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Dibenzo[*a*,*c*]cyclooctadiene lignans of the genus *Schisandra*: importance, isolation and determination

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Abstracts

The drug Wuweizi (dried fruits *of Schisandra chinensis* or *S. sphenantherd*) is one of important medicinal means used in the Oriental medicine. The lignans of dibenzo[*a*,*c*]cyklooctadiene type are major constituents, a volatile oil with mono- and ses-quiterpens, an oil, organic acids and small amounts of additional compounds are also present. The content of major lignans (schizandrin, deoxyschizandrin, gomisin A, gomisin N, γ-schizandrin, wuweizisu C) in commercially available drugs ranges usually between 3 and 5%. The present paper biefly comments the isolation and biological activity of the lignans and is especially concerned with analytical methods (TLC and HPLC) for the determination of the drug fingerprint and methods for the determination of constituents in drugs, mixtures and biological materials. HPLC methods using RP-silica bonded phases and diluted methanol, acetonitrile (or a mixture of both), are most important for these purposes. Electromigration methods are less suitable and the importance of hyphenation procedures is practically negligible.

Keywords: *Schisandra chinensis*; Dibenzo[*a*,*c*]cyclooctadiene lignans; Isolation

1. Introduction

Lignans represent a relatively large group of secondary metabolites in taxons of divisions Pteridophyta, Coniferophyta and Magnoliophyta. According to their structures they have been classified into six major structural classes; they occur practically in all morphological parts of the plants (they are an important constituent of wood). Some compounds are widely distributed, others are concentrated in a relatively narrow taxonomic domain. Several hundreds of lignans have been reported so far. Their pharmacological properties have constantly attracted attention as some of them became important drugs (e.g. the lignans of *Podophyllum* sp., the flavonolignans of *Silybum marianum*).

Medicinal preparations of the Oriental medicine, especially the Chinese ones, have been important sources of new drugs since 1960s not only in Europe and the USA, but also in the countries where the *Materia medica* has originally been used. Schisandra berry, fruits of *Schisandra chinensis* (TURCZ.) BAILL. (Schisandraceae), containing dibenzo[*a*,*c*]cyclooctadiene lignans has been intensively studied from the phytochemical and pharmacological points of view. In the Chinese medicine, the taxon belongs to the top ten most widely used plants. Two taxons are used in the therapeutic practice. Whilst pharmacopoeial drug Wuweizi (Beiwuweizi) consists of fruits *of S. chinensis* from the northern parts of China, Nanwuweizi contains fruits *of Schisandra sphenanthera* from the southern China provinces [1]. Regarding the content of active constituents both drugs are practically equivalent. In traditional Chinese and Japanese medicines the drug is used as an antihepatotoxic, antiasth-

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matic, antidiabetic, sedative and tonic agent [2]. Besides these effects adaptogenic activity, i.e. the ability to increase the resistance against unfavourable influence of stress factors on the animal organism [3], has been reported. Sheng Mai San [4,5], a composite preparation with adoptogenic and anti-oxidant effects is well known. Presently, several lignans with interesting and therapeutically useful properties are available. These compounds undergo additional studies and there has been a great interest in using both isolated pure substances and standardized extracts as drugs and functional foods. Therefore, effective isolation and determination have been intensively studied. Analytical studies also play an important role in the evaluation of a raw material, since it has been shown that it can easily been adulterated with the fruits of the relative taxon *Kadsura japonica* or with completely different cheap impurities, such as the fruits of genus *Euonymus* or *Vitis* [2]. The occurrence of dibenzo[*a*,*c*]cyclooctadiene lignans in nature is rather rare: within the class Magnoliopsida they are concentrated to the phylogenetically old order Illiciales that comprises two families—Illiciaceae and Shisandraceae. Both of them include a small number of genera: Illiciaceae family is monotypic (*Illicum*), Shisandraceae family includes the *Kadsura* genus and *Schisandra* genus with 47 species altogether.

2. Structure of constituents

The constituents of *S. chinensis* fruits (seeds) can be divided into four classes:

i. dibenzo[*a*,*c*]cyclooctadiene lignans (schisandrin, schisantherines, gomisins) [6];
ii. monoterpenes (borneol, 1,8-cineol, citral, *p*-cymol, α- and β-pinene) [7];
iii. sesquiterpenes (sesquicarene, α-ylangene, chamigrenal, α- and β-chamigrene, β-bisabolene, etc.) [8–10] and
iv. other compounds of various structures (thymoquinol, thymoquinol 5-*O*-β-glucopyranoside, kaempherol 3-*O*-β-D-rutinoside, 5-hydroxymethyl-2-furaldehyde, anwulalignan, β-sitosterol, schisandrolic acid, isoschisandrolic acid, citrostadienol) [11].

Lignans, predominantly the sesquiterpenic ones, have synergistic effect and are responsible for the majority of the declared biological activities. Biological effects of a number of substances (especially those belonging to the forth group) have not been reported in relation to the activity of Schisandra berry.

Several independent teams were engaged in the studies of lignans in the 1970s. Consequently, some compounds have more synonyms. The names are mostly derived from the name of the drug in original languages; according to Ikeya et al. [12], the names are spelled “Hoku-gomishi” or “Kita-gomishi” in Japan and “Wu-wei-zi” in China.

2.1. Spectral analysis

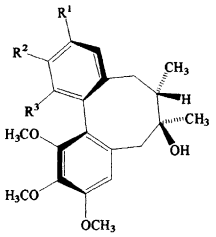
The plane structure of schizandrin was elucidated by Kochetkov et al. [13]. After the separation by means of column chromatography on aluminium oxide and subsequent paper chromatography, the structure was determined, using the UV, a IR spectroscopy. Partial stereo structure of schizandrin (presence of a *cis*-dimethyl) was proposed by Chen et al. [14]. Kochetkov and co-workers continued in elucidating the structure of the lignans and determined the molecular structure of deoxyschizandrin and pseudo-γ-schizandrin by means of chemical analysis and the UV, IR and ^{1}H NMR spectroscopy [15].

In the late 1970s and early 80s, the team of Tsumura company (Ikeya et al.) has significantly contributed to determining structures of Schisandra lignans. Their papers published predominantly in the journal Chem. Pharm. Bull., give a detailed overview how the structures (Fig. 1) can be resolved in a fashionable and convincing way, namely, by the combination of chemical decomposition and spectral methods, e.g. Refs. [12,16–20]. Among the spectral methods, NMR is crucial. In determining the absolute structure of schisandrin [12], the authors used the original procedures by Kochetkov et al., performed oxidation with potassium permanganate followed by methylation with diazomethane that gave dimethyl-4,4′,5,5′,6,6-hexamethoxydiphenate. Schizandrin was identified on the basis of these results. The conformational arrangement of schizandrin was elucidated by measurements of intramolecular nuclear Overhauser effects (NOE) that enlightens configuration of biphenyls. Structures of gomisins (gomisin A, gomisin O, epigomisin O) were elucidated by the UV and IR spectroscopy, circular dichroism and by chemical analysis [18]. The absolute structure of gomisin D was elucidated by Ikeya et al. [16], using X-ray crystallographic analysis after bromination of gomisin D and heavy atom method. Determination of stereostructures of (−)gomisin L_1 and L_2, (±)-gomisin M_1 by means of ^{1}H NMR and ^{13}C NMR spectral analysis was reported by Ikeya et al. [21]. Some of the lignans, their alternative names and papers on their properties are listed in Table 1.

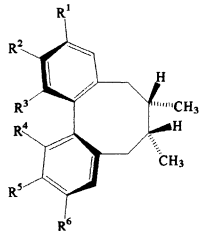
Determining conformation by means of “high-tech” 2D NMR can be exemplified by the conformation analysis of several dibenzocyclooctadiene lignans from *Kadsura matsudai* and *Schisandra ariesanensis*. Their conformations were elucidated by 2D NMR techniques including ^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H–^{13}C heteronuclear multiple quantum coherence (HMOC) and ^{1}H–^{13}C heteronuclear multiple bonds coherence (HMBC) [30].

