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Communication:

Extraction and isolation of the salidroside-type metabolite from zinc (Zn) and cadmium (Cd) hyperaccumulator *Sedum alfredii* Hance*

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The active metabolite in the post-harvested biomass of zinc (Zn) and cadmium (Cd) hyperaccumulator *Sedum alfredii* Hance from phytoextraction is of great interest in China. The current study demonstrates that a salidroside-type metabolite can be yielded from the Zn/Cd hyperaccumulator *S. alfredii* biomass by means of sonication/ethanol extraction and macroporous resin column (AB-8 type) isolation. The concentrations of Zn and Cd in the salidroside-type metabolite were below the limitation of the national standards.

Key words: Salidroside-type metabolite, Isolation, Hyperaccumulator, *Sedum alfredii* Hance

1 Introduction

Post-harvest processing of toxic biomass from phytoremediation of heavy metal contaminated soil is a worldwide unsolved problem (Sas-Nowosielskaa *et al*., 2004). The term "phytomining" was first proposed by Chaney (1983) for toxic biomass from phytoextraction. The phytomining of nickel, thallium, and gold involves planting a hyperaccumulator over a low-grade ore body or mineralized soil, harvesting the toxic biomass for incineration to produce a viable bio-ore, from which nickel, gold, and other metals can be recycled (Anderson *et al*., 1999). Pyrolysis in the range of 750–950 °C was shown to be better than incineration for volatilization of zinc (Zn) and cadmium (Cd) in the leaves of Zn/Cd hyperaccumulator *Thlaspi caerulescens* from phytoextraction, hence Zn and Cd can be recovered from the gas phase, and then the bottom ash can be recycled as fertilizer (Keller *et al*., 2005). Fast pyrolysis at 673 K enhanced the volatilization of heavy metals in the toxic biomass of birch and sunflower produced from phytoremediation (Lievens *et al*., 2008), while for fast pyrolysis at lower temperature of 623 K, heavy metals were mainly left in the bottom ash of toxic willow biomass (Lieven *et al*., 2009). Chinese native hyperaccumulator *Sedum alfredii* Hance of Crassulace family, grown in an old lead (Pb)/Zn mine area in southern China, can hyperaccumulate above 20000 mg/kg Zn, as high as 2% of the dried biomass of plant, and also more than 500 mg/kg Cd in its shoot tissues (Yang *et al*., 2002; 2004; Guo *et al*., 2009). A field trial of phytoextraction of heavy metal contaminated soil showed that a biomass of 1800 kg/hm^2 of this hyperaccumulator was reached under favourable growing conditions in around one year. A hydrometallurgical technique was applied to leach heavy metals from *S. alfredii* biomass, with an ammonia-ammonium chloride solution as a leaching agent, which resulted in a total Zn leaching efficiency under optimum conditions of approximately 98.0% (Yang *et al*., 2009). With the hydrothermal upgrading technique, removal efficiency for Zn, in water phase,

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was greater than 99.0%, and extraction efficiency for crude bio-oil, in oil phase, was greater than 60.0%, from the hyperaccumulator *Sedum plumbizincicola* biomass (Yang *et al*., 2010), where phenolic hydrocarbons and derivatives were the main constituents. Development of post-harvest processing was of great significance for the hyperaccumulator *S. alfredii* biomass from phytoextraction in China (Lone *et al*., 2008). The pharmacological significance of the salidroside metabolite has been described as anti-fatigue and anti-drug, the regulation of nervous and endocrine, as well as anti-aging, anti-cardiovascular, and anti-tumor. The pharmacological salidroside is a main metabolite in roots of *Rhodiola* from the Crassulace family, and interestingly, it has been discovered in the extract of the hyperaccumulator *S. alfredii* biomass in a previous study (unpublised data). However, no report is available at present on the extration or isolation of this pharmacological metabolite from this hyperaccumulator. In this study, hence, sonication/ethanol extraction and macroporous resin column (AB-8 type) isoaltion will be applied to the production of metabolite of salidroside or salidroside-type from the post-harvested hyperaccumulator *S. alfredii* biomass, and concentrations of Zn and Cd in this metabolite will be controlled below the limitations of the national standards.

