to the amino group stretching near the chiral centre. These and the other VCD spectral characteristics of **1** and **2** are shown in Table 1 and Table 2, respectively.

Table 2. VCD Characteristic of **1**.

CD Band	Vibrational frequency (cm^{-1})	Possible Vibrational origin	CE sign
1	1700	Stretching C=O of five-membered heterocycle	Negative
2	1623	Stretching C=C aromatic	Negative
3	1451	Bending C18-H, C6-H, C21-H, C3-H	Negative
4	1384	Bending N-H, C3-H, C14-H, C21-H	Negative
5	1305	Bending C3-H, C14-H, C15-H, C17-H, C19-H, C20-H, C17-H	Negative
6	1220	Bending C-H aromatic, N-H, C3-H, C15-H, C20-H; twisting C6-H, and C5-H; wagging C14-H	Negative
7	1211	Stretching C-O with bending C15-H, C19-H	Negative
8	1195	Stretching C-N, C-C, C-O carboxylic methyl ester with bending C5-H, C6-H, O-CH_3	Positive
9	1178	Stretching C-N and C-C with bending C21-H, O-CH_3	Negative
10	1092	Stretching C-N, C-C, C-O-C with bending C-H aromatic	Negative

Table 3. VCD Characteristic of **2**.

CD Band	Vibrational frequency (cm^{-1})	Possible Vibrational origin	CE sign
1	1691	Stretching C=O five-membered heterocylic, C=C aromatic	Positive
2	1655	Stretching C-C aromatic, and unsaturated C=C	Negative
3	1476	Bending C14-H, C15-H, C21-H	Negative
4	1442	Bending C6-H, C21-H, C18-H	Positive
5	1383	Bending C3-H, C5-H, C6-H, C14-H, C15-H, C18-H, C19-H, C20-H, C21-H	Negative
6	1329	Bending C-H aromatic, C3-H, C5-H, C19, C20-H	Positive
7	1278	Stretching C=C aromatic, C-N, C-C with bending C3-H, C5-H, C6-H, C14-H, C15-H, C19-H C20-H	Negative
8	1160	Stretching C-N, C-C with bending C-H aromatic, C5-H, C6-H	Positive
9	1118	Stretching C-N, C-C with bending C18-H, C21-H	Negative
10	1102	Stretching C-N, C-C, C-O with bending C5-H, C6-H, C14-H, C18-H, C21-H	Positive
11	1066	Stretching C-C with bending C18-H	Positive

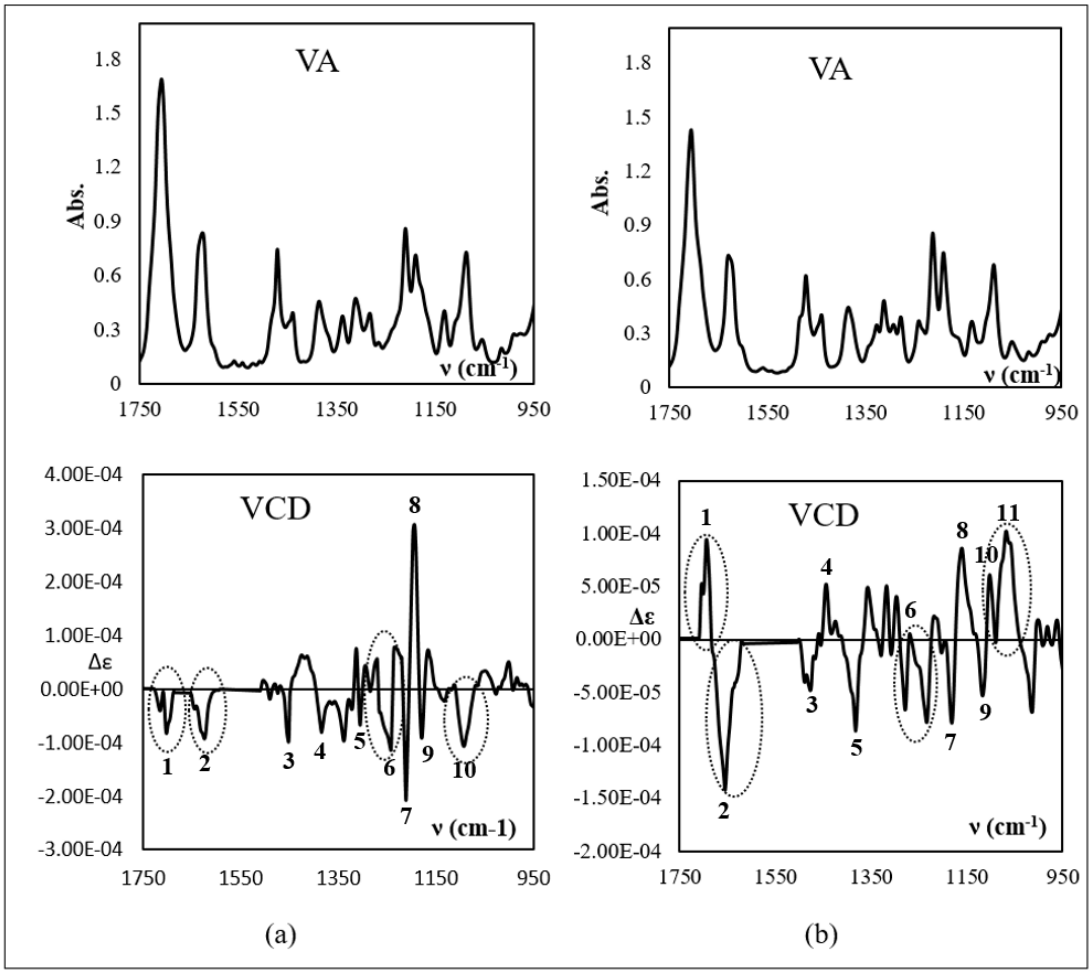


Figure 3. VA spectra (Top Panel) and VCD spectra (Lower Panel) of **1** (a) and **2** (b) acquired in chloroform-*D*.

CONCLUSION

In this paper, r-HPLC in two complete cycles successfully purified the diastereomers isopteropodine (**1**) and pteropodine (**2**) with purities of 96.44% and 97.52%, respectively. It was also demonstrated that the VCD characteristics of both alkaloids would unambiguously distinguish their C-7*R* and C-7*S* diastereomeric configurations. The findings from this study revealed that the combination of r-HPLC and VCD analyses are able to successfully separate, characterize and distinguish the diastereomers.

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