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Review

Recent advances on HPLC/MS in medicinal plant analysis

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ABSTRACT

With gaining popularity of herbal remedies worldwide, the need of assuring safety and efficacy of these products increases as well. By nature they are complex matrices, comprising a multitude of compounds, which are prone to variation due to environmental factors and manufacturing conditions. Furthermore, many traditional preparations compose of multiple herbs, so that only highly selective, sensitive and versatile analytical techniques will be suitable for quality control purposes. By hyphenating high performance liquid chromatography and mass spectrometry (LC–MS) these high demands are fulfilled, providing the user with a multitude of technical options and applications. This review intends to reflect the impact of LC–MS for medicinal plant analysis focusing on most relevant reports published within the last five years. Commenced by introductory remarks to the different MS approaches most commonly used (e.g. ion trap and time of flight mass analyzers, fragmentation and ionization modes), respective LC–MS applications on the analysis of natural products in medicinal plants, commercial products and biological samples are presented. Methodological aspects like stationary and mobile phase selection or MS settings are discussed, and advantages or limitations of the described techniques are highlighted.

Contents

1. Introduction

The inception of mass spectrometry (MS) can be traced back to the beginning of the last century, when Joseph John Thomson observed the fact that magnetic and electrostatic fields deflect positive rays depending on their mass. Yet, it took many years of intense research by scientists like Alfred Nier (1940s: improvement in vacuum technologies and ion sources) or Wolfgang Paul (1953: quadrupole mass analyzer) till this technique became of practical relevance for the analysis of organic compounds. The following years (1960–1980) were highlighted by a multitude of technical developments like MALDI (matrix-assisted laser desorption), FTMS (Fourier transform mass spectrometry), FAB-MS (fast atom bombardment mass spectrometry) or ion-trap instruments, just to name a few [1,2]. Today, a mass spectrometer is nearly standard equipment in a modern analytical laboratory and indispensable for

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scientific research on a high level. Due to different instrumental characteristics in ionization, resolution, scan speed, and fragmentation MS is a versatile technique with described applications from astronomical studies and geophysics to biology and medicine.

The analysis of secondary metabolites in plants is a challenging task because of their chemical diversity, usually low abundance and variability even within the same species. It is estimated that 100,000–200,000 metabolites occur in the plant kingdom [3], and considering the fact that many traditional herbal preparations of Chinese or Indian origin contain not one but several medicinal plants, only highly selective and sensitive methods will be suitable for controlling their composition and quality. Sensitivity is the disadvantage of using proton nuclear magnetic resonance analysis (^{1}H NMR) for this purpose, yet it is the only technique which produces signals directly correlating with the amount of analytes in the sample [4]. Thus, most commonly chromatographic or electrophoretic techniques in combination with different detectors are employed for this purpose. Due to extremely small sample volumes capillary electrophoresis (CE) often faces sensitivity problems as well [5], and gas chromatography (GC) is a powerful tool for volatile constituents only (otherwise derivatization of the analytes is required). Thus, high performance liquid chromatography (HPLC) was and still is the preferred separation technique for the analysis of natural products. Recent developments of sub 2 μm stationary phases and pumping devices enabling pressures up to 1300 bar further supported this trend [6]. The type of detector used, ranging from photodiode array (DAD) or UV–vis detectors, evaporative light scattering (ELS) and fluorescence detectors to MS-detection, largely depends on the analytes investigated. Compared with the foremost mentioned techniques, the latter offers excellent sensitivity and selectivity, combined with the ability to elucidate or confirm chemical structures (depending on the instrument used) [7,8]. Therefore, it is not surprising that the use of LC–MS for the qualitative and quantitative analysis of constituents in medicinal plants steadily increased over the last years.

This review intends to summarize the most interesting and relevant applications of HPLC–MS for natural products analysis reported within the last five years (2006–2010); GLC–MS reports are not covered as they are outside the scope of this manuscript. A confinement to certain plants or applications is necessary due to the extent of the topic. The chapters are grouped by compound classes, and special emphasis will be put on applicability of the described procedures on real life samples, methodological features and possible advantages or disadvantages compared to alternative approaches. In order to familiarize the reader with the topic, in the next chapter the basic principles of MS are briefly discussed and major differences of the most commonly used types are highlighted.

2. Instrumentation

2.1. Ionization source

Any mass spectrometer comprises three major components, which are ionization source, analyzer and detector. Ions, which are easier to manipulate than molecules in neutral stage, can be obtained using different ionization techniques, with electrospray ionization (ESI) and atmospheric pressure ionization (APCI) being most widely used [9]. ESI is a soft ionization technique (little fragmentation but often adducts are observed) for a wide range of compounds, and ionization is achieved by applying a high electric charge to the sample needle. The latter has an inner part for the LC eluent and an outer part for a nebulising gas (e.g. nitrogen). A fine aerosol is produced, in which the droplet size continuously decreases due to evaporation of the solvent. Once a critical limit is reached (Rayleigh limit) ions are formed by a process called "Coulombic explosion", and they enter the analyzer due to a potential or pressure difference. The setup of an APCI source is similar but charge is applied via a corona pin, which is located at the exit of a heated tube. This technique is especially suitable for non-polar compounds, and just like ESI it can be operated in positive and negative mode [10]. A more recent development is atmospheric pressure photoionization (APPI). It is considered as alternative for compounds that are poorly ionized by ESI and APCI, and is based on the interaction of a photon beam produced by a kypton or xenon lamp with vapors formed by the nebulisation of a liquid solution [11]. This technique usually requires the addition of a dopant, a preferentially ionized substance that acts as intermediate between photons and analytes. Solvents like acetone, toluene or anisole have been used for this purpose, mostly leading to the formation of positively charged compounds [12].

2.2. Mass analyzer

Four parallel aligned metal rods are the core of any single quadrupole, the most economic type of mass analyzer used nowadays (Fig. 1). The opposing rods are connected and an RF voltage is applied between one pair of rods and the other. Direct current is then superimposed, so that a quadrupolar electric field is created, which only ions of a certain masses can pass through [9]. Like all other analyzers it has to be maintained under vacuum in order to enable transition of the ions. A quadrupole can be considered as a mass filter, not permitting the fragmentation of ions (MS/MS). This is possible by arranging three quadrupole cells in sequence (triple quadrupole), where the first serves as mass filter, the second as collision cell, and the third as filter again. Fragmentation occurs in the collision cell with the help of a collision gas (e.g. argon or nitrogen) and applied energy (collision energy). Different data acquiring modes are possible with this setup, for example the highly sensitive selected reaction monitoring (SRM), also known as multiple reaction monitoring (MRM) [13,14].

A different concept is followed in ion trap analyzers, which are especially suitable for multiple fragmentation steps (MS^n) rather than quantitative studies. In linear ion traps, ions are isolated and accumulated due to a special arrangement of hyperbolic and ring shaped electrodes as well as oscillating electric fields. Then the ions can be fragmented in a similar way as described above by collision-induced decomposition (CID). This process can be repeated in sequential manner, so that valuable structural information is obtained [15]. More complex and elaborate trapping technologies are so-called 3D traps, for which Fourier transform ion cyclotron resonance-MS (FT-ICR-MS) and Orbitrap are examples. Both confine ions in cyclotron motion, either by strong magnetic fields (FT-ICR-MS) or electrostatic forces (Orbitrap), resulting in high levels of resolution (typically from 100,000 to 1,000,000) and mass precision (1–2 ppm) [16].

An increasingly popular type of mass analyzer is time of flight (TOF). It is based on the fact that molecular mass is related to velocity, and therefore compounds will reach the detector with increasing m/z values. Crucial parameters are position of the ion source (orthogonal or linear), length of the flight tube and the reflectron [17]. The latter, also called ion mirror, consists of a set of rings, is accountable for the high resolution power of TOF. They increase flight distance and further focus the ions, so that mass accuracies of 5 ppm are possible. This permits the verification of elemental composition and differentiation of isobaric compounds (with the same nominal mass but different elemental compositions, and therefore different exact mass). In addition to good sensitivity and resolution the technique allows high scan speeds making it suitable for fast separations [18] and it can be coupled to quadrupole (QTOF-MS) or ion trap (IT-TOF-MS) mass fil-