2 Materials and methods

2.1 Plant materials

The non-hyperaccumulator (NHE) specimens were harvested from Jiuxi tea plantations in Zhejiang Province of China, the mined ecotypes of hyperaccumulator *S. alfredii* (HE1) were directly collected from the old Pb/Zn mine areas of Southeast China, and the agricultural ecotypes of hyperaccumulator *S. alfredii* (HE2) were from the contaminated agricultural soil located at Cixi County of Zhejiang Province of China, which were transplanted for two to three growing seasons with the seedlings from the HE1.

2.2 Plant extraction and isolation

2.2.1 Plant extraction

The matured HE2 biomass was harvested during May 2010 from contaminated agricultural soil located at Cixi County. The above-ground tissues (stems and leaves) were air-dried at 65 °C and/or vacuum-lyophilized at −80 °C, respectively, and then ground to powder in a high-speed mixer-grinder (Retsch RS100, Germany), and sieved by a 100-mesh screen. The powders (100 g) of HE2 were extracted in 40% ethanol, followed by sonication extraction, magnetic stirring extraction, or heat reflux extraction (Table 1). After filtration, the filtrate was concentrated to give the crude Extract 1 (Fig. 1). The Extract 1 was dissolved in distilled water and treated with petroleum ether. After the petroleum ether layers removal, the water layers were concentrated to obtain a pellet. An exaction of 0.50 g/ml (in 100 ml), based on the dried biomass of plant, was made of the pellet and distilled water, and then used for the isolation via macroporous resin column.

2.2.2 Plant isolation via macroporous resin

Macroporous resin (AB-8 type) (Shanghai Mosu Chemicals Co., Ltd., China) of 40 g was cleaned, and the isolation condition of the macroporous resin column was optimized according to the method of Xing *et al*. (2012). The water layers of the Extract 1 (Fig. 1) were mounted on top of the cleaned macroporous resin column of the AB-8 type, with the

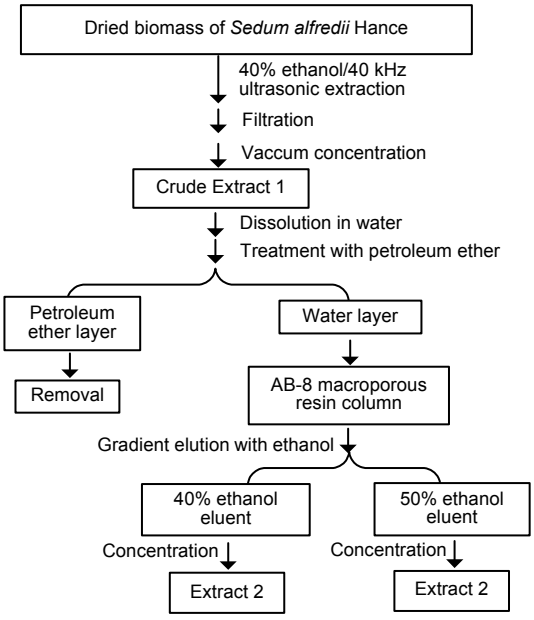

Fig. 1 Optimized process for preparation and isolation of metabolites of salidroside and salidroside-type from dried hyperaccumulator *Sedum alfredii* (HE2) biomass

Table 1 Extraction method for the compounds from the dried biomass (stems and leaves) of the hyperaccumulator *Sedum alfredii* (HE2)

Plant sample	Extraction method	Weight of biomass (g)	Extraction solvent	Ethanol volume (L)	Extraction time	Extraction yield (%)
Air-dried at 65 °C	Sonication extraction	100	40% ethanol	1	1 h	13.7±0.5
	Magnetic stirring extraction	100	40% ethanol	1	3 d	11.3±0.7
	Reflux extraction	100	40% ethanol	2	1.5 h	13.1±0.7
Vacuum-dried at −80 °C	Sonication extraction	100	40% ethanol	1	1 h	13.4±0.7

