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Review

Separation procedures applicable to lignan analysis

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Abstract

Lignans are a class of secondary plant metabolites produced by oxidative dimerization of two phenylpropanoid units. They have been found in many plants of Oriental medicine. In consequence of recent knowledge it is held that lignans are responsible for the key pharmacological activities of these plants. This review surveys the chromatographic, electromigration and hyphenated methods so far applied for the separation of lignans in Oriental plants used in phytotherapy as well as for the analyses of these lignans and their metabolites in biological matrices and food samples. In addition, the sample clean-up procedures—solvent extractions and supercritical fluid extractions—are also included.

Keywords: Reviews; Lignans

Contents

1. Introduction

Lignans are a class of secondary plant metabolites produced by oxidative dimerization of two phenylpropanoid units. The term *lignan* is applied to the optically active dimers of phenylpropanoids linked by the central carbon atoms of their side chains, while the term *neolignan* means dimers of two phenylpropanoids that are not connected by the bond between the central carbons of the side chains.

Lignans are mostly present in nature in the free form, while their glycoside derivatives create only a minor form. They are widely distributed in the plant kingdom and have been found in species belonging to more than seventy families

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being represented in pteridophytes, gymnosperms and angiosperms. In plant species from the *Podophyllaceae* family, they are the principal organic inclusions. Lignans are found in roots, rhizomes, stems, leaves, seeds and fruits. Not all these sources with some exceptions provide their commercially useful quantities. The exception could be the wound resins of the trees, since lignans occur here in simple mixtures with other natural products and are readily separated in substantial quantities. To some extent, this is also true for heartwood sources [1]. The extraordinary high concentrations of lignans (6–24%, w/w) have been recently found in wood knots of *Picea abies* [2].

In spite of their extensive distribution, their biological functions in plants are unclear. Because of potent antimicrobial, antifungal, antiviral, antioxidant, insecticidal and antifeeding properties of some of them, lignans play probably an important role in plant defense against various biological pathogens and pests. Furthermore, they may participate in the plant growth and development [1]. In addition to their purpose in nature, lignans also possess significant pharmacological activities including antitumor [3–5], antiinflammatory, immunosuppressive, cardiovascular [6], antioxidant [7–9] and antiviral [10] actions. There is a correlation between the presence of the "mammalian" lignans—enterolactone and enterodiol—formed by the bacterial transformation of plant lignans in colon of mammals, and the reduced incidence rates of breast, prostate and colon cancers [11,12]. However, some recent studies indicate that a high serum concentration of enterolactone is not associated with reduced prostate [13] and breast cancer risks [14]. In addition, a latest prospective population-based study showed that a high serum enterolactone level is associated with reduced coronary heart disease and cardiovascular disease related mortality [15].

Among Oriental plants and corresponding lignans, the greatest attention is focused above all on aryltetrahydronaphthalene lignans from *Podophyllum* species, dibenzocyclooctadiene lignans from *Schisandra chinensis*, neolignans from *Magnolia officinalis*, arylnaphthalene, aryltetrahydronaphthalene and diarylbutane lignans from *Phyllanthus* species and furofuran lignans from sesame (*Sesamum indicum*).

Podophyllotoxin, an aryltetrahydronaphthalene lignan, is a well-known natural plant lignan. Podophyllotoxin and similar lignans are found in high concentrations in roots and rhisomes of *Podophyllum* species (*Berberidaceae*), e.g. *Podophyllum peltatum* or *Podophyllum emodi* (Fig. 1A). *Podophyllum emodi* is used to treat snake-bites, ulcer and rheumatism in China for centuries. An alcoholic extract of *Podophyllum peltatum*, termed podophyllin, or pure podophyllotoxin is a topical remedy for venereal and nasal warts caused by papilloma virus. Podophyllotoxin is a well-known potent inhibitor of microtubules assembly. The inhibition of tubulin polymerization disturbs the dynamic equilibrium between microtubules and tubulin and arrests of cell division at mitosis. Podophyllotoxin is above all a source for synthesis of widely used anti-tumor agent etoposide (Fig. 1A). This lignan derivative displays the different mechanism of action through irreversible inhibition of topoizomerase II, which induced DNA strand breaks and the cell death [16]. The determination of etoposide in biological fluids was recently summarized in this journal [17].

Schisandra chinensis (*Schisandraceae*) is a well-known medicinal herb with the exclusive position in traditional Chinese medicine where it has been used as tonic, antitussive and hepatoprotective remedy. *Schisandra* fruits are also prescribed in some Kampo medicines in Japan and have been employed in treatment viral hepatitis in China. *Schisandra chinensis* contains at least 30 dibenzo[*a*,*c*]cyclooctadiene lignans (Fig. 1B), some of them show strong antihepatotoxic action [18,19]. Lignans increase glutathione status in the tissue and hence preserve the cells from the oxidative damage [20]. The antioxidative action is not limited only to the hepatocytes, but it also plays the crucial role in cardioprotection against ischemia-reperfusion [21]. The results show that the methylenedioxy group and the dibenzocyclooctadiene skeleton is an important structural determinant of the protective effect [22]. In the course of search for natural anti-AIDS agents, it was found that some dibenzocyclooctadiene lignans significantly inhibit the HIV replication [23,24]. Some of them have also cytostatic, antiinflammatory, antiulcer, vasorelaxant and neuroleptic activities [25].

The stem bark of *Magnolia officinalis* has been used in traditional Chinese medicine for the treatment of thrombotic stroke, fever and headache. Magnolol and honokiol, isomers of neolignans, have been isolated from the bark of this plant and other plant of family *Magnoliaceae* (Fig. 1C). These neolignans are reported to have various biological activities, including anti-oxidative properties [26] and antidepressant-like effects [27]. They inhibit intracellular calcium mobilization in platelets [28], relax vascular smooth muscles by inhibition calcium influx through voltage-gated calcium channels [29]. Recent studies indicate that honokiol inhibits angiogenesis and the growth of transformed epithelial cells in vitro, thus demonstrating that it has both anti-angiogenic and anti-tumor activity [30].

The plants of the genus *Phyllanthus* (*Euphorbiaceae*) grow in most tropical and subtropical countries and are used widely for the treatment of jaundice, hepatitis, intestinal infections, diabetes and kidney disorders [31]. Lignans phyllamyricin B and retrojusticidin B, obtained from *Phyllanthus myrtifolius* strongly inhibit HIV-1 reverse transcriptase [32], phyllanthin and hypophyllanthin showed cytotoxic activity to multidrug-resistant cells [33] (Fig. 1D).

Sesame seeds (*Sesamum indicum*) have been used for centuries as a traditional health food in East Asian countries. Sesame oil contains a significant quantity (up to 1.5%) of furofuran lignans, sesamin and sesaminol [34] with antioxidant and cardioprotective activities (Fig. 1E). They display a multiple effects on the lipid metabolism, decrease plasma cholesterol level and inhibit lipid peroxidation and low-density lipoproteins oxidation. Moreover, sesame lignans inhibit Δ5-desaturase and 3-hydroxy-3-methylglutaryl coenzyme A reductase activities [34–36].