3. Biological activities of lignans

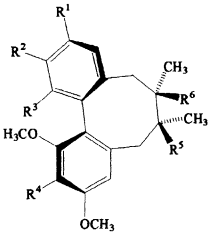
In China, Schisandra berry has been used for the treatment of patients suffering from the chronic virus hepatitis B since 1970s. Their application was successful in the areas strongly afflicted with the infection and resulted in a decrease



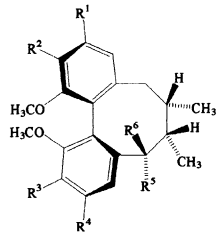
	R^1	R^2	R^3	R^4	R^5	R^6
deoxyschizandrin	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
(+)-gomisin K_2	OH	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
(+)-gomisin K_3	OCH_3	OCH_3	OH	OCH_3	OCH_3	OCH_3
(±)-gomisin M_1	OCH_3	OCH_3	OH	OCH_3	$-OCH_2O-$	
(+)-gomisin M_2	OCH_3	OCH_3	OCH_3	OH	$-OCH_2O-$	
(±)-γ-schizandrin	$-OCH_2O-$		OCH_3	OCH_3	OCH_3	



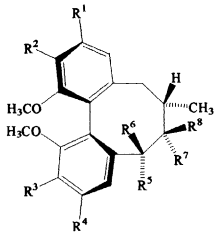
	R^1	R^2	R^3	R^4	R^5	R^6
(-)-gomisin L_1	OCH_3	OCH_3	OH	OCH_3	$-OCH_2O-$	
(-)-gomisin L_2	OH	OCH_3	OCH_3	OCH_3	$-OCH_2O-$	
gomisin N	$-OCH_2O-$		OCH_3	OCH_3	OCH_3	OCH_3
gomisin J	OH	OCH_3	OCH_3	OCH_3	OCH_3	OH
(-)-gomisin K_1	OH	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
wuweizisu C	$-OCH_2O-$		OCH_3	OCH_3	$-OCH_2O-$	



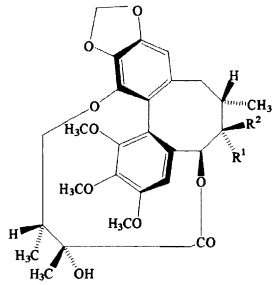
	R^1	R^2	R^3	R^4	R^5	R^6
schizandrin	OCH_3	OCH_3	OCH_3	OCH_3	OH	H
isoschizandrin	OCH_3	OCH_3	OCH_3	OCH_3	H	OH
gomisin A	$-OCH_2O-$		OCH_3	OCH_3	OH	H
gomisin H	OCH_3	OCH_3	OH	OCH_3	OH	H
angeloylgomisin H	OCH_3	OCH_3	O-Ang	OCH_3	OH	H
tigloylgomisin H	OCH_3	OCH_3	O-Tgl	OCH_3	OH	H
benzoylgomisin H	OCH_3	OCH_3	O-Bz	OCH_3	OH	H
gomisin T	OCH_3	OCH_3	OCH_3	OH	OH	H



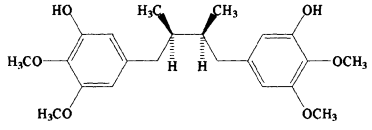
	R^1	R^2	R^3	R^4	R^5	R^6
gomisin O	$-OCH_2O-$		OCH_3	OCH_3	H	OH
gomisin R	$-OCH_2O-$		$-OCH_2O-$		H	OH
benzoylgomisin R	$-OCH_2O-$		$-OCH_2O-$		H	O-Bz
epigomisin O	$-OCH_2O-$		OCH_3	OCH_3	OH	H
angeloylgomisin O	$-OCH_2O-$		OCH_3	OCH_3	H	O-Ang
angeloylisogomisin O	OCH_3	OCH_3	$-OCH_2O-$		H	O-Ang
benzoylisogomisin O	OCH_3	OCH_3	$-OCH_2O-$		H	O-Bz



	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8
gomisin B	$-OCH_2O-$		OCH_3	OCH_3	H	O-Ang	OH	CH_3
gomisin C	$-OCH_2O-$		OCH_3	OCH_3	H	O-Bz	OH	CH_3
gomisin F	OCH_3	OCH_3	$-OCH_2O-$		H	O-Ang	OH	CH_3
gomisin G	OCH_3	OCH_3	$-OCH_2O-$		H	O-Bz	OH	CH_3
angeloylgomisin Q	OCH_3	OCH_3	OCH_3	OCH_3	H	O-Ang	OH	CH_3
angeloylgomisin P	$-OCH_2O-$		OCH_3	OCH_3	O-Ang	H	CH_3	OH
tigloylgomisin P	$-OCH_2O-$		OCH_3	OCH_3	O-Tgl	H	CH_3	OH
gomisin S	OH	OCH_3	OCH_3	OCH_3	OH	H	CH_3	H
schizantherin D	$-OCH_2O-$		$-OCH_2O-$		H	O-Bz	OH	CH_3



	R^1	R^2
gomisin D	OH	CH_3
gomisin E	CH_3	H



pregomisin

Fig. 1. Stereostructures of lignans in *S. chinensis*.

Table 1
Major lignans and some of minor lignans in *S. chinensis* (Turcz.) Baill

Lignan	Synonyms	Isolation, properties spectra
Schizandrin	schisandrin, schisandrol A, wuweizi alcohol A, wuweizichun A	Ikeya et al. [12] Kochetkov et al. [13,22] Chen et al. [14]
Isoschizandrin		Ikeya et al. [23]
(±)-y-Schizandrin	schizandrin B, deoxygomisin A, wuweizisu B	Kochetkov et al. [13,15] Ikeya et al. [21]
Deoxyschisandrin	dezoxischizandrin, schizandrin A, wuweizisu A, dimethylgomisin J	Kochetkov et al. [15] Ikeya et al. [18]
Wuweizisu C	schizandrin C	Chenet al. [14] Ikeya et al. [24]
Gomisin A	schisandrol B, wuweizi alcohol B, wuweizichun B, TJN 101	Ikeya et al. [12]
Gomisin B	schisantherin B, schizandrer B, wuweizi ester B	Ikeya et al. [12]
Gomisin C	schisantherin A, schizandrer A, wuweizi ester A	Ikeya et al. [12]
Gomisin D		Ikeya et al. [16]
Gomisin E		Ikeya et al. [18]
Gomisin F		Ikeya et al. [12]
Gomisin G		Ikeya et al. [12]
Gomisin H	norschizandrin	Ikeya et al. [17]
Angeloylgomisin H		Ikeya et al. [17]
Benzoylgomisin H		Ikeya et al. [17]
Tigloylgomisin H		Ikeya et al. [17]
Gomisin J		Ikeya et al. [25]
Gomisin K_1		Ikeya et al. [26]
Gomisin K_2		Ikeya et al. [26]
(+)-Gomisin K_3	schisanhenol, schizantherol	Ikeya et al. [26]
Gomisin L_1		Ikeya et al. [21]
Gomisin L_2		Ikeya et al. [21]
Gomisin M_1		Ikeya et al. [21]
Gomisin M_2		Ikeya et al. [21]
Gomisin N		Ikeya et al. [27]
Gomisin O		Ikeya et al. [18]
Angeloylgomisin O		Ikeya et al. [28]
Angeloylisogomisin O		Ikeya et al. [28]
Benzoylgomisin O		Ikeya et al. [28]
Benzoylisogomisin O		Ikeya et al. [28]
Epigomisin O		Ikeya et al. [18]
Angeloylgomisin P	schisantherin C	Ikeya et al. [19]
Tigloylgomisin P		Ikeya et al. [19]
Angeloylgomisin Q		Ikeya et al. [20]
Gomisin R		Ikeya et al. [24]
Gomisin S		Ikeya et al. [29]
Gomisin T		Ikeya et al. [29]
Schisantherin D		Ikeya et al. [24]
Pregomisin		Ikeya et al. [25]

