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Review

Review of the analytical techniques for sesquiterpenes and sesquiterpene lactones

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Abstract

In this paper the analytical techniques of about the last 2 decades for sesquiterpenes including their lactones are reviewed. For sesquiterpenes, methods like GC, GC–EI-MS, GC–CI-MS, GC–MS–MS, GC–FT-IR, GC–UV, GC–AES, ^{13}C-NMR, PY–GC–MS, HPLC, HPLC–TSP, SFE, SFC, SFC–UV are available, GC combined with MS is the most widespread. Sesquiterpene lactones can be analysed by HPLC, HPLC–TSP, HPLC–APCI, HPLC–ESI, HPLC–PB, HPLC–NMR, SFC, MEKC, GC, GC–MS, TLC and OPLC. Here HPLC is the method of choice. The usefulness of the individual methods are briefly discussed.

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Keywords: Reviews; Terpenoids; Sesquiterpenes; Sesquiterpene lactones

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1. Introduction

Sesquiterpenes are C-15 terpenoids which occur as hydrocarbons or in oxygenated forms such as alcohols, ketones, aldehydes, acids or lactones in nature. They are important constituents of essential oils, which have many applications in medical, but also in soap and perfume formulations. Moreover, they are found as flavour compounds in aroma mixtures. Essential oils as well as aroma mixtures have very complex compositions from the chemical standpoint. Most often the single constituents only occur in traces. Therefore effective chromatographic techniques are required for optimal separation and identification or isolation of the individual components.

Within the group of sesquiterpenes their lactones deserve special interest. These secondary metabolites derive from the basic sesquiterpene carbon skeleton, in which one of the methyl groups of the isopropyl group is oxidized to the lactone group [1]. They are mainly found in several genera of Asteraceae, but also in Umbelliferae and Magnoliaceae [2]. These compounds are of interest not only from chemical and chemotaxonomical standpoints, but also because many of them possess biological and therapeutic activity including anti-inflammatory, antitumoral, antimicrobial, anthelmintic and anti-feeding [3]. Because of these effects, preparations of plants containing sesquiterpene lactones are often used in traditional medicine. To guarantee the same quality of the medicinal plants chromatographic techniques including qualitative and quantitative analysis are used. Moreover, these methods are also helpful tools in isolation and identification of these compounds.

In the past several papers have focused on chromatographic methods used in the analysis of sesquiterpenes [4,5]. In the meantime some new analytical techniques, especially combined ones, have been developed. In this review all the methods of about the last 2 decades successfully applied to the analysis of these secondary metabolites are summarized and discussed. Because of the importance mentioned above their lactones are discussed in a separate chapter.

2. Analytical methods for sesquiterpenes (without lactones)

Recently, Kubeczka [4] proposed that the analytical methods applied to the analysis of essential oils can be classified into three different groups. Concentrating only on sesquiterpenes this classification can be adopted, too:

—Chromatographic methods like gas chromatography (GC), high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC), including multidimensional and chromatographic coupling techniques resulting in the separation to individual components.

—Hyphenated techniques that means instrumental on-line couplings of chromatographic separation devices to spectrometers like coupling of GC with mass spectrometry (MS), Fourier transform infrared spectrometry (FT-IR), UV or atomic emission spectroscopy (AES) as well as coupling of HPLC with MS. The advantage of those techniques is that more information about the structure of the separated components is available and often identification is possible.

—Methods like MS spectrometry and especially ^{13}C-NMR spectroscopy resulting in the identification of a multi-component sample without previous separation.

2.1. Gas chromatography

GC is the most efficient chromatographic technique for separating volatile mixtures, because of its high resolving power and the availability of universal detection using flame ionization detection (FID). Mostly capillary columns with dimethyl polysiloxane (methyl silicone) non-polar and Carbowax 20M polar phases are used. Carbowax 20M phases include DB-Wax, BP-20, PEG 20M and HP 20, while methyl silicone phases include SE-30, SF-96, OV-1, OV-101, BP 1, CPSIL 5CB, SP 2100, DB 1, DB 5 and HP 1 [6]. Among these fused-silica capillary GC columns DB 1 or DB 5 and CPSil 5 are mostly preferred. Identification based on direct comparison of retention times with standards or precise knowledge of retention indices, e.g., Kováts' retention indices [6–9]. Recently some 900 Kováts' indices of

400 individual compounds were summarized from the general literature [6].

One of the most important developments in gas chromatography has been the introduction of enantioselective capillary columns with a high separation efficiency in the mid-1960s by Gil-Av et al. [10]. Hereby a great number of chiral substances including many essential oil constituents can be separated. For more than 10 years, phases exclusively based on chiral diamide structures and concomitant hydrogen bonding interactions. The first optimum performance was reached in the development of Chirasil-Val, a methyl–polysiloxane phase containing about 6% branched aliphatic side chains with L-valine in diamide linkage, and similar polymeric chiral diamide stationary phases [11,12] which possessed high thermal stability. Specific derivatization procedures, such as formation of carbamates, cyclic carbonates or oximes, introduced sites for hydrogen bonding interactions and enhanced the range of applications (for literature see Ref. [13]). The analysis of patchoulol oil used in perfume may serve as an example that the resolution of a sesquiterpene mixture is sometimes better on Chirasil-Val than on methyl polysiloxane such as OV-101 [14].