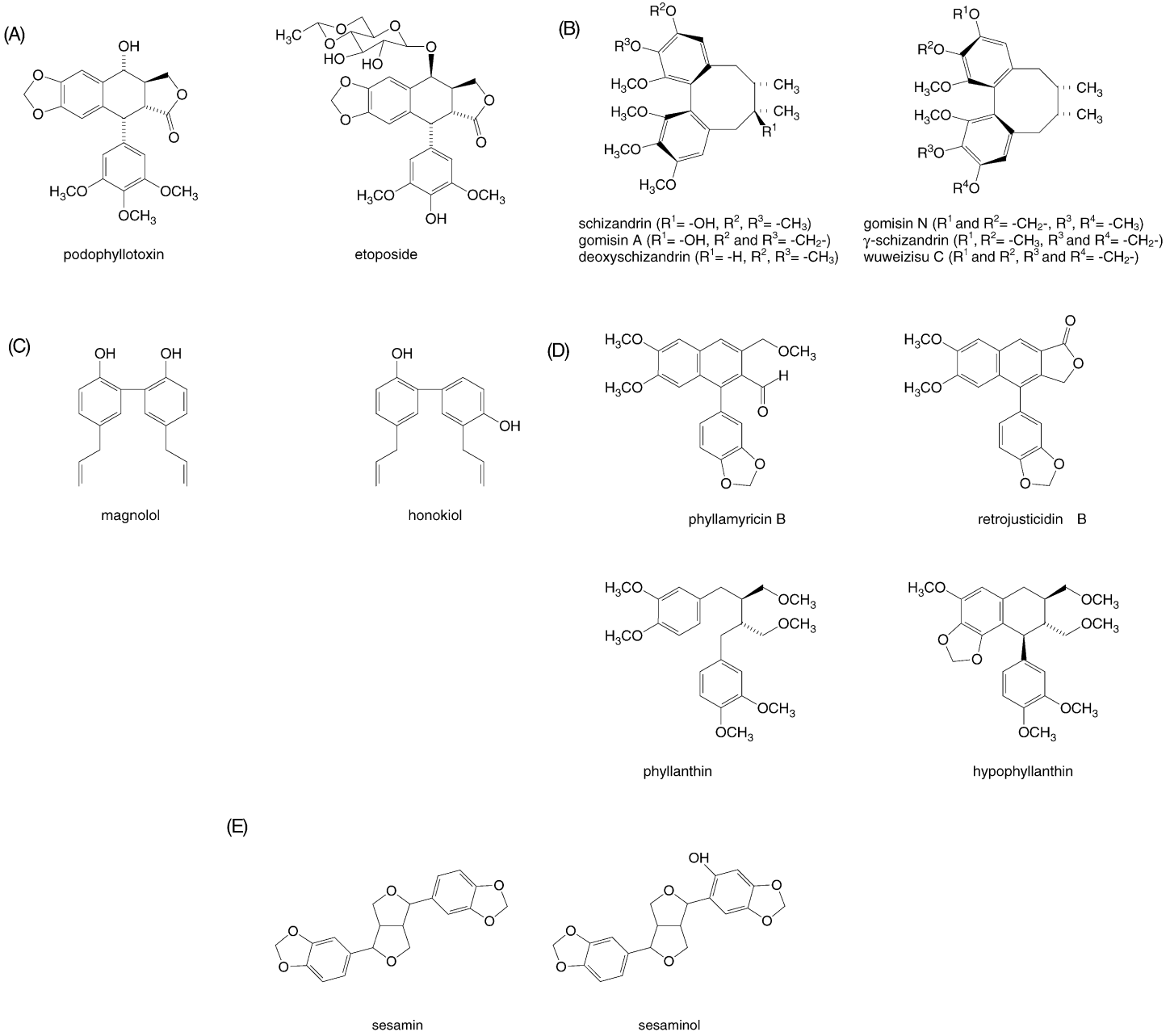

Fig. 1. Main lignans from *Podophyllum* (A), *Schisandra* (B), *Magnolia* (C), *Phyllanthus* (D) and *Sesamum* (E) species.

Lignans have been found in many medicinal plants of Oriental medicine. In consequence with recent knowledge it is held that they are responsible for the key pharmacological activities of these plants. This review surveys the chromatographic, electromigration and hyphenated methods so far applied for the separation of lignans in Oriental plants used in phytotherapy as well as for the analyses of these lignans and their metabolites in biological matrices and food samples.

2. Determination of lignans in plant samples

2.1. Sample clean-up

2.1.1. Solvent extraction

Large amount of lipid substances occurs in all plants especially in seeds and also as the coating and wax of fruits and leaves. Beyond the deleterious effects that they may have on given analytical instrumentation (for example column or capillary clogging) their presence will interfere with the lignan analysis. Since lipids are generally soluble in nonpolar organic solvents, it is convenient to separate them from plant sources by extraction with hexane or petroleum ether etc. mostly in Soxhlet apparatus. Lignans are subsequently extracted from raw material with a polar solvent such as acetone, ethanol or methanol. However, the seeds may contain the nonpolar lignans that are extracted with hexane or petroleum ether. The lignan rich fraction is then obtained by re-extraction of seed oils with methanol [37]. Recently, an accelerated solvent extraction—the extraction performed at elevated temperature and pressure for short time periods—was used to obtain lignan rich fraction from wood samples of *Picea abies* [2]. For analytical purposes lipids and terpenoids can be removed easily from oils by solid phase extraction (SPE) on C_{18} cartridges [38,39].

Another approach is the direct treatment of plant source with a hot polar solvent, usually methanol [40,41] or ethanol [42]. The pre-treatment of sample with a hot water also occurred [43]. Methanolic extract can be directly analysed or concentrated and diluted with water. Methanol–water solution is further fractionated with hexane to remove nonpolar compounds including lipids, terpenes and chlorophylls and subsequently extracted with chloroform, dichloromethane [44,45] or ethyl acetate [42] to obtain the lignan fraction. The methodology using 95% ethanol seems to be better for screening plant leaves for lignans [44].

2.1.2. Supercritical fluid extraction

Supercritical fluid extraction (SFE) is a rapid, selective and convenient method for sample preparation prior to the analysis of compounds in natural product matrices [46–48]. The unique properties of supercritical solvents (high diffusivities, lower viscosities, almost zero surface tensions, etc.) bring several advantages over classical methods of sample preparation like shorter extraction times, enhanced selectivity and lack of residual organic solvent in the final extract. The most widely used supercritical solvent is pure or modified carbon dioxide because of low values of its critical parameters, which allow mild extraction parameters especially the extraction temperature. Moreover, it is non-toxic, non-flammable and, what is also important, environmentally friendly.

For the first time SFE has been used as an alternative to conventional liquid extraction of lignans by Lojková et al. [38]. They applied supercritical carbon dioxide to extract lignans from seeds and leaves of *Schisandra chinensis*. The extraction yields were approximately 96% of lignans from seeds but only 26% from leaves (based on the total lignan content determined from Soxhlet extraction with petroleum ether and methanol) probably due to the strong interaction of lignans with plant matrix. Choi et al. further investigated the effect of temperature and pressure on the carbon dioxide SFE of lignans from the fruits of *Schisandra chinensis* [49]. They found that the variation of both parameters had a little effect on the extraction yield which were approximately 80% that by organic solvent extraction. As the leaves and stems (caulomas) of *Schisandra chinensis* contain significant amounts of lignans Kim et al. tried to improve their extraction yield from these materials [50]. The simple addition of 10% ethanol to carbon dioxide increased the yield up to 87% for leaves. The kinetics of carbon dioxide extraction of lignans from *Schisandra chinensis* was studied by Dean [51] and by Bártlová et al. [52]. In addition, the optimised carbon dioxide SFE was used for the lignan extraction from *Schisandra sphenathera* fruits [53].

The biologically active compounds including lignans were also extracted by supercritical carbon dioxide from fruits, leaves and stems of *Forsythia koreana* [54]. The efficiency of extraction was optimised to get the highest yield of arctigenin, the lignan with highest anti-inflammatory activity produced in this plant. Although pure carbon dioxide extracted only 66% of that obtained by simple extraction with methanol, the extraction yield was significantly enhanced (to 110%) by application of carbon dioxide-methanol mixture (80:20).

Recently even the on-line coupling of SFE with supercritical fluid chromatography (SFC) was used for the extraction and separation of neolignans—magnolol and honokiol—in *Magnolia* cortex [55]. The extraction was performed for 1 min with carbon dioxide containing 5% methanol. Extract was passed directly to the SFC NH_2 column and pre-concentrated on its head. After SFE, the extraction vessel was bypassed, the concentration of methanol in carbon dioxide was raised up to 15% and the trapped analytes were analysed (Fig. 2A). The combination of extraction, pre-concentration and analysis were thus completed within 5 min (Fig. 2B). As seen from Table 1 SFE can be successfully applied for the extraction of lignans from different plants even from their different parts.

