



Authentication of the botanical origin of Western herbal products using *Cimicifuga* and *Vitex* products as examples

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Abstract Various herbal medicines have been developed and used in various parts of the world for thousands of years. Although locally grown indigenous plants were originally used for traditional herbal preparations, Western herbal products are now becoming popular in Japan with the increasing interest in health. At the same time, there are growing concerns about the substitution of ingredients and adulteration of herbal products, highlighting the need for the authentication of the origin of plants used in herbal products. This review describes studies on *Cimicifuga* and *Vitex* products developed in Europe and Japan, focusing on establishing analytical methods to evaluate the origins of material plants and finished products. These methods include a polymerase chain reaction-restriction fragment length polymorphism method and a multiplex amplification refractory mutation system method. A genome-based authentication method and liquid chromatography-mass spectrometry-based authentication for black cohosh products, and the identification of two characteristic diterpenes of agnus castus fruit and a shrub chaste tree fruit-specific triterpene derivative are also described.

Keywords Herbal product · Quality control · DNA identification · LC/MS · *Cimicifuga* · *Vitex*

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Introduction

Herbal medicines have been used for thousands of years to treat illness in various parts of the world. Kampo medicines (traditional Japanese medicines) were developed in Japan using herbal plants native to Asia, whereas Western herbal plants native to Europe have traditionally been used as crude drugs. The raw materials and extracts are now standardized to be used for medicines in many countries. Thus, closely related plants are used worldwide for different pharmaceuticals with different treatment applications, depending on their background. For example, the flowering aerial part of *Leonurus cardiac* L. is used to relieve symptoms of nervous tension under the name ‘motherwort’ in Europe, whereas the flowering aerial part of *L. japonicas* Houtt. and *L. sibiricus* L. is used as a crude drug named Leonurus Herb (益母草 in Japanese) as a component of Kampo formulations for the treatment of women’s disorders in Japan (Table 1). When herbal medicines are made from locally grown plants there is less concern, but misidentification and confusion may occur when both Western and the Asian herbal medicines are used in the same area.

In Japan, Western herbal preparations have only been used as health food products because of the lack of usage experience but they now are becoming more popular due to the changes in cause-specific morbidity and the increasing interest in self-medication. After guidelines for the approval of pharmaceuticals including Western herbs were established based on a notification from the Japanese Ministry of Health, Labour and Welfare [1], two products prepared from Western herbs have been approved as over-the-counter (OTC) drugs in Japan, and more products are expected to become available to the Japanese market in the near future.

Table 1 Examples of the Western herbs and crude drugs in Japan derived from plants of the same genus

Genus	Crude drug in Japan							
	Western herb ^a	Name	Botanical origin	Part of use	Name	Japanese name	Botanical origin	Part of use
Artemisia	Wormwood		<i>A. absinthium</i> L.	Leaf, flowering top	Artemisia capillaris flower	茵陳蒿	<i>A. capillaris</i> Thunb.	Flower head
Cimicifuga	Black cohosh		<i>C. racemosa</i> (L.) Nutt	Rhizome, root	Cimicifuga Rhizome	升麻	<i>C. simplex</i> (DC.) Turcz. <i>C. dahurica</i> (Turcz.) Maxim. <i>C. heracleifolia</i> Kom. <i>C. foetida</i> L.	Rhizome
Leonurus	Motherwort		<i>L. cardiac</i> L.	Flowering aerial part	Leonurus herb	益母草	<i>L. japonicas</i> Houtt. <i>L. sibiricus</i> L.	Flowering aerial part
Quercus	Oak bark		<i>Q. robur</i> L. <i>Q. petraea</i> (Matt.) Liebl. <i>Q. pubescens</i> Willd	Bark	Quercus bark	栲櫟	<i>Q. acutissima</i> Carruth. <i>Q. serrata</i> Murray, <i>Q. mongolica</i> Fisch. ex Ledeb. <i>Q. uariabilis</i> Blume	Bark
Rosa	Dog rose		<i>R. cania</i> L., <i>R. pendulina</i> L. and other <i>Rosa</i> species	Receptacle and sepal	Rose fruit	薔果	<i>R. multiflora</i> Thunb	Fruit, pseudocarp
Vitex	Agnus castus fruit		<i>V. agnus-castus</i> L.	Fruit	Shrub chaste tree fruit	蔓荊子	<i>V. rotundifolia</i> L. f. <i>V. trifolia</i> L.	Fruit

^a Herbal substances defined in the European Pharmacopoeia

Practical methods for verifying the botanical sources of herbal products are thus required to ensure the safety and efficacy of such products [2–4]. This review describes some of our recent studies on the genetic and chemical authentication of the original plant species in herbal products.

Studies on the botanical origins of *Cimicifuga* products

Black cohosh (*Cimicifuga racemosa* (L.) Nutt.) is one of the most commonly used Western herbs today. Its roots and rhizomes have traditionally been used for the treatment of women's disorders in North America and Europe, and it is now used globally to alleviate menopausal symptoms, being one of the top 10 best-selling herbs in the USA. Black cohosh extracts or materials are officially prescribed in the European Pharmacopoeia (EP) and standardized as an active ingredient of OTC drugs in Europe. In the USA, black cohosh extracts and materials are prepared for use as dietary supplements, and The United States Pharmacopoeia and The National Formulary (USP–NF) provides the standards.

Because the regulation of herbal products as dietary supplements in the USA is less strict than for OTC drugs in Europe, the substitution and adulteration of black cohosh dietary supplements with related *Cimicifuga* plants has frequently occurred in North America [5–7]. Additional reports have described hepatotoxicity associated with the use of black cohosh products, especially from products derived from the wrong plant parts instead of the roots and rhizomes or different *Cimicifuga* species [8–12]. The Ministry of Health, Labour and Welfare of Japan as well as other national ministries have updated their notifications about the risk of adverse liver reactions caused by the use of products containing black cohosh.

One possible reason why *Cimicifuga* plants native to Asia have been found in dietary supplements in the USA is that Chinese crude drugs were mistakenly recognized as black cohosh and were mixed with it. *Cimicifuga* plants are also used as traditional medicines in East Asia including Japan; the rhizome of *C. dahurica* (Turcz.) Maxim., *C. foetida* L. and *C. heracleifolia* Kom. is defined in the Chinese Pharmacopoeia (CP) as a crude drug named Cimicifuga Rhizome with heat-clearing and detoxifying effects, whereas the Japanese Pharmacopoeia (JP) describes *C. simplex* (DC.) Wormsk. in addition to the above three species as the botanical origin of Cimicifuga Rhizome (or “升麻” in Japanese) [13]. It is illegal in Japan for any health foods to contain materials or extracts prepared from *C. simplex*, *C. dahurica*, *C. foetida* or *C. heracleifolia* instead of *C. racemosa*, because Cimicifuga Rhizome is a pharmaceutical ingredient used exclusively for drugs.

Because of these problems, we developed genetic and chemical methods to simultaneously discriminate *C. racemosa* from other related species for the evaluation of black cohosh products that are commercially available in Japan.

Genome-based authentication of black cohosh products

Molecular biological techniques are useful and powerful methods to detect genetic differences among closely

Table 2 Genetic identification of referential *Cimicifuga* plant samples

Sample ID	Plant species	ITS region		Registered GenBank accession no.	trnL-F region		Registered GenBank accession no.
		GenBank accession no.	Sequence identity (%)		GenBank accession no.	Sequence identity (%)	
C1	<i>C. rubifolia</i>	Z98297	99	AB987680	AJ223004	98	AB987688
C2	<i>C. japonica</i>	Z98291	98	AB987681	AJ222999	98	AB987689
C3	<i>C. simplex</i>	Z98299	99	AB987682	AJ223008	98	AB987690
C4	<i>C. simplex</i>	Z98299	99	AB987683	AJ223008	98	AB987691
C5	<i>C. simplex</i>	AB044408	96	AB987684	AJ223008	98	AB987692
C6	<i>C. dahurica</i>	Z98284	99	AB987685	AJ222992	98	AB987693
C7	<i>C. heracleifolia</i>	Z98290	97	AB987686	EU186382	97	AB987694
C8	<i>C. racemosa</i>	Z98296	99	AB987687	AJ223003	98	AB987695

Fig. 1 Partial ITS sequences observed in referential *Cimicifuga* plants. Sequence data are deposited in GenBank under accession nos. AB987680–AB987687. **Bold** the sequence recognized by *Bst*BI