Finally, the introduction of capillary columns using different hydrophobic cyclodextrin derivatives with a higher thermal stability considerably improved the separation facilities [4,13]. Cyclodextrins were modified by the introduction of alkyl and acetyl groups giving hydrophobic cyclodextrin derivatives, which interact enantioselectively with a great variety of chiral constituents of essential oils by forming diastereomeric inclusion complexes. Thus, separation of saturated hydrocarbons is possible as well as that of compounds with several functional groups [13]. Sesquiterpene enantiomers can be preferably resolved with 25 m capillaries with heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin or heptakis(6-*O*-*tert*.-butyldimethyl-silyl-2,3-di-*O*-methyl)-β-cyclodextrin (both 50%, w/w, in OV-1701) [13] as shown with a enantiomeric mixture of α-cubebene, β-cubebene, and β-elemene (see Fig. 1). Carrying out GC analysis it became evident that many of the chiral constituents were present in a certain proportion of the enantiomers. Pure chiral compounds were found only in a few cases. Sometimes the selectivity of a single column is not sufficient, especially when the mixture is very complex. Then two-dimensional capillary gas chromatography is a valuable tool for reliable stereochemical assignments. At first the essential oil is pre-separated on a non-chiral stationary phase (e.g., a 25 m capillary with CPSil 5). Afterwards small fractions are automatically or manually transferred to a second column [e.g., a 25 m capillary with heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin]. For this combined technique two FIDs and a ''live switching'' coupling piece for peak transfer from the polysiloxane to the cyclodextrin column are necessary [4,13]. Enantioselective capillary gas chromatography can be a valuable tool in quality control of essential oils, such as that from chamomile. It is possible to separate all four stereoisomers of α-bisabolol. Thus natural α-bisabolol can be differentiated from nature-identical, racemic, material in pharmaceutical and cosmetic formulations [15].

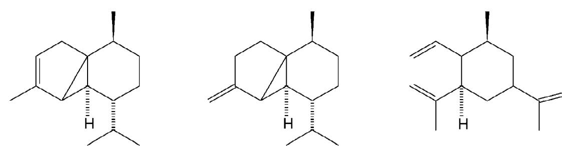

Fig. 1. Structures of enantiomeric compounds separated by a 25 m fused-silica capillary column with heptakis(6-*O*-*tert*.-butyldimethyl-silyl-2,3-di-*O*-methyl)-β-cyclodextrin (20% in OV 1701) [13].

In gas chromatography, FID is the most common detection method used. Nitrogen-containing compounds can be identified by nitrogen–phosphorus detection (NPD) [8] and ^{3}H-labeled sesquiterpenes by a radioactivity monitor [16]. A serious problem in essential oil mixtures is the complete separation of the hydrocarbons from the oxygenated compounds, which sometimes possess very low polarities and are obtained in the hydrocarbon fraction. This problem can be solved by solid-phase extraction (SPE) or solid-phase microextraction (SPME) followed by GC analysis [17,18], e.g., SPE with silica gel as stationary phase and hexane and diethyl ether as mobile phases gives a good separation between sesquiterpenes and their oxygenated derivatives. Another

possibility is the use of oxygen flame ionization detection (O-FID) for the selective determination of oxygenates [4,14,19]. This application seems to be particularly useful if very small samples have to be analyzed, e.g., from tissue cultures or oil glands. The O-FID analyzer works in such a way that first a cracking reaction forms carbon monoxide which is reduced in a second reactor yielding equimolar quantities of methane. The latter product can be sensitively detected by FID. The GC analysis of peppermint oil reported by Kubeczka [4] demonstrates the usefulness of this detection method.

2.2. Gas chromatography–mass spectrometry (EI-MS, CI-MS, MS–MS)

With the advent of high-resolution capillary GC using fused-silica columns, separation of complex mixtures of sesquiterpenoids was possible and the number of sesquiterpenoids isolated from essential oils has increased into the hundreds in recent years. Against this background identification only by GC retention data and Kováts' retention indices alone has become uncertain. Therefore, nowadays the combination of gas chromatography and mass spectrometry in the electron impact mode (GC–EI-MS) is a well established technique for the routine analysis of essential oils. This technique offers the possibility to gain additional information by mass spectra [4]. However, it has to be emphasized that identification of sesquiterpene hydrocarbons based only on mass spectra is also virtually impossible. Molecular rearrangement and isomerization processes of unsaturated hydrocarbons result in very similar mass spectra lacking characteristic fragmentation patterns. Only combined data of retention times, Kováts' or Sadler retention indices [20] and mass spectral data offer the possibility of an unambiguous identification of sesquiterpenoid constituents. In the last decade numerous papers have been published using these combined GC–MS techniques [9,21–28].

Nevertheless, sometimes mass spectra measured in EI mode are problematic, because they miss the molecular ions, specially with esters. In this case, the application of GC–MS in the chemical ionization (CI) mode using various reagent gases often yields valuable additional information. For sesquiterpenes it could be proven that the use of ammonia was by far superior to isobutane [29]. An additional method is negative ion chemical ionisation (NCI) with OH^- as the reactant ion [30–32]. However, these techniques have the disadvantage that there is no characteristic fragmentation pattern in the mass spectra. This problem can be solved for sesquiterpenes with one and two non-conjugated double bonds by using trioxo(*tert*.-butylimido)osmium(VIII) [33]. The reagent forms cyclic osmate ester amides as intermediates which gave after reduction vicinal monoamino alcohols, bisamino alcohols and aminotriols. These derivatives show characteristic mass spectra which can be used as a fingerprint for identification of the respective parent compound.

GC–MS is a very useful tool for the analysis of complex mixtures, but sometimes identification is limited when a single chromatographic peak contains several compounds so that the recorded mass spectra are difficult to interpret. There are several possibilities to solve this problem. One is MS–MS (tandem mass spectrometry), which, when coupled with GC, allows separate analysis of each component of such complex peaks. Moreover, the presence of minor constituents can also be confirmed [31,34]. The analysis of vetiver oil consisting of 150 components shows, that the combination of GC with different MS techniques (EI-MS, CI-MS, MS–MS) gives the best results in the resolution of a complex mixture. Altogether, 118 compounds have been characterized [34].

2.3. Gas chromatography–FT-IR spectroscopy

GC–MS is considered to be the method of choice in the identification of volatile compounds including sesquiterpenoids. However, in distinguishing isomers, which often occur in the terpene group, capillary GC–FT-IR coupling offers a useful supplementation [35]. Herres and Kubeczka have written an excellent review on this method [35,36]. Advantages of FT-IR spectroscopy are high resolution and sensitivity. The more time-consuming interpretation and the absence of a database of reference vapour phase spectra may be reasons why this technique is not generally accepted for the analysis of volatile compounds. However, a GC–FT-IR-MS instrument is available, whereby simultaneously IR and mass

spectra can be obtained. Thus, the unambiguous identification of critical isomeric sesquiterpenes is possible.