of transaminase levels, in an increase of hepatic proteins and glycogen and in the hepatocyte membrane stabilization. The need of the Schisandra drug was so high that immediate research activities were initiated. A synthetic lignan analogue known as DDB (Xian Nong Tan Brand®) [31] is the most potent antihepatotoxic substance and has probably been used in the treatment of hepatitis B till nowadays. A great attention was also paid to gomisin A that inhibits experimentally induced liver damage, stabilizes cell membranes and seems to inhibit an acute hepatic failure as well [32]. It is recommendable as an antihepatotoxic agent for peroral administration, improves hepatic functions, prevents abnormal cell ploidization [33] and protects liver from the influence of deoxycholic acid, an endogenous risk factor for the development of hepatic carcinoma [34]. The lignan accelerates hepatocyte proliferation and renewal of liver functions after partial hepatectomy. It enhances hepatic blood flow and proliferation of endoplasmatic reticulum which finally results in a liver enlargement [35]. A basic pharmacological study has been reported for gomisin A [36]. Wuweizisu C exerts similar ef-

fects [37]. Schisandrin B can be used as a hepatoprotective drug decreasing toxicity of xenobiotic agents in organism with diabetic impairment [38]. The connection between immunity and liver diseases were proven previously [39,40]. Favourable effects of gomisin A that are given rather by its protective influence on the membranes of hepatocytes than by the direct lymphocyte activation and complement inhibition have been found in a model of immune liver damage [41]. At least a half of the published papers devoted to the lignans is concerned with their influence on hepatocytes. This effect has always been considered to be principal one and the relationships between the structure of lignans (effect of substituents in aromatic rings, configuration of the biphenyl moiety, influence of the C-6 and C-7 oxygen groups and other substituents in the molecule) and their hepatic effects were already defined 20 years ago [42]. Schizandrol A inhibits the activity of the central nervous system and contributes to the sedative effect of the drug; the inhibitory activity of the lignan in the brain relates to dopaminergic system [43]. Some gomisins exhibit antipyretic properties (gomisin A [44], H [44], J [45], N [46] and schisandrin [44]), analgetic effect (gomisin A and predominantly schisandrin) [47] and muscle relaxant activity (in this case gomisin A is more potent than schisandrin) [47].

Many of Schisandra lignans not only inhibited lipid peroxidation in cell membranes, but also reinforced the ability of the organism to eliminate reactive oxygen species by increasing superoxid dismutase (SOD) and catalase activities [48]. Their activity seems to be comparable to that of vitamin E, especially in liver microsomes where schisanhenol was most potent [49]. Free radical scavenging acitivity (FRSA) of schisandrol A and schisandrin B for the hydroxyl radical and superoxide anion was compared with that of vitamin E and C. Their efficiency for the hydroxyl radical was higher than that of the two vitamins, for the superoxide anion they were more potent than vitamin E [50]. According to literature FRSA against oxygen radicals is dependent on the configuration of biphenyls; Iignans with *S*-configuration have better activity than those with *R*-configuration [51]. Gomisin C inhibits the respiratory burst in rat neutrophils [52]. Constituents of Schisandra berry in the above-mentioned Sheng Mai San contribute to the myocard protection due to their antioxidative properties [5]. This effect of the preparation was demonstrated both in vitro and in vivo [53,54].

Anti-asthmatic effect relates to PAF activity: high potency was shown with lignans lacking ester group on C-6, hydroxyl on C-7 or methylenedioxy grouping and with *R*-configuration of biphenyl. 6(7)-dehydroschisandrol A exhibited the highest activity [55]. Schisandrin A having IC_{50} of 1.7×10^{-5} M proved to be the strongest antagonist at PAF receptors (IC_{50} of ginkgolide $B = 1.9 \times 10^{-7}$ M) [56].

Evaluation of the direct adaptogenic effect, i.e. the influence of compounds on the hypothalamus–pituitary–adrenocortical axis (HPA), can be complicated by a number of experimental problems. Though experimental models for studying adaptogenic properties do exist, many authors accept the results with some scepticism. The models are complex and do not cover general adaptation syndrome as a whole. Presently, a view is held that adaptogenic activity should be evaluated in relation to other biological effects of administered drugs, not only as the influence on the HPA. One of suitable models for the evaluation of adaptogenic activity of Schisandra berry (in the form of a standardized extract from fruits) consists in the monitoring of NO concentration in saliva and can serve for both the evaluation of physical burden on the organism and expression of preventive effects against stress [57]. Compounds with adaptogenic properties can among other things improve resistance against cardiotoxic effects of some antineoplastic agents, e.g. adriamycin (mixture of lignans) [58] or puromycin (gomisin A) [59]. Gomisin A (and also gomisin J and wuweizisu C) prevented mutagenic effects of 12-*O*-tetradecanoylphorbol-13-acetate [60,61].

The lignans of Schisandra berry exhibit also influence on various enzyme systems. They inhibit aldose reductase and can be effective in the treatment of diabetes [62]. They can also modulate metabolism of cholesterol and lipids by their inhibitory effect on the acylCoA: cholesterol acyltransferase (ACAT); gomisin N was the most potent inhibitor of the enzyme (IC_{50} = 25 mM) [63]. A number of lignans can inhibit 5-lipoxygenase in therapeutically applicable doses and exhibit anti-inflammatory activity [64,65]. Important anti-inflammatory properties were recorded for gomisin A [66].

4. Isolation

As Schisandra berry has not been used as a drug any more and serves presently only as a raw material, the need of an effective procedure for the isolation of active constituents emerged. Four major factors must be considered to propose the optimal separation conditions:

- physico-chemical properties of lignans,
- character of the raw material and spectrum of active constituents and their content in it,
- methods of isolation,
- other components and their amount in the raw material.

4.1. Physico-chemical properties of lignans

Dibenzo[*a*,*c*]cyclooctadiene lignans of Schisandra berry are colourless, odourless, usually crystalline substances (approximately 50 lignans have been isolated from the taxons of the Schisandra genus so far). They form either prisms (schisandrin) or needles (gomisin F). Sometimes they separate from solutions as amorphous powders (tigloylgomisin P, angeloylgomisin P). Their melting points are in the range of 80–190 °C. Sometimes diethyl ether can be bound in crystals (gomisin B: $C_{28}H_{34}O_9 \cdot 1/2C_2H_5OC_2H_5$) [12]. They are optically active. Generally, they belong to compounds with low to medium polarity. For their crystallization, mixtures of solvents, such as diethyl ether–*n*-hexane (gomisin B) or

diethyl ether–acetone (gomisin G), are suitable. It is also possible to use methanol (gomisin A). Solubility of the compounds depends on the presence of free oxygen functions (especially phenolic hydroxyls). Binding of these substituents in the methylenedioxy group and/or esterification of the tertiary alcoholic group (benzoyl-, tigloyl-, angeloyl-) results in the increase of lipophilicity. In the complex mixture individual lignans influence solubility of one another and often cannot been separated by means of solvents with graduated polarity. Two aromatic systems form a series of conjugated double bonds, and the compounds therefore absorb UV light (λ_{max} of major lignans is ~220 nm at $\varepsilon \sim 80{,}000$).