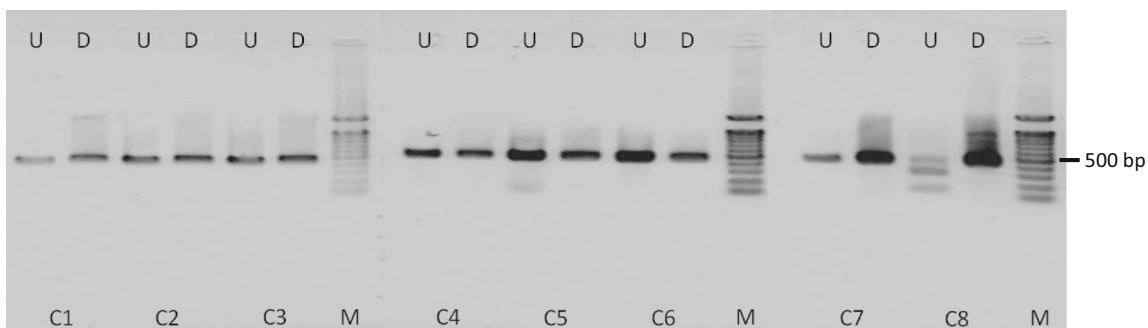
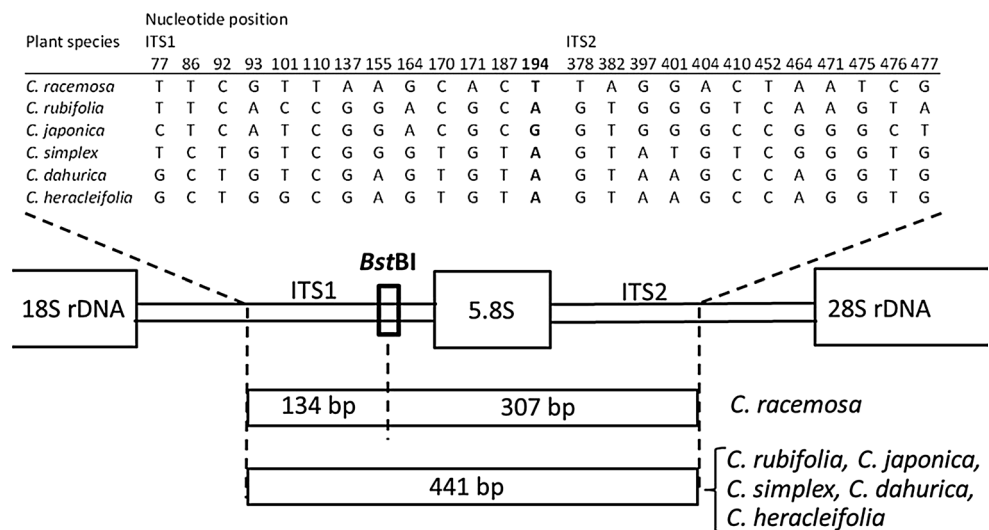


Fig. 2 PCR-RFLP analysis of *Cimicifuga* plants showing the restriction enzyme *Bst*BI on a partial ITS region. Data taken from reference 20. The forward primer: 5'-ATCATCCATTGTCGGGT

CAT-3', the reverse primer: 5'-GTTTACAACCACCGCAGACC-3'. Sample IDs are shown in Table 2. *M* 100 bp DNA ladder, *D* digested, *U* undigested

Fig. 3 Partial *trnL*-F gene sequences observed in referential *Cimicifuga* plants. The sequence data were deposited in GenBank under accession nos. AB987688–AB987695

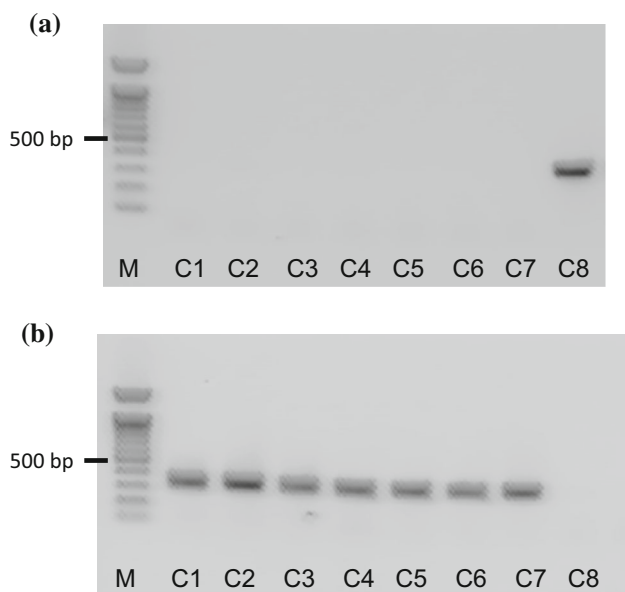
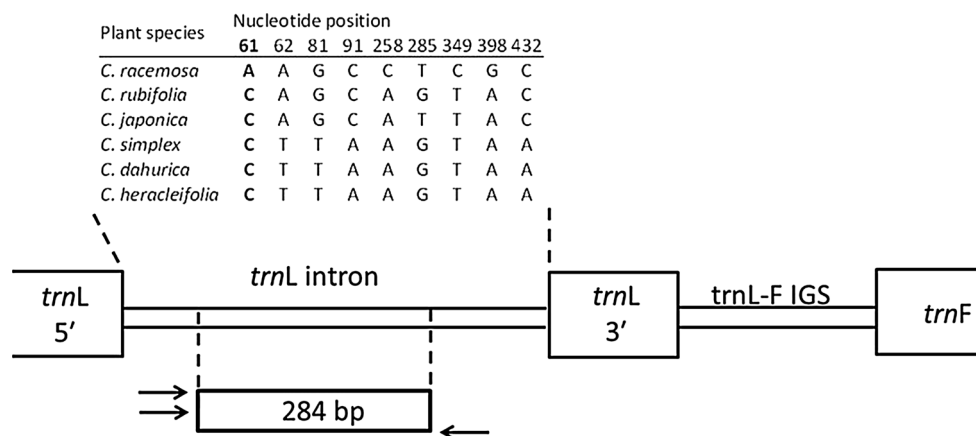


Fig. 4 ARMS analysis of *Cimicifuga* plants in *trnL*–*trnF* IGS region. Data taken from reference 20. **a** PCR amplification using a specific primer set for *C. racemosa*. **b** PCR amplification using a primer set for other *Cimicifuga* species. The sequences of a species-specific sense primer for *C. racemosa* and another five *Cimicifuga* species: 5'-C CTGAGCCAAATCCTGTCTA-3' and 5'-CCTGAGCCAAATCCT GTTCTC-3'. The sequence of a common anti-sense primer: 5'-TCA AATCAGCCACTCCATAGTATGA-3'. Sample IDs are shown in Table 2. *M* 100 bp DNA ladder

related plant species [14–17]. For *Cimicifuga* plants, a random amplified fragment length polymorphism fingerprinting method was developed to identify the four sympatric species distributed in eastern North America (*C. racemosa*, *C. americana* Michx., *C. pachypoda* Elliott and *C. rubifolia* Kearney) [18], and a multiplex amplification refractory mutation system (ARMS) method was developed to identify four Asian species (*C. dahurica*, *C. foetida*, *C. heracleifolia*, and *C. simplex*) [19]. To the best of our knowledge, there has been no report about the simultaneous identification of both North American and

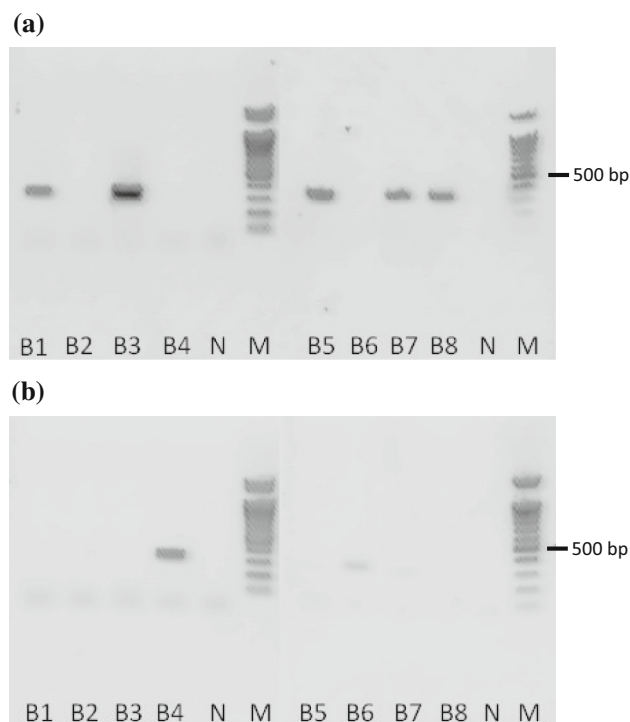


Fig. 5 ARMS analysis of black cohosh products using the partial *trnL* gene. Data taken from reference 20. **a** PCR amplification using a specific primer set for *C. racemosa*. **b** PCR amplification using a primer set for other *Cimicifuga* species. B1, 2, 3, 4 and 6 contained powdered dried material within the capsules, whereas B5, 7, and 8 contained both powdered material and dry extract within the capsules. *N* no template control, *M* 100 bp DNA ladder

Asian species. To establish a DNA analytical method that can discriminate *C. racemosa* from possible contaminated species, we prepared six *Cimicifuga* plants, identified each species by sequencing, and attempted to develop polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and ARMS methods [20].