Sonication extraction: powders extracted by 40% ethanol in sonication apparatus (40 kHz, 25 °C); Magnetic stirring extraction: powders extracted by 40% ethanol (25 °C) at magnetic stirring rate of 2 500 r/min; Heat reflux extraction: powders extracted by 40% ethanol at the reflux temperature

elution using a water-ethanol step gradient of decreasing polarity (0%, 10%, 20%, 40%, and 50% ethanol). The elutions of 40% and 50% ethanol through the macroporous resin were collected, respectively. Each replicate was sampled in 400 μl, then diluted to a volume of 2 ml, and filtrated through 0.22 μm membrane filters for measurement of high performance liquid chromatography (HPLC; Agilent, USA), with salidroside as standard. Elutions of 40% and 50% ethanol were concentrated separately to give the Extract 2 (Fig. 1), and then vacuum-lyophilized.

2.3 Salidroside metabolite in plant tissues

The powders (100 g) were subjected to an ethanol extraction (40%, 1 L, 25 °C) for 24 h followed by sonication extraction (40 kHz, 20 °C) for 1 h. The 40% ethanol extraction was concentrated until its volume was 0.2 L, and then 0.2 ml samples were diluted to 2 ml, and filtered through 0.22 μm membrane filters for measurement of HPLC, with salidroside as standard. HPLC was performed on an Agilent Technology 1200 series (USA), equipped with an eclipse XDB C_{18} column (Agilent, USA) (4.6 mm×250 mm, 5 μm) in the G1316A TCC thermostat-monitored column compartment and the G1314B VWD ultraviolet (UV) detector (Agilent, USA), with methanol/water (15/85; v/v) as the mobile phase at a flow rate of 1 ml/min, and samples detected at 278 nm.

2.4 Concentrations of Zn and Cd in metabolites and plant tissues

All the chemicals used were of analytical grade. The 10 mg metabolite extracted through the macroporous resin of AB-8 type from the hyperaccumulator *S. alfredii* was dissolved completely in a mixture of HNO_3-H_2O_2 (5 ml:2 ml), and the concentrations of Zn and Cd were measured by an Agilent 7500a (USA) inductively coupled plasma-mass spectroscopy (ICP-MS).

The shoot tissues of *S. alfredii* species were harvested and vacuum-lyophilized, and then ground to power. The samples (0.1 g) of the powdered dried stems and leaves were digested completely with a mixture of HNO_3-H_2O_2 (5 ml:2 ml), and concentrations of Zn and Cd in the digestion were measured by an inductively coupled plasma-optical emission spectroscopy (ICP-OES) (IRAS-AP, Thermo, USA).

2.5 Statistical analysis

All data were statistically analyzed in SigmaPlot (Version 11) using analysis of variance (ANOVA), with least significant difference (LSD, $P<0.05$) to identify significant results.

3 Results and discussion

This study was originally aimed at obtaining high value-added salidroside metabolite from post-harvested toxic hyperaccumulator *S. alfredii* biomass. In this study, 40% ethanol extraction (2 L, 25 °C) for 24 h followed by sonication extraction (40 kHz, 20 °C) for 1 h was applied to the powders (200 g) of HE1, HE2, and NHE. Higher amount of salidroside was found in the shoots of HE1 and HE2 than in NHE (Table 2), with HE1 showing much higher levels than HE2. This finding emphasizes the importance of isolating salidroside metabolite from

powdered HE2 biomass (stems and leaves) via phytoextraction. As shown in Table 1, extractions with 40% ethanol (25 °C) were applied (1) at sonication extraction (40 kHz, 20 °C) for 1 h (sonication extraction method), (2) at the magnetic stirring rate of 2500 r/min for 3 d (magnetic stirring extraction method), and (3) at the reflux temperature for 1.5 h (heat refluxes extraction method). After the sonication extraction method, markedly fewer metabolites were found in the extraction of 40% ethanol from the vacuum-dried biomass than from the air-dried biomass (Figs. 2a and 2d). The heat reflux extraction method, comparatively, can achieve more metabolites as well as crude Extract 1 from the dried HE2 biomass, but with the possibility of poor thermal stability for the extracted plant metabolite. The sonication extraction method resulted in the extracted metabolite with greater thermal stability.