2.4. Further combined techniques: GC–UV and GC–AES

The combined techniques GC–UV or GC–AES mentioned in the review of Kubeczka [4,37] have not gained much importance up to now. GC–UV does not possess any significant advantage compared to GC–MS. UV spectra only offer low information, even less than FT-IR spectra. The coupling of capillary gas chromatography with atomic emission spectroscopy offers the possibility to the analyst to provide information on the elemental composition of the individual components of a mixture and, more importantly, on the percentual elemental composition of a component [38]. However, this is also possible by precise mass measurement. Altogether, both techniques can give additional information being to some extend complementary to MS and/or IR data.

2.5. Combination of ^{13}C-NMR or pyrolysis with GC–MS

Sometimes it may be useful to confirm by an additional analytical method the results of capillary GC and GC–MS, as it was shown with the rhizome oil of *Piper betle* [39]. Here ^{13}C-NMR followed GC and GC–MS analysis to confirm the structure assignments proposed by retention data and mass spectra. Structurally, closely related molecules such as stereoisomers, which exhibit insufficiently resolved mass spectral patterns, and compounds inseparable by GC, such as T-muurolol and T-cadinol can be identified in this way. However, this analysis has sometimes limitations, too. Spectra containing an immense density of individual lines, especially in the aliphatic region, are difficult to elucidate. Here the ambiguous assignment of signals may be very difficult. To make specific assignments more certain, distorsionsless enhancement by polarization transfer (DEPT) experiments can be carried out [4]. By this method, primary, secondary, tertiary or quarternary carbon atoms can be distinguished. In both cases identification is based on comparison of the sample spectrum with spectra of pure compounds. Qualitative as well as quantitative analysis is possible.

Furthermore, PY–GC–MS can be used as a microtechnique for the simultaneous analysis of various classes of compounds shown with *Vitex agnus-castus* and *Origanum heracleoticum* [40,41]. Samples in the submilligram range are heated at typically 500–1000 °C in the inert atmosphere of the GC carrier gas. The pyrolysis products are swept directly into the gas chromatograph and result in a pyrogram which is characteristic of the original sample. On the other hand, flash vaporization for molecules of low mass can be carried out by pyrolysis. The molecules are tightly adsorbed on the sample matrix, thereby extraction and concentration steps are saved.

2.6. High-performance liquid chromatographic analysis

The relatively good separation obtained by GC has delayed the application of HPLC to the analysis of volatile compounds, such as sesquiterpenoids (Table 1). However, HPLC analysis, which is has been well reviewed previously [5,42], offers some advantages compared to GC. HPLC can be carried out when GC analysis of thermolabile and/or polar compounds is difficult to achieve, involatile materials has to be handled or preparative isolation of volatile compounds is required. The coupling with diode array detection (DAD) can be useful. Restricting factors for application of HPLC in sesquiterpene analysis are the limitations inherent in the commonly available detectors for HPLC. Many volatile sesquiterpenoid components lack chromophoric groups and cannot be analysed by HPLC with UV detection at 254 nm. In these cases refractive index or low UV monitoring are necessary. Often low UV monitoring is preferred, because refractive index (RI) detection is not sensitive enough and low concentrations are not detectable. These problems limit the applicability of several solvents, because considerable background absorption at low wavelengths has to be avoided. Another problem in HPLC is the restricted peak capacity and the relatively small range of k' values of a liquid chromatographic system, by which effective separation of multicomponent mixtures in one operation is often not possible. Temperature is an important factor which controls k' values. Therefore analysis at

Table 1
Analytical separations of sesquiterpenes by HPLC

Sample	Column	Detection	Ref.
Solanum tuberosum (sesquiterpenoid stress metabolites)	μBondapak C_{18} (10 μm) (30 cm×3.9 mm)	255 nm	[47]
Valeriana officinalis	RP 18 (Knauer) (25 cm×4.6 mm)	225 nm	[52]
	Superspher (5 μm) (25 cm×4 mm)	254 nm	[53]
Lime oil (mono- and sequiterpenes)	Whatman Partisil-PXS (5 or 10 μm) (25 cm×4.6 mm)	Refractometer	[45]
Sesquiterpene hydrocarbons, sesquiterpene alcohols	Nucleosil C_{18} (7 μm) LiChrosorb RP 18 (10 μm) Zorbax C_{18} (7–8 μm) (25 cm×4.6 mm)	220 nm	[44]
Echinacea species (sesquiterpene ester)	Hibar 125-4 with LiChrospher 100	280 nm	[54]
Cotton stele (sesquiterpenoid stress metabolites)	Radial PAK C_8 (10 μm) (10 cm×8 mm)	254 nm	[46]
Sesquiterpene (phytoalexins)	Spherisorb CN (5 μm) (10 cm×3 mm)	205 nm	[48]
Polygonum hydropiper (polygodial)	Microsorb C_{18} (5 μm) (15 cm×4.6 mm)	116 nm	[50]
Petasites hybridus (sesquiterpenes)	Nucleosil-100 (3 μm) (25×4 mm)	254 nm	[49]
Ylang ylang oil *Solidago altissima* (germacrene-D derivative)	Chiralcel OB (25 cm×4.6 mm)	255 nm	[51]

low temperatures, e.g., −15 °C afforded better separations [5]. This could be proven on a LiChrosorb Si 60 (7 μm) column with *n*-pentane as solvent [42]. By these conditions a mixture of the sesquiterpene hydrocarbons, containing α-copaene, δ-elemene, β-elemene, β-caryophyllene, α-bergamotene, β-bisabolene, α-humulene and δ-cadinene was separated. Silica gel was at first deactivated by adding 4.8% water, because otherwise dry or fully activated adsorbents would possess undesirable properties such as sample alteration and irreversible adsorption [37,38].

The applications of HPLC in the analysis of volatile compounds can be placed in three major groups:

—Prefractionation of complex volatile mixtures prior to GC or GC–MS analysis.

—Qualitative and quantitative detection of specific volatile constituents.

—Semipreparative or preparative isolation of volatile compounds.