Eight referential *Cimicifuga* plants were kindly provided by the Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, Health and

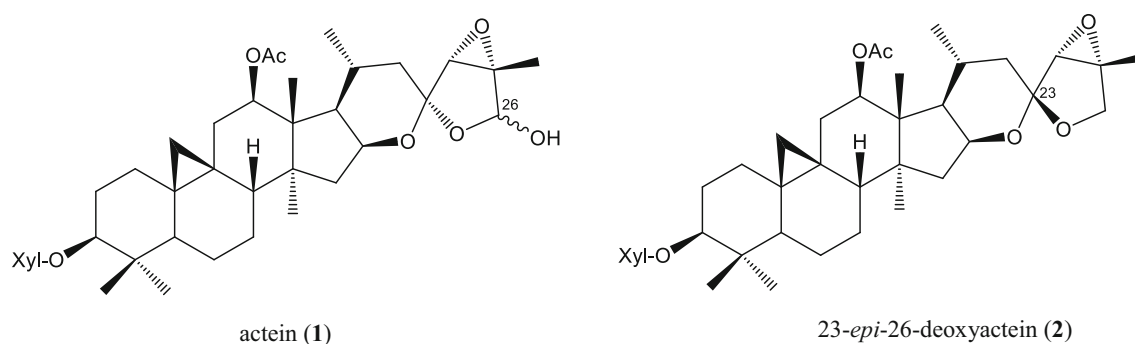


Fig. 6 Chemical structures of actein and 23-*epi*-26-deoxyactein. Xyl β -D-xylopyranosyl

Table 3 The *Cimicifuga* products applied for LC/MS analysis

Sample ID	Class ^a	Content	Source plant ^b	XIC pattern	Predicted species
BC-1	A	Powdered plant material	<i>C. racemosa</i>	I	
BC-2	A	Dry extract		I	<i>C. racemosa</i>
BC-3	A	Powdered plant material	<i>C. racemosa</i>	I	
BC-4	A	Powdered plant material and dry extract	<i>C. racemosa</i>	I	
BC-5	A	Dry extract		IV	Others
BC-6	A	Powdered plant material	<i>C. dahurica</i>	II	
BC-7	A	Powdered plant material and dry extract	<i>C. dahurica</i>	III	
BC-8	A	Dry extract		I	<i>C. racemosa</i>
BC-9	A	Powdered plant material and dry extract	<i>C. racemosa</i>	I	
BC-10	A	Dry extract		I	<i>C. racemosa</i>
BC-11	A	Dry extract		I	<i>C. racemosa</i>
BC-12	A	Powdered plant material	<i>C. dahurica</i>	II	
BC-13	A	Dry extract		I	<i>C. racemosa</i>
BC-14	A	Dry extract		I	<i>C. racemosa</i>
BC-15	A	Powdered plant material	<i>C. racemosa</i>	I	
BC-16	A	Powdered plant material and dry extract	<i>C. dahurica</i>	III	
BC-17	A	Dry extract		I	<i>C. racemosa</i>
BC-18	A	Dry extract		IV	Others
BC-19	A	Powdered plant material and dry extract	<i>C. dahurica</i>	III	
BC-M1	B	Dry extract		I	<i>C. racemosa</i>
BC-M2	B	Dry extract		I	<i>C. racemosa</i>
BC-M3	B	Dry extract		I	<i>C. racemosa</i>
BC-M4	B	Dry extract		I	<i>C. racemosa</i>
BC-M5	B	Dry extract		I	<i>C. racemosa</i>
BC-M6	B	Dry extract		I	<i>C. racemosa</i>
CR-1	C	Cut crude drug	<i>C. dahurica</i>	II	
CR-2	C	Cut crude drug	<i>C. dahurica</i>	II	
CR-3	C	Cut crude drug	<i>C. dahurica</i> , <i>C. heracleifolia</i>	II	
CR-4	C	Cut crude drug	<i>C. dahurica</i>	II	
CR-5	C	Cut crude drug	<i>C. dahurica</i>	II	

^a A health food, B OTC drug of EP grade, C crude drug of JP grade

^b The species of plant materials were identified by DNA sequencing

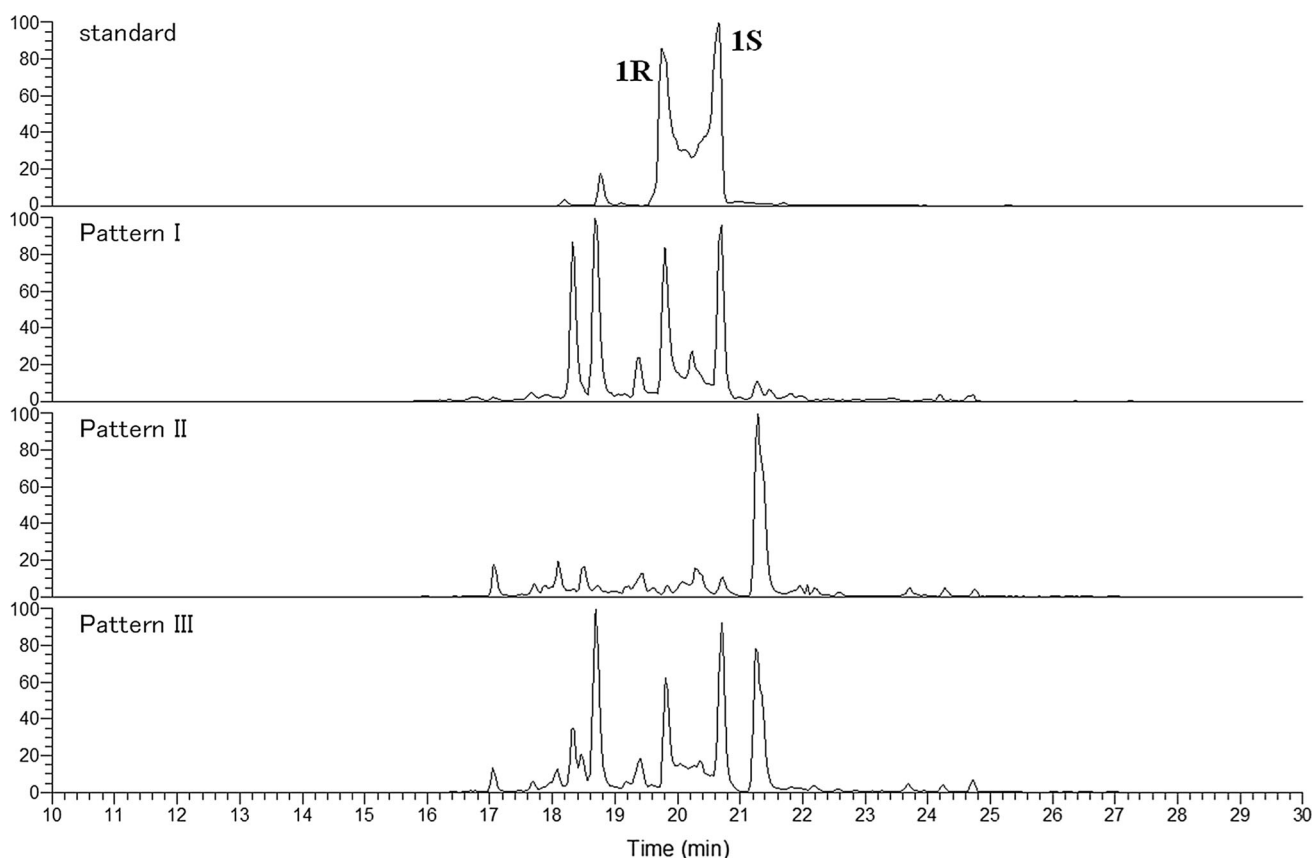


Fig. 7 Representative XIC of black cohosh samples classified as patterns I, II, and III. Data taken from reference 22. **1R**: (R)-actein, **1S**: (S)-actein

Nutrition, and were identified as six species (one *C. racemosa*, three *C. simplex* and one each of *C. japonica* (Thunb.) Spreng, *C. dahurica*, *C. heracleifolia* and *C. rubifolia*) by DNA sequencing on the ITS and *trnL*-F regions (Table 2). Based on these sequences and sequences obtained from the DNA databases, we first developed a novel PCR-RFLP method for the identification of *C. racemosa* targeting on its unique *Bst*BI restriction site (5' TT*CGAA 3') on the ITS1 region (Fig. 1). When the 441 bp of a DNA fragment on the ITS1 region was amplified by PCR from referential plant samples and digested with *Bst*BI, two unique fragments of 134 and 307 bp were observed only from the referential *C. racemosa* plants (Fig. 2).