As shown in Fig. 3e, standard salidroside peaked around 15.5 min retention time (RT). There was no signal for the salidroside (Figs. 3a and 3b) in the HPLC graph of the 40% ethanol extraction from the dried HE2 biomass. Isolation via the macroporous resin column of AB-8 type was applied to the crude Extract 1, with gradient elution using different ethanol (0%, 10%, 40%, and 50%) (Fig. 3), and then the peak around 15.8 min RT was found, which was close to the peak around 15.5 min RT of the standard salidroside. The lower concentration of targeted metabolite, with a peak around 15.8 min RT in both 40% and 50% ethanol effluents, resulted in a smaller signal for UV detection, which is reflected in the relatively large baseline noise for its HPLC graph (Figs. 3c and 3d). As shown in Fig. 3, the majority of impurities in the crude Extract 1 were washed off by water elution through the macroporous resin column (AB-8 type) (Fig. 3a). An unknown metabolite, which peaked around 9.5 min RT, appeared in the 10% ethanol effluents that were run through the column (Fig. 3b), but it was not our targeted metabolite. The targeted metabolite with the peak around 15.8 min RT was observed in both 40% and 50% ethanol effluents that were run through the column (Figs. 3c and 3d). Taking into account the peak broadening in Figs. 3c and 3d as compared to that of standard salidroside in Fig. 3e, salidroside, along with other compounds, was possibly present in Extract 2. We assumed, from HPLC/MS spectra, that the metabolites peaked around 15.8 min RT contained a salidroside-type compound with a lower molecular weight than that of standard salidroside. Further, measurement by ICP-MS showed that concentrations of Zn and Cd in Extract 2 were (0.31±0.01) and (0.09±0.01) mg/kg, respectively, both of which were below the limitations of the relevant national standards. The HE1 and HE2 ecotypes demonstrated a greater uptake and accumulation of Zn and Cd in the above-ground than NHE ecotype, and thus concentrations of Zn and Cd were significantly higher in HE than in NHE. Interestingly, in this study, HE contains higher levels of the targeted metabolites of salidroside and salidroside-type than NHE, as shown in the peak area in HPLC graphs. However, the relationships between soil heavy metal content, shoot heavy metal content, and the targeted metabolite levels of salidroside and salidroside-type are still unknown in both HE and NHE species.

Table 2 Concentrations of Zn and Cd in the soil and plant shoots, and salidroside contents in the shoots of hyperaccumulator (HE1 and HE2) and non-hyperaccumulator (NHE) of *Sedum alfredii*

Plant type	Zn (mg/kg)		Cd (mg/kg)		Salidroside (g/kg)
	Soil	Shoot	Soil	Shoot	
NHE	63.6±3.1a	185.3±1.9a	0.38±0.04a	6.1±0.2a	0.22±0.08a
HE1	3100±140b	12533±408c	34.6±3.8b	1024±197c	2.6±1.1b
HE2	81.7±16.4a	8318±163b	0.65±0.17a	149.5±14.2b	1.1±0.4b

Data are represented as mean±standard error (SE) (n=3). Different letters in the same column indicated significant differences at P<0.05 as determined by the Duncan's multiple range test. Sonication extraction (40 kHz) in 40% ethanol (2 L, 25 °C) was employed to the dried biomass (stems and leaves) of *Sedum alfredii* for 1 h. NHE: non-hyperaccumulator, from Jiuxi non-contaminated soil; HE1: mined ecotype of hyperaccumulator, from Quzhou Pb/Zn mined soil; HE2: agricultural ecotype of hyperaccumulator, from Cixi contaminated agricultural soil