2.2. Chromatographic methods

Various chromatographic modes—thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC) have been found as

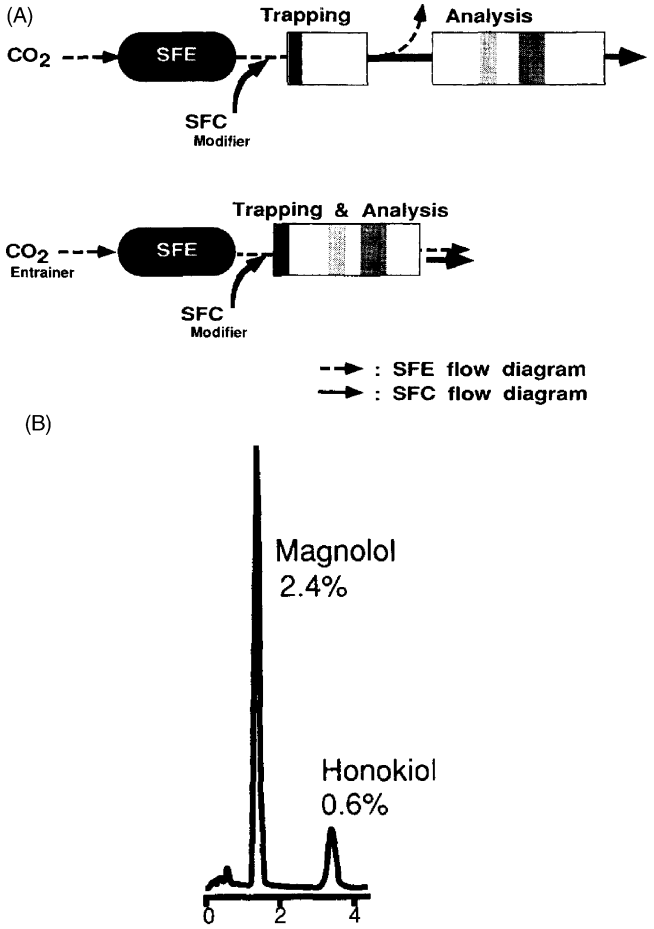

Fig. 2. Determination of magnolol and honokiol in *Magnolia* cortex using SFE on-line coupled with SFC by on-column trapping. On-line SFE-SFC system (A) and separation of magnolol and honokiol (B); reprint from [55] with permission.

valuable tools for the separation of lignans. Whereas TLC has been mainly used for the analysis of these compounds, even their structural identification could be performed by the combination of HPLC or GC with mass spectrometry (MS) detection. Recently, Kamal-Eldon et al. compared different chromatographic modes (TLC, GS, GS–MS and normal- and reversed-phase HPLC) for their ability to separate lignans in seed oils from *Sesamum* species [56]. The authors discussed the advantages and limitations of each method and made following statements: (i) 2D-TLC is a valuable qualitative technique; (ii) 1D-TLC is useful for preparative purpose; (iii) GC is a supplement for qualitative analysis having many limitations as a quantitative tool, (iv) GC–MS is a necessary technique to confirm the lignan identity and finally (v) HPLC is a one step technique suitable for quantitative analysis. These conclusions could be taken as a valuable recommendation for the application of given chromatographic methods in the lignan analysis.

2.2.1. Thin-layer chromatography

TLC is the simplest and inexpensive technique and consequently it was the first method used for the lignan separation [57]. Since that time it has been applied not only for the qualitative analyses but also in combination with densitometry for their quantitative determinations. Moreover, depending on the sample load and plate thickness, TLC is also useful as a semi-preparative technique [58].

Different types of silica gel TLC plates were applied as stationary phases for these analyses [56–64]. Recently special HPTLC plates allowing better separation with sharper zones and reduced development times were used for this purpose [65]. The composition of developing solvents—mobile phases were selected in dependency on the chemical structure of certain class of lignans and the overall sample composition (for examples see Table 2). Besides 2D-TLC with multiple solvent development was used to improve the separation [56].

The visualization of TLC spots or bands were normally carried out with a UV lamp on commercially available fluorophore (e.g. dichlorofluorescein) impregnated plates (GF_{254}) [58–60,62,65] or by spraying the plates with sulfuric acid in ethanol (acetic anhydride, acetone or methanol) [57,58,63], anisaldehyde–sulfuric acid [63], ammonium ceric sulfate–nitric acid [64] or phosphomolybdic acid [65] reagents followed by destructive carbonisation.

Because of its simplicity and relatively high throughput, TLC is now mainly used for initial examination of plant materials and for monitoring of various stages of the lignan purification. As mentioned above the quantitative evaluation then may be performed by densitometry.

2.2.2. High-performance liquid chromatography

The most frequently used analytical technique for determination of lignans so far is HPLC (see Tables 3–5). The majority separations were carried out in the reversed-phase mode; however, analyses on conventional silica-gel columns were

Table 1
Parameters of the lignan SFE from different plant materials

Plant	Plant part	SFE solvent	SFE parameters	References
Schisandra	Seeds, leaves	CO_2	40 MPa, 55 °C	[38]
	Fruits	CO_2	34 MPa, 60 °C	[49]
	Leaves, stems	10% EtOH in CO_2	34 MPa, 60 °C	[50]
	Fruits	CO_2	21 MPa, 37 °C	[53]
Forsythia	Fruits, leaves, stems	20% MetOH in CO_2	34 MPa, 80 °C	[54]
Magnolia	Cortex	SFE—5% MetOH in CO_2	20 MPa, 45 °C	[55]
		SFC—15% MetOH in CO_2	20 MPa, 45 °C	

Table 2
Some examples of TLC mobile phases used for the lignan analyses

Plant	Mobile phase	Reference
Podophyllum	Chloroform-acetone (2:1)	[57]
	Chloroform-methanol (25:1)	[58]
Schisandra	Ethyl acetate-pertoleum ether (1:1)	[59]
	Toluene-ethyl acetate (6:4)	[60]
	Toluene-ethyl acetate (9:1), (4:6)	[61]
	Petroleum ether-ethyl formate-formic acid (15:5:1)	[62]
	Toluene-ethyl acetate-glacial acetic acid (70:33:3)	[63,65]
Diphylleia	Dichloromethane-diethyl ether (4:1)	[64]
Sesamum		
1D-TLC[a]	Petroleum ether-diethyl ether-acetic acid (70:30:1)	[56]
	Chloroform-benzene-methanol (60:40:1)	
	Chloroform-diethylether (90:10)	
2D-TLC[b]	1D—hexane-diethyl ether (70:30)	
	2D—chloroform-diethylether (90:10)	

[a] 1D-TLC—one-dimensional TLC.
[b] 2D-TLC—two-dimensionl TLC.