4.2. Content of lignans in plant organs

The major lignans are usually obtained from the fruits of plants imported from China. Locally, a drug of the Japanese, Korean or other origin can be used. There is only a subtle morphological difference between the Beiwuweizi and Nanwuweizi (two different taxons and processing), but the two drug have different spectrum of lignans. The drug from *S. chinensis* contains predominantly schisandrols A and B, gomisins B and C, schisanhenol, deoxyschisandrin, γ-schisandrin and schisandrin C. As much as 20% of the lignans were found in the drug from *S. chinensis* [67]. However, the data were published in a local journal original of which is not available and cannot be critically reviewed. Whilst the profile and content of the lignans in the drugs from *S. chinensis* are relatively stable, the drugs from *S. sphenanthera* may significantly differ from one another regarding the composition and content of the lignans [68]. Hence, the drug of Chinese origin seems to be the best one. The drugs of European origin contain lower amounts of lignans. The content of five major lignans, schisandrin, gomisin A, deoxyschisandrin, gomisin N and wuwеizisu C (Fig. 2), was monitored in the samples of seeds from various geographic regions (the Czech Republic 5, Austria 1, Germany 1, Poland 1, Estonia 2, Ukraine 1, Russia 2) in 1992–1995. The samples obtained from natural populations of the Russian Far East contained in average 3.33% of the lignans. The average value for all remaining samples was 2.90% of lignans [69]. Similar results yielded an analysis of the Japanese (Korean and Tai-wan) drugs [70]. The results of the analyses performed with samples of various morphological parts (defoliated cauloms, leaves and seeds) of *S. chinensis* grown in the Northern Bohemia (six year culture, collected 1994), in which the content of six major lignans (schisandrin, gomisin A, deoxyschisandrin, γ-schisandrin, gomisin N, wuweizisu C) was determined, were comparable with above-mentioned data: in average the seeds contained 2.294%, cauloms 0.480% and leaves 0.201% of the lignans [71].

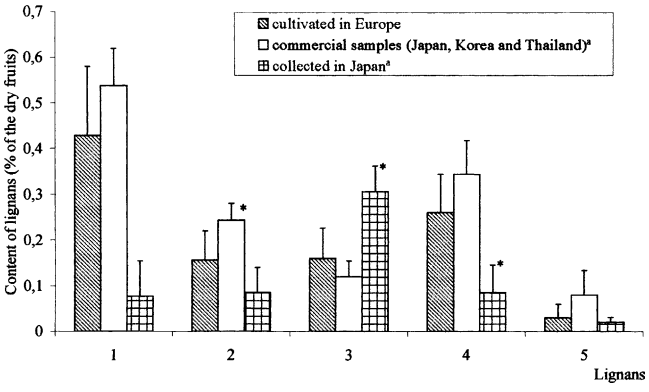


Fig. 2. The contents of lignans 1–5 in the fruits of *S. chinensis* cultured in Europe and in Japan, Korea and Thailand [69]. Lignans: schizandrin (1), gomisin A (2), deoxyschizandrin (3), gomisin N (4), wuweizisu C (5). Number of samples per group: cultured in Europe 7, commercial 13, collected in Japan 8. (a) Values determined by K. Nakajima [70]. (b) Significant difference with respect to the fruits cultured in Europe; $p < 0.01$ (Student's *t*-test).

4.3. Isolation procedures

Extract for the isolation of lignans can be prepared by means of two procedures: classical extraction with a suitable solvent(s) or supercritical fluid extraction. The major problem during the isolation process from both generative parts and defoliated cauloms represents the presence of lipophilic components (higher aliphatic hydrocarbons, fatty acids and their esters). Leaves are not usable for this purpose as vegetative organs contain also quercetin, kaemferol and (*E*)-cinnamic acid. The content of these compounds in leaves is substantially higher than in cauloms (Table 2), which poses a problem in the separation process. Another complicating factor is the presence of pigments commonly occurred in photosynthesizing organs (chlorophylls and their degradation products and carotenoids). Besides the content of major lignans is too low.

4.3.1. Classical extraction ("wet way")

The seeds (or fruits) are surely the most suitable morphological part for the isolation. In comparison with seeds, fruits contain small amounts of pigments and significant amounts of organic acids (a mixture of tartaric, citric, succinic and malic acid). The content of citric and malic acid is almost as high as that in lemon [73], nonetheless, their presence causes no trouble. The Japanese autors [12] used a standard procedure by means of which they obtained practically all Schisandra lignans. dried fruits were ground and extracted with petroleum ether, the residue was then re-extracted with hot methanol. Petroleum ether was evaporated and then the extract was distilled with steam (to remove terpenic components), the residue was extracted with diethyl ether and the solvent evaporated. Methanolic extract was evaporated, dissolved in water and extracted with ethyl acetate. The solvent was then removed, the residue dissolved in methanol and submitted to chromatography on Celite 535 (*n*-hexane, dichloromethane and methanol). Hexane eluate was added to the diethyl ether fraction obtained from the petroleum ether extract after the steam distillation and the resulting mixture was separated

Table 2
Analysis of major compounds in leaves and caulomas of *S. chinensis* (Turcz.) Baill. [72]

Part of plant	Harvest (year)	Content[a] (mg/g d.w.) ± S.D. (n = 9)		
		Quercetin	Kaempferol	*E*-cinnamic acid
Leaves	1997	1.535 ± 0.027	0.359 ± 0.009	3.824 ± 0.069
	1998	1.140 ± 0.038	0.268 ± 0.004	3.131 ± 0.020
	1999	1.294 ± 0.049	0.298 ± 0.005	2.515 ± 0.041
Caulomas	1997	0.341 ± 0.01	Not detected	0.116 ± 0.002
	1998	00.303 ± 0.006	Not detected	0.099 ± 0.002
	1999	0.389 ± 0.004	Not detected	0.260 ± 0.003

[a] Content is average from three individuals, each injected in triplicate; d.w.: dry weight.

on silica gel, using binary mixtures of solvents with increasing polarity (*n*-hexane–benzene, benzene–acetone). Repeated chromatography then yielded individual lignans. The procedure is simple and effective, but requires a lot of work and time. The original efforts to separate lignans after a simple removal of lipids with petroleum ether failed: although lignans themselves are not soluble in petroleum ether the presence of additional compounds has solubilizing effect. Besides lipids, content of which is ∼30%, the separation process can be influenced with terpenic volatile oils (the content in seeds is ∼2.7%) [10]. These must then be removed by steam distillation that has no significantly harmful effects on lignans. On the basis of these facts it would seem reasonable to extract the drug directly with hot methanol, which would simplify the extraction process [71]. The lipophilic substances would then be removed in the first fractions of the chromatography on silica gel and would not further interfere with the isolation of lignans [12]. Minor lignans can be isolated from the enriched fractions (column chromatography on silica gel) by TLC separation, using various mixtures of eluents, especially benzene - diethyl ether, *n*-hexane–diethyl ether, *n*-hexane–acetone, *n*-hexane–ethyl acetate and chloroform–ethanol [16,17,19].

The processing of defoliated cauloms is more difficult. They contain only ∼0.30% of volatile oils [71] but the profile of secondary metabolites is more complex: in addition to kaempherol and (*E*)-cinnamic acid, *β*-sitosteroI, 24-methylencycloartanon and especially aliphatic hydrocarbons (C-20 to C-30), higher fatty acids (C-15 to C-30) and their methyl and ethyl esters are present [71]. In this case, nonpolar substances could not been removed at the beginning of chromatography (using light petroleum + chloroform). They are eluted throughout the chromatographic process together with lignans and other metabolites [71]. Pre-concentration of extracts using other cheap stationary phases, e.g. polyamide for the separation of phenolic substances was not successful, since the lipophilic extracts are only partly soluble in water that is normally used for the chromatography on polyamide. The procedure does not enable to separate the lignans lacking free phenolic groups, such as gomisin A, gomisin N, deoxyschisandrin, schisandrin, wuweizisu C and (±)-γ-schisandrin that belong to important active constituents.