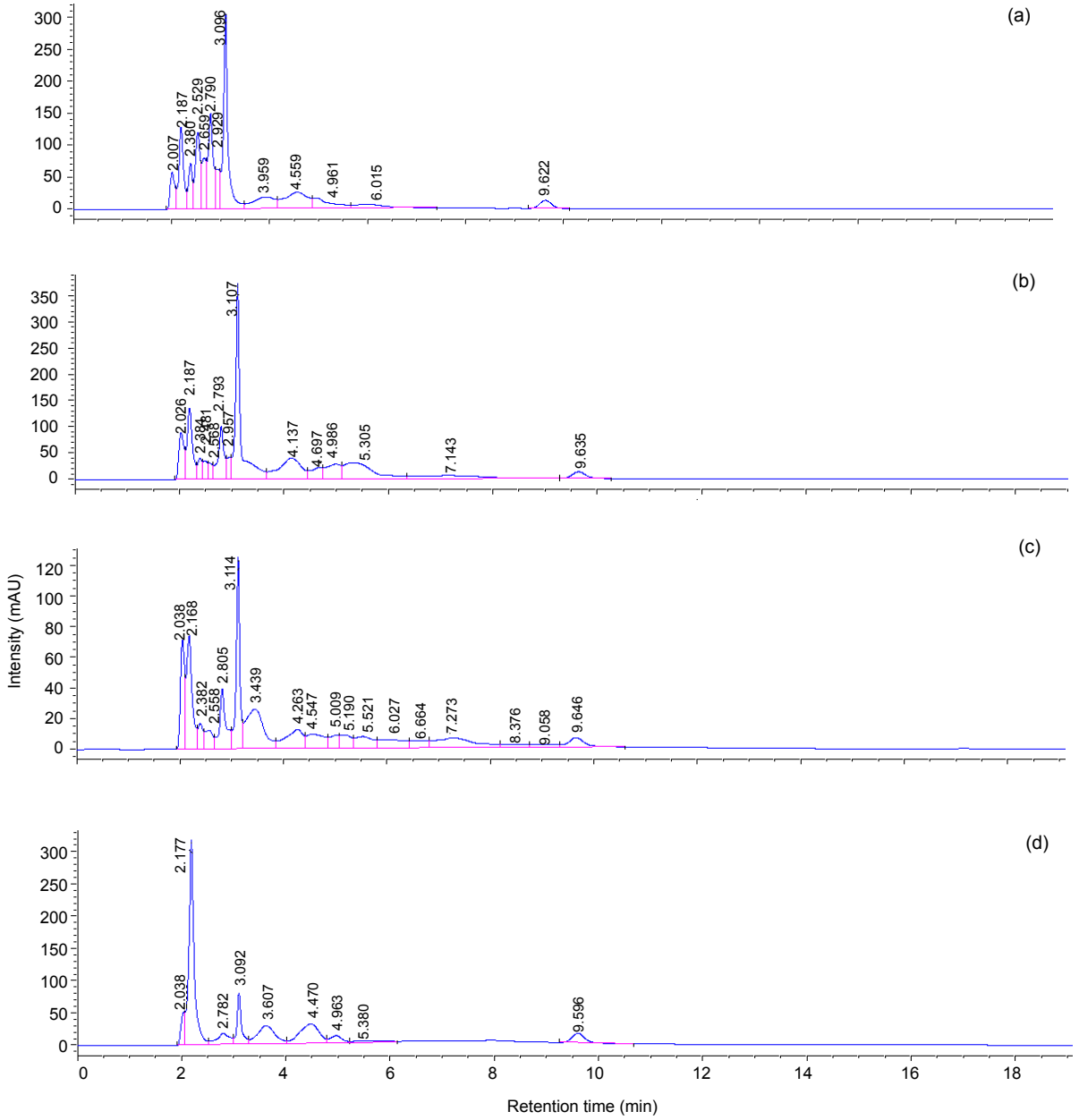

Fig. 2 HPLC graphs of the 40% ethanol extraction for air-dried biomass by means of sonication extraction (a), magnetic stirring extraction (b), and heat reflux extraction (c), and for the vacuum-dried biomass by means of sonication extraction (d) of the dried hyperaccumulator *Sedum alfredii* (HE2) biomass

4 Conclusions

The targeted salidroside-type metabolite can be obtained from post-harvested Zn/Cd hyperaccumulator *S. alfredii* biomass by means of sonication/ethanol extraction and macroporous resin column (AB-8 type) isolation, with the concentrations of Zn and Cd in these metabolites below the limitation of the national standards.