Table 3
Examples of HPLC conditions used for determination of lignans in *Podophyllum* plants

Compounds	Sample	HPLC conditions	Detection	Reference
Podophyllotoxin, α-peltatin, β-peltatin	*Podophyllum* resin	Column: Perkin-Elmer silica A (550 × 2.5 mm). Mobile phase: $CHCl_3$ with 1.8% ethanol. Flow rate: 0.8 ml min^{-1}.	UV 206 and 280 nm	[66]
Seven diastereoisomers of podophyllotoxin	Mixture of diastereoisomers	Column A: Hypersil silica gel column (250 × 5 mm, 5 μm). Mobile phase: *n*-heptane–CH_2Cl_2–CH_3OH 90:10:4. Flow rate: 1.0 ml min^{-1}. Column B: ODS-Hypersil (250 × 5 mm). Mobile phase: CH_3CN–CH_3OH–H_2O 22.5:22.5:55 or CH_3CN–DMSO–CH_3OH–H_2O 20:20:10:50. Flow rate: 1.0 ml min^{-1}	UV 280 nm	[67]
Podophyllotoxin	*Podophyllum* resins of different source	Column: LiChrospher Si 100. Mobile phase: CH_3OH–tetrahydrofuran–acetic acid–hexane 10:4:1:100. Flow rate 2.0 ml min^{-1}	UV 280 nm	[68]
Eight podophyllum lignans and glucosides	Resins of *P. peltatum* and *P. emodi*	Column: ODS-Hypersil (250 × 5 mm, 5 μm). Mobile phase: CH_3OH–H_2O 40:60. Flow rate: 1.5 ml min^{-1}	UV 280 nm	[69]
Eight *Podophyllum* lignans	*Podophyllum peltatum*	Column: Taxsil (250 × 4.6 mm). Mobile phase: complex gradient elution mode containing three solvents. Solvent A: reagent alcohol (Fisher Scientific Reagent Alcohol: 90.6% ethanol, 4.5% CH_3OH, 4.9% *iso*-propanol)—tetrahydrofuran—methyl-*t*-butyl ether 4:3:1. Solvent B: CH_3OH–H_2O–acetic acid (15:84:1) containing 0.1% ammonium acetate pH = 3.46. Solvent C: CH_3CN. Flow rate: 1.1 ml min^{-1}	Photodiode-array detection over the 210–300 nm range, quantitation at 240 nm	[42]
Podophyllotoxin	Fifty plants and callus cultures of *Podophyllum hexandrum*	Column: Nova Pak C18 (250 × 4.6 mm). Mobile phase: CH_3CN–CH_3OH–H_2O 37:5:58. Flow rate: 1.5 ml min^{-1}	UV 235 nm	[45]

Table 4
Examples of HPLC conditions used for determination of lignans in *Schisandra* plants

Compounds	Sample	HPLC conditions	Detection	Reference
Ten *Schisandra* lignans	*Schisandra chinensis*	Column: μ Bondapak C_{18}. Mobile phase: stepwise gradient CH_3CN–CH_3OH–H_2O (11:11:16), 9 min. CH_3CN–CH_3OH–H_2O (10:10:10). Flow rate: 1.0 ml min^{-1}	UV 254 nm	[70]
Seven *Schisandra* lignans	Seeds of *Schisandra chinensis*	Column: YWG-C-18 (300 × 5 mm). Mobile phase: CH_3OH–H_2O (77:23, v/v). Flow rate: 0.9 ml min^{-1}	UV 254 nm	[71]
Six *Schisandra* lignans	Sheng Mai San	Column: YWG-C-18 (300 × 3.9 mm, 10 μm). Mobile phase: CH_3OH–H_2O (72:28, v/v). Flow rate: 1.0 ml min^{-1}	UV 254 nm	[72]
Eight *Schisandra* lignans	Fruits of *S. chinensis* and *S. sphenanthera*	Column: LiChrospher 100 RP 18 (125 × 4 mm, 5 μm). Mobile phase: A—H_2O with 1% (v/v) 0.1 M H_3PO_4; B—CH_3CN with 1% (v/v) 0.1 M H_3PO_4. Gradient elution 40–80% B 0–20 min. Flow rate: 1.0 ml min^{-1}	UV 210 nm	[63]
Schisandra lignans	Fruits of *Schisandra chinensis*	Column: Prodigy ODS 5 (150 × 2 mm, 5 μm). Mobile phase: A—H_2O; B—CH_3OH. Gradient elution 60–100% B 0–15 min, 100% B 15–20 min, v/v). Flow rate: 0.2 ml min^{-1}	UV 225 nm or MS electrospray interface	[43]
Five *Schisandra* lignans	Seeds and fruits of *Schisandra chinensis*	Separon SGX C_{18} (Tessek, 150 × 3 mm, 5 μm). Mobile phase: CH_3OH–H_2O (73:27, v/v). Flow rate: 0.3 ml min^{-1}	UV 254 nm	[39]
Six *Schisandra* lignans	Leaves and caulomas of *Schisandra chinensis*	Column 1: Nucleosil 100 C_{18} endcapped (250 × 4 mm, 5 μm). Mobile phase A: CH_3CN–H_2O isocratic 50:50 (v/v) 0–5 min, gradient 60:40 (v/v), 5–35 min, gradient 70:30 (v/v), 35–55 min, isocratic 70:30 (v/v), 55–70 min. Mobile phase B: CH_3OH–H_2O isocratic 70:30 (v/v), 0–1 min, gradient 95:5 (v/v), 1–35 min, isocratic 95:5 (v/v), 35–40 min. Flow rate: 0.75 ml min^{-1}. Column 2: Merck-Lichrospher 100 RP 18 (250 × 4 mm, 5 μm). Mobile phase: gradient CH_3CN–H_2O from 40:60 to 70:30 (v/v), 0–60 min, isocratic 70:30 (v/v) 60–90 min. Flow rate: 1.0 ml min^{-1}	UV 254 nm	[52]
Schizandrin and its three metabolites	Rat bile and urine	Column: Nucleosil C_{18} (300 × 4.6 mm, 5 μm). Mobile phase: CH_3CN–CH_3OH–H_2O 2:2:5. Flow rate: 1.0 ml min^{-1}	UV 254 nm	[73]
Four *Schisandra* lignans	*Schisandra* fruits and fruit extracts	Column: Luna C18 (2) (250 × 4.6 mm, 5 μm). Mobile phase: A—H_2O containing 0.1% formic acid; B—CH_3CN. Gradient elution 45–60% B 0–12 min, 60% B 12–24 min, 60–90% 24–40 min, 90–100% 40–45 min. Flow rate: 1.0 ml min^{-1}	UV 255 nm and MS electrospray interface	[41]

Table 5
Examples of HPLC conditions used for determination of lignans in *Magnolia* plants

Compounds	Sample	HPLC conditions	Detection	Reference
Magnolol and honokiol	*Magnolia officinalis*	Column: Nucleosil 7C_{18} (250 × 4 mm, 7 μm, (Macherey-Nagel). Mobile phase: CH_3CN–H_2O–H_3PO_4 (65:35:0.1, v/v/v). Flow rate: 1.0 ml min^{-1}	Photodiode-array detection, quantitation at 290 nm for honokiol and 218 nm for magnolol	[74]
Magnolol, honokiol and six nonlignan constituents	Chinese herbal formula Hsiao-cheng-chi-tang	Column: Cosmosil 5C_{18} (250 × 4.6 mm, 5 μm, Nacalai Tesque). Mobile phase: A—0.02 M sodium acetate and 0.42 M acetic acid–CH_3CN (9:1), B—CH_3OH–CH_3CN–1% acetic acid (9:9:2). A–B profile: 0 min, 90:10; 14–22 min, 75:25; 30 min, 70:30; 35 min, 20:80; 45–55 min, 0:100; 60 min, 90:10. Flow rate: 0.8 ml min^{-1}	Photodiode-array detection (280 nm)	[75]
Magnolol, honokiol and five nonlignan constituents	Chinese herbal formula Wuu-Ji-San	Column: Cosmosil 5C_{18} (150 × 4.6 mm, 5 μm, Nacalai Tesque). Mobile phase: A—0.03% H_3PO_4 (v/v), B—CH_3CN. Gradient elution 0 min, 95:5; 52 min, 30:70. Flow rate: 1.0 ml min^{-1}	UV 254 nm	[76]
Honokiol	Rat plasma	Column: Cosmosil 5C_{18}-AR (250 × 4.6 mm, 5 μm, Nacalai Tesque). Mobile phase: CH_3CN–H_2O 70:30 (v/v), adjusted to pH 2.5–2.8 with H_3PO_4. Flow rate: 1.0 ml min^{-1}	Photodiode-array detection	[77]
Magnolol and its metabolite 8,9-dihydroxydihydromagnol	Human urine	Column 1: LiChrosorb Si-60 (250 × 4 mm, 5 μm). Mobile phase: acetic acid–diethyl ether–*n*-hexane (0.2:17:82.8, v/v). Flow rate: 1.5 ml min^{-1}. Column 2: CapcellPak C_{18} SG120 (250 × 4.6, 5 μm). Mobile phase: 0.1 M phosphate–CH_3OH 2:3, v/v. Flow rate: 1.0 ml min^{-1}	Multi-channel UV-detector	[78]
Magnolol and honokiol	Rat plasma	Column: Cosmosil 5C18 (150 × 4.6 mm, 5 μm, Nacalai Tesque). Mobile phase: CH_3OH–CH_2Cl_2–H_2O–H_3PO_4 81:3:16:0.12, v/v/v/v). Flow rate: 0.5 ml min^{-1}	UV 290 nm	[79]
Magnolol and its metabolites	Rat urine and feces	Column: μs Finepack SIL C_{18} (250 × 1.5 mm). Mobile phase: CH_3CN–H_2O–H_3PO_4 50:50, v/v). Flow rate: 0.1 ml min^{-1}	UV 250 nm and LC–MS interface	[80]