Although we found this to be a convenient and useful method to identify species of plant samples and to detect the presence of *C. racemosa* in black cohosh products, we could not detect contaminated or adulterated products by this method because undigested fragments remained. Therefore, we next developed an ARMS method for discriminating *C. racemosa* from other related species, using two types of specific sense primers targeting the nucleotide substitution at position 61 on the *trnL* gene (Fig. 3). The species-specific and the common fragments (284 bp) were

Fig. 8 Chromatograms of ACF and SCTF samples. Data taken from reference 24. Two samples of ACF available in Germany and five samples of SCTF in Japan were derived from *V. agnus-castus* and *V. rotundifolia* or their mixture with *V. trifolia*, respectively. **a** TIC of ACF and SCTF samples. **b** XIC of ACF and SCTF samples at *m/z* 335.22–335.23. The MS and MS/MS spectra of peak ID 1552 from ACF-2 are shown in the upper-right window

successfully observed from *C. racemosa* and the other five species when the PCR was performed with DNAs from referential plants using the specific primer sets for *C. racemosa* and those for others, respectively (Fig. 4). Our results indicated that the ARMS analysis was a more accurate method than the PCR-RFLP method to identify *C. racemosa* from the related species, although the ARMS method takes a longer time.

We then applied the ARMS method to test the original plants of eight black cohosh products available on the Japanese market, and the results demonstrated that three of the eight products were not derived from *C. racemosa* (Fig. 5; Table S1). We also performed TLC and HPLC analyses for detecting two marker compounds—actein (**1**) and 23-*epi*-26-deoxyactein (**2**)—(Fig. 6) from black cohosh products, and we found that the three of the eight products

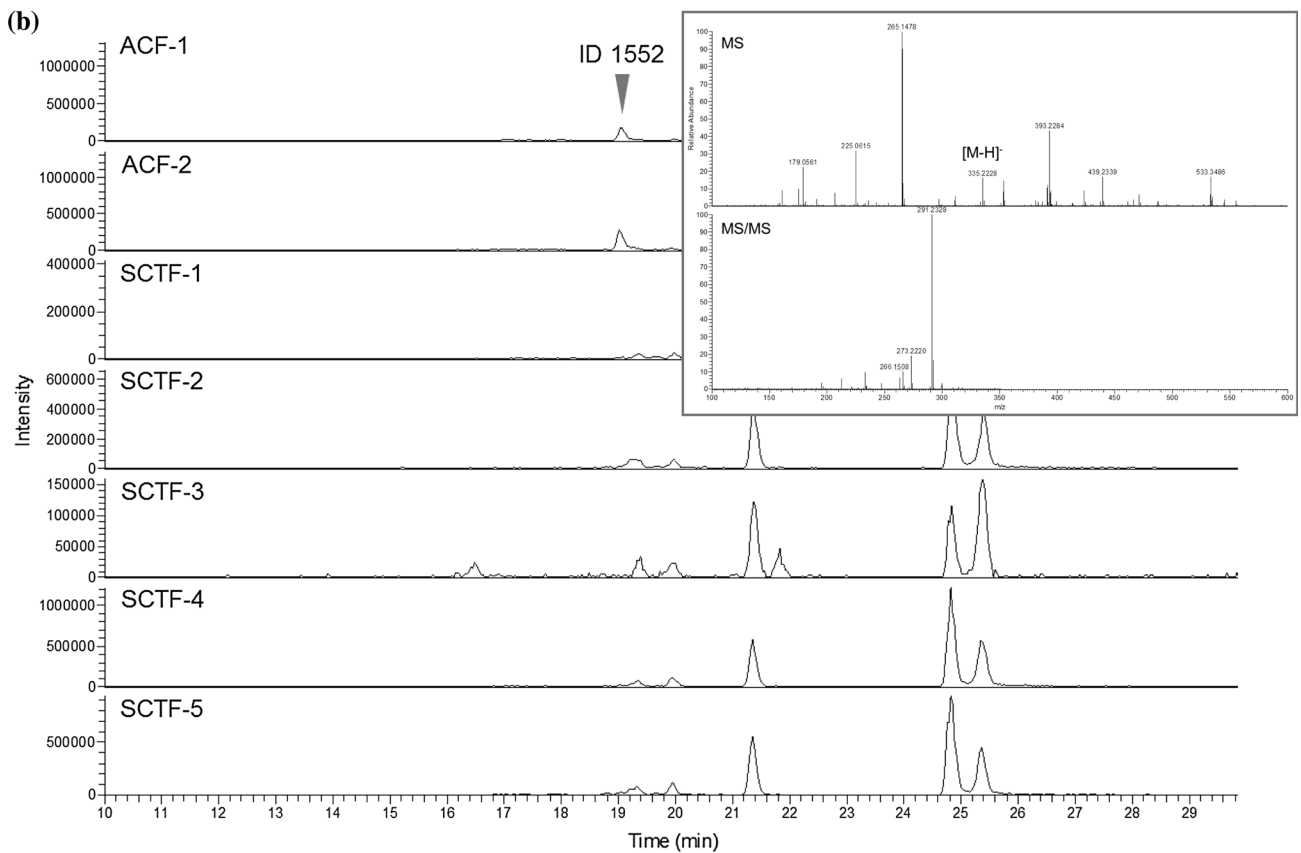
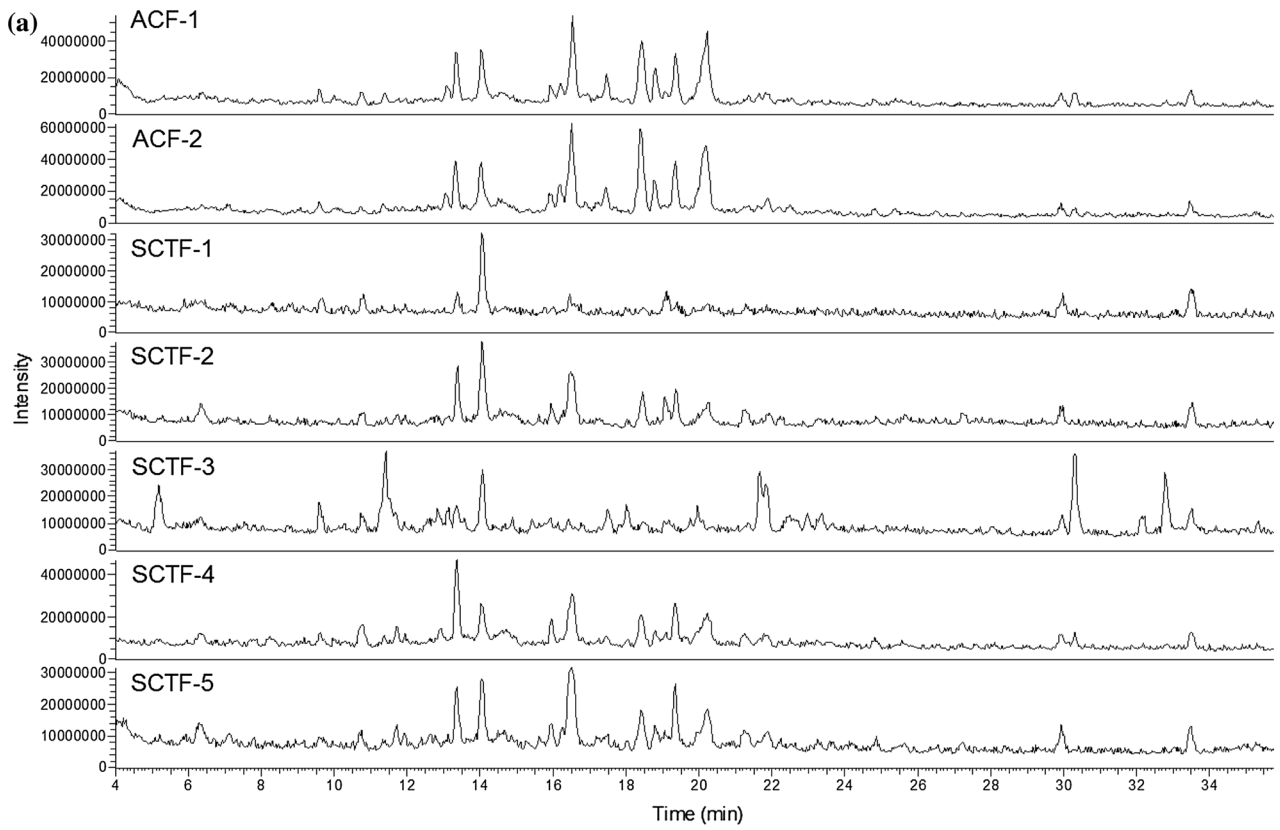