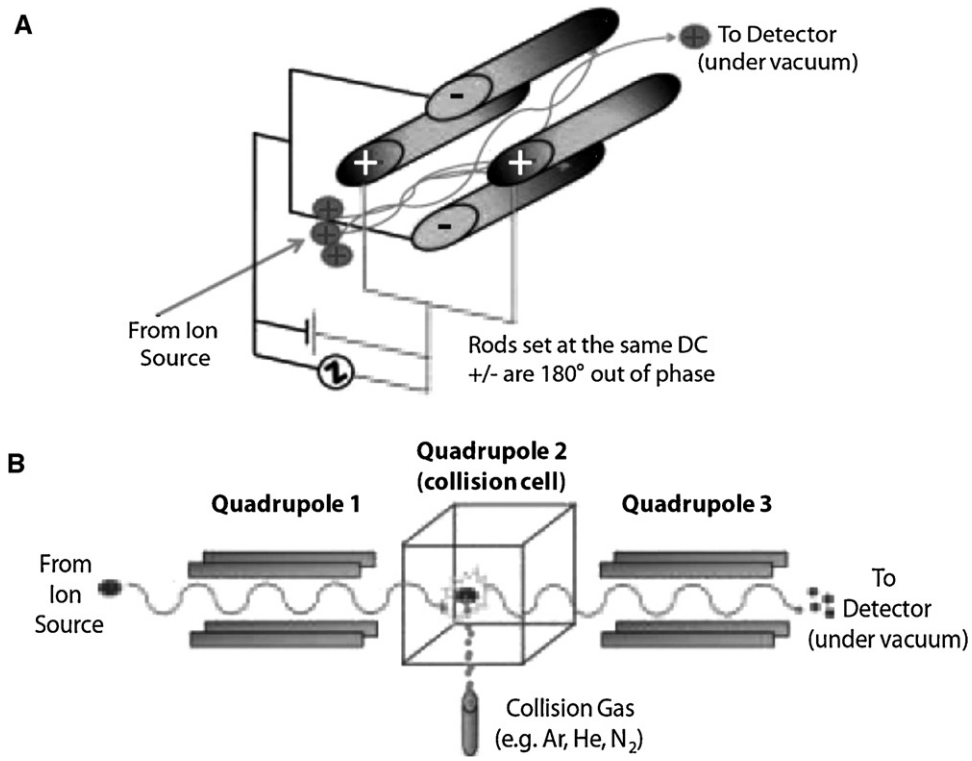

Fig. 1. Schematic design of a single quadrupole (A) and triple quadrupole (B) mass analyzer. Reproduced with permission from [9].

ters. Thus, high resolution MS/MS or MS^n analyses are possible as well [8].

2.3. Detector

MS detectors record either a current or charge produced by the ions. In modern instruments this is commonly achieved using the micro-channel plate (MCP) technology. It consists of a thin plate perforated by tiny tubes or slots typically 6–10 μm in diameter. In the presence of an electric field each channel acts as electron multiplier and whenever hit by an ion or photon a cascade of electrons starts [19]. The electrons then exit the channel on the opposite end, and the resulting current is measured on an anode. After each cascade the MCP has to be recharged before it can detect another signal. For much higher gain not just one but two or three plates, arranged in V or Z shape, are used in many instruments [20].

3. Applications

3.1. Acids

Good examples for the relevance of quality control of herbal preparations are aristolochic acids. They are a mixture of nephrotoxic and carcinogenic nitrophenantrene carboxylic acids occurring in the genus *Aristolochia*. Due to a similar name in Chinese the non-toxic *Stephania tetrandra* ("Fangji") is sometimes wrongly substituted with *Aristolochia fangchi* ("Guang Fangji"); both are traditionally used as diuretic and for treating rheumatic conditions. These confusions led to the poisoning of more than 50 women in Belgium ("Chinese herbs nephropathy"), who took an adulterated herbal slimming preparation [21]. But despite the fact that this incident happened nearly twenty years ago the problem still persists. In 2006 Koh et al. analyzed ten samples of "Fangji", and in nine of them the presence of aristolochic acid I (AA I) was confirmed by LC–MS/MS [22]. The methanolic plant extracts were separated on reversed phase material (Luna C18; 5 μm) using methanol and water as mobile phase. For MS studies a Finnigan LCQ ion trap mass spectrometer with APCI source was utilized, with AA I being assignable in positive mode as $[M+NH_4]^+$ adduct. For fragmentation helium was used as collision gas and the relative collision energy was set to 35%. In another study Zhao et al. investigated the distribution of aristolochic acids in *Herba Asari* including the safest preparation method [23]. They concluded if an aqueous roots is used it can be considered a safe remedy, as the amounts of AA I are low (0.08 μg/g compared to 0.32 μg/ml in methanol extract). LC–MS was not only used for identification but also quantification of AA I, which was recorded in SIM mode at $m/z = 359.1$, corresponding to the same adduct as mentioned above (see Table 1 for further methodological details).

A more comprehensive investigation of the different aristolochic acids in *Aristolochia* species (e.g. *A. contorta*, *A. fangchi* or *A. mollissima*) was described by Yuan et al. in 2007 [24]. They studied the occurrence of six acids (AA I, AA II, AA C, AA D, 7-OH AA I and aristolochic acid) together with four aristololactams, and quantified these constituents using diode array detection (DAD) and MS (Micromass ZQ 4000 in positive ESI mode). A comparison of both approaches showed that "aristolochic acids are in nature not provided with high ionization efficiency", as indicated by similar LOD values determined (e.g. for AA I by MS: 12 ng/ml; by UV at 254 nm: 15 ng/ml). Nevertheless, the authors considered the described LC–MS assay suitable for quality control purposes because of its good selectivity. They also studied the ionization characteristics of aristolochic acids (with ESI multiple products like $[M-H_2O+H]^+$or $[M-NO_2+H]^+$ were observed) and fully validated the procedure. Other LC–MS studies targeting aristolochic acids to a smaller extend are those on the biochemical effects induced by AA I [25] or a cooperative study using MS for confirmation purposes only [26].

Numerous reports on the analysis of phenolic acids (e.g. caffeic acid and its derivatives) by LC–MS have been published. They

Table 1
Selected LC–MS assays for the analysis of acids in herbal medicines.

Analytes	Matrix	LC-conditions	Source	Quant.	Val.	Appl.	Ref.
Aristolochic acid I	*Asarum* spp.	Alltech C18 (5 μm), 10 mM ammonium acetate, methanol, acetonitrile, water (+0.1% formic acid)	ESI	MS	1, 2	S,SA	[23]
Aristolochic acids (AA I, AA II, AA C, AA D)	*Aristolochia* and *Asarum* spp.	XDB C18 (4 μm), 0.2% acetic acid in water and methanol	ESI	DAD + MS	1–4	S,SA	[24]
Chlorogenic acids and geniposidic acid	*Eucommia ulmoides*	Agilent C18, 0.5% acetic acid in methanol	APCI	–	2	SA	[27]
Caffeic, chlorogenic, protocatechuic acid	*Salvia miltiorrhiza*	Kromasil C18 (5 μm), 0.2% formic acid, methanol and acetonitrile	ESI	MS	1–4	S,SA	[28]
Chlorogenic acids and metabolites	Human plasma	Inertsil ODS-2 (5 μm), 50 mM acetic acid in water and acetonitrile	ESI	MS	1–4	S,SA	[30]
Hydroxycinnamic acids	Human plasma and urine	Luna C18 (5 μm), 0.1% formic acid in water and acetonitrile	ESI	–	2	S,SA	[31]
Caffeic acids	*Echinacea* product	Alltima C18 (5 μm), water and methanol	GD EI	MS	1–4	S,SA	[32]
Amino acids	Barley	Luna SCX 100 Å (5 μm), 30 mM ammonium acetate (pH 6), 5% acetic acid	ESI	MS	1–4	S,SA	[34]
Amino acids (derivatized)	*Gentiana dahurica*	Hypersil BDS-C18 (5 μm), 30 mM formic acid, water, and acetonitrile	ESI	FD	1–4	S,SA	[35]

Quant.: quantification; Val.: validated (1: sensitivity; 2: specificity; 3: accuracy; 4: precision); Appl.: application; S: standard; SA: sample.

describe their determination in medicinal plants and preparations thereof (e.g. *Eucommia ulmoides* [27], *Salvia miltiorrhiza* [28]), in food items (e.g. propolis [29]), and in biological matrices like plasma [30] or urine [31]. As can be seen in Table 1, in most reports comparable analytical conditions were described, which are based on RP stationary phases and acidic mobile phases. MS, either ESI or APCI were employed as ionization sources, was used for identification and/or quantification purposes. Matsui et al. for example studied nine chlorogenic acids and some of their metabolites (ferulic acid and caffeic acid) in human plasma [30]. Volunteers were asked to drink a green coffee bean extract and, based on a newly developed LC–ESI-MS/MS assay, the pharmacokinetics of the above mentioned compounds were investigated. After carefully optimizing separation conditions, collision energies and by using a triple quadrupole API-4000 (Applied Biosystems) in MRM mode the method showed excellent sensitivity with LOD values between 0.1 and 0.6 ng/ml. By selecting three diagnostic product ions for each of the compounds their unequivocal assignment was possible. Thus, the authors considered their assay superior to previously reported ones.

