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Countercurrent separation assisted identification of two mammalian steroid hormones in *Vitex negundo*

Qingfei Fan[a,b], Yang Liu[b,c], Daniel Kulakowski[b,c], Shaonong Chen[b,c], J. Brent Friesen[b,c,d], Guido F. Pauli[b,c,*], Qishi Song[a,**]

[a] *Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, 650223, PR China*
[b] *Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL, 60612, USA*
[c] *UIC/NIH Center for Botanical Dietary Supplements Research, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL, 60612, USA*
[d] *Physical Sciences Department, Rosary College of Arts and Sciences, Dominican University, River Forest, IL, 60305, USA*

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ABSTRACT

Countercurrent separation (CCS) has been widely used for the separation of high abundance compounds. However, the identification of low abundance compounds, such as mammalian steroid hormones, from natural sources is still a challenging task. A mixture of 14 human steroid hormone reference compounds was prepared for the development of a CCS enrichment strategy. The TLC-based GUESS (Generally Useful Estimate of Solvent Systems) method along with partitioning experiments were implemented to develop a process for the enrichment of these low abundance compounds with CCS. The application of CCS to the steroid hormone enrichment of *Vitex negundo* extracts was demonstrated by the identification of progesterone and estriol. This method provides a CCS-driven strategy to mine plant sources for low abundance compounds.

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1. Introduction

1.1. Previous investigations of mammalian steroid hormones in plants

Steroid hormones are a class of congeneric steroidal compounds with generally high stability, which have important regulatory functions in mammals and other organisms. Pregnane derivatives, such as estrogen, estradiol and diethylstilbestrol, tend to have estrogenic effects. Steroid hormones were first discovered in humans and other mammals. However, these steroids have been found in a variety of organisms, most notably in the plant kingdom. In 1964, Leboeuf, Cavé, and Goutarel reported that progesterone was found in *Holarrhena floribunda* leaves [1]. Since then progesterone and its congeners were successively reported to be present in apple seeds [2], loblolly pine [3], common foxglove (*Digitalis purpurea* L.), tobacco (*Nicotiana tabacum* L.), elecampane (*Inula helenium* L.) [4], *Juglans regia* [5], *Adonis aleppica* [5], and wheat [6], mostly using chromatographic methods, but also via isolation [5]. Other mammalian steroid hormones have been reported in plants. For example, estrone (1,3,5(10)-estratrien-3-ol-17-one) has been found in the seeds of the date palm, *Phoenix dactylifera* L. [7]. Estriol was tentatively identified in willow flower extract by UV absorption and melting point in 1933 [8]. Analytical techniques including GC–MS and ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) have been powerful tools to identify these low abundance compounds [4,9].

1.2. Challenges to identifying mammalian steroid hormones in plants

Despite of the current development of highly sensitive MS techniques, the identification of mammalian steroid hormones in natural sources is still a challenging task. For example, Gaw-

Abbreviations: CCS, countercurrent separation; TLC, thin-layer chromatography; K, partition coefficient; Rf, retention factor for TLC; SS, solvent system; HEMWat, hexanes-ethyl acetate-methanol-water biphasic solvent system; ChMWat, chloroform-methanol-water biphasic solvent system; SSE, TLC solvent systems based on ethyl acetate and hexane; GUESS, generally useful estimate of solvent systems; Sf, stationary phase volume retention ratio.

* Corresponding author.
** Corresponding author at: Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, 650223, PR China.
E-mail addresses: gfp@uic.edu (G.F. Pauli), songqs@xtbg.ac.cn (Q. Song).