also published [66–68,78]. In reversed-phase HPLC, mobile phase generally consists of a mixture of organic solvents, typically methanol or acetonitrile, and an aqueous phase, which consists of water, acidic buffer or diluted acid. Lim and Ayres [67] showed that the selection of organic modifier strongly affected the retention behavior of aryltetrahydronaphthalene lignans in reversed-phase chromatography. Methanol was essential for the separation of the diastereoisomers of *Podophyllum* lignans by reversed-phase chromatography, but is less useful for the separation of the functional-group derivatives of podophyllotoxin. Acetonitrile was superior for the resolution of functional derivatives, but was unable to separate the diastereoisomers. A ternary mobile phase of acetonitrile–methanol–water (22.5:22.5:55) or a quaternary solvent mixture of acetonitrile–dimethylsulphoxide–methanol–water (20:20:10:50) [67] was necessary for the separation of diastereoisomers and functional group derivatives. With acetonitrile–water as an eluent on reversed-phase column, obvious order of elution on reversed-phase was obtained, the polar lignan glycosides were eluted before the unconjugated lignans [67]. The using of methanol induced the reversal of elution order for some compounds and the improvement in the resolution, demonstrating its superiority. The enhancement of the separation ability is attributed to hydrogen bonding of methanol, adsorbed on stationary nonpolar phase, with lignans depending on their structure. The hydrogen bonding effect of methanol causes the greater retention of lignans with a glucoside moiety or alcoholic hydroxyls compared to acetonitrile, and results in the unique elution order of lignans [67]. Our unpublished results reached with HPLC analysis of *Schisandra* lignans also showed that the better resolution is obtained with methanol, nevertheless the better peak shape is achieved with acetonitrile [52] (Fig. 3) and therefore the combination of both these organic modifiers may be the best choice [45,70,73,75]. A HPLC method for the simultaneous determination of magnolol

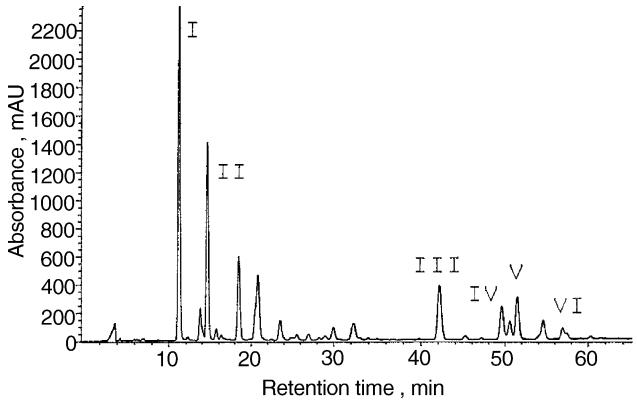

Fig. 3. HPLC separation of SFE-CO_2 extract from *Schisandra* caulomas. Peak identification: (I) schizandrin; (II) gomisin A; (III) deoxyschizandrin; (IV) γ-schizandrin; (V) gomisin N; (VI) wuweizisu C; reprint from [52] with permission.

and honokiol in rat plasma was recently developed. The mobile phase contained 3% of dichloromethane beside methanol and water [79]. A simple method using ion-pair HPLC involving reversed phase column and 10 mM tetra-*n*-amylammonium bromide–acetonitrile (4:6) as a mobile phase was established for the determination of magnolol and honokiol in *Magnolia* bark [81]. To achieve better resolution and shortening of analyses, the gradient elution mode is frequently implemented [41–43,52,63,70,75,76].

The mobile phase with acidic pH was frequently used; it suppresses ionization of weakly acidic phenolic groups of lignans and reduces of peak tailing by decreasing of interaction with residual metal ions in the stationary phase. The most employed acidic modifier was phosphoric acid [63,74,76,77,79,80], but acetic buffer was also used [75]. Addition of ammonium acetate to the mobile phase decreased the retention of *Podophyllum* lignans, while keeping the resolution [42]. The acidic modifier was marginally employed for quantitation of nonphenolic *Schisandra* lignans [41,63], but was always used for analyses of *Magnolia* lignans—magnolol and honokiol—which contain weakly acidic phenolic groups [74,76,77,79,80].

Composition of sample solvent also influences the peak shape. Lim dissolved the samples in mixture methanol–water (9:1) [69]. Our unpublished results with determination of *Schisandra* lignans proved that optimal concentration of methanol for sample injection is between 40 and 80%. Lower concentration of methanol is detrimental to peak shape of highly nonpolar lignans, whereas use of anhydrous methanol for injection of sample causes the deformation of peaks of relatively polar lignans.

Detection of lignans by UV absorption is frequently applied. The selectivity of detection can by enhanced by photodiode-array detection. No doubt, the selectivity and sensitivity of UV detection is sufficient for determination of lignans in plant extracts, but is also used for analyses of lignans in biological fluids where more sensitive methods are usually adopted. The use of HPLC interfaced with MS is often recommended for detection of analytes in biological fluids that generally requires methods of high selectivity and sensitivity. An escalated number of papers appeared in the literature in the last decade has described HPLC coupled with MS for the lignan detection, mostly oriented on analysis of mammalian lignans [41,43,80,82–85].

Electrochemical detection is another high sensitive method that is frequently used for determination of plant polyphenols (e.g. flavonoids or phenolic acids) and their metabolites in biological fluids. Applications of this detection method for the plant lignan analyses are limited due to relatively small group of lignans with free phenolic hydroxyl(s) because phenolic groups of lignans are mostly methylated or lignans contain methylendioxy groups. Gamache and Acworth [86] published the analysis of mammalian lignans—enterolactone and enterodiol—in tissue homogenates and rat plasma. They used a serial of the coulometric detection cells with increasing potentials with limits of detection of 5–50 pg. Nurmi and Adlercreutz [87] developed the validated HPLC method for profiling 13 phytoestrogens and their metabolites in plasma using coulometric electrode array detection, including plant lignans–secoisolariciresinol and *O*-desmethylangolensin. The detector response variation was eliminated by frequent calibration. Detection limit was 3.4 pg for secoisolariciresinol.

Lignans are chiral plant metabolites, which occur in vascular plants in an enantiomeric excess or enantiomerically pure. Determination of the chiroptical properties of plant lignans, usually involves their isolation. Halls and Lewis used HPLC coupled with the laser polarimeter for the rapid analysis of plant extracts containing chiral lignans [40].

2.2.3. Gas chromatography

GC is used mainly for analysis of volatile compounds; otherwise these have to be derivatised. Although GC and especially GC–MS were applied mainly for the determination of lignans in various biological matrices and foods samples (see below), several methods have been also developed for the lignan analysis in plant materials. Whereas the determination of lignans from biological matrices and food samples involves the production of for example trimethylsilyl ether (TMS) derivatives; no derivatisation was usually needed for plant samples. Mostly helium was used as a carrier gas in these analyses.