Only recently Castro et al. used liquid chromatography particle beam mass spectrometry (LC–PB/MS) to determine caffeic acid derivatives in *Echinacea* extracts [32]. The particle beam served as a "transport-type" interface enabling continuous introduction of sample solutions into the sources without residual solvent vapors or changing the natural chromatographic characteristics. Two ionization sources, glow discharge (GD) or electron impact (EI), were then alternately employed followed by MS detection on a Thermabeam LC/MS quadrupole mass spectrometer. In GD the molecules collide with a cathodic surface, are released into the gas phase by thermal vaporization, followed by ionization within the negative glow region; in EI the molecules are hit by electrons produced in a wire by thermionic emission and thereby are ionized [33]. A comparison of both ionization techniques revealed similar MS spectra in terms of fragmentation patterns. Considering sensitivity divergent observations were made, for example the LOD of caftaric acid was 0.64 ng/ml by EI and 7.70 ng/ml by GD, whereas for chlorogenic acid just an opposite trend was observed (4.85 ng/ml versus 3.10 ng/ml). Quantitative results were nearly identical by both sources, but in respect to repeatability EI was advantageous (GD showed relative standard deviations of up to 16.8%).

Two methods for the LC–MS determination of amino acids in plant material should be mentioned as well. Thiele et al. described the direct determination of amino acids in barley [34], Sun et al. identified previously derivatized compounds in *Gentiana dahurica* [35]. The former separated 20 amino acids on a strong cation exchange HPLC column, and then quantified them in positive ESI mode on a triple quadrupole MS (Fig. 2). By using an internal standard (deuterated phenylalanine) and selecting the MRM mode they were able to detect the analytes as low as 0.1 μM with acceptable precision (σ_{rel} interday ≤8.8%) and accuracy (recovery rates between 64 and 115%). With LOD-values in the low femtomole range the method described by Sun was advantageous in terms of sensitivity, but it required derivatization with 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate prior to analysis. The obtained derivatives were then quantified by fluorescence detection. LC–MS was used for confirmatory purposes only, for which the authors also discussed the fragmentation characteristics in detail.

3.2. Alkaloids

Excellent reviews on the analysis (incl. LC–MS) of ergot alkaloids [36], *Vinca* alkaloids [37], and pyrrolizidine alkaloids in *Delphinium* [38] have been published already. The latter are also found in two other important medicinal plants, *Symphytum officinale* (comfrey) and *Tussilago farfara* (coltsfoot). Comfrey is a traditional remedy for the treatment of arthritis and sprains, coltsfoot decoctions are used against coughs and as an anti-inflammatory drug. Besides desired bioactive constituents both herbs contain liver toxic pyrrolizidine alkaloids, so that in countries like Austria only extracts free of 1,2-unsaturated derivatives are commercialized [39]. Liu et al. published a LC–MS method for the determination of several alkaloids (e.g. lycopsamine, echimidine and lasiocarpine) in *S. officinale* [40]. For qualitative and quantitative studies (only lycopsamine was quantified, without method validation) a LCQ-Duo ion trap mass spectrometer from Thermo Finnigan was used, structural confirmation and the identification of additional constituents was achieved on a LTQ Orbitrap hybrid mass spectrometer. All alkaloids were directly assignable at m/z values corresponding to $[M+H]^+$ in positive ESI mode. MS^2 and MS^3 spectra were found to be especially helpful in differentiating symviridine N-oxide and echimidine, two structurally closely related minor alkaloids. Additionally, the effect of different extraction procedures (pressurized hot water extraction and refluxing) on the content of lycopsamine was studied. Its amount was markedly decreased if the samples were extracted under pressure at 60 °C compared to a higher temperature without applying pressure. This observation is in contrast to a similar study of the same group on the determination of senkirkine and senecionine in *Tussilago farfara* [41]. These compounds are also pyrrolizidine alkaloids, but the results were comparable with both extraction procedures. A discrepancy that the authors explained by the instable nature of lycopsamine. Otherwise similar analyt-

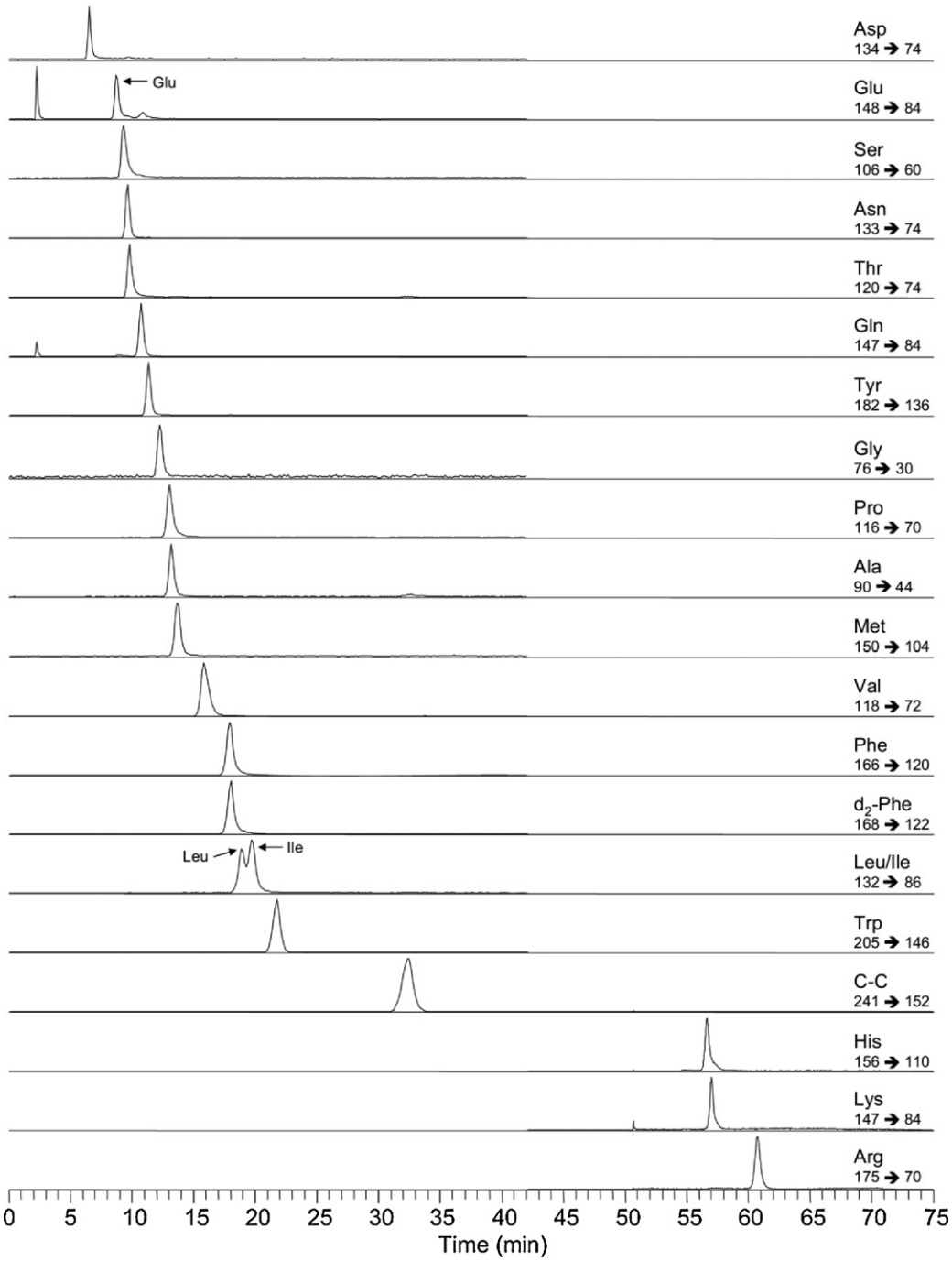

Fig. 2. Selected ion chromatograms of 20 underivatized amino acids (20 μM each) and the internal standard d_2-Phe (10 μM) recorded in MRM mode. Reproduced with permission from [34].

ical approaches as mentioned before were utilized. Joosten and colleagues studied pyrrolizidine alkaloids in *Jacobaea vulgaris*. As this plant is not of medicinal interest but responsible for livestock losses and the contamination of milk and honey it only is mentioned briefly here [42].