HPLC is often used as prefractionation of complex mixtures into groups of components in order to simplify GC and GC–MS analysis. Several examples are given in Ref. [42,43]. Using reversed-phase materials, e.g., RP18, fractionation is carried out according to the polarity and the chain length of essential oil constituents [44]. Most often three groups containing either oxygenated terpenes, monoterpene hydrocarbons or sesquiterpene hydrocarbons are obtained. The use of a reversed-phase column involves aqueous solvents, isocratic or gradient elution and UV detection. The pattern of elution is reversed on silica gel, that means starting with

hydrocarbons, etc. [45]. In this approach isocratic non-aqueous solvent mixtures and, in most cases, RI detection is necessary [45]. The obtained fractions can be subsequently separated by GC analysis. In Ref. [5] numerous examples are given, e.g., prefractionation of *Citrus*, or *Valeriana* essential oil.

Another field of HPLC application is the qualitative or quantitative determination of specific constituents in complex mixtures. In Ref. [46] a HPLC procedure is described for the determination of four antifungal sesquiterpenoid stress metabolites, hemigossypol, 6-methoxyhemigossypol, desoxyhemigossypol and 6-methoxydesoxyhemigossypol (for structures see Fig. 2), in cotton stele infected with *Verticillium dahliae*. Analysis was carried out on a Radial-PAK reversed-phase C_8 column (10 μm), eluted with a 0.1% aqueous phosphoric acid–methanol gradient, and monitored at 254 nm. Stress-induced sesquiterpenes of the potato can be determined on a reversed-phase μBondapak C_{18} column using methanol–water (7:3) and a detection wavelength of 200 nm [47]. Other systems used for sesquiterpene analysis are: a Spherisorb CN column (5 μm) and hexane–isopropanol as solvent [48] or a Nucleosil-100 column (3 μm) and a hexane–diisopropylether–acetonitrile gradient [49]. In Ref. [50] the quantitative analysis of polygodial in *Polygonum hydropiper* leaves is described using a Microsorb 5 μm C_{18} stationary phase and acetonitrile–water as isocratic solvent. Additional examples are given in Ref. [5].

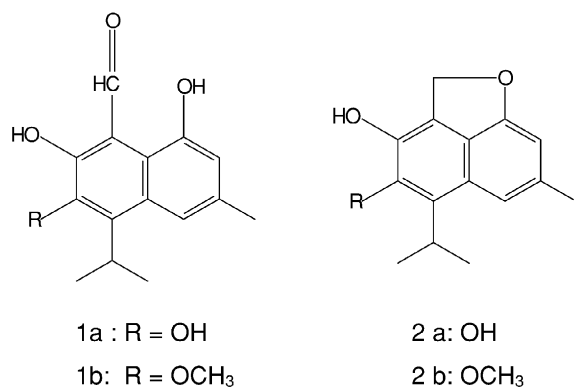

Fig. 2. Structures of sesquiterpenoid stress metabolites from cotton stele, hemigossypol (1a), 6-methoxyhemigossypol (1b), desoxyhemigossypol (2a) and 6-methoxyhemigossypol (2b), separated by HPLC analysis [46].

Previously it was shown that enantiomeric resolution can also be achieved by HPLC analysis. This was demonstrated with germacrene-D, which was separated in its enantiomers by a Chiralcel OD column [51]. HPLC technique of sesquiterpenes can be used in quality control of medicinal plants, when these compounds can serve as lead compounds. Valerenic acid and valerenal (for structures see Fig. 3) are sesquiterpenes which only occur in roots from *Valeriana officinalis* [52,53]. They can be used to exclude the occurrence of other *Valeriana* species. This is the same with *Echinacea* species which can also be differentiated by sesquiterpenes [54].

A further application of HPLC which has to be mentioned is its use in the isolation of pure compounds. Here often a combination of reversed-phase HPLC pre-fractionation followed by liquid–solid chromatographic HPLC separation can afford pure compounds. Another successful way for difficult separations is modifying commercial prepacked preparative silica gel columns by treatment with silver ions [55–57]. To achieve quantitative coating of the silver salt on the support it seems the best to dissolve silver perchlorate in a non-polar solvent such as toluene. Thus, a couple of isomeric non-polar sesquiterpene olefins from tolu balsam could be isolated (see Fig. 4).

2.7. Combined techniques with HPLC

As mentioned in Section 2.6. HPLC can be used as pre-fractionation followed by further chromatographic analysis, such as GC–MS. This procedure can be recommended when the essential oil is a very complex mixture, so that overlap of some peaks occur and minor compounds are not detected. This analytical performance is described in Ref. [44] for Vervain essential oil or in Ref. [45] for lime oils.

In Ref. [58] this technique is more developed. An automated HPLC–high resolution GC (HRGC) analysis is described for the volatile fraction of citrus oil. HPLC was used to separate the oil in three fractions, which were automatically transferred to the GC without interferences. Furthermore, it is possible to couple GC and MS so that the components can be identified by their retention time as well as by their mass spectrum. The results of this work show that