Table 4 Comparison of ^1H - and ^{13}C -NMR data between Ref. and compounds **3/3a** in chloroform- d (800 and 200 MHz, δ in ppm, J in Hz)

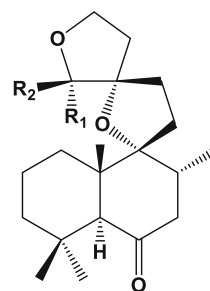
Position	LA/LB ^{*1} (5:2 mixture) δ	3/3a (4:1 mixture) δ
1	1.86 (1H, m)	1.56 (overlapping)
	1.44 (1H, dt, 12.5, 3.7)	1.41 (1H, dd, 10.4, 4.8)
	CH ₂ (29.9/28.7)	CH ₂ (30.4/30.3)
2	1.64–1.68 (1H, m)	1.57 (overlapping)
	1.59 (1H, dd, 9.1, 4.3)	1.50 (1H, m)
	CH ₂ (26.5/26.5)	CH ₂ (17.2/17.3)
3	3.11 (1H, dd, 11.6, 4.3)/3.07 (1H, dd, 11.3, 4.3)	1.20 (overlapping)
	–	0.97 (1H, ddd-like, 12.8, 6.3, 4.0)
	CH ₂ (78.2/78.4)	CH ₂ (41.5/41.5)
4	C (37.4/37.4)	C (31.2/30.9)
5	2.63 (1H, s)/2.64 (1H, s)	2.51 (1H, s)/2.62 (1H, s)
	CH (58.5/58.1)	CH (58.2/57.6)
6	C (210.3/210.6)	C (210.5/210.6)
7	2.41 (1H, td, 13.1, 0.9)/2.36 (1H, td, 13.1, 1.0)	2.43 (1H, m)
	2.06 (1H, dd, 13.1, 4.3)/2.01 (1H, overlapping)	2.00 (1H, td, 12.8, 4.8)
	CH ₂ (48.7/48.9)	CH ₂ (47.6/47.8)
8	2.10–2.19 (1H, m)	2.09 (m)
	CH (38.6/37.8)	CH (37.4/36.6)
9	C (92.7/93.0)	C (91.9/91.8)
10	C (47.3/47.5)	C (46.4/46.8)
11	2.14 (1H, dd, 9.4, 3.6)	2.17 (1H, ddd, 13.0, 8.8, 3.2)
	1.83 (1H, m)	1.83 (1H, ddd, 13.0, 9.6, 4.8)
	CH ₂ (29.0/31.2)	CH ₂ (28.0/27.6)
12	2.02 (1H dd, 9.1, 3.6)	1.99 (1H ddd, 12.8, 8.8, 3.2)
	1.92 (1H, dd, 9.1, 4.3)	1.92 (1H, m)
	CH ₂ (36.6/37.2)	CH ₂ (35.4/35.4)
13	C (91.3/92.5)	C (90.3/91.7)
14	2.43 (1H, dt, 11.9, 9.8)	2.43 (1H, ddd, 12.5, 9.6, 2.4)
	1.92 (1H, ddd, 11.9, 7.3, 2.7)	1.94 (1H, ddd, 12.5, 7.2, 3.2)
	CH ₂ (35.5/31.1)	CH ₂ (34.6/35.9)
15	4.11 (1H, ddd, 9.8, 8.7, 2.7)/4.04 (1H, td, 8.5, 3.9)	4.13 (1H, ddd, 9.6, 8.8, 3.2)/4.06 (1H, m)
	3.79 (1H, td, 8.7, 7.3)/3.97 (1H, td, 8.5, 7.0)	3.77 (1H, ddd, 8.8, 7.2, 2.4)/4.00 (1H, m)
	CH ₂ (64.8/66.3)	CH ₂ (64.0/65.5)
16	4.81 (1H, s)/5.33 (1H, s)	4.78 (1H, s)/5.37 (1H, s)
	CH (99.0/101.6)	CH (98.1/100.6)
17	0.95 (3H, d, 6.4)/0.96 (3H, d, 6.4)	0.93 (3H, d, 6.4)
	CH ₃ (16.9/17.6)	CH ₃ (16.0/16.7)
18	1.08 (3H, s)/1.07 (3H, s)	0.91 (3H, s)/0.89 (3H, s)
	CH ₃ (27.7/27.6)	CH ₃ (31.7/31.6)

Table 4 continued

Position	LA/LB ^{*1} (5:2 mixture) δ	3/3a (4:1 mixture) δ
19	1.16 (3H, s)/1.15 (3H, s)	1.14 (3H, s)/1.18 (3H, s)
	CH ₃ (14.9/15.0)	CH ₃ (20.8/21.7)
20	0.88 (3H, s)/0.87 (3H, s)	0.81 (3H, s)/0.86 (3H, s)
	CH ₃ (20.0/20.2)	CH ₃ (19.0/19.2)
16-OH		3.18 (1H, br s) ^{*2}

^{*1} LA leucasperol A, LB leucasperol B; a pair of signals from tautomers is shown as 'LA/LB'

^{*2} Disappeared upon treatment with heavy water



$\text{R}_1=\text{OH}$ $\text{R}_2=\text{H}$ chastol (**3**)

$\text{R}_1=\text{H}$ $\text{R}_2=\text{OH}$ epichastol (**3a**)

Fig. 9 Chemical structures of chastol and epichastol

did not contain marker compounds, as was also shown by the results of the ARMS analysis (data not shown). These TLC and HPLC results completely followed the results of the ARMS analysis and strongly demonstrated that the ARMS analysis is a useful method for authenticating the botanical source of black cohosh products.

LC/MS-based authentication of black cohosh products

Although the ARMS analysis was a very precise and straightforward way to evaluate the botanical origin of black cohosh products as described above, it had a disadvantage in that hardly any DNA was obtained from the products consisting of dry extracts. On the other hand, chemical analyses using marker compounds were also difficult to use for evaluating the botanical origin because most of the metabolites from closely related plant species overlap. The EP sets an identification test for black cohosh to confirm the presence of actein (**1**) and 23-*epi*-26-deoxyactein (**2**) by TLC. However, since these compounds are present in other *Cimicifuga* species as well as *C. racemosa*, the EP sets three additional TLC tests for substitution and adulteration to compare the chromatogram