Several reports on the analysis of alkaloids in *Aconitum carmichaeli* (Ranunculaceae) have been published within the last few years. The plant is widely distributed in Asia and Northern America and, despite of the presence of toxic diterpene alkaloids, its tuber plays an important role in TCM as analgesic and cardiotonic herbal medicine. In the Chinese Pharmacopeia only the use of processed plant material (by soaking in salt water or pressure steaming; the product is called "Fuzi") is allowed, with a maximum alkaloid content of 0.15% (determined by titration) [43]. Nevertheless, the alkaloids need to be carefully monitored because they suppress the inactivation of sodium channels and lead to neurotoxic and cardiotoxic degenerations. Kaneko et al. described the analysis of major *Aconitum* alkaloids (e.g. aconitine, mesaconitine) in human plasma [44], Wang their determination in urine by LC–MS/MS [45]. Both required a sample cleanup on SPE cartridges (Oasis MCX or BondElut certify HF) prior to analysis. LC separations were performed on C-18 phases, either using an ammonium acetate buffer or an ammonium bicarbonate solution with pH 9.5 for elution (see Table 2). From practical perspectives reference [45] is more relevant, because Kaneko only used spiked plasma samples for developing and validating the assay. Wang reported

Table 2
Selected LC–MS assays for the analysis of alkaloids in herbal medicines.

Analytes	Matrix	LC-conditions	Source	Quant.	Val.	Appl.	Ref.
Pyrrolizidine alkaloids (lycopsamine, echimidine)	*Symphytum officinale*	Luna C18(2) (3 μm), water and acetonitrile both with 0.1% formic acid	ESI	MS	2	S,SA	[40]
Aconitum alkaloids	Human plasma	Shiseido UG80 C18, 20 mM ammonium acetate solution in ACN	ESI	–	1–4	S	[44]
Yunaconitine, crassicauline A, foresaconitine	Human urine	XTerra RP18 (3.5 μm), 10 mM ammoniuim bicarbonate (pH 9.5), ACN	ESI	MS	1–4	S,SA	[45]
Aconitine, hypaconitine, mesaconitine	*Aconitum carmichaeli*	Varian ODS-3 (5 μm), 0.1% formic acid in water and acetonitrile	ESI	MS	1–4	S,SA	[46]
Aconitum alkaloids	*Aconitum carmichaeli* (processed)	XTerra RP18 (5 μm), 2.5 mM ammonium bicarbonate (pH 10), ACN	ESI	DAD	1–4	S,SA	[47]
Verticine, verticinone	Rat plasma	Zorbax StableBond C18 (1.8 μm), 0.1% formic acid in water and acetonitrile	ESI	MS	1–4	S,SA	[48]
Steroidal alkaloids	*Fritillaria* spp.	Zorbax extend C18 (5 μm), water and ACN both with 0.03% diethylamine	ESI	MS	1–4	S,SA	[49]
Oxoprotoberberine alkaloids	*Coscinium fenestratum*	Zorbax Eclipse XDB C18 (3.5 μm), 0.1% formic acid in water and methanol	ESI	–	2	SA	[50]
Tryptanthrin, indigo, indigorubin	*Isatis indigotica*, *Strobilanthes cusica*	Hypurity-Advance C18 (5 μm), water and ACN both with 0.005% TFA	APCI	MS	1–4	S,SA	[51]

Quant.: quantification; Val.: validated (1: sensitivity; 2: specificity; 3: accuracy; 4: precision); Appl.: application; S: standard; SA: sample.

the following alkaloid levels in the urine of a patient: 50.9 ng/ml yunaconitine, 8.9 ng/ml crassicauline A, and 1.2 ng/ml foresaconitine. An interesting study comparing two different MS approaches for the determination of *Aconitum* alkaloids was published 2009 [46]. Plant material was extracted with acetonitrile, and then the resulting solutions assessed by MALDI-TOF and LC-QTOF; the latter using an ESI source in positive mode. In a semi-quantitative matter MALDI-TOF allowed the identification of hypaconitine in 10 out of 14 "Fuzi" samples, aconitine and mesaconitine were found in eight. LC-QTOF results were alike, but at the same time allowing the monitoring of a total of 57 peaks. By using an internal standard the amounts of individual compounds were compared, and based on correlation coefficients close to 0.8 the authors concluded that both MS-approaches are suitable for semi-quantitative profiling. The relevance of different processing procedures on the alkaloid content was studied by Lu et al. [47]. They utilized LC–MS for confirmatory purposes only; quantification was done by DAD at 240 nm. Concerning the alkaloid levels enormous differences were observed among different samples, e.g. for mesaconitine ranging from 1.9 to 291.9 mg/kg.

Other reports on the analysis of alkaloids by LC–MS describe the determination of steroidal alkaloids in *Fritillaria* species [48,49], berberine type alkaloids in *Coscinium fenestratum* [50], and tryptanthrin, indigo and indigorubin in *Isatis indigotica* and *Strobilanthes cusica* [51]. The approaches and methods described do not differ much from already mentioned ones, except for [48]. Xin et al. used turbulent-flow chromatography (TFC) for analysis of three alkaloids (verticine, verticinone and isoverticine) in *Fritillaria thunbergii*. TFC is a column switching technique in which the sample is introduced to a first column filled with coarse packing material (in this case 25 μm Oasis HLB), the column is flushed (to remove unbound constituents), and then the retained compounds are eluted to and separated on a second column (Zorbax StableBond C-18). This technique enabled the quantification of the alkaloids in rat plasma in 7 min without sacrificing resolution or sensitivity (LLOQ between 0.1 and 0.6 ng/ml).

3.3. Coumarins

The analysis of coumarins and coumarin glycosides by LC–MS is rarely described in literature. Coumarins are natural benzopyrone derivates, which are commonly found in plants belonging to the Apiaceae, Fabaceae, Rutaceae, Asteraceae and Umbelliferae families. Both desirable (e.g. analgesic, spasmolytic or anti-coagulant) and toxic (e.g. phototoxic) effects are reported among their pharmacological properties. Carcinogenic aflatoxins are also considered as coumarins [52].

The use of coumarins for the differentiation of herbal material was reported by Li et al. [53]. Eight different derivatives (nodakenin, nodakenetin, bergaptol, isoimperaorin, notoptol, notopterol, 6′-*O*-trans-feruloylnodakenin, and p-hydroxyphenetylanisate) were determined in *Notopterygium incisum* and *N. forbesii*. Both plants species are listed in the People's Republic of China Pharmacopeia under the name "Qiang Huo". Method development was performed by using HPLC-DAD, for identification of analytes an HPLC-DAD–MS^n system (ThermoQuest Finnigan LCQDECA system with ESI source) was employed. Positive and negative ionization modes were recorded using the following settings: Sheath gas: N_2 (80 units), auxiliary gas: N_2 (20 units), capillary: 350 °C, spray voltage: 4.5 kV, capillary voltage: −13 V/+25 V, lens voltage: +18 V/−16 V, and collision energy from 35 to 50%. With a Diamonsil C18 column as stationary phase, and a methanol/water gradient all eight coumarins could be well separated and identified in plant samples. Most of the compounds were assignable via pseudo molecular ion formation in positive mode ($[M+H]^+$, $[M+Na]^+$ and $[2M+Na]^+$), with the exception of bergaptol, which readily was ionized in negative ESI mode.

In 2007 Qian et al. reported on improved analytical procedures for the above mentioned two plant species by using High Performance Thin Layer Chromatography (HPTLC) and LC–MS–MS [54]. The latter experiments were performed on an Alltima C18 column and water/acetonitrile as mobile phase. Detection was achieved at 255 nm and by using a Perkin-Elmer Sciex API 365 triple-quad MS with ion-spray interface, respectively. An identification of analytes was possible by their retention times, UV- and MS-spectra, as well as MS–MS data. Three marker substances (isoimperatorin, notopterol and bergapten) were quantified in 16 batches of *Rhizoma* and *Radix Notopterygii* collected in China. Because of the low content of bergapten the authors considered it not suitable as a marker compound, while isoimperatorin and notopterol can be used for quality assessment and differentiation.