GC has been for the first time applied to the lignan separation in 1969 by Ayres and Chater [88]. On the other hand they worked only with a model mixture of lignans and used this methodology to differentiate their main classes and to distinguish their geometrical isomers. In 1989, Sohn et al. reported the application of GC–MS method for the identification of lignans in *Schisandra* [89]. They used a SPB-1 fused silica capillary column, the column temperature 200–300 °C (rate 4 °C/min) and the electron impact (EI) ionisation with an ionisation potential of 70 eV. Totally 11 lignans were detected under given conditions. The same research group modified

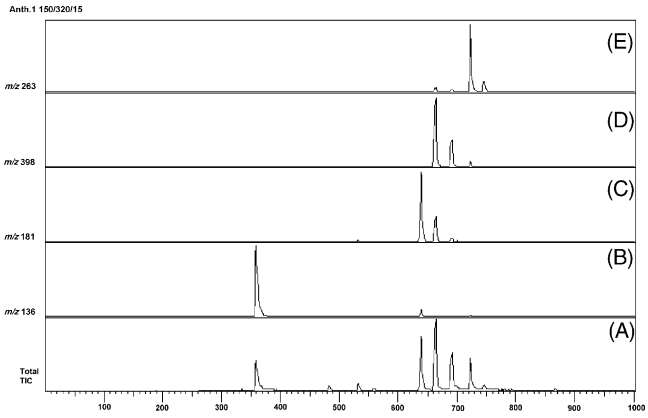

Fig. 4. GC–MS analysis of *Anthriscus sylvestris* (A). The other chromatograms are extracted ion currents, for the I.S. (B), yatein (C), deoxypodophyllotoxin (D), and anhydropodorhizol (E); reprint from [94] with permission.

the developed method for simple flame-ionisation detector (FID) using the identical column and the same separation conditions; only seven lignans were detected in this case [90]. Besides Ono et al. published simple GS–MS method for determination of schizandrin in human plasma [91]. A SPB-1 fused silica capillary column and the column temperature 60–290 °C (rate 20 °C/min) were used for the analyses. The MS detector was operated in the EI mode (70 eV). The method requires only 100 μl of plasma and may be used for pharmacokinetic studies. Two GC–MS methods were also published to analyse the essential oils of *Schisandra* fruits and seeds [92,93].

Recently, Koulman et al. reported a new GC–MS method for the lignan profiling in *Anthriscus sylvestris* and related plant species [94]. The analyses were performed on a non-polar WCOT column with a high temperature programme 150–320 °C (rate 15 °C/min), EI ionisation (70 eV) was used to obtain mass spectra. The method gave clear lignan profile for deoxypodophyllotoxin, yatein and anhydropodorhizol in *Anthriscus sylvestris* (Fig. 4) and was applied for comparison their concentrations in plants from different locations. Moreover it allowed to identify another lignans—arctigenin and phylligenin—in *Anthriscus sylvestris* and to demonstrate the presence 5-methoxypodophyllotoxin and β-peltatin in *Linum flavum* and pinoresinol in *Forsythia intermediana* and *Pastinaca sativa*.

2.3. Electromigration methods

In the last decade, capillary electrophoresis (CE) has become powerful separation technique that can provide highly efficient separations and large peak capacities [95–98]. The availability of sophisticated and automated instruments together with above-mentioned advantages predict this technique for analyses of complex matrices like plant extracts. Consequently CE became a complementary and/or additional method to chromatography in analyses such type of samples. Due to the neutral character of lignans at normal conditions, only limited CE modes could be applied for their separation without any manipulation of physical and chemical characteristics of samples. The detection was performed by means of UV absorption, the most common detection mode in CE.

2.3.1. Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) is the simplest and widely used CE mode [99], where the separation is based on differences in the charge-to-mass ratio and analytes migrate as the discrete zones of different velocities. Whereas the anions and cations are separated due to their electrophoretic mobilities and the mobility of the electroosmotic flow (EOF), the neutral species co-migrate with EOF. To overcome this limitation numerous approaches may be applied–derivation or complexation reactions, extreme pH, etc.

The last approach has been adopted by Chou el al. for the simultaneous determination of magnolol and honokiol in *Magnolia officinalis* cortex [100]. The high pH of background electrolyte—5 mM phosphate-10 mM tetraborate (pH 9.1–9.2) caused the deprotonation of hydroxyl groups of magnolol and honokiol allowing their separation by mean of CZE. The content of these two active principles in *Magnolia officinalis* could be successfully determined within 12 min. Zhang et al. further optimised this method by addition of 20% methanol to background electrolyte—20 mM tetraborate-20 mM phosphate buffer (pH 9.0) [101] and finally by changing pH of background electrolyte in the range 5.0–11.7 [102]. The effects of temperature and applied separation voltage on migration mobility, resolution, efficiency and peak shape were also investigated. Better CZE separation and determination of magnolol and honokiol was achieved in 9 min by increasing of pH (10 mM borate-10 mM phosphate buffer) up to 11.6 (Fig. 5).

2.3.2. Micellar electrokinetic capillary chromatography

Micellar electrokinetic capillary electrochromatography (MEKC) is CE mode introduced by S. Terabe in 1984 primarily for the analyses of neutral solutes [103,104]. In MEKC, one or more surfactants are added to the background electrolyte above their critical micellar concentration to form micelles and these act as the separation medium (pseudostationary phase) for uncharged analytes.

Song et al. [105] have reported the first MEKC separation of lignans in 1994. They applied this CE mode for the analysis of crude chloroform extract of *Podophyllum emodi* roots, but only two lignans—podophyllotoxin and 4′-desmethylpodophyllotoxin—were identified. Since then several studies have been performed on MEKC separation of *Podophyllum* lignans. The main lignans including their glucoside derivatives were separated by Ganzera et al. using 25 mM sodium dodecylsulfate (SDS) and 15% methanol in 40 mM phosphate-15 mM tetraborate (pH 7.25) as a background electrolyte [106]. The method was applied to compare different samples of *Podophyllum peltatum* collected in various part of USA, however just one lignan—podophyllotoxin

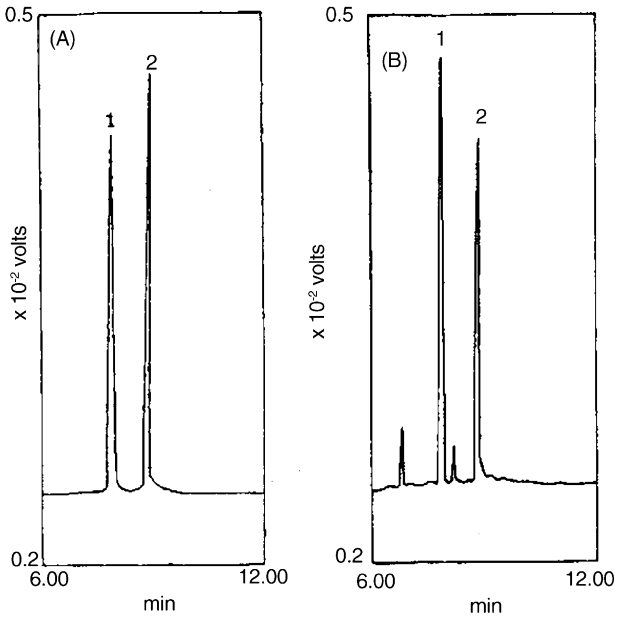

Fig. 5. CZE separation of a mixture of authentic magnolol — 1 and honokiol — 2 (A) and an ethanol extract of *Magnolia officinalis* bark (B); reprint from [102] with permission.

was quantify. The quantitative results obtained by MEKC were in good agreement to those obtained by HPLC.