Hence, there are three possibilities of obtaining lignans in a larger scale. The first consists in the saponification with alkali hydroxide. According to Kochetkov et al. [13], the seeds were extracted with petroleum ether, the dried extract dissolved in methanol, dried and dissolved again in 85% aqueous methanol. On the basis of paper chromatography, it was found that all schisandrin and almost all other lignans named by the autors as α-, β-, γ-, δ- and *ε*-schisandrins and schizandrol dissolved. Only negligible amounts of α- a β-schizandrin remained undissolved. The dissolved fraction was saponified with potassium hydroxide solution and after additional processing it yielded a yellow substance that was divided into nine fractions by the column chromatography. From the fractions obtained, the major lignans schizandrin, schizandrol a γ-schizandrin were isolated. The name γ-schizandrin was used by these authors for the first time, whilst one of the first reported lignans, schizandrin, was named by Balandin [74]. The non-saponified fraction can be chromatographically processed easier than the original extract. However, the procedure is only suitable for obtaining concentrate of lignans (approximately 15 compounds) in which hydroxyls in cyclooctane ring are not esterified. Acetal as well as ether group in the aromatic part of the skeleton are stable in the alkaline environment. From the practical point of view, the procedure may be suitable, since none of the major and required lignans has an ester group. The procedure for the purification of schisandrin in kilogram amounts was proposed by Tsumura company: the extract of fruits was saponified with water–alcoholic solution of potassium hydroxide. After removing methanol, the mass was washed with aqueous potassium hydroxide solution and re-crystallized from the mixture acetone–ethyl acetate–*n*-hexane. The purity of the resulting product was minimum 99% [75].

The second method includes, extraction of the ground plant material with hot methanol, concentration and dilution with water. The mixture is then eluted with water and organic solvents on the column of Diaion HP-20 and fractions with lignans are separated. The divinylstyrylbenzene resin is commonly used for the SPE separations of hydrophobic compounds, for desalination and isolation of fungi and plant metabolites. Nonetheless, the direct application of an extract with a certain content of lipid substances and the following elution with water can increase dispersion of lipids on the

grains of adsorbent. The method has been applied for the isolation of schisandrin [76].

The third method concerns the preparation of gomisin A from the raw extract of Schisandra cauloms and roots. The original extract was obtained in a classical way with solvents and after pre-separatory adjustment separated by means of preparative HPLC. Reportedly, gomisin A can be obtained in a large scale [77]. Using the modified HPLC (flash method) for the isolation of natural products is presently quite common and accessible from the financial point of view.

4.3.2. Supercritical fluid extraction (SFE)

SFE has potential as an alternative to the use of organic solvents for the extraction of biologically active components from natural products. At present SFE is in practice almost exclusively oriented on the use of pure or modified carbon dioxide. SFE is preferable to Soxhlet extraction or sonication in those cases in which the resulting recoveries of target analytes are comparable with those achieved by a classical method. SFE offers a cleaner extract, containing a low content of ballast compounds, gained in a significantly shorter extraction time. As SFE utilizes no or only minimal amount of an organic solvent (such solvent, the modifier, being typically used to increase the solubility of more polar compounds that are insoluble in non-polar CO_2) for extraction, a more environmentally friendly extraction process results. This important difference from traditional organic solvent approaches is beneficial both at analytical scale and more importantly at the pilot plant or industrial scale where it could provide economic advantages.

The efficiency of SFE *of S. chinensis* lignans depends particularly on the type of matrix. The effect of extraction conditions at pressures above 20 MPa and at temperatures 40–80 °C on extraction rate is not very pronounced [78]. Recovery of lignans from seeds extracted with pure CO_2 was 96–98% after 60-min of extraction [69,79]. Similarly, 91% of lignans [80] or 80% of lignans [78,81] were obtained from fruits and more than 90% of lignans were obtained from cauloms [81] after only 30 min of extraction. On the other hand, lignan recovery from leaves was only 26% with pure CO_2 after 60-min of extraction [79]. When ethanol modifier was added to carbon dioxide in volumetric concentration up to 10% with the aim to overcome this difficulty [81], the recovery of lignans from leaves increased substantially to 87%, while no increase in extraction yield from fruits and cauloms was observed in the course of extraction. In another work [82], however, no increase in lignan yield from leaves after CO_2 modification with ethanol was observed.

5. Chromatographic methods

The work was aimed at elaborating reproducible procedures for the evaluation of drug samples and methods enabling correct and accurate determination of their constituents not only in extracts and pharmaceutical products but also in biological (animal) materials. It is important to minimize operations during the sample adjustment, since each such an intervention decreases the accuracy of the results. The papers accessible with difficulties due to the language barriers were cited through the review "*S. chinensis*: Chemistry and Analysis" published recently by Wang et al. [83], which mediates knowledge of East-Asian literature on this topic.

5.1. TLC—chromatographic fingerprint

Qualitative TLC was applied to elaborate the fingerprint of Schisandra fruit [2]. Silica gel 60 F_{254} Merck, toluene–ethyl acetate–glacial acetic acid (70:33:3, v/v/v) was used for the analysis. 20 μl of methanolic extracts (corresponding to approximately 7 μg of the drug) were applied. The plates were detected in UV 254 nm, UV 366 nm and with *p*-anisaldehyde + sulphuric acid reagent. Five major lignans (gomisins A and C, deoxyschisandrin, γ-schisandrin and schisandrin) are detectable in UV 254 in the presence of a fluorescent indicator. With *p*-anisaldehyde they give multi-coloured spots after heating (∼5 min at 100 °C). The method cannot be used for the evaluation of cauloms, defoliated cauloms and leaves. Vegetative morphologic parts contain relatively big amount of other secondary metabolites and have much lower content of lignans. Hence, the detection is not sufficiently conclusive. Coloured pigments and flavonoids can be removed on a pre-separatory column with the neutral aluminium oxide. Reading of the resulting chromatogram is then substantially easier. Nonetheless, such a method cannot be used for the characteristic fingerprint. Though some lignans have free phenolic groups, their acidic properties are not important under common conditions. Eluents without addition of an acid (benzene or *n*-hexane–ethyl acetate or *n*-hexane–acetone in various ratios) can be successfully used for preparative separations. UV light, preferably in combination with a fluorescent indicator added to the adsorbent, is most suitable means of detection. This non-destructive method with neutral solvent solvents enables transition to preparative TLC.

Suprunov and Samoilenko [84] developed a thin layer chromatography and colorimetric method to determine the more polar major lignans, schizandrin and schizandrol (gomisin A) in ethanolic extracts from *S. chinensis* fruit or seed. The extract was after evaporation of a part of ethanol mixed with Al_2O_3, dried and eluted with chloroform using an Al_2O_3 column. Sample of the solution was spotted on silica gel plate and eluted with ethyl acetate-petroleum ether (1:1, v/v). The spots of schizandrin and schizandrol (gomisin A) were removed from the plate, extracted with chloroform, treated with concentrated H_2SO_4 and acetone and measured using a dark violet filter.

Zhu et al. [85] applied a simple, rapid and accurate method for TLC-densitometric detection of schisandrol A and B (gomisin A and schizandrin) in Sheng Mai San. The lignans

were extracted with ether at room temperature. The sample solution and that of mixed authentic standard lignans were spotted on silica gel plate and developed with toluene–ethyl acetate (6:4, v/v). The separated spots were scanned, using Shimadzu TLC Scanner. Another TLC densitometry method to analyze lignans in Schisandra was published by Wang et al. [86]. The samples were extracted with *n*-hexane, the hexane extracts were then evaporated to dryness and the samples were re-dissolved in methanol and spotted on a silica gel GF254 plate. They were developed with toluene–ethyl acetate (9:1, v/v) for schisandrin A, B and C, with toluene-ethyl acetate (4:6, v/v) for schisandrol A, schisandrol B, wuweizi ester and gomisin K_3. Zhao et al. [87] reported another TLC method to determine quantitatively schisandrin B ((±)-γ-schizandrin). The sample was first extracted with ether under reflux, dried to dryness and re-dissolved in methanol. The methanol extract passed through a neutral Al_2O_3 column and was spotted on a silica gel GF_{254} plate, which was developed with petroleum ether–ethyl formate–formic acid (15:5:1, v/v/v).