3.4. Phenols and flavonoids

The LC analysis of phenols and flavonoids is well established and many respective reports are found in literature. These compounds

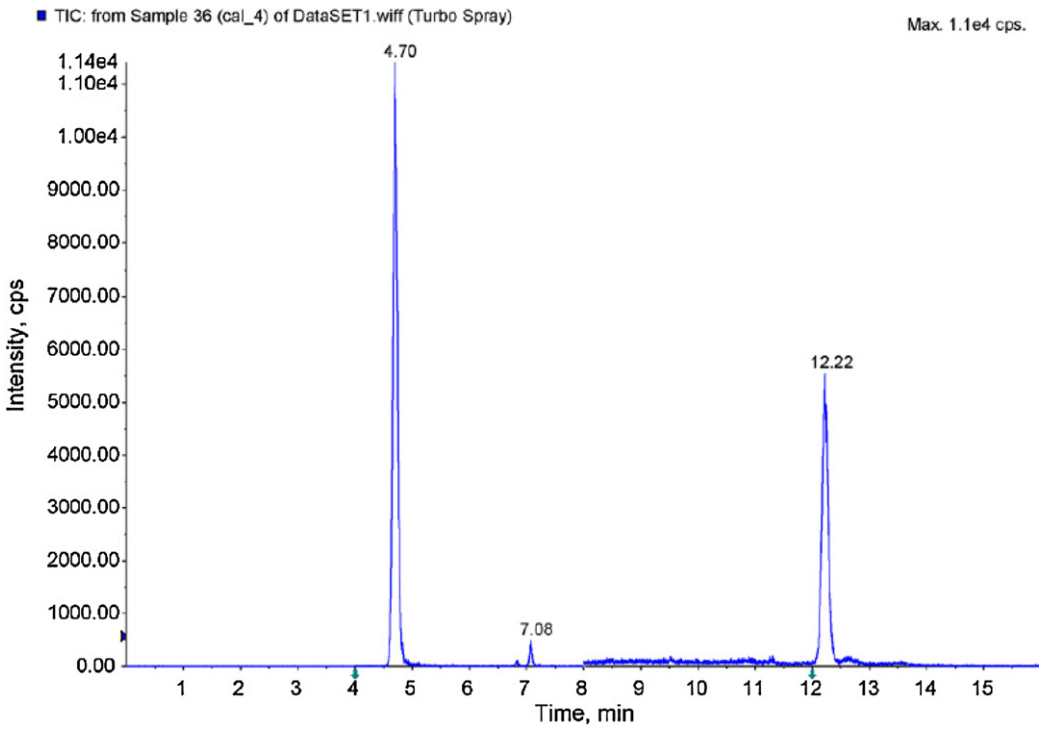

Fig. 3. Typical mass chromatograms of dansyl-icariin (Rt = 4.7) and dansyl-d5-E2 (Rt = 12.2); concentration of icariin in the standard solution was 4 ng/ml. Reproduced with permission from [69].

are of special interest because of their enormous structural variability (5000 derivatives are known up to now), which explains their broad spectrum of pharmacological effects and medicinal uses [5]. Vukics and Guttmann reviewed their biological properties ranging from antioxidant, antimicrobial or estrogenic activities, up to tumor, AIDS and cancer treatment, just to name a few [55]. But besides this recent publication, the separation and pharmacological properties of flavonoids were the topic of many reviews in the past already [55–60]. Thus, in this review only a few most recent reports are discussed in detail.

Based on the LC–MS analysis of flavonoids the differentiation of closely related plant species or those which are listed under equal names has been described [61–63]. Furthermore, the identification of (unknown) plant constituents is still of interest [64–66], as it is the first step to explain the benefits of traditionally used medicinal plants by scientific means [67,68]. As an example for the latter the study of Surveswaran et al. should be mentioned, who investigated phenolic compounds in Indian medicinal plants by LC–ESI-MS. They selected twelve species of the Asclepiadoideae and Periplocoideae subfamilies, plants which are relevant not only for medicinal but also for food purposes [64]. Methanolic extracts were prepared and separated on a Shimadzu LC–MS-2010EV system using a VP-ODS C18 column (250 mm × 2.0 mm, 4.6 μm) as stationary phase. The mobile phase consisted of 0.1% formic acid in water and methanol, applied with a flow rate of 0.2 ml/min. ESI-MS spectra were recorded from *m*/*z* 160 to 800, using ESI voltages of 4.5 kV in positive and 3.5 kV in negative ionization mode, respectively. Among other compounds tentatively identified by their mass spectra, chlorogenic acid and rutin were then selected for quantitative studies. Based on the obtained results the authors pointed out that the identified phenolic compounds significantly contribute to the antioxidant capacity of these plants. The obtained fingerprints were useful for authentication and quality control purposes.

Epimedium species (Berberidaceae) are widely used in TCM to improve menopausal symptoms, bone health, erectile function, cardiovascular failures, and hypertension [65,69,70]. One of the main bioactive constituents in the plant is the flavonol icariin. In 2007 Gong et al. [69] developed a method for the trace analysis of icariin in human serum after oral administration of an *Epimedium* decoction. Qualitative and quantitative results are reported, but due to the low amount of icariin in the samples a derivatization step with dansyl chloride was required prior to analysis. This greatly improved ionization efficiency in positive ESI mode. The chromatographic system consisted of a Phenomenex Synergi Max-RP 80A (150 mm × 2 mm, 4 μm) column, and a binary mobile phase (water/acetonitrile in the ratio of 95/5 and 5/95, acidified) applied in gradient mode. At a flow rate of 0.25 ml/min the required analysis time was only 16 min. Tandem MS spectra were recorded on an API 4000 triple quad MS equipped with a turbo ion spray source in SRM mode. The specific transitions for dansyl-icariin and dansyl-d5-E2 (internal standard) were recorded at *m*/*z* 910 → 764 and *m*/*z* 511 → 171. Fig. 3 shows the mass spectra of both compounds at a concentration of 4 ng/ml, indicating excellent sensitivity of the assay. Using a similar setup, the determination of icariin in rat plasma [65] and urine [70] was described.

The already mentioned review by Vukics and Guttmann also summarized multi-stage MS applications of flavonoid analysis, which were published within the last years [55]. Typical examples are the identification of phenylethanoid glycosides in *Cistanche deserticola*, which were isolated by high-speed counter-current chromatography [71], or the differentiation of diverse *Celastraceae* species [72]. An innovative approach is the use of multi-stage MS coupled to a comprehensive LC system (LC × LC) for the characterization of mate extracts [73]. Mate is a tea-like beverage especially popular in South America, which is prepared from dried and minced leaves of *Ilex paraguariensis*. The plant is known for numerous health benefits (e.g. hepatoprotective, choleretic, diuretic, and hypocholesterolemic). Because of a complex matrix, interferences and co-elutions single stage LC-separations are usually not sufficient for an accurate characterization of the respective extracts. Thus, Dugo and his colleagues developed a two-dimensional LC separation system followed by MS detec-

Table 3
Selected LC–MS assays for the analysis of phenols and flavonoids in herbal medicines.

Analytes	Matrix	LC-conditions	Source	Quant.	Val.	Appl.	Ref.
Flavonoids, phenolic acids	*Polygonum capitatum*, *P. chinensis*, and other spp.	VP-ODS C18 (5 μm), 0.1% formic acid in water and in methanol	ESI	MS	2	S,SA	[61]
Flavonoid aglyca and glycosides	*Chrysanthemum morifolium*, *C. coronarium*, *Artemisia annua*,	Zorbax SB-C18 (3.5 μm), 0.1% formic acid in water and ACN	ESI + APCI	–	2	S,SA	[62]
Apigenin, chrysoeriol, acacetin, phenolic acids	*Saussurea laniceps*, *S. medusa*	Alltima C18 (5 μm), 0.1% formic acid in water and in acetonitrile	ESI	DAD + MS	1–4	S,SA	[63]
Flavonoids (e.g. rutin) and phenolic acids	Asclepiadoideae and Periplocoideae species	VP ODS C18 (4.6 μm), 0.1% FA in water and in methanol	ESI	–	1, 2	S,SA	[64]
Icariin	*Epimedium* spp.	Shimadzu C18 RP (5 μm), 0.05% acetic acid in water and ACN	ESI	MS	1–4	S,SA	[65]
Flavonoids, lignanoids, phenolic acids	*Saussurea involucrata*	Alltima C18 (5 μm), 0.1% formic acid in water and in acetonitrile	ESI	DAD + MS	1–4	S,SA	[66]
Liquiritin, liquiritigenin, isoononin	*Glycyrrhiza uralensis*	Zorbax C18 (5 μm), 0.1% formic acid in water and in acetonitrile	ESI	DAD	2	S,SA	[67]
Isoflavonoids, phtalides	*Radix Astragali*, *Radix Angelica sinensis*	Zorbax SB-C18 (1.8 μm), 0.1% formic acid in water and ACN	ESI	–	2	S,SA	[68]
Icariin	*Epimedium* spp.	Synergi Max-RP 80A (4 μm), ACN and water with FA	ESI	MS	1–4	S,SA	[69]
Icariin, icariside I, icaritin, desmethylicaritin	*Epimedium* ssp.	Synergi Max-RP 80A (4 μm), ACN with 0.1% formic acid and 0.5 mM ammonium formate in water	APCI	MS	2	SA	[70]
Flavonol-3-O-glycosides	*Maytenus aquifolium*, *M. ilicifolia*	Symmetry C18 (5 μm), 0.5% formic acid in water, ACN	ESI	–	2	SA	[72]
Flavonoids and phenolic acids	*Ilex paraguariensis*	Ascentis RP-Amide (5 μm) and Ascentis Express C18 (2.7 μm), water and acetonitrile	ESI	–	1, 2	S,SA	[73]
Flavonoids and phenolic acids	*Geranium robertianum*, *Uncaria tomentosa*	Symmetry Shield C8 (5 μm), 0.1 M formic acid in water and MeOH	ESI	–	2	S,SA	[74]
Curcumin and derivatives	*Curcuma domestica*	Synergi Fusion RP80 (4 μm), 0.1% acetic acid in water and ACN	ESI	MS	1, 2	SA	[75]
Oxyresveratrol	*Smilax china*	Zorbax SB-C18 (5 μm), water and acetonitrile	ESI	–	2	SA	[76]
Flavonoids (gossypetin, herbacetin glycosides)	*Rhodiola rosea*	Symmetry C18 (3.5 μm), 0.1% formic acid in water, ACN	ESI	–	2	SA	[77]
Rhoifolin	*Uraria picta*	Spherisorb ODS-2 (10 μm), 1% TFA in water, ACN	ESI	DAD	1–4	S,SA	[79]
Antioxidant phenolics	*Tanacetum parthenium*	YMC ODS (5 μm), 0.05% TFA in water and methanol	ESI	–	2	SA	[80]