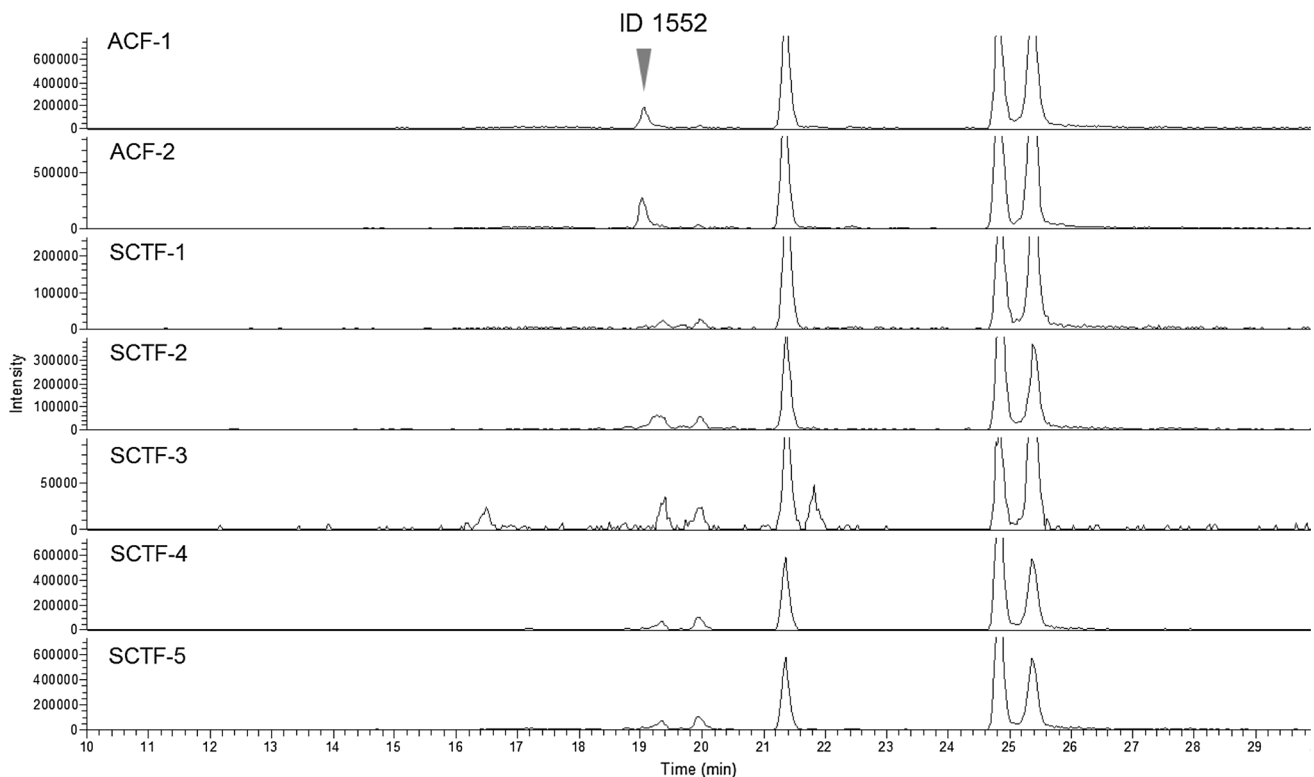


Fig. 10 Extracted ion chromatograms of the processed ACF samples at m/z 335.22–335.23. Data taken from reference 24. ACF-11, -12 and -13 were available in Europe as OTC drugs of EP grade and ACF-21 and -22 produced in the USA were available in Japan as health food products

obtained from the reference solution with each chromatogram from *C. americana*, *C. foetida*, and the mixture of *C. dahurica* and *C. heracleifolia* [21].

In the course of our studies on the botanical origins of black cohosh products, we found and reported a simple way to detect substitution and adulteration [22]. We prepared 25 black cohosh products including six OTC drugs purchased in Europe (Table 3: BC-1 to -19, BC-M1 to -M6) and five Cimicifuga Rhizome crude drugs of JP grade (Table 3: CR-1 to -5), and we genetically identified their origins by DNA sequencing and analyzed their constituents by liquid chromatography-mass spectrometry (LC/MS). The sequencing analysis of the ITS region revealed that five of the 10 tested health food products containing powdered plant material contained undeclared *C. dahurica*, and that Cimicifuga Rhizome products were derived from *C. dahurica* except for CR-3, which contained both *C. dahurica* and *C. heracleifolia* (Table 3).

When these 15 samples were analyzed by LC/MS for the detection of actein (**1**) and 23-*epi*-26-deoxyactein (**2**), they showed three chromatographic patterns (I, II and III) based on the extracted ion chromatograms (XIC) at m/z 735.4 corresponding to actein (Fig. 7). All of the black cohosh products derived from *C. racemosa* (BC-1, -3, -4, -9, and -

15) definitely contained marker compounds and showed pattern I. Of the 10 products that contained plant materials of *C. dahurica*, all of the Cimicifuga Rhizome samples (BC-6 and BC-12) showed pattern II, whereas the chromatograms of the other black cohosh samples (BC-7, -16, and -19) showed pattern III.

Pattern III appears to overlap with patterns I and II, and the products of pattern III consisted of powdered materials and dry extracts; it was thus strongly suggested that BC-7, -16 and -19 consisted of powdered materials of *C. dahurica* and an extract prepared from *C. racemosa*. Taking these results together with our above-described findings, we speculated that patterns I, II, III represent their botanical origins as *C. racemosa*, *C. dahurica* and the above-described mixture, respectively. We therefore established a simple method to detect substitution and adulteration of black cohosh products by monitoring a specific mass range on LC/MS.

We then analyzed the other black cohosh samples (which consisted only of dry extracts) by LC/MS to evaluate their botanical origins, and we found that 13 products including OTC drugs correctly used the dry extract of *C. racemosa*, and we observed that the XIC from BC-5 and BC-18 were out of synchronization with all three patterns (Table 3). Our finding that all the OTC drugs of EP grade

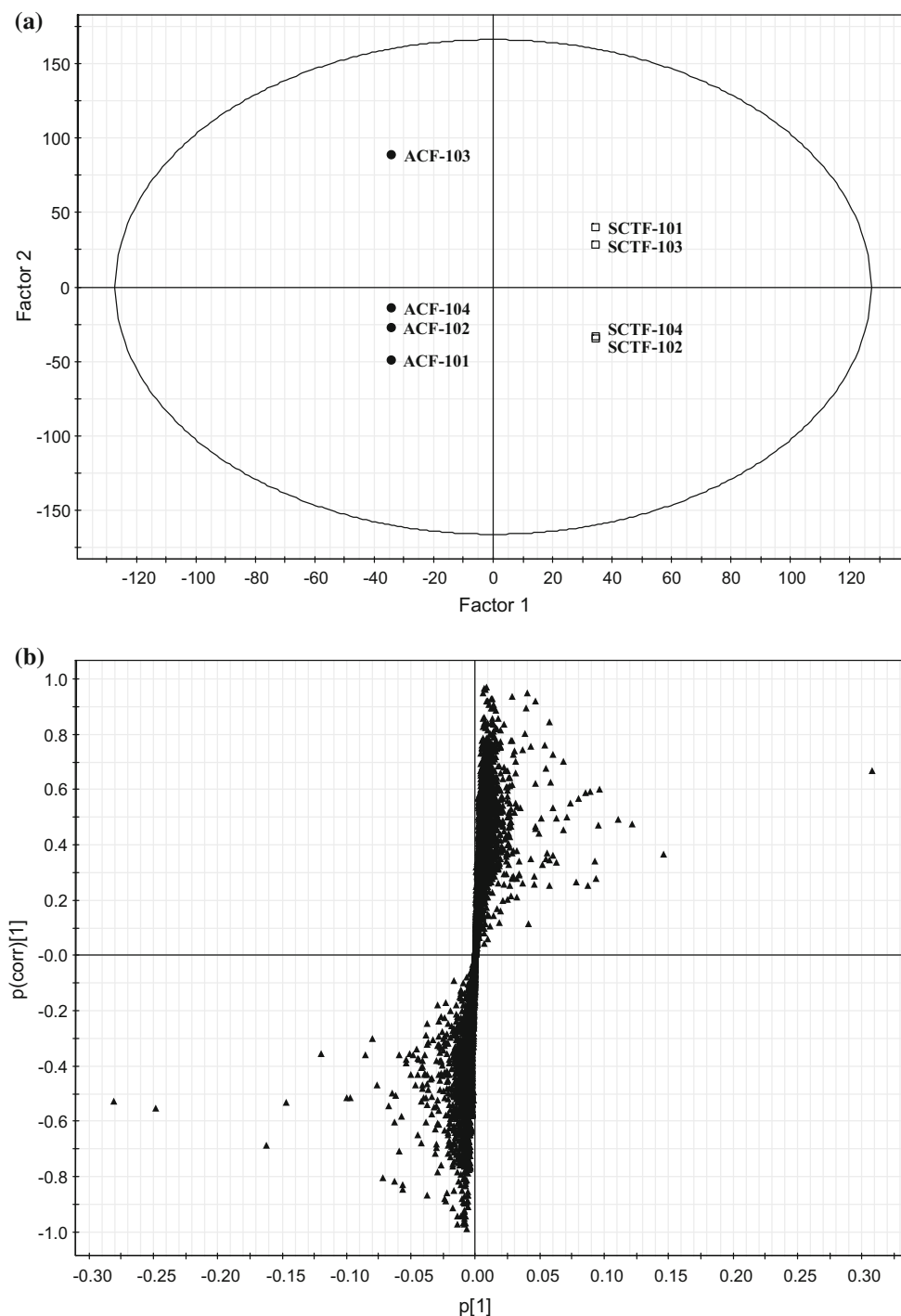


Fig. 11 OPLS-DA/O2PLS plots based on the LC/MS data of the SCTF and ACF products. Data taken from reference 30. Four samples of SCTF were crude drugs of non-JP grade with dried fruit form. ACF-101 and -102 were crude drugs of EP grade with dried fruit

form. ACF-103 and -104 were health food with capsule form. **a** Score plot of SCTF and ACF products. *Black circles* ACF. *Open squares* SCTF. **b** S-plot of SCTF and ACF products

were classified in the pattern I group strongly supports the usefulness of this method. The use of our new method could thus be a possible solution to the problem of adulteration of black cohosh products available in Japan.