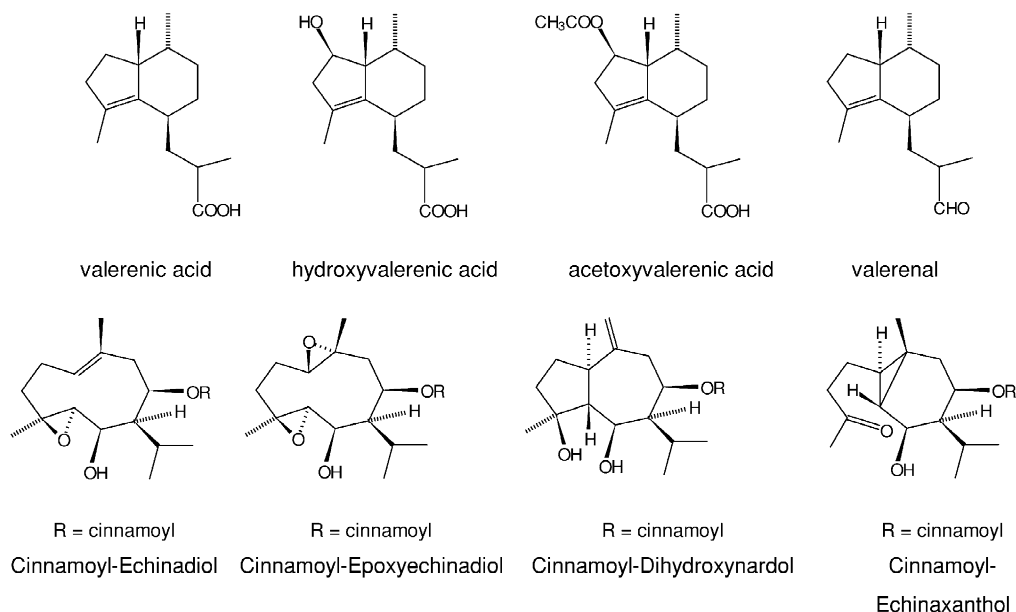

Fig. 3. Sesquiterpenes from the medicinal plants *Valeriana officinalis* and *Echinacea purpurea*—HPLC analysis for quality control [52–54].

combined HPLC–HRGC has a great potential for the analysis of complex mixtures, specially valuable for quality control of essential oils in industrial laboratories. The technique enables identification and separation of compounds of the same polarity from a mixture of components of different polarity, even if the relative concentrations of the various classes of components vary considerably. This was shown by Mondello et al. [58] who separated aliphatic, monoterpene and sesquiterpene aldehydes from sweet orange oil by this technique.

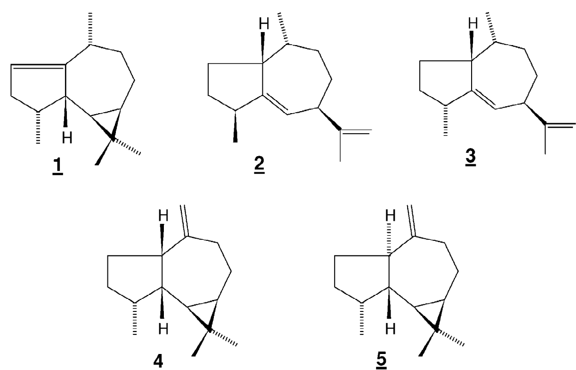

Fig. 4. Structures of aromadendrene (1, 4, 5) and guaiane (2, 3) derivatives from tolu balsam separated by HPLC on silver-loaded LiChrosorb Si 60 [55].

For the solution of special problems, e.g., when thermosensitive sesquiterpenes have to be separated and identified, HPLC coupled with mass spectrometry can be used. This is demonstrated with prehelminthosporol, a phytotoxin formed by the plant parasitic fungus *Bipolaris sorokiniana*. Quantification was possible using reversed-phase on-line HPLC–MS and plasma spray ionization [59]. A detection limit of ≈5 ng was obtained using selected ion monitoring at m/z 219. HPLC combined with thermospray mass spectrometry is a further coupling technique often used in phytochemical analysis for screening of extracts. The usefulness was shown with crude extracts of the pathogenic basidiomycete *Armillaria* which were screened for sesquiterpene aryl esters [60]. However, it has to be mentioned that mass spectra obtained by ionisation methods lack characteristic fragmentation pattern. Therefore, unambiguous identification of unknown compounds is not possible using only retention time, UV spectra and molecular mass, which is obtained from the mass spectra. Liquid chromatography–mass spec-

trometry has to be considered only as a suitable method for screening of crude fractions or for comparative analysis.

2.8. Supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC)

Analysis of sesquiterpenes may often cause problems because of their thermosensitivity. This property may have a negative effect in the isolation procedure as well in analysis carried out for identification, e.g., GC or GC–MS. Liquid phase solvent extraction, steam distillation or headspace are frequently used, even though they are time consuming, may yield low extraction efficiencies, and may result in loss or degradation of the components. These problems can be solved by using SFE. It has been reported that this technique is selective and highly efficient in the isolation of sesquiterpenes [61,62] and even superior to distillation–solvent extraction [62]. Many supercritical fluids, such as carbon dioxide, have low critical temperatures which enables extractions to be performed at relatively low temperatures, thus possible degradation of thermally labile compounds is reduced. Concentration steps are simplified, because carbon dioxide is a gas at room temperature. Therefore it is possible to couple the extraction step directly with chromatographic analysis, e.g., with capillary gas chromatography [61]. Nevertheless, by using SFE and on-line GC degradation may occur during GC analysis as already mentioned above.

An analytical method which also uses supercritical carbon dioxide and bare silica as stationary phase is SFC. Detection can follow by on-line coupling with UV spectroscopy [63] or FT-IR [64–66]. These attractive techniques at low temperatures are suitable for the analysis of extreme thermally labile compounds such as sesquiterpene hydrocarbons. The advantages as well as disadvantages of on-line coupling with FT-IR, have already been discussed in Section 2.3 and are excellently summarized in Ref. [64]. Carbon dioxide offers large transparency regions in infrared, so that many functional groups can be detected. Chromatograms are reconstructed either from interferograms by Gram-Schmidt algorithm or through an infrared absorbance window [65]. Combined SFC–UV offers an alternative to the existing HPLC techniques [63]. By modifying carbon dioxide with 0.5% of methanol sesquiterpene alcohols, such as nerolidol, α-bisabolol and (2E,6E)-farnesol were separately eluted in a rather short time (10 min). Addition of methanol is essential, otherwise the sesquiterpene alcohols would not be eluted from the silica-packed column. This is due in part to the carbon dioxide, which is not a good enough solvent for sesquiterpene alcohols, but also to the absorption of hydroxyl groups of sesquiterpene alcohols on residual silanol sites of the stationary phase.

3. Analytical methods of sesquiterpene lactones (SLs)

Following the classification of the sesquiterpenes in different groups analytical methods of SLs can be divided into two groups:

—Chromatographic methods like HPLC; SFC, GC, TLC, OPLC and MEKC for the separation in individual components.

—Hyphenated techniques such as GC–MS, HPLC–UV, HPLC–NMR or SFC–UV by which characterization and often identification of the separated compound is possible.