Quant.: quantification; Val.: validated (1: sensitivity; 2: specificity; 3: accuracy; 4: precision); Appl.: application; S: standard; SA: sample.

tion. The first separation was performed on an Ascentis RP-Amide phase (250 mm × 1.0 mm, 5 μm; flow rate 10 μl/min), eluting fractions were trapped, and then re-assayed on an Ascentis Express C18 (30 mm × 4.6 mm, 2.7 μm; flow rate 4 ml/min) in the second dimension. By coupling these columns with different separation modes high-resolution separations were possible. The LC system was hyphenated with a Shimadzu ion trap-time of flight (IT-TOF) MS equipped with ESI source. Parent ions were acquired from *m*/*z* 170 to 200 in positive, and from *m*/*z* 330 to 680 in negative ionization mode, respectively. MS/MS fragments were recorded at *m*/*z* values from 50 to 400, and MS^3 fragments ions from *m*/*z* 50 to 200. With this technique the characterization of 26 polyphenols and xanthines in mate extracts was feasible, including hydroxycinnamoyl quinic acids, methylxanthines and flavonol glycosides.

Table 3 lists these and other applications including experimental details (phenolic acids were discussed in Section 3.1 already). The described approaches range from single stage [61–68,79] and tandem mass spectrometry [69,70,74–78,80] to multi stage MS systems [71–73], indicating the versatility of LC–MS. Because of technical variability and the amount of information obtained it is obvious that LC–MS^n can be considered as state of the art technique for the analysis of phenols and flavonoids in complex matrices such as herbal extracts.

3.5. Quinones and xanthones

Quinones and xanthones are classified as oxidation products of aromatic compounds, with usually intense color (yellow or red) and smell (quinones). They are found in many medicinally used plants as biologically active constituents, and this review focuses on their LC–MS determination in relevant species belonging to the Iridaceae [81], Lamiaceae [82,83], Gentianaceae [84] and Liliaceae [85] families.

Paramapojn et al. reported on the first procedure for the analysis of five naphthopyrone derivates (eleutherinoside A, eleuthoside B, isoeleutherin, eleutherin and eleutherol) in methanolic extracts of *Eleutherine americana* [81]. Widely cultivated in China and Thailand, this plant is traditionally used for the treatment of coronary diseases, as diuretic, emetic and purgative. HPLC separations were performed on a Synergi MAX-RP C12 column, with water and acetonitrile as mobile phase. Separation temperature, injection volume and detection wavelength were set to 40 °C, 10 μl, and 254 nm, respectively. For LC–MS analysis an Agilent 1100 HPLC was connected to a Bruker Esquire 3000 plus ion trap, introducing the LC eluent with a split ratio of 1:3. MS spectra, which served for confirmatory purposes only, were recorded in alternating ESI mode (probe temperature: 350 °C, nebuliser gas: 30 psi, dry gas: 10 l/min). The compounds of interest were unambigu-

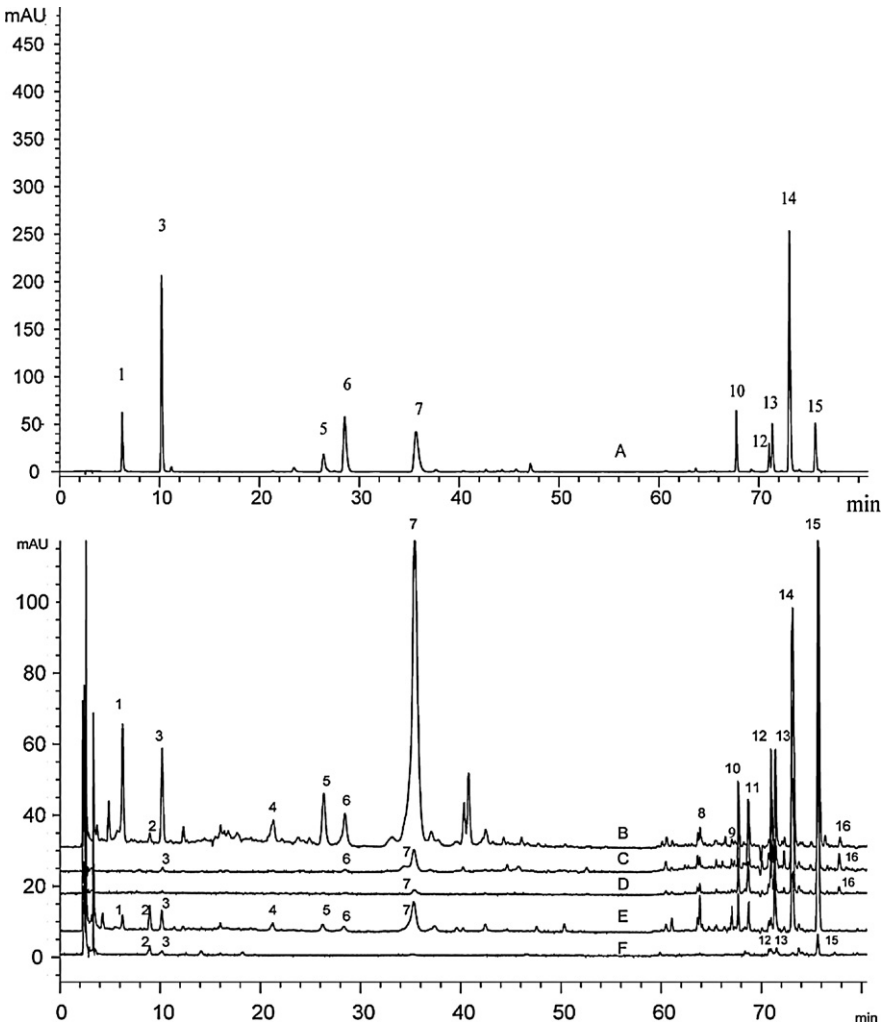

Fig. 4. HPLC chromatograms of different cell types incubated with an *S. miltiorrhiza* extract. Mixture of reference compounds (A), extract of *Radix Salviae miltiorrhizae* (B), extract of endothelial cells (C), extract of myocardial cells (D), extract of blood platelets (E), and extract of red blood cells (F).
Reproduced with permission from [83].

ously identified in positive (isoeleutherin, eleutherin, eleutherol and eleutherinoside A) or negative mode (eleuthoside B). Method validation and quantification of the five marker compounds in plant specimens were accomplished by HPLC-UV. With a concentration ranging from 0.11 to 0.20% eleutherol was found to be the major naphthopyrone in most of the samples analyzed.

"Danshen" (*Radix Salviae miltiorrhizae*) is listed in the Chinese Pharmacopeia for the treatment of coronary heart diseases and for suppressing tumor necrosis. Major bioactive compounds in the plant are diterpenequinones like tanshinone I, dihydrotanshinone I, tanshinone IIA, and cryptotanshinone, which possess anti-bacterial, anti-inflammatory and anti-coagulative properties [82,83]. Liu et al. studied the metabolism of these compounds in rats by LC–MS [82]. The described analytical conditions are comparable to the previously mentioned ones (e.g. RP-stationary phase, water and acetonitrile as mobile phase, ion trap MS in alternating mode). MS/MS spectra, which were recorded at collision energies between 0.33 and 0.39 V, allowed the identification of unknown metabolites of tanshinone I and dihydrotanshinone I, and they permitted the postulation of respective metabolic pathways. An interesting approach for the prediction of possible bioactive compounds in the same species was published by Qu et al. [83]. They prepared an aqueous ethanolic extract of *S. miltiorrhiza*, and added it to different types of target cells (e.g. myocardial or endothelial cells). After incubation the cells were washed, denaturated and then extracted with 75% ethanol. Based on the compounds found in these extracts (for this purpose LC–MS was used) the authors attempted to find possible bioactive constituents. Once again optimum separations were obtained on RP-material (Zorbax ODS C18) and the mobile phase comprised water (with 0.1% formic acid) and acetonitrile. For MS analysis an Agilent G1969 LC/MSD TOF system with ESI source was used. The following compounds could clearly be identified (see Fig. 4): danshensu (1), protocatechuic aldehyde (3), salvianolic acid D (4), rosmarinic acid (5), lithospermic acid (6), salvianolic acid B (7), danshexinkun D (9), dihydrotanshinone I (10), danshexinkun B (11), cryptotanshinone (12), tanshinone I (13), methylene tanshinone (14), tanshinone II (15) and miltirone (16). In their concluding remarks the authors stated that their proposed strategy was in coincidence with the holistic characteristics, known constituents and acting sites of "Danshen".