Ikeya et al. [16,17,19] applied preparative layer chromatography to purify concentrated fractions obtained by column chromatography and isolated minor lignans. The plates were 20 cm × 20 cm, 0.75 mm thick, coated with Kiesegel PF_{254} (Merck).

5.2. HPLC—chromatographic fingerprint and other separations

This method is decisive for the evaluation of the quality of Schisandra drugs. Whilst the results of TLC are not always conclusive, HPLC gives better evidence, since the evaluation from the quantitative point of view can be simultaneously done. LiChrospher© 100 RP 18 Merck (5 μm) was used for both pre-separation and separation. For analysis gradient elution [A: water-1% 0.1 NH_3PO_4, (v/v)], [B: acetonitrile + 1% 0.1 NH_3PO_4, (v/v)] was applied. Linear gradient system was 40% B to 80% B within 20 min. 2 μl of basic methanol extract were applied to the column and detection was performed with photodiode array detector (λ = 210 nm) [2]. Samples from various localities can be very precisely differentiated using this method.

In Fig. 3 it can be seen, that the Japanese sample is characterized by schisandrol A (peak 1), schisandrol B (peak 2), schisandrin A (peak 5) and schisandrin B (peak 6). Schisantherin A (peak 3) and schisantherin B (peak 4) are present only in lower concentrations. The chromatogram in Fig. 4 differs from that in Fig. 3. Polar lignans (R_t < 10 min) schisandrol A/B and schisantherin A/B were not detected, schisandrin A and B are missing; chromatogram is characterized by the major peaks at R_t 10–16 min. Hence, for the HPLC fingerprint analysis of *S. chinensis* (Beiwuweizi) the lignans schisandrol A and B as well as schisandrin A and B are best suited for identification, whereas schisantherin A and B are less typical because of their very low concentrations. *S. sphenanthera* (Nanwuweizi) can be differentiated from *S. chinensis* by the

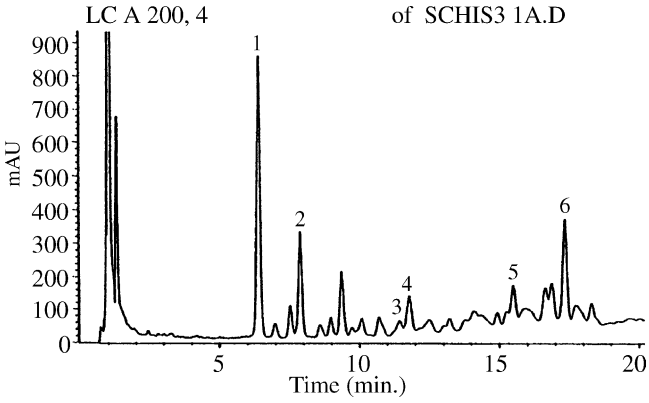


Fig. 3. HPLC fingerprint analysis of a commercial drug sample from Japan (*S. chinensis*) [2]: schisandrol A (1), schisandrol B (2), schisantherin A (3), schisantherin B (4), schisandrin A (5) and schisandrin B (6).

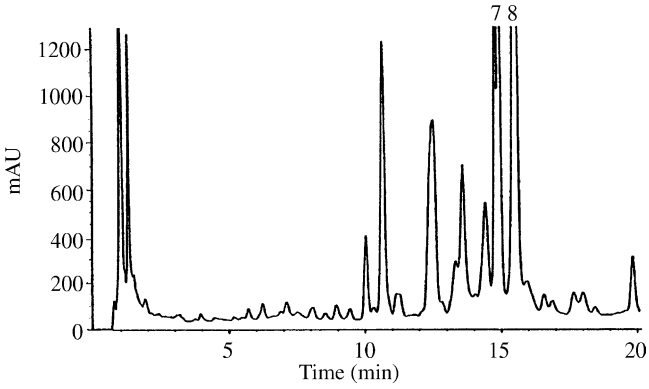


Fig. 4. HPLC fingerprint analysis of a drug sample of Chinese origin (Sichuan Province); (*S. sphenanthera*) [2].

deficiency of schisandrol A/B, as seen in the HPLC and TLC-chromatograms, respectively [2]. Comparing mobile phases used for the analytical HPLC shows that autors almost always used reversed-phase mode (RP-HPLC) with the bonded phase of octadecylsilane (C18) and detection in UV λ = 254 nm. The mobile phases are formed with a mixture of methanol–water or acetonitrile-water, the former being more usual. The mixture methanol–acetonitrile–water was used less frequently. With regard to the polarity of the lignans, the procedure is quite understandable.

5.2.1. RP-HPLC with methanol–water mobile phase

Wang and Li [88] published an HPLC method to separate wuweizisu C, schizandrol A, schizandrol B, schisandrin A and schisandrin B. Detection was at 254 nm. With a mobile phase methanol–water (77:23, v/v) good separation was achieved. Zhu et al. [89] applied an HPLC method to separate six active lignans (schizandrin, wuweizichun B, wuweizi ester A, wuweizi ester B, deoxyschizandrin and γ-schizandrin) in Sheng Mai San and for determination of two major lignans, schizandrin and wuweizichun B. They used a column

packed with YWG C18 (10 μm), methanol–water (72:28, v/v) as mobile phase and detection at 254 nm. They found that peaks of the compounds from other two plants contained in the preparation do not interfere with the peaks of lignans and thus the assay provides a rapid method for the quality control of Sheng Mai San. A similar method was reported by Tong and Song [68] who used 77% methanol as mobile phase to determine lignans in the fruits of *S. chinensis* and *S. sphenanthera*.

Lignans in *S. chinensis* seeds were determined using a HPLC system with Separon SGX C18 (5 μm) column by Lojková et al. [79], Slanina et al. [69] and Kvasničková et al. [90]. Samples were prepared by extraction with supercritical CO_2, dissolved in either double distilled water or methanol and a sample loop of 20 μl was used for sample injection. The mobile phase was methanol–water (75:25 v/v) or (73:27, v/v). Five major lignans (schizandrin, gomisin A, gomisin N, deoxyschizandrin and wuweizisu C) were detected at 254 nm and identified by comparing their retention times with those of standards and on the basis of co-injection of individual standards with the analyzed sample. The signals were evaluated using the method of absolute calibration with a set of solution in the concentration range 0.01–0.17 mg/ml [69]. A linear relationship of the concentration and peak area was obtained for all lignans at the concentration range studied.

The reversed-phase HPLC method on the C18 bonded phase with mobile phase consisting of the mixture methanol–water and with the detection at λ = 254 nm was also applied for the quantitative determination of γ-schizandrin in Schisandra syrup [91]. The samples were concentrated, cleaned and dissolved in methanol before the analysis. The average recovery was 99.38% with R.S.D. of 0.92%.

5.2.2. RP-HPLC with mobile phase containing acetonitrile

Five major lignans, schizandrol A, schisandrol B, schisandrin A, schisandrin B and schisandrol C, were separated on column filled with a YMC-Pack ODS-A (5 μm) in the works of Choi et al. [78] and Kim et al. [81]. The samples were CO_2 extracts from different parts of *S. chinensis*, dissolved in methanol. Isocratic elution was performed with acetonitrile–water (60:40, v/v) at a flow rate of 1.0 ml/min and monitored at λ = 240 nm. Dean and Liu [80] used an HPLC system containing a C_{18} ODS2 column and a UV–vis detector to separate and quantify deoxyschisandrin in CO_2 extract from *S. chinensis* fruit dissolved in methanol. Flow rate of the mobile phase, acetonitrile–water–acetic acid (70:29:1, v/v/v), was 1 ml/min. Detection was at 254 nm. Linear calibration graphs were produced for the lignan over the concentration range 0.43–43 μg/ml and gave a correlation coefficient of 0.9999 (n = 6).