3.1. HPLC with UV detection

SLs are compounds with low volatility and many of them are thermolabile. Because of these properties HPLC, mainly on reversed phase, is the analytical method of choice for SL analysis in crude plant extracts (see Table 2 and Fig. 5). However, normal-phase gradient run HPLC by *n*-hexane–acetonitrile–isopropanol may be sometimes preferable [67,68]. HPLC has been applied successfully to the analysis of 33 pseudoguaianolides and xanthanolides of the genus *Parthenium* [69] as well as 21 pseudoguaianolides of *Arnica chamissonis* and 15 of *A. montana* [70,71]. Acetonitrile–water or methanol–water gradients are used, respectively. In a comprehensive study 37 SLs of the *Melampodiinae* have been investigated [72]. Based on this method and combined with GC (see Section 3.3) a crude extract of *Melampodium cinereum* was analysed. Further studies revealed the usefulness of qualitative HPLC analysis for the detection of Sls in plant extracts,

Table 2
Analytical separations of sesquiterpenes lactones by HPLC

Sample	Skeleton*	Column	Solvent	Detection	Ref.
Parthenium species	PGU, XA	Ultrasphere-ODS (15×4.6 mm)	CH_3CN–water	215 nm	[69]
Laurus nobilis	GE, GU	LiChrosorb RP 18 (5 μm, 25×4 mm) LiChrosorb RP 8 (5 μm, 25×4 mm) LiChrosorb RP 8 (5 μm, 12.5×4 mm)	Water–CH_3CN	210 nm	[78]
Cichorium intybus	GU	Radial-PAK C_{18} (10×8 mm)	MeOH–water	258 nm	[74]
Arnica chamissonis	PGU	Shandon Hypersil ODS (5 μm, 25×4.6 mm)	MeOH–water	225 nm	[70]
Arnica montana	PGU	Shandon Hypersil ODS (5 μm, 25×4.6 mm)	MeOH–water	225 nm	[71]
Ambrosia maritima	PGU	Nucleosil C_8 (5 μm, 12.5×4 mm)	Water–CH_3CN	220 nm	[73]
Helianthus species	GE, GU	Shandon Hypersil ODS (5 μm, 25×4 mm)	MeOH–water	225 nm 265 nm	[80]
Cichorium intybus	GU	Spherisorb C_{18} (5 μm, 20×4.6 mm)/ LiChrosorb Si (10 mm, 25×4.6 mm)	MeOH–water, Water–CH_3CN, THF–water/ CH_2Cl_2–MeOH, EtOAc–hexane, *tert.*-butyl-methyl ether–MeOH	258 nm	[75]
Melampodium cinereum species of subtribe *Melampodiinae*	GE	Shandon Hypersil ODS (5 μm, 15×4.6 mm)	MeOH–water	210–254 nm	[72]
SLs from different plants	GE EU	RP 8 (10 μm, 20×4.6 mm) Si-100 (10 μm, 20×4.6 mm)	MeOH–water *n*-Hexane–CH_3CN–isopropanol	230 nm	[67,68]
Artemisia annua		CLC-ODS (25×4.6 mm) and Bondclone 10 CN (30×3.9 mm)	Water–CH_3CN (1% TFA)	220 nm	[84]
Artemisia leucodes	GU	Microcolumn of KAKh-2 (2×62 mm)	MeOH–0.5 acetate buffer or THF–0.01 *M* phosphate buffer	254 nm	[79]
SLs from different plants	GE, EU, GU PGU	LiChrosorb RP 18 (7 μm, 25×4.6 mm)	MeOH–water	254 nm	[87]
Tanacetum species	GE, EU, GU	LiChrosorb RP 18 (5 μm, 62×2 mm)	MeOH–water	250 nm	[77]
Artemisia caerulescens	EU	LiChrospher 100-RP (24×4 mm)	Water–CH_3CN	236 nm	[82]
Parthenium species	PGU	Nucleosil C_{18} (5 μm, 25×4 mm)	MeOH–water	210 nm	[76]

*EU: Eudesmanolide; GE: germacranolide; GU: guaianolide, PGU: pseudoguaianolide; XA: xanthanolide.

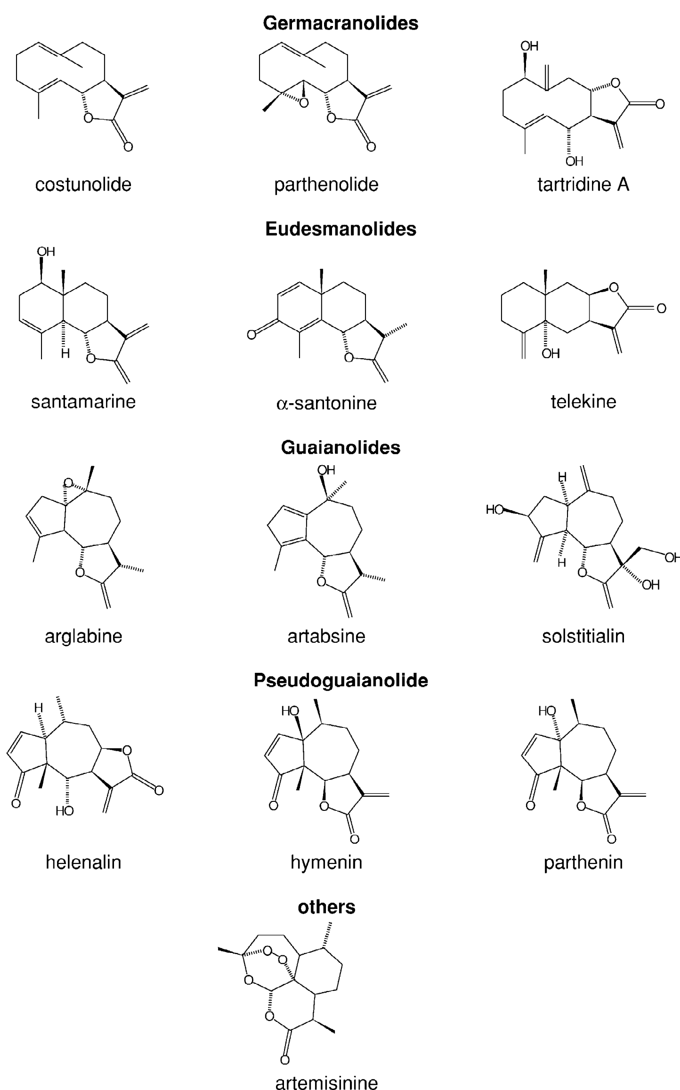

Fig. 5. Structures of some sesquiterpene lactones analysed by HPLC.