Swertia chirata (Gentianaceae) is used in traditional Ayurvedic medicine because of its febrifuge, anthelmintic, tonic and laxative properties. Suryawanshi et al. assessed the bioactive constituents in the plant by LC–ESI-MS/MS, including xanthone (mangiferin) and secoiridoid glycosides (amarogentin, amaroswerin, sweroside and swertiamarin) [84]. Due to selectivity and specificity of tandem MS the authors did not attempt to separate all compounds by chromatographic means, but recorded them in MRM mode on

Table 4
Selected LC–MS methods for terpene analysis in herbal medicines and medicinal products.

Analytes	Matrix	LC-conditions	Source	Quant.	Val.	Appl.	Ref.
Ginsenosides (e.g. Rb1, Rf, K, F1, F2)	*Panax ginseng*, *P. notoginseng*, *P. japonicus*, *P. quinquefolium*	ACQUITY BEH C18 (1.7 μm), 0.05% formic acid in water and ACN	ESI	–	2	S,SA	[86]
Ginsenosides (e.g. Rb1, Rc, Rd, Rg1, Re, Rf)	*Panax ginseng*, *P. notoginseng*, *P. japonicus*	ACQUITY BEH C18 (1.7 μm), 0.05% formic acid in water and ACN	ESI	MS	2	S,SA	[87]
Ginsenosides (e.g. Rb1, Rd, Rg1, Rh1)	*Panax ginseng*	Supelcosil LC-8-DB (3 μm), 10 mM ammonium acetate in water and methanol	ESI	–	2	S,SA	[88]
Diterpenes (kansuinines and ingerols)	*Euphorbia kansui*	Apollo C18 (5.0 μm), water and methanol	ESI	–	2	S,SA	[89]
Asiatic acid, asiaticoside, madecassoside	*Centella asiatica*	Eclipse C18 (5 μm), 0.1% acetic acid in water and acetonitrile	ESI	DAD	1–4	S,SA	[90]
Toosendanin	*Melia toosendan*	Zorbax SB-C18 (53.5 μm), water and acetonitrile	ESI	MS	1–4	S,SA	[91]
Atractyloside	*Callilepis laureola*	XTerra Phenyl (5 μm), 25 mM ammonium acetate, water, ACN	ESI	MS	1–4	S,SA	[92]

Quant.: quantification; Val.: validated (1: sensitivity; 2: specificity; 3: accuracy; 4: precision); Appl.: application; S: standard; SA: sample.

an API 4000 triple quadrupole mass spectrometer. Therefore, the required analysis time under isocratic conditions was 3.5 min only. The method was validated (no detailed results are presented) and used for the quantitative analysis of four sample batches. Additionally, the fragmentation of $[M+H]^+$ and $[M+Na]^+$ ions of the analytes was studied based on their collision-induced dissociation (CID) spectra. The latter were recorded using the information-dependent acquisition (IDA) method, which allows on-column selective product ion MS/MS data acquisition by using nitrogen as collision gas. Xanthone-C-glycosides showed characteristic fragment ions because of the opening of the C-glycosidic unit, while iridoid-O-glycosides revealed typical fragments due to cleavage of the glycoside linkage and retro-Diels-Alder (RDA) reaction within the iridoid moiety.

Another recent report described the analysis of xanthones and saponins from *Anemarrhena asphodeloides* in rat urine [85]. After oral administration of an aqueous root decoction, the samples were purified on C18 SPE cartridges and then assayed by LC–MS^2. HPLC separations were performed at 20 °C on a Diamonsil C18 (200 mm × 4.6 mm, 5 μm) column using a linear gradient of acetonitrile and water containing 0.4% formic acid (analysis time 35 min). An APCI interface in negative mode enabled ionization, and argon was used as collision gas (3 mTorr), with corona discharge voltage set to 4000 V and a collision energies ranging from 20 to 60 V. Eleven compounds including two xanthones, three of their metabolites (glucuronide and methyl conjugates of magniferin), and six steroidal saponins were identified in the samples based on their UV spectra and MS fragmentation patterns. As bioavailability of these constituents in *A. asphodeloides* was confirmed for the first time the authors pointed out that their results are a significant contribution in explaining the use of this valued medicinal plant.

3.6. Terpenes

Among the most popular herbal remedies is ginseng. In this respect it has to be mentioned that different ginseng varieties are commercially available, which often are not differentiated. They are *Panax ginseng* (Chinese or Korean ginseng), *P. notoginseng*, *P. japonicus* and *P. quinquefolius* (American ginseng). An UPLC–QTOF/MS method for the differentiation of those species was published by Xie et al. in 2008 [86]. It is based on the assessment of specific, bioactive triterpenes, the so-called ginsenosides. The described setup included a Waters ACQUITY UPLC system equipped with an ACQUITY BEH C18 column (100 mm × 2.1 mm; 1.7 μm), and a Micromass Q-TOF Premier TM, operating in positive ion electrospray mode (see Table 4 for further details). For comparison purposes the authors also analyzed the samples on a "normal" HPLC column (Symmetry Shield RP18; 250 mm × 4.6 mm, 5 μm), but in addition to prolonged analysis time (20 min versus 80 min) the number of identified triterpenes was much higher by UPLC (25 versus 11 compounds). Thus, the UPLC approach was preferred. All triterpenes were tentatively identified in 70% aqueous methanolic extracts by their high resolution TOF-MS spectra with mass accuracies of 2 ppm and below. For internal MS calibration lock masses were used (leucine-enkephalin), which were introduced by a specific interface. The obtained UPLC–QTOF/MS raw data was then statistically analyzed (principal component analysis and partial least square-discriminant-analysis) to find possible variables. Indeed, it was possible to clearly distinguish among the five species, with ginsenoside Rf, 20(S)-pseudoginsenoside F11, malonyl ginsenoside Rb1, and ginsenoside Rb2 being accountable for distinction. As this interesting study focused on differentiation of *Panax* species no method validation or quantitative results are presented. In a second publication the same authors used a similar approach for metabolite profiling in three ginseng varieties [87].

The determination of ginsenosides in biological matrix (horse urine after oral administration) by LC–MS was reported by Chung et al. [88]. This study is of relevance because these compounds are prohibited feed additives for racing horses and therefore controlled by anti-doping agencies. Investigations were focused on the analysis of seven analytes belonging to two different aglyca subtypes, (20S)-protopanaxadiol (ginsenosides Rb1, Rd, Rg3 and Rh2) and (20S)-protopanaxatriol (ginsenosides Re, Rg1and Rh1). The authors also evaluated the use of GC–MS^2 for this purpose, but only after enzymatic hydrolysis and derivatization both aglyca were found in spiked urine samples. LC–MS^2 and LC–MS^3 on the other hand allowed the direct determination of all seven intact ginsenosides in the same matrix (LOD values from 5 to 100 ng/ml in EI mode). For respective experiments a Thermo Finnigan LCQ Advantage MS in negative ESI mode was selected, optimum LC-separations were obtained on a Supelcosil LC-8-DB column (75 mm × 3.0 mm; 3 μm). Further in vitro (Rb1 and Rg1 were incubated with horse liver microsomes) and in vivo experiments (1 g of Rg1 was orally administered to two horses) investigated the metabolism of ginsenosides. By LC–MS/MS it was possible to identify the glucuronides of (20S)-protopanaxatriol and Rg1 as major metabolites in urine.

Another important medicinal plant in TCM is *Euphorbia kansui*, known as "Kansui" in Chinese. The roots of this plant are utilized to treat many ailments like cancer, leukemia, or pancreatitis; yet, the plant is also known for toxic effects (e.g. skin irritation). Zhang et al. reported on an HPLC-DAD–MS–MS assay to investigate the inflammatory constituents in the plant [89]. An ethanolic plant extract was first separated by column chromatography using silica gel, the obtained fractions were tested for their inflammatory potential in

Table 5
Mixed analytes determined by LC–MS.