Wang et al. [83] developed and validated a reverse phase HPLC method to analyze the lignans in methanolic extracts from Schisandra fruits. A pre-packed 250 mm × 4.6 mm i.d. (5 μM particle size) Luna C18 column (Phenomex, Torrance, CA) was selected for HPLC analysis. The absorption spectra were recorded from 200 to 400 nm for all peaks; quantification was carried out at a single wavelength of 255 nm. The mobile phase included water (containing 0.1% formic acid, solvent A) and acetonitrile (solvent B) in the following gradient system: initial 45% B, linear gradient to 60% B in 12 min, hold at 60% for 12 min, linear gradient to 90% B in 16 min and linear gradient to 100% B in 5 min. The total running time was 45 min and the post running time was 12 min; flow rate was 1 ml/min. Four reference standards, schisandrol A, schisandrol B, schisandrin A and schisandrin B, were used. The method showed excellent linearity, accuracy and precision.

Bártlová et al. [82] tested two chromatographic systems and three gradient systems to separate and quantify six lignans (schizandrin, gomisin A, deoxyschizandrin, γ-schizandrin, gomisin N and wuweizisu C) in CO_2 extracts from cauloms and leaves of *S. chinensis*. UV detection was at λ = 254 nm and a mixture acetonitrile (A)–water (B) was applied as a mobile phase in gradient systems. In the first chromatographic system, the column was filled with Nucleosil 100 5 μm C18; mobile phase flow rate was 0.75 ml/min. Splitting of the peaks occurred occasionally when the first gradient system (50% A for 5 min, gradient to 60% A in 30 min, gradient to 70% A in 20 min and 70% A isocratic for 15 min) was applied. Using the second gradient system (70% A for 1 min, gradient to 95% A in 34 min and 95% A isocratic for 5 min) the resolution of γ-schisandrin and gomisin N was slightly worse, but no peak splitting occurred. Resolution of lignan peaks and the number of theoretical plates of the column were evaluated for both gradient systems. In the second chromatographic system, the column was MERK-Lichrospher 100 RP 18 (5 μm), flow rate was 0.5 ml/min and the gradient system was 40% A to 70% A in 60 min, consecutively 70% A for 30 min. The results obtained using both chromatographic systems were comparable.

5.2.3. RP-HPLC with mobile phase combining methanol and acetonitrile

Nakajima et al. [70] separated and quantitatively analysed lignans in the fruits *of S. chinensis*, using a rapid reversed-phase HPLC stationary phase. The column was filled with bonded phase octadecylsilane Bondapak C_{18} (10 μM m) and two mobile phases, acetonitrile–methanol–water (11:11:16, v/v/v) and (10:10:10, v/v/v), were applied, the first one for 9 min and the second one for 30 min, with flow rate of 1 ml/min. Lignans were detected at 254 nm. Ten lignans were separated and identified (schizandrin, gomisin D, gomisin J, gomisin A, angeloylgomisin H, gomisin G, deoxyschizandrin, (±)-γ-schizandrin, gomisin N and wuweizisu C). The average recoveries were 96.9% for gomisin A, 102.2% for deoxyschizandrin and 98.7% for wuweizisu C. The method was later used by Zhang et al. [92] with the difference that the first mobile phase was applied for 12 min. The average recoveries of 93.3% for schisandrin A, 99.1% for schisandrol

B and 101% for gomisin N are reported. The method was applied also in the next work [93] to separate and quantify eleven lignans in the decoctions from *S. chinensis* fruits. The first mobile phase was applied for 10 min and the total separation was 42 min. The lignans were identified as schizandrin, gomisin A, angeloylgomisin H, gomisin G, deoxyschizandrin, (±)-γ-schizandrin, gomisin N and wuweizisu C and quantified comparing their peak areas with those of standards. The remaining three lignans were biphenyl lignans belonging to the same group as the other eight lignans.

5.2.4. HPLC in normal-phase mode

Adachi and Isobe [94] separated lignans from the fruit-extract *of S. chinensis* by preparative chromatography in the normal-phase mode. Almost identical chromatographic profiles were obtained using polymethacrylic adsorbent with particle sizes 5, 17 and 31 μm. The adsorbent with particle size of 31 μm was used in preparative chromatography and the separation was carried out at a flow-rate of 4.7 ml/min with an eluent *n*-hexane–ethanol (90:10, v/v). Injection volume of the sample in concentration 20–400 mg/ml was 1280 μl and the wavelength of UV detection was 254 nm. It was proved that the separation of schizandrin at the maximum sample concentration corresponding to the loading volume of 3.3 g/1 adsorbent was still sufficient for high purity separation.

5.2.5. Countercurrent chromatography

This method was naturally used only for the isolation of schisanhenol and schisanhenol acetate from the ethanolic extract of the *Schisandra rubriflora* seeds [95]. The solvent mixture *n*-hexane–ethyl acetate–methanol–water (10:5:5:1, v/v/v/v), flow rate 2 ml/min, with a table-top model high speed planner centrifuge equipped with a multilayer coiled column were used, as described in detail by Ito [96]. The report is sporadic and though the method was later coupled with thermospray MS, it has never been used for the isolation of other Schisandra lignans.

6. Electromigration methods

Kvasnickova et al. [90] developed a capillary electrochromatography (CEC) method using macroporous polyacrylamide monolitic columns and applied it successfully to analyze and quantify schizandrin, gomisin A, gomisin N and wuweizisu C in the extract from *S. chinensis* seeds. The columns were prepared by in situ copolymerisation of acrylamide, *N*,*N*′-methylenebisacrylamide, vinylsulfonic acid and lauryl acrylate in presence of polyethylene glycol as a porogenic agent. The polymer monolitic stationary phases represent prospective separation media for capillary electrochromatography as the tedious packing of particles into narrow capillaries and the fabrication of frits is avoided. Good separations were achieved in less than 35 min. The calibration graphs were linear in the range 0.025–1.0 mg/ml of given lignan with correlation coefficients higher than 0.995. The inter-day reproducibility of the peak area was below 3.9% and the inter-day reproducibility of the migration time were below 4.2%. The results of quantitative CEC analyses were in a good agreement with those determined by HPLC.

Sterbova et al. [97] published a micellar electrokinetic capillary chromatography (MEKC) as a promising method for the determination of lignans in plant samples. The separation conditions were optimized with respect to different parameters, including sodium dodecyl sulfate (SDS) and acetonitrile concentration, pH of the background electrolyte, separation voltage and capillary temperature. The background electrolyte consisting of 40-mm SIDS and 35% acetonitrile in 10-mm tetraborate buffer (pH 9.3) was found to be the most suitable electrolyte for this analysis. The applied voltage of 28 kV (positive polarity) and the capillary temperature 25 °C gave the best separation of lignans. The inter-day reproducibility of the peak areas and the migration times was below 2.0%. The results of MEKC analyses were compared with those obtained by CEC HPLC. The possibilities of using this method for the determination of lignans in drug and in serum samples were also tested.