e.g., pseudoguaianolides from the ambrosanolide-type from *Ambrosia maritima* [73], bitter SLs with a guaianolide skeleton from chicory roots [74,75], pseudoguaianolides (ambrosanolides and parthenolides) from *Parthenium* species [76] or germacranolides and eudesmanolides from *Tanacetum* species [77]. In Ref. [78] some hints for application are given. To avoid severe peak leading, especially in early peaks, SLs should be solved in the same ratio of the solvent mixture rather than in only one solvent. It was shown that there is no difference in separation quality of some structural different SLs using either an RP 8 column of 125×4 mm or 250×4 mm. However, because of lower back pressure and shorter time of analysis the shorter column is preferred. For special applications a microcolumn may be useful [79].

For a better sample preparation avoiding undesirable compounds Spring developed a micro-sampling technique [80]. It was demonstrated that it is possible to directly sample 20 glandular trichomes of leaves and flower heads, where SLs are sequestered, under a

dissection microscope and prepare a water/water extract for analysis. This method is suitable for chemotaxonomical studies as shown with the genus *Helianthus* [80].

HPLC is a valuable tool for quantitative analysis, too. The content of single SLs as well as the total amount can be determined shown with the complex mixture of SLs in flowers of *Arnica chamissonis* and *A. montana* [70,71] which are used for Arnicae flos in the German pharmacopoea. Quality control of the flower drug as well as standardization of preparations used in traditional medicine are possible by this method. Further reports on the same topic, but with less complex mixtures are the quantitation of hymenoxon in *Hymenoxys odorata* and of helenalin, mexicanin E and helenalin in *Helenium* species [81] as well as santonin in *Artemisia caerulescens* [82]. In Ref. [83] it is reported that the combination of ODS and CN columns results in a good resolution with maximum recovery of the three SLs artemisinin, arteannuin-B and artemisinic acid in the chinese medicinal plant *Artemisia annua* (''qinghao''). Because of the importance of artemisinin as a novel antimalarial agent, several reports on quantitation can be found in literature (see Ref. [84]). One problem in HPLC analysis of artemisinin, specially in plant extracts, is its absorption at the low end of the UV spectrum, between 210 and 220 nm. Therefore, ElSohly et al. [84] published a HPLC method with postcolumn derivatization. Artemisinin is modified by 0.2% NaOH resulting in an UV absorption at 292 nm. The mentioned problem of low absorption maximum in UV can be applied to most sesquiterpene lactones. This property is often a limited factor in carrying out successful HPLC analysis of SLs in crude plant extracts especially of minor components [85,86]. A solution of this problem, but only for SLs containing α-methylenebutyrolactone functions, is derivatization by 9-thiomethylanthracene [85]. The thiol containing reagent reacts in a Michael type addition with the α-methylenebutyrolactone of the SL and increases the sensitivity, so that routinely nanogram quantities of the lactones can be detected by HPLC and monitoring at 369 nm. Parthenolide, the effective compound in *Tanacetum parthenium* used for the treatment of migraine and arthritis has been determined in such a way [85].

3.2. Combined techniques with HPLC: HPLC–MS and HPLC–NMR

As mentioned above, UV detection is sometimes unsuccessful for SL analysis, because several SLs show weakly absorbing chromatophoric groups or no chromophores at all. Therefore, other detection methods, such as RI or evaporative light scattering detection (ELSD) can be used to overcome this limitation [87,88]. Whereas these detection techniques are not yet widespread in HPLC analysis of SLs the combination with mass spectrometry has become extremely helpful in the detection and identification of SLs in raw plant extracts. The thermospray (TSP) interface was one of the first which was developed in this field [89]. The mass spectra obtained by HPLC–TSP yield different quasi-molecular peaks, e.g., $[M+H]^+$, $[M+NH_4]^+$, or ions formed from solvent adducts. Further fragment ions resulting from the loss of water or acid residues may be obtained. The mass spectra are comparable with those from the direct chemical ionization (DCI) technique in the positive ion mode [90].This method was successfully applied to the identification of artemisinin in *Artemisia annua* [90] or of chlorinated SLs being formed in the isolation procedure in the neurotoxic thistle *Centaurea solstitialis* [91]. Further techniques which give similar results as with TSP are atmospheric pressure chemical ionization (APCI) or electrospray (ESP) coupled with HPLC. Stuppner et al. showed that APCI technique has to be considered more suitable for the detection of pseudoguaianolides in extracts from *Arnica montana* [92]. Minor constituents could only be detected by APCI. Nevertheless, HPLC–ESI gains more and more importance in the structure elucidation of isolated SLs for information on molecular mass [93], because the molecular ion is often missed in EI mass spectra. A further coupled technique worth to be mentioned is HPLC combined with particle beam (PB) mass spectrometry [87]. One of the main advantages of a PB interface is the possibility of recording mass spectra in both EI and CI modes, through a single analyser mass spectrometer with medium-high sensitivity. In Ref. [87] 22 structurally different SLs were separated and mass spectra in the EI as well as in CI mode, positive-ion

CI (PICI) and electron-capture negative ionization (ECNI) with methane as reagent gas, recorded. The HPLC–PB-EI mass spectra of the investigated SLs showed exactly the same patterns as those obtained by direct introduction. HPLC–PB-CI mass spectra often gave intense SL molecular or quasi-molecular ions, together with some other diagnostic ions. Based on these data fractions from *Artemisia umbelliformis* were analysed.

LC–NMR is a further technique which combines high-performance separation techniques with a structurally informative spectroscopic method (for literature see Ref. [94]). It allows extracts to be screened not only for structural classes but also for compounds without isolation of individual compounds. In the beginning this method has achieved limited success due to the lack of sensitivity. Recently, this situation has changed as new solvent suppression techniques have been introduced. The successful application of LC–NMR is demonstrated with *Vernonia fastigiata* extracts, in which nine SLs including minor constituents could be identified [94].