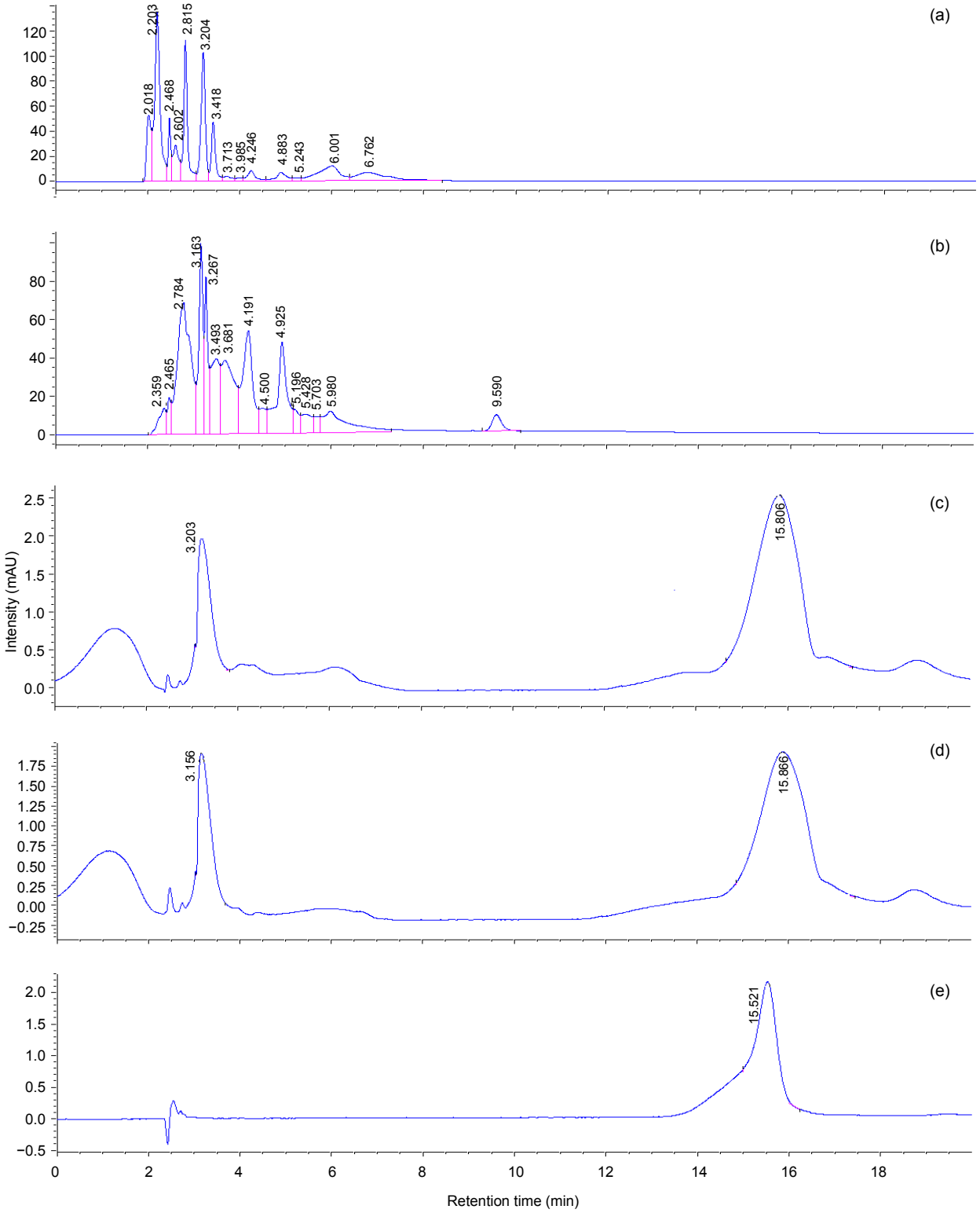

Fig. 3 HPLC graphs of the salidroside-type metabolites in the gradient effluents through the macroporous resin column from dried hyperaccumulator *Sedum alfredii* (HE2) biomass

(a) Water elution; (b) Ethanol elution of 10%; (c) Ethanol elution of 40%; (d) Ethanol elution of 50%; (e) Salidroside standard

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