As each lignan could have its own pharmacological function Liu et al. developed MEKC method for the quantitative analysis of seven lignans in *Podophyllum emodi* [107]. The effects of several parameters including pH, SDS and modifier concentration on the resolution were studied. A 100 mM SDS and 30% 2-propanol in 10 mM phosphate-5 mM borate (pH 7.2) were found as the optimal background electrolyte for these analyses. Whereas normal detection mode was applied for determination of fully resolved compounds, the second derivative electropherogram was used in the case of low content and not fully resolved compounds.

The same research group applied MEKC for the separation of diastereoisomers of *Podophyllum* lignans. Seven pairs of diastereoisomers at C2-position including two pairs of spin-labelled compounds were successfully separated within 35 min by means of background electrolyte containing 120 mM SDS and 30% 2-propanol in 5 mM sodium tetraborate-20 mM phosphate buffer (pH 6.5–7) [108]. Similarly the separation of seven pairs of diastereoisomers at C4-position including three pairs of spin-labelled compounds was achieved within 20 min by means of background electrolyte containing 30 mM SDS and 10% 2-propanol in 20 mM tetraborate buffer (pH 9.5–9.7) [109]. The methods developed can be used to identify the lignans during isolation and to monitor their configuration changes, chemical reactions and pharmaceutical metabolisms.

Kuo et al. developed MEKC method for the separation of 12 lignans from *Phyllanthus* plants [110]. The different micellar systems—SDS and sodium deoxycholate (SDC) were investigated and optimised. Whereas tetrahydrofuran (THF) had to be added to the SDS micellar system—9.5% THF and 20 mM SDS in 35 mM Tris buffer (pH 9.0), no organic modifier was needed with SDC micellar system—15 mM SDC in 15 mM tetraborate-10 mM phosphate (pH 9.0). Twelve lignans were separated in 17 min. The selectivity in both systems was significantly different since the retention in SDC system was greatly influenced by hydrogen bonding interactions in contrast with dominated hydrophobic interactions in SDS system. Although both micellar systems gave a satisfactory separation within a reasonable time only the method based on SDS system was further validated. The method was finally used for the determination of lignans in *Phyllanthus urinaria* and *Phyllanthus niruni*. Recently even the MEKC sweeping, an on-capillary sample concentration technique developed Quirino and Terabe [111], was used to enhance the concentration sensitivity for the analysis of phyllanthusols A and B from extract of *Phyllanthus acidus* roots [112]. This pre-concentration strategy allowing the sampling of approximately hundred times larger volumes in contrast with conventional MEKC, brought excellent quantification and detection limits of 0.55 and 0.24 μg/ml, respectively. The concentrations of both phyllanthusols thus could be determined in root extract directly without another sample manipulations by MEKC with background electrolyte of 50 mM phosphate (pH 2) containing 80 mM SDS and 30% methanol.

Štěrbová et al. reported MEKC method with SDS as a micellar phase for the determination of lignans in *Schisandra chinensis* [113]. Because of the low solubility of lignans up to 35% acetonitrile had to be added to the background electrolyte consisting of 40 mM SDS in 10 mM tetraborate buffer (pH 9.3). The effect of sample composition on the lignan solubilities and separation was also investigated. The best overall results were achieved with 20 mM SDS and 35% acetonitrile in 10 mM tetraborate buffer (pH 9.3). The electropherogram of *Schisandra* extract under optimal conditions is shown in Fig. 6. As can be seen MEKC allowed baseline resolution of all lignans. Moreover, it brought not only three times higher separation efficiency in contrast with CEC (see below) and more that eleven times higher than with isocratic reversed-phase HPLC but also a substantial reduction of analysis time.

The applicability of the developed method was further demonstrated by the analysis and determination of lignans in drugs (Fyto Kaps Schisandra capsules). In addition the possibility of using the developed method for the pharmacokinetics studies was tested by the analyses of serum samples spiked with *Schisandra* extract. A simple procedure with SPE on a C_{18} cartridge was used for the sample deproteination and concentration.

2.3.3. Capillary electrochromatography

The other CE mode mainly dedicated for the analysis of neutral molecules is capillary electrochromatography (CEC) [114–116]. It is a hybrid between CE and HPLC combining the high efficiency and miniaturization of CE with the selectivity of HPLC. As with CE, the separation is performed

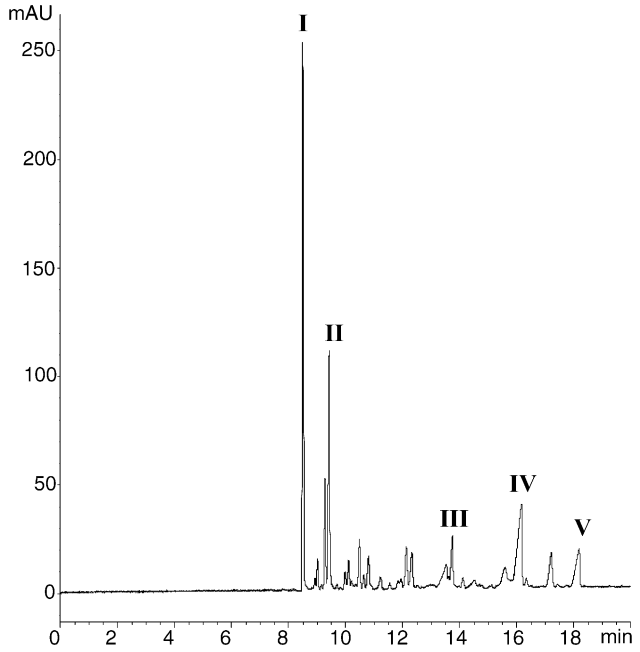

Fig. 6. MEKC electropherogram of *Schisandra* seeds extract. Peak identification: (I) schizandrin; (II) gomisin A; (III) deoxyschizandrin; (IV) gomisin N; (V) wuweizisu C; reprint from [113] with permission.

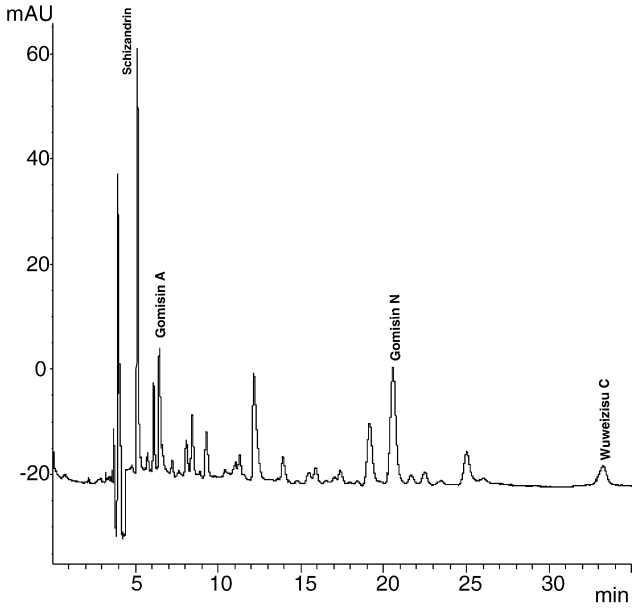

Fig. 7. Typical electrochromatogram of *Schisandra* seeds extract on a polyacrylamide monolithic column; reprint form [117] with permission.

in a capillary with EOF as the driving force for background electrolyte—mobile phase movement; however, the capillary contains a stationary phase as in HPLC.

The reverse-phase variant of CEC using monolithic polymer-based stationary phase has been developed for the determination of lignans from *Schisandra chinensis* [117]. The stationary phase was prepared by in situ copolymerisation of acrylamide, *N*,*N*′-methylenebisacrylamide, vinylsulfonic acid and lauryl acrylate in presence of poly(ethylene glycol) as a porogenic agent. The columns were successfully used to analyse and quantify the major lignans from SFE extract of *Schisandra chinensis* seeds. Elution was performed isocratically by means of 30% acetonitrile in 10 mM Tris-15 mM borate (pH 8.2). Fig. 7 shows that the good separation was obtained in less than 35 min. The CEC method was compared to a standard reversed-phase HPLC. The levels of schizandrin, gomisin A, gomisin N and wuweizisu C determined by CEC were in a good agreement with those determined by HPLC using UV detection, no significant differences in selectivity were observed. Moreover, CEC offers better resolution and higher efficiency, satisfactory precision and reduced operation cost. All these are advantages over traditional chromatographic procedures.