3.3. Supercritical fluid chromatography and micellar electrokinetic chromatography (MEKC)

Sesquiterpene lactones can be separated by SFC as already shown with sesquiterpenes in Section 2.8 [86,95]. This method is especially suitable to thermolabile SLs. Packed column SFC has a sample capacity similar to HPLC, operates with the same columns, but requires shorter analysis times and allows more consistent column conditions during repeated analysis. SFC has the disadvantage that the polar stationary phase can retain polar components [86]. In Ref. [96] a method is described how a synthetic mixture of three difficult-to-separate SLs was separated by optimization on a short column using the three-parameter simplex and then transferring the method to a longer column. Detection can be carried either by UV spectroscopy, demonstrated with *Artemisia umbelliformis* and *Carduus benedictus* [86] extract or by ELSD as proven with bilobalides in *Ginkgo biloba* and *Artemisia annua* [88,95]. The latter paper also describes detection of artemisinin and artemisinic acid by SFC–FID. In Ref. [86] a couple of stationary columns were tested: LiChrospher 100 RP 18, LiChrosorb RP 8, LiChrosorb Diol, S3W Silica Spherisorb, S3-nitrile Spherisorb (CN). A CN separation phase with MeOH–water (95:5) as modifier for supercritical CO_2 was the most successful combination in the SLs investigated.

A further analytical method which gives similar results as proven for the qualitative analysis of SLs of *Arnica* flowers is MEKC [97]. MEKC is a rapid analytical method which saves both time and solvent compared with HPLC. However, retention times, especially the longer ones, are somewhat difficult to reproduce by MEKC.

3.4. Gas chromatography

GC analysis are often used in the routine analysis of plant extracts which are screened for SLs. However, thermal degradation has to be considered. Owing to this property, many heteroatom-substituted sesquiterpene lactones cannot be analysed by GC without derivatization and trimethylsilyl ethers have to be prepared [98]. Nevertheless, successful application of GC and GC–MS to underivatized SLs not being thermolabile have been reported in the literature [70–72]. Qualitative and quantitative analysis of extracts from *Arnica* flowers were carried out on a 25 m fused-silica capillary column coated with OV-01-CB-0.25. Capillary GC analysis on a bonded polymethylsilicone stationary phase (RSL-150) of 37 SLs of the *Melampodiinae* revealed good separations, but several of the compounds under study decomposed, either in the injector port or in the column itself [72]. This was evidenced by multiple, poorly shaped peaks. Comparing separation power of GC and HPLC analysis it was concluded that the use of both systems in conjunction with one another is a powerful technique for the rapid analysis of plant samples for these SLs.

3.5. Thin-layer chromatography

TLC was one of the first chromatographic methods and was also applied to analysis of SLs. Its importance has decreased nowadays. However, in combination with special spray reagents TLC can provide

valuable information about the type of SL. Visualization reagents are numerous, e.g., vanillin/*o*-phosphoric acid, anisaldehyde or *p*-dimethylaminobenzaldehyde-sulfuric acid, sulfuric acid, resorcin-sulfuric or phosphoric acid, aluminium chloride or hydroxylamine [67,68,99–104]. SLs lacking an exocyclic-α-methylene group can be visualized by dimethylamine followed by Dragendorff reagent [68]. In general, it depends on the skeleton which of the reagents is more favourable. TLC can be used for isolation control in column chromatography, e.g., open column chromatography (CC) or middle-pressure liquid chromatography (MPLC) and identity control of medicinal plants as published for *Arnica* flowers [105]. TLC is here advantageous, because of its rapid, easy and cheap performance without the need of a large instrumental equipment. However, compared with HPLC– or GC–MS this chromatographic method provides less information. Moreover, TLC can be used to separate diastereoisomers, such as parthenin and hymenin by multiple development of TLC plates in the same solvent system [106]. These two SLs only differ in the configuration of the cylopentenone ring. In contrast, screening of complex plant extracts by TLC often causes problems [67,68], because the wide range of colors produced with all types of SLs makes its interpretation difficult.

A special TLC method, worth mentioning is overpressured layer chromatography (OPLC) [67,68]. As the separation in OPLC chamber is much faster than in normal TLC, the separation time is relatively short and in the closed system there is no or limited chance providing artefacts. Therefore, OPLC is especially suitable for studying big number of samples. The two directional development allows the separation of up to 34 samples in a single run, while HPLC using RP 8 or Si 100 phases by isocratic and gradient elutions, respectively, may be the preferred technique when rapid qualitative and quantitative informations are needed. However, it was demonstrated that OPLC combined with direct densiometry also allows rapid quantitative analysis of SLs, e.g., parthenolide derivatives in different plants of *Zoegea leptaurea*. Parthenolide can also quantitatively determined in *Chrysanthmum parthenium* by using TLC–FID [107].

4. Nomenclature

AES	Atomic emission spectroscopy
APCI	Atmospheric pressure chemical ionization
CC	Column chromatography
CI	Chemical ionization
DAD	Diode array detection
DCI	Direct chemical ionization
ECNI	Electron-capture negative ionization
EI	Electron impact
ELSD	Evaporative light scattering detection
ESI	Electrospray ionization
DEPT	Distortionless enhancement by polarization transfer
FID	Flame ionization detection
FT-IR	Fourier transform infrared spectrometry
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HRGC	High-resolution gas chromatography
LC	Liquid chromatography
MEKC	Micellar electrokinetic chromatography
MPLC	Middle-pressure liquid chromatography
MS	Mass spectrometry
MS–MS	Tandem mass spectrometry
NICI	Negative chemical ionization
NPD	Nitrogen–phosphorus detection
O-FID	Oxygen flame ionization detection
OPLC	Overlay pressure liquid chromatography
PB	Particle beam
PICI	Positive ion chemical ionization
PY	Pyrolysis
RI	Refractive index
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SL	Sesquiterpene lactone
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
TLC	Thin-layer chromatography
TSP	Thermospray

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