7. Hyphenation procedures

7.1. Hyphenated GC separations

Sohn et al. [98] used a gas chromatography-mass spectrometry (GC/MS) method to identify lignans in *S. chinensis*. They used a SPB-1 fused silica capillary column; the column temperature was 200–300 °C at the rate 4 °C/min and the mass ionization voltage was 70 eV (EI mode). A total of 11 lignans including gomisin J, schisandrin A, gomisin N, schisandrol A, schisandrin C, schisandrol B, angeloylgomisin H, tigloylgomisin H, angeloylgomisin Q, schisantherin B and benzoylgomisin have been detected. Only seven of these lignans, however, could be detected, using the same separation method with FID detector [99]. Matsuzaki et al. [100] determined gomisin A and its major metabolite (Met. B) in rat serum. A highly sensitive and precise method for the determination was developed by selected ion monitoring (SIM) with GC/MS using a fused silica capillary column (SPB(TM)-1, Supelco. The calibration curves of gomisin A and Met. B both showed a good linearity between 2.0 and 2000 ng/ml. The analytical precision (intra-assay, C.V. <4.7%), recoveries (98.4 ± 10.1%) and detection limit (2 ng/ml) of gomisin A indicated that this system was suited for its determination in biological fluid. The same results were obtained for Met. B. The method was applied later for the determination of schizandrin in human plasma [101]. A 0.1-ml plasma sample was used for solid-phase extraction. A good linear relationship was obtained in the concentration range studied (2.0–500 ng/ml) and the method was sufficiently

accurate and precise to support clinical pharmacokinetic studies.

7.2. Hyphenated HPLC and other separation methods

Lee et al. [102] applied countercurrent chromatography with a newly developed analytical high-speed centrifuge system coupled with thermospray MS to identify and validate lignans from a crude extract of *Schisandra rubriflora*, another *Schisandra* species used in traditional Chinese medicine for treatment of hepatitis. The CCC/UV chromatograms showed four distinct peaks. Both negative and positive ion detection, using ion evaporation ionization and filament on chemical ionisation, were employed for the analysis and six lignans, including pregomisin, schisanhenol and deoxyschizandrin, were tentatively identified. He et al. [103] applied an electrospray high-performance liquid chromatography–mass spectrometry interface coupled with a photodiode-array detector to identify 15 lignans from ethanolic extract of the fruits of *S. chinensis*. They used a Prodigy ODS (5 μm) column and gradient method with mobile phase methanol–water. Gradient elution was with 60 to 100% methanol in 15 min, 100% methanol for 5 min and 100–60% methanol in 5 min; the flow rate was 0.2 ml/min. Six peaks were unambiguously identified as schizandrin, schisantherin A, schisantherin B, schisanhenol, deoxyschizandrin and (-schizandrin, based on their abundant $[M + H]^+$, $[M + Na]^+$ ions, UV spectra and retention times, compared with those of reference compounds. Another nine peaks were tentatively identified, based on their intense $[M + H]^+$ and $[M + Na]^+$ ions and UV spectra. Liquid chromatography–electrospray–mass spectrometry (HPLC–ES–MS) was proved to be a powerful tool for direct, on-line qualitative identification of previously known compounds in plant extracts.

Wang et al. [83] analyzed lignans in Schisandra fruits using an LC/MSD system. The mobile phase was water (containing 0.1% formic acid, solvent A) and acetonitrile (solvent B) in the following gradient system: initial 45% B, linear gradient to 60% B in 12 min, hold at 60% for 12 min, linear gradient to 90% B in 16 min and linear gradient to 100% B in 5 min. The total running time was 45 min and the post running time was 12 min; flow rate was 1 ml/min. A 2 to 1 stream splitting was used for the electrospray mass spectrometer operated under positive ion mode. Total six major lignans (schisandrol A, schisandrol B, angeloylgomisin H, schisandrin A, gomisin N and schisandrin B) were tentatively identified by comparing the UV and MS spectra with the reference standards and by their $[M + 1]^+$ and $[M + Na]^+$ ions.

8. Conclusion

Presently, it is clear that the seeds are most suitable material for the isolation of dibenzo[*a*,*c*]cyklooctadiene lignans from Schisandra, since, compared to other morphological parts, they have the highest content of these substances and the lowest amount of impurities. The papers published during the last 40 years show that approximately six major lignans are of practical importance from the total number of ∼40. These include schisandrin, deoxyschisandrin, γ-schisandrin, gomisin A, gomisin N, wuweizsu C. For the isolation, sequential extraction with petroleum ether and methanol, evaporation of solvents, dissolution of the combined extracts in water, filtration and sorption on the ion exchanger (Diaion HP20) seems to be most suitable. After the elution of ballast substances mixture of lignans is separated by means of preparative HPLC (in the case of minor lignans) or flash chromatography, that is suitable for the concentrates enriched with a major lignan. The isolation of minor lignans (concentration of which is around 0.001%) is only achievable using sequential column chromatography (CC)-preparative TLC-preparative HPLC. The separation can also be successful when countercurrent chromatography (true CCC) is used. In 1980s, CCC became very popular for separations of natural products and many highly effective procedures eliminating formation of artefacts (e.g. eliminations and rearrangements due to bonding to adsorbents) were developed. In spite of the advantage, the method is used only rarely nowadays. If only petroleum ether extract is used for isolation of lignans, separation is easier, since impurities are present in minor amounts. However, our experience shows that maximum percentage of the total extractive amount of lignans comes to petroleum ether. Saponification of petroleum ether extract with methanolic solution of potassium hydroxide also gives satisfactory results. Nonetheless, spectrum of compounds obtained is narrower and yields are lower as the method is more aggressive.

The method proposed by Wagner [2] is optimal for obtaining chromatographic fingerprint of Schisandra berry. It comprises both TLC and HPLC fingerprint; in the latter reproducible results can be obtained in about 30 min. The method is especially suitable for a routine analysis of fruit drugs. In analyses of other materials (cauloms, leaves, roots) and in controling technological processes good results can be obtained by means of hyphenation methods, predominantly (HPLC–ES–MS). The resulting information is directly proportional to the number of reference lignans for which the retention times and UV spectra are at disposal. Of course, lignans with the same molecular weight but different stereochemistry cannot be reliably identified on the basis of $[M + H]^+$ and $[M + Na]^+$ values. Connection with photodiode-array detection can be successfully applied. GC/MS separations on fused silica capillary columns proved to be useful for the determination of some major lignans (schisandrin, gomisin A and its metabolite B) in a biological matrix, e.g. the serum (pharmacokinetic studies). For the biological material GC/MS seems to be more suitable than HPLC/MS; it is highly sensitive and precise. It can be successfully applied also for the determination of lignans in extracts from fruits. Some ester lignans were determined using this method.

It follows from the literature that maximum eight lignans (and possibly their metabolites) are commonly deter-

mined. RP-HPLC (ODS silica) with UV detection using usually gradient elution proved to be ideal for this purpose, since it enables a good separation of lignans according to their polarity. The samples are mostly applied in methanol. The mixtures methanol–water, acetonitrile-water or methanol–acetonitrile–water either neutral or acidified (HCOOH, H_3PO_4) are used as eluents. The method is suitable not only for analysing extracts from the various morphological parts of Schisandra but also medicinal preparations containing the extracts (e.g. Sheng Mai San). Methods show usually good linearity, accuracy and precision.

A HPLC method in normal phase mode using polymethacrylate adsorbent and *n*-hexane–ethanol as the eluent has also been reported for the determination of constituent in herbal drugs. However, it does not seem to be more advantageous than the above-mentioned RP-HPLC methods.

Electromigration procedures using capillary electrochromatography (CEC) or micellar electrokinetic capillary chromatography (MEKC) were used both for the determination of major lignans in plant material (CEC) and monitoring the compounds in plasma. Although, they yield good results they have not been used in a broader extent.

Finally, it is necessary to mention attempts to determine the summary content of the lignans in routine analyses of herbal drugs by means of a reaction giving a coloured product resulting selectively from dibenzo[*a*,*c*]cyklooktadiene skeleton substituted in the aromatic rings (one of the major lignans would be used as a standard). The amount of the product would serve as measure of the total content of lignans. Methods of this type were developed in the then Soviet Union in 1970s. For example, chloramines were used and the product of the colour reaction was extracted with ethyl acetate. Another example represents a colour reaction with sulfuric acid. The methods were not further developed and it is difficult to evaluate the accuracy and precision of determinations. If such a method would be successfully developed it would surely attracted a great attention.

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