2.4. Special hyphenated methods

Since MS is now becoming a quite common detection approach in the lignan analysis by means of HPLC or GC, the methods using the MS detection were mentioned in the parts dedicated to the given methodologies and not in the part devoted to hyphenated methods. Although HPLC–MS and GC–MS has simplified the lignan identification, on several occasions MS data alone are insufficient for their structure elucidation. Further data especially NMR spectra are therefore demanded. The combined HPLC–NMR technique has been developed during the last years. Its application has been expanded to several fields including the lignan identification in plant samples. The combination of reversed-phase HPLC with NMR spectroscopy was used to the rapid detection and identification of lignans from *Torreya jackii* [118]. Analysis consisted of gradient reversed-phase HPLC and directly coupled ^{1}H NMR (500 MHz) spectroscopic detection in a stopped-flow mode. Seven lignans were detected and HPLC–^{1}H NMR identified their structures, which were later confirmed by convenient ^{1}H NMR and MS spectra of the isolated compounds and reference samples. The entire procedure is very rapid; it involves 2 h of sample pre-purification, a preliminary HPLC run and 30 min for running each of HPLC–^{1}H NMR spectra in the stopped-flow mode. Similarly, on-flow HPLC-^{1}H NMR was used for the study of an antioxidant fraction from *Orophea enneadra* and isolation of a polyacetylene, lignans and a tocopherol derivative [119]. The on-line information allowed rapid identification of three closely related lignans. However, this approach necessitated large quantities of the sample to be injected to obtain satisfactory on-flow HPLC–^{1}H NMR spectra; in addition isolation of the compounds was necessary to get complete NMR data.

Countercurrent chromatography (CCC) based on the principle of liquid–liquid partition in combination with a thermospray MS has been used for the identification of lignans from *Schisandra rubriflora* [120]. The separation was performed using an analytical high-speed planet centrifuge with a two-phase solvent system composed of *n*-hexane, ethanol

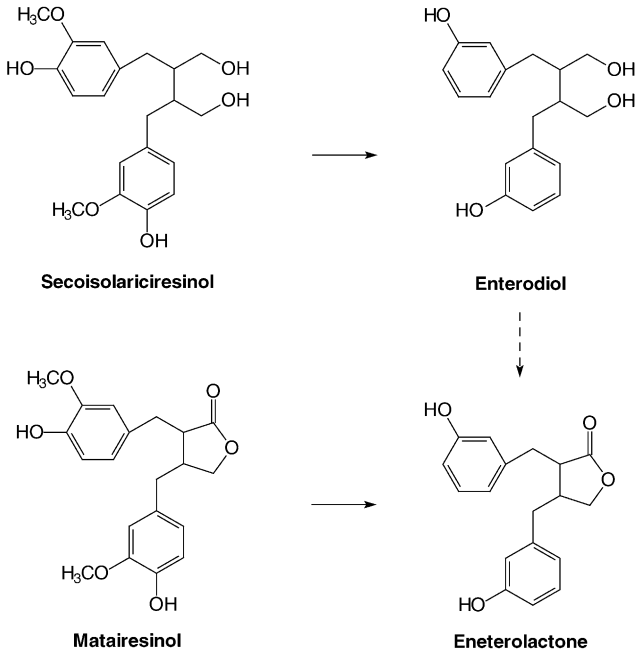

Fig. 8. Structure of plant lignans—secoisolariciresinol and matairesinol—and mammalian lignans—enterolactone and enterodiol.

and water (6:5:5). Lignans were analysed in both positive ion and negative ion MS modes using ammonium acetate buffer added post-column. As in the case of other phenolic compounds, the negative mode was found to be 10–100 times less intense. Consequently the selected ion chromatograms of $[M + H]^+$ and $[M + NH_4{}^+]^+$ in positive ion mode were used to detect the minor lignans. Moreover, due to the high selectivity of the method, lignans could be identified even if they could be not completely separated. CCC–MS offers a number of advantages in analyses such samples. The absence of column eliminates the complication arising from stationary phase adsorption, deactivation and contamination often observed in HPLC. Because the two-phase solvent system that can be employed for CCC is unlimited, coupling of CCC to MS provides a complementary methodology to HPLC–MS. Additionally the coupling of CCC to MS is also suitable for preparative work.

3. Determination of lignans in biological matrices and food samples

As mentioned above, the plant lignans—secoisolariciresinol and matairesinol—are after ingestion deglycosylated and partly converted by colonic bacteria to the mammalian lignans—enterolactone and enterodiol—also called enterolignans (Fig. 8) [121–123]. Recently, another plant lignans have been identified as further precursors [124,125]. Enterolignans possess a range of biological activities including antioxidant, antitumor, weakly estrogenic and anti-estrogenic properties, etc. [126]. They are present in most of body fluids—urine, plasma, saliva, semen, prostatic fluid. Because of complexity of these matrices in comparison with the plant samples, the analyses often require more complicated sample pre-treatment. The samples are typically extracted with organic solvent or by SPE on C_{18} cartridge that is in many cases followed by ion-exchange chromatography to remove various interfering substances. Since enterolignans are present in biological fluids usually as the glucuronide and/or sulfate derivatives, they are liberated enzymatically or chemically. The quantitative determination is finally performed by means of HPLC with different detection approaches (see above) [86,87,127–130], or by means of GC or GC–MS [4,131]. Recently, even an immunoassay method has been applied for this purpose [132]. For the reason that this subject is a little out of the scope of this review, two excellent reviews recently published by Hoikkala et al. [133] and by Raffaelli et al. [134] are recommended for more detail informations. The first review covers both the different methods of enterolignan analysis in biological matrices, and the methods of sample pre-treatment. In addition, their biological activity, biosynthesis and metabolism are reviewed in the second one.

In consequence with established biological functions of enterolignans, there is growing interest in determination of their precursors in foods. As in the case of biological matrices, these analyses are a difficult task. Two main approaches were applied for this purpose. The direct method developed by Mazur et al. involves five-step extraction and purification procedure (rehydratation with water, enzymatic and acid hydrolysis, two solid-phase ion-exchange chromatographic extraction, derivatisation) followed by isotope-dilution GS–MS in selected ion monitoring mode [135]. The indirect in vitro methodology simulates colonic fermentation with fecal microflora. Fresh human fecal inoculum is used and the food samples fermented for 48 h under anaerobic conditions. The samples are further extracted and purified (C_{18} solid-phase extraction, enzymatic hydrolysis, C_{18} solid-phase extraction, ion-exchange-chromatography), derivatised and analysed by GS–MS [4]. Recently, Meagher and Beecher estimated that in vitro fermentation gave higher values for most food samples compared to direct analysis [136] and found secoisolariciresinol and matairesinol in many cereals, grains, fruits and vegetables, but the richest sources was linseed (flaxseed) and other oilseeds such as sesame. What is more secoisolariciresinol and matairesinol were found even in wine [137], coffee and tea [138].

4. Conclusion

Traditional Oriental medicines have attracted great interest in the recent pharmacological research. The continuous search for new drugs of plant origin is important direction in many civilization diseases treatments. This review is focused on the separation of lignans found in the plants of Oriental medicine, which are responsible for the essential pharmacological activities of these plants including antitumor, antiinflammatory, immunosuppressive, cardiovascular

and antiviral actions, as well as on the separation of these lignans and their metabolites in biological matrices and food samples. The chromatographic, electromigration and hyphenated methods so far applied for this purpose are summarised, the sample clean-up are also included.

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