Studies of the botanical origins of *Vitex* products

The dried ripe fruit of *Vitex agnus-castus* L. is defined under the names of agnus castus fruit (ACF) and chaste

Table 5 Comparison of ^1H -NMR data between compound **4** and Ref. in methanol- d_4 (800 MHz, δ in ppm, J in Hz)

Position	4		Ref. ^{*1}	
	δ		δ	
2	3.84	(1H, ddd, $J = 4.0, 10.4, 11.2$)	3.85	(1H, ddd, $J = 4.4, 9.9, 11.0$)
3	4.64	(1H, d, $J = 10.4$)	4.64	(1H, d, $J = 9.9$)
12	5.29	(1H, t, $J = 3.0$)	5.30	(1H, br s)
18	2.51	(1H, s)	2.51	(1H, br s)
23	0.90	(3H, s)	0.90	(3H, s)
24	0.95	(3H, s)	0.96	(3H, s)
25	1.05	(3H, s)	1.06	(3H, s)
26	0.82	(3H, s)	0.81	(3H, s)
27	1.36	(3H, s)	1.97 ^{*2}	(3H, s)
29	1.20	(3H, s)	1.20	(3H, s)
30	0.93	(3H, d, $J = 6.4$)	0.93	(3H, d, $J = 6.6$)
2'	6.43	(1H, d, $J = 16.0$)	6.43	(1H, d, $J = 16$)
3'	7.62	(1H, d, $J = 16.0$)	7.63	(1H, d, $J = 16$)
2''	7.20	(1H, d, $J = 1.6$)	7.21	(1H, br s)
5''	6.81	(1H, d, $J = 8.0$)	6.81	(1H, d, $J = 7.7$)
6''	7.07	(1H, dd, $J = 1.6, 8.0$)	7.08	(1H, br d, $J = 7.7$)
OCH ₃	3.89	(3H, s)	3.90	(3H, s)

Data taken from reference 30

^{*1} Zhao Q-C, Cui C-B, Cai B, Yao X-S, Osada H, *Molbank*, M328 (2003)^{*2} May be mistyped**Table 6** ^{13}C -NMR data of compound **4** in methanol- d_4 (200 MHz, δ in ppm)

Position	4 δ	Ref. ^{*1} DEPT	Position δ
1	48.2	CH ₂	48.55
2	67.8	CH	67.65
3	85.8	CH	85.64
4	40.8	C	40.65
5	56.6	CH	56.46
6	19.8	CH ₂	19.58
7	34.2	CH ₂	34.03
8	42.9	C	42.68
9	48.8	CH	8.71 ^{*2}
10	39.4	C	39.00
11	24.9	CH ₂	24.75
12	129.2	CH	129.14
13	140.5	C	140.16
14	41.3	C	41.14
15	29.8	CH ₂	29.59
16	26.8	CH ₂	26.60
17	49.3	C	48.72
18	55.3	CH	55.09
19	73.8	C	73.57
20	43.3	CH	43.09
21	27.5	CH ₂	27.30
22	39.3	CH ₂	39.22

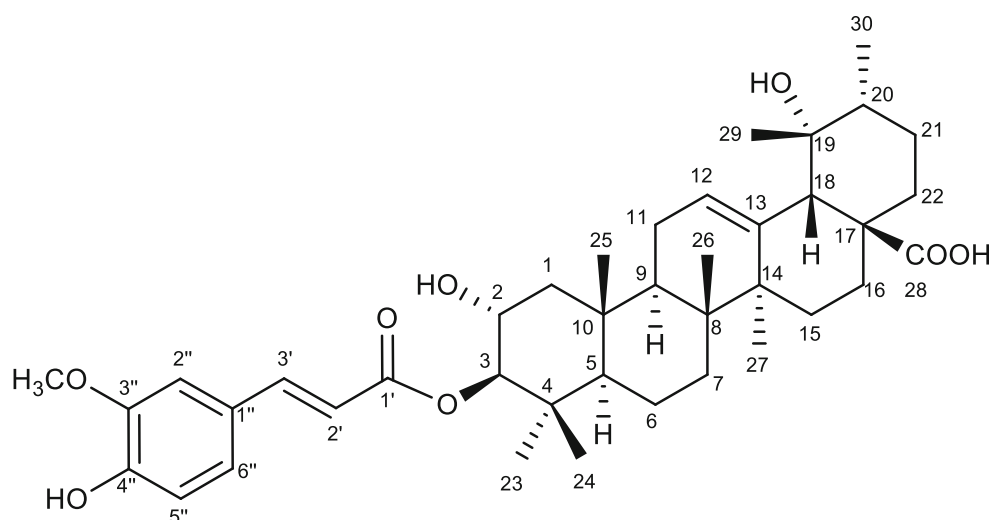
Table 6 continued

Position	4 δ	Ref. ^{*1} DEPT	Position δ
23	29.4	CH ₃	29.25
24	18.5	CH ₃	18.32
25	17.3 ^{*3}	CH ₃	16.60
26	17.7 ^{*3}	CH ₃	17.07
27	25.0	CH ₃	24.83
28	182.9	C	182.29
29	27.2	CH ₃	27.05
30	16.8 ^{*3}	CH ₃	17.50
1'	169.8	C	169.61
2'	116.3	CH	116.16
3'	146.7	CH	146.50
1''	128.1	C	127.90
2''	111.8	CH	111.58
3''	149.6	C	149.40
4''	150.7	C	150.53
5''	116.7	CH	116.49
6''	124.3	CH	124.04
OCH ₃	56.7	CH ₃	56.51

Data taken from reference 30

^{*1} Zhao Q-C, Cui C-B, Cai B, Yao X-S, Osada H, *Molbank*, M328 (2003)^{*2} May be mistyped^{*3} Assigned from HMBC correlations

Fig. 12 Chemical structure of 3-*O*-*trans*-feruloyl tormentic acid



tree, respectively, in the EP and USP–NF. ACF is used to treat symptoms of premenstrual syndrome and climacteric disorder, and it is becoming popular in Japan as the active ingredient of OTC drugs and health food products. On the other hand, the fruit of its congeneric species, *V. rotundifolia* L. f. and *V. trifolia* L., has been used as a crude drug named shrub chaste tree fruit (SCTF) (蔓荆子 in Japanese) for its pain relief, sedative and anti-inflammatory effects.

Although the EP sets an identification test for ACF to confirm the presence of aucubin and agnuside by TLC as well as a purity test by macroscopic analysis based on the size of the fruit to distinguish *V. agnus-castus* from other *Vitex* species [23], the chemical components and morphological characteristics of *Vitex* species are too similar to discriminate between them. Considering the internationalization of the supply chain of herbal plants, this situation could lead to misidentification of the botanical origin of these crude drugs, resulting in unexpected adverse reactions as well as a decline in their pharmaceutical efficacy. We therefore attempted to identify the marker compounds that distinguish ACF and SCTF with the goal of establishing quick methods to evaluate their origins.

Identification of characteristic diterpenes of ACF

We first prepared crude drugs of ACF of EP grade and SCTF of Japanese standards for non-pharmacopoeial crude drugs (non-JP) grade to confirm their origins by DNA sequencing, and we applied them in a differential analysis using LC/MS data [24]. The sequencing analysis of the *LFY/FLO* gene revealed that two samples of ACF available in Germany and five samples of SCTF in Japan were derived from *V. agnus-castus* and *V. rotundifolia* or its mixture with *V. trifolia*, respectively (Table S2). To identify candidate marker compounds for ACF against SCTF, we

performed a high-resolution LC/MS analysis of crude drug samples in an automated differential analysis, and we obtained 23 peaks as candidates. We then filtered these candidates on the basis of each formula and intensity, and seven peaks were finally picked up as characteristic compounds for ACF.