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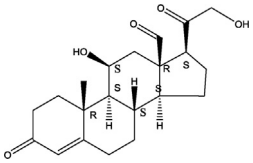
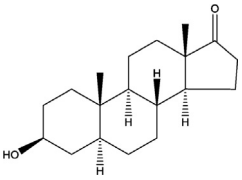
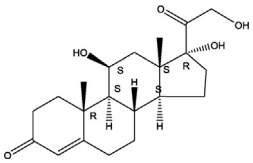
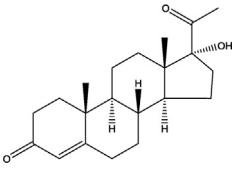
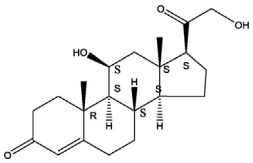
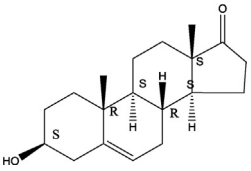
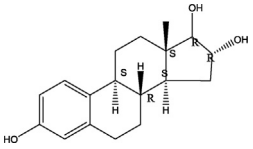
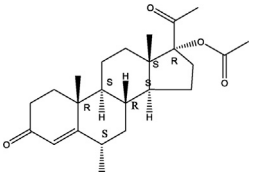
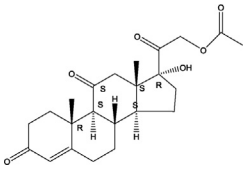
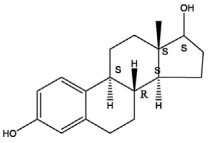
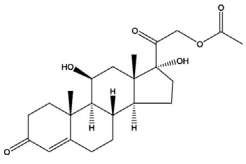
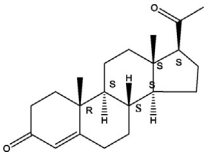
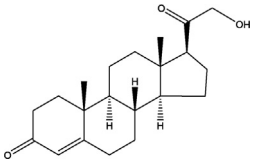
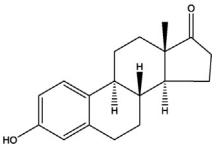
ienowski and Gibbs estimated the amount of progesterone was 500 ng/g in apple seeds using thin layer and gas chromatography methods [2]. The concentration of progesterone in the leaves of *Prunus virginiana* L. was reported to be 13 ng/g of dry weight [7]. Using GC–MS, Iino et al. reported finding progesterone in the shoots and inflorescences of in *Arabidopsis thaliana* L. in concentrations of 160 and 400 ng/kg of fresh weight, respectively [9].

1.3. Sample preparation and CCS for enrichment of target compounds

Countercurrent separation (CCS) is an advanced liquid–liquid chromatographic separation technique that is performed by instruments such as high-speed countercurrent chromatography (HSCCC) and centrifugal partition chromatography (CPC). CCS has been widely used for the preparative separation and isolation of (typically high-abundance) constituents due to its technical merits, such as low solvent consumption, highly selective solvent systems, and high recoveries [10]. Thus, this technique has been widely used for natural product separation and isolation [11–13]. However, the solvent system (SS) selection process presents a considerable challenge for CCS applications, especially when trying to target low-abundance constituents. Choosing a suitable SS is a critical operation in CCS [14,15]. The Generally Useful Estimate of Solvent Systems (GUESS) method, introduced in 2005, is a one of the few alternatives to SS selection with extensive use of partitioning experiments [16]. The GUESS method employs TLC to identify suitable SSs with minimal labor and has been shown to enable the rapid purification of target compounds [17–19]. The use of separatory funnel liquid–liquid separations with biphasic SSs of three or more solvents has been used to prepare samples for CCS fractionation [20,21]. A CCS-driven method of systematically fractionating sam-

Table 1
A list of 14 reference compounds (mammalian steroid hormones and their derivatives).

no	name	MW	structure	No	name	MW	structure
1	**aldosterone**	360.44		8	**epiandrosterone**	290.44	
2	**hydrocortisone**	362.46		**9**	**17α- hydroxyl progesterone**	330.46	
3	**corticosterone**	346.46		**10**	***trans*-dehydro androsterone**	288.42	
4	**estriol**	288.38		**11**	**medroxyprogesterone acetate**	386.52	
5	**cortisone acetate**	402.48		**12**	**β-estradiol**	272.38	
6	**hydrocortisone acetate**	404.50		**13**	**progesterone**	314.46	
7	**21-hydroxyprogesterone**	330.46		14	**estrone**	270.37	

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