The chemical formulas calculated based on the accurate mass and MS fragmentation pattern of these peaks were collated with the phytochemical metabolite database, and we selected a putative diterpene with a novel structure—ID 1552 (RT 19.02 min, m/z 335.2228, formula $C_{20}H_{31}O_4$ for $[M-H]^-$)—as the first target to identify the chemical structure (Fig. 8). An ACF health food product was used as a source material of peak ID 1552. The repeated column chromatography and preparative TLC of a $CHCl_3$ extract of the ACF health food product afforded compound **3/3a** as a mixture. Compound **3/3a** was obtained as a white powder. The 1H -NMR and ^{13}C -NMR spectroscopic data of **3/3a** (Table 4) were very similar to those of known compounds, leucasperols A and B [25], and the complete structures of **3/3a** were determined on the basis of the spectroscopic data from HMBC, COSY, NOESY and differential NOE NMR experiments (Fig. 9). Since there have been no reports on the isolation of compounds bearing this structure other than leucasperols A and B from *Leucas aspera* L., we proposed the trivial names chastol and epichastol for compounds **3** and **3a**, respectively.

We analyzed additional ACF products by LC/MS to verify the usefulness of **3/3a** as marker compounds, and we successfully confirmed their detection (Fig. 10; Table S3). Since avoiding contamination from closely related species is a significant requirement for pharmaceuticals of natural origin, this information will be valuable for the quality control of both ACF and SCTF products from the viewpoint of regulatory science.

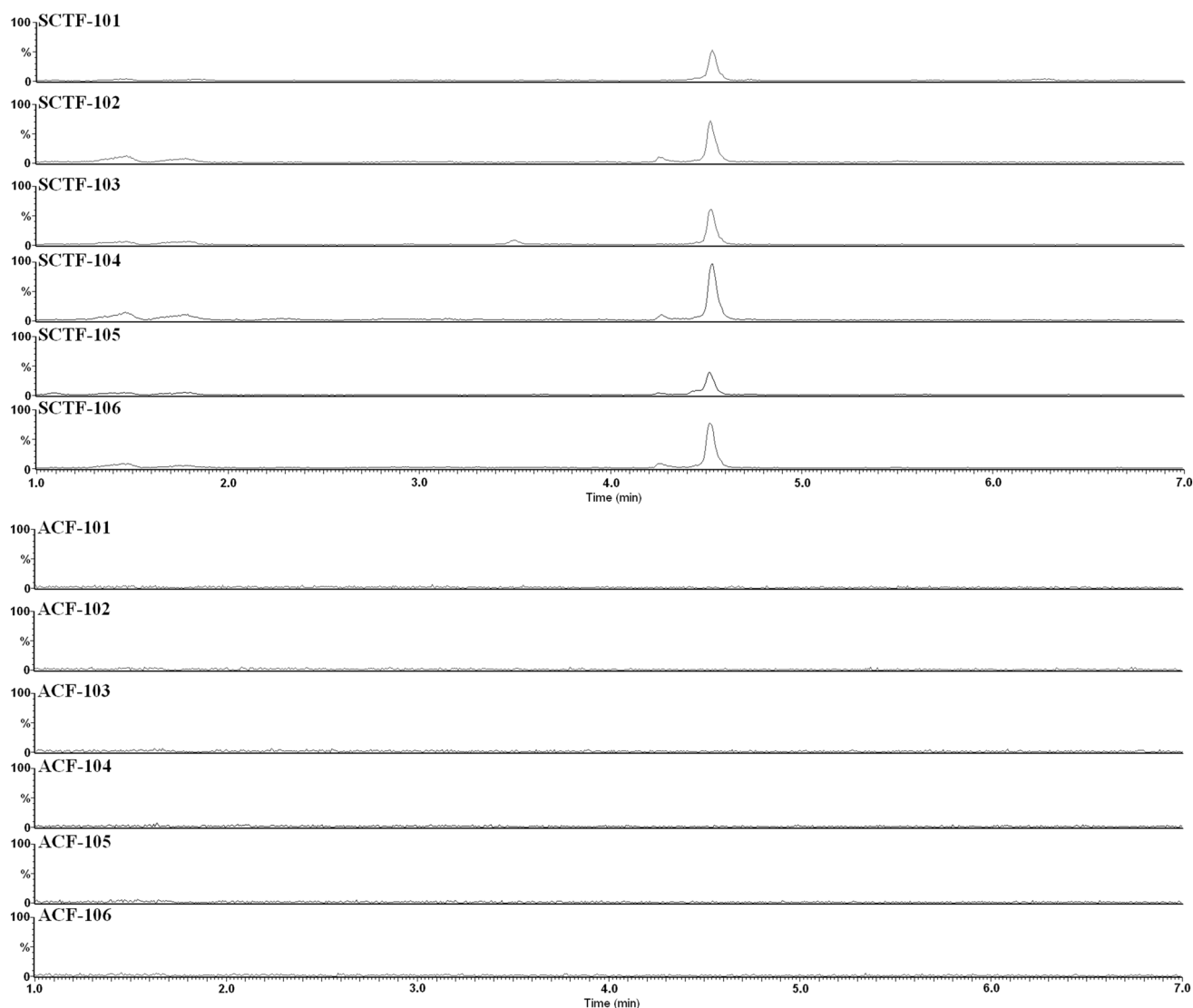


Fig. 13 Extracted ion chromatograms of SCTF and ACF samples at m/z 663.38–663.40. Data taken from reference 30. Six samples of SCTF were crude drugs of non-JP grade with dried fruit form. ACF-101 and -102 were crude drugs of EP grade with dried fruit form.

ACF-103, -104, -105 and -106 were health food with capsule form. The vertical axes represent the relative signal intensity to 60,000 and 6,000 for SCTF and ACF, respectively

Identification of an SCTF-specific triterpene derivative

Metabolomics have recently been adopted for the classification of variables and the identification of marker compounds based on the characteristics of their metabolic profiles in phytochemical investigations. For example, chemometric analyses such as a principal component analysis, a partial least-squares discriminant analysis, and an orthogonal partial least-squares discriminant analysis (OPLS-DA) are frequently used as multivariate tools for discrimination analyses [26–29]. In order to identify marker compounds for SCTF against ACF, we analyzed SCTF

and ACF products by LC/MS to apply to multivariate analyses [30].

When the LC/MS data set was subjected to OPLS-DA, the clusters of SCTF and ACF were clearly separated on the score plots, and 28 ions were picked up as the most influential variables that contribute to the segregation of SCTF and ACF samples on the S-plots (Fig. 11; Table S4). We then filtered these candidates on the basis of each formula and intensity, and seven peaks were finally picked up as specific compounds for SCTF. The chemical formulas calculated based on the accurate mass and MS fragmentation pattern of these peaks were collated with the phytochemical metabolite database, and we selected a

putative triterpene—ID 6436 (RT 4.59 min, m/z 663.3920, formula $C_{40}H_{55}O_8$ for $[M-H]^-$)—as the first target to identify the chemical structure.

The SCTF crude drug was extracted with $CHCl_3$ and fractionated using normal and reversed-phase column chromatography and prep-HPLC, monitoring the marker compound on LC/MS as a guide to obtain compound **4**. Compound **4** was isolated as an amorphous colorless powder, and its molecular formula was determined as $C_{40}H_{56}O_8$ by high-resolution electrospray ionization mass spectrometry, with 13 degrees of unsaturation. Its complete structure was determined as 3-*O-trans*-feruloyl tormentic acid on the basis of spectroscopic data from 1H -NMR, ^{13}C -NMR, and HMQC and HMBC analyses (Tables 5, 6; Fig. 12); this is the first time that this compound has been isolated from a *Vitex* plant.

We also analyzed additional SCTF and ACF products by LC/MS to evaluate the usefulness of compound **4** as a marker compound for SCTF and successfully verified its specificity (Fig. 13; Table S5). The limit of detection of compound **4** in SCTF raw materials was 0.02 mg/ml. These results demonstrated that monitoring a marker compound for SCTF—3-*O-trans*-feruloyl tormentic acid—should help prevent the use of adulterated materials in ACF products, and would also be a useful means of identifying the origin of raw materials in SCTF products.

Future perspective

With the changes in cause-specific morbidity and the increasing interest in self-medication, Western herbs are becoming popular in Japan. Because herbal products and their ingredients contain thousands of constituents, the authentication of original species from herbal products is the first and most important task to ensure their quality and thus their safety and efficacy. This review described the importance of quality control in the regulation of herbal products, using *Cimicifuga* and *Vitex* products as examples. There are many other Western herbs being developed for OTC drugs, foods with functional claims and health foods, and the proper use of the correct origin for each herb is necessary. It is important to continue to work on the quality control of herbal products from the viewpoint of regulatory science.

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