Analytes	Matrix	LC-conditions	Source	Quant.	Val.	Appl.	Ref.
Phenols, alkylphthalides, phthalide dimers	*Ligusticum chuanxiong*, *Angelica sinensis*, *A. acutiloba*, *Cnidium officinale*	Alltima C18 (5 μm), acetonitrile and water	ESI	DAD	1–4	S,SA	[93]
Hyperforin, and polyprenylated derivatives	*Hypericum perforatum*	ODS-80TM (5 μm), 0.01% phosphoric acid in water and acetonitrile	ESI	DAD	2	S,SA	[94]
Podophyllotoxin lignans	*Linum scabrellum*, *Hyptis suaveolens*	Prevail RP C18 (5 μm), 0.2% acetic acid in water and ACN	ESI	–	2	S,SA	[95]
Glucosinolates	*Isatis tinctoria*, *I. indigotica*	Aqua C18 125 Å (5 μm), 10 mM aqueous ammonium formate (pH 4.6) and acetonitrile	ESI	MS	1, 2	SA	[96]
Alkamides	*Echinacea purpurea* products	Zorbax SB-C18 (3.5 μm), 0.2% formic acid in water and ACN	ESI	MS	2	S,SA	[97]
Adulterants (e.g. sildenafil, famotidine, ibuprofen)	Herbal products	Megachem C18 (5 μm), TFA in water and methanol	TIS	–	2	S,SA	[98]
Adulterants	Herbal product	ACQUITY UPLC HSS T3 (1.8 μm), 0.1% formic acid in water, ACN	ESI	–	2	SA	[99]
Amlodipine, indapamide, valsartan	Gold Nine Soft Capsules	Zorbax Eclipse Plus C18 (1.8 μm), 0.1% formic acid in water and acetonitrile	ESI	DAD	2	S,SA	[100]

Quant.: quantification; Val.: validated (1: sensitivity; 2: specificity; 3: accuracy; 4: precision); Appl.: application; S: standard; SA: sample.

two biological assays, and the constituents in the most active (toxic) one were then determined. By LC–MS–MS it was found that this fraction mainly contained diterpenes (kansuinines and deoxyingenol derivatives). In 2009 Shen published a method for the analysis of major triterpenes (asiatic acid, asiaticoside and madecassoside) in *Centella asiatica* (Apiaceae) [90]. Main emphasize of this study was the evaluation of different extraction procedures (microwave-assisted extraction was found to be the best in terms of extraction efficiency), so that from analytical perspectives a mostly routine application is presented. Under the same chromatographic conditions the performance of two different mass analyzers (Agilent 6320 Ion Trap and Agilent G1969A TOF) was tested, and the authors considered both equally suitable to identify the three markers in plant samples. All compounds were assignable in positive ESI mode at *m*/*z* values corresponding to $[M+Na]^+$ (ion trap) or $[M+H]^+$ (TOF).

A few studies emphasized on the identification of single terpenoids in plant material by LC–MS. For example, Ong and Ong quantified toosendanin in *Melia toosendan* by ion trap MS (calibration by external standard, method precision for sample analysis 4.3–7.6%; [91]). Steenkamp et al. published an assay for the determination of atractyloside, a toxic diterpene from *Callilepis laureola* (Asteraceae), in plant material (content 9 μg/g) and human viscera (venomed, dead patient: 280 ng/g) [92].

3.7. Others

The LCMS analysis of analytes not covered in the previous chapters is compiled in Table 5. Yi et al. used HPLC-DAD–ESI–MS for the determination of phenolic compounds, alkylphthalides and phthalide dimers in the rhizomes of *Ligusticum chuanxiong* and three other related umbelliferous medicinal plants [93]; Charchoglyan et al. analyzed hyperforin and three polyprenylated acylphloroglucinol derivatives in in vitro cultures of *Hypericum perforatum* [94]. Another study focused on Mexican medicinal plants and the occurrence of podophyllotoxin type lignans therein [95]. Out of 50 species initially selected, two (*Linum scabrellum* and *Hyptis suaveolens*) were found to contain such compounds, and therefore they were considered by the authors as potential anticancer drugs. In all of the above mentioned studies LC–MS or LC–MS/MS were used for the identification of compounds. However, Mohn and Hamburger used LC–ESI-MS and LC–MS/MS techniques for the qualitative and quantitative analysis of glucosinolates in *Isatis tinctoria* and *I. indigotica* seeds [96]. Eight respective derivatives could be identified and based on the occurrence of two of them, namely glucoisatisin and epiglucoisatisin, both *Isatis* species could be differentiated. Quantitative results were obtained on a micrOTOF ESI-MS from Bruker Daltronics in negative ionization mode, using sinigrin as internal standard. Woelkart et al. investigated the oral bioavailability of alkamides from *Echinacea purpurea* formulations (Echinaforce™) in human plasma [97]. With an LOD of 0.025 ng (20 μl injection) a LCQ Deca XP Plus ion trap mass spectrometer from Finnigan was ideal for quantitative studies, which revealed that dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides are quickly absorbed within 15 min after oral administration.

Even it cannot be considered natural products analysis in a narrower sense, another type of applications should be mentioned as well, the analysis of adulterants (e.g. illegally included synthetic drugs) in herbal medicinal products [98–100]. In 2006 Liang et al. presented a semi quantitative method for the rapid determination of nine most common adulterants like sildenafil, diazepam, captopril and amoxicillin in herbal products purchased from the Chinese market [98]. Using a mobile phase comprising methanol, water and TFA the samples were separated on C-18 material within 5 min, and the selected compounds monitored in MRM on a SCIEX API 3000 triple-quad MS/MS equipped with a Turbo Ion Spray Interface (TIS). Over 200 samples were investigated, with 81 of them being advertised for "enhancing sexual potency"; in 28 of them (35%!) positive results for sildenafil (viagra™) were found. In a similar study Uchiyama et al. examined one herbal product, illegally sold in Japan for its narcotic effects, for the presence of cannabimimetic compounds [99]. By using GC–MS and LC–ESI-MS they found two respective compounds, one cannabinoid analog and one cannabimimetic indole. Just recently, Kesting et al. published a study on adulterants in a TCM preparation called "Gold Nine Soft Capsules", a "herbal based" medicine intended for treating hypertension [100]. The authors investigated the product by LC-HRMS (Agilent G1969A LC/MSD TOFMS) and LC–MS-SPE-NMR (Esquire 4000 ion trap with Bruker/Spark Prospect II LC–SPE–NMR interface; a schematic design of the setup is presented in Fig. 5). Three synthetic antihypertensive drugs (amlodipine, indapamide, and valsartan; content of valsartan: 40 mg/capsule) were found. This and the previously mentioned studies clearly indicate that the quality of herbal products is still a major concern for consumers and

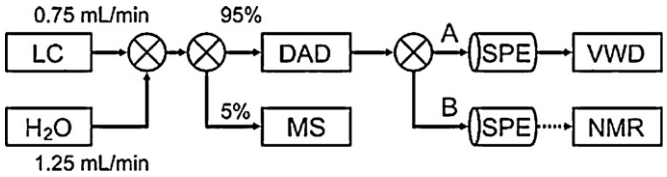

Fig. 5. Schematic design of the LC–MS-SPE/NMR system described by Kesting et al. Reproduced with permission from [100].

regulatory authorities. Especially, as the use of TCM and Ayurvedic preparations from questionable sources is still popular. Pretending to be of natural origin they sometimes contain a mixture of synthetic adulterants, which explain their (unexpected) potencies but also are responsible for side effects of "unknown" reason.

3.8. Fingerprinting

Chromatographic fingerprinting is recommended by the Chinese Pharmacopeia as a potential and reliable strategy for the quality control of complex mixtures like herbal medicines. Many traditional preparations are composed of multiple herbs, so that the analysis of selected constituents might not reflect their overall quality or efficacy. Therefore, in fingerprinting the whole chromatographic/electrophoretic/spectroscopic pattern is recorded and compared to a reference (e.g. sample with proven efficacy). By using specific software like CASES (computer aided similarity evaluation system) this can be done based on the occurrence of common peaks, and obtained correlation coefficients indicate the degree of similarity. The successful use of this approach has been described for authentication, quality assurance, stability and similarity studies. Most commonly the identification of compounds is achieved by LC–MS or LC–MS^n, for actual fingerprint analysis either UV or MS-traces can be used. Typical examples for single herb fingerprinting are *Euonymus alatus* [101], *Echinops latifolius* and *E. grijsii* [102], or *Dysosma versipellis* and *D. pleiantha* [103], just to name a few. Multi herb preparations that have been studied by this technique include "Ba Zhen Yi Mu" (a mixture of nine herbs used for regulating blood circulation; [104]), "Gan Lu Yin" (ten herbal constituents, including *Artemisia capillaris*, *Rhemannia glutionsa* and *Eryobotrya japonica*; [105]), or "Shen Mai injection" (*Radix Ginseng rubra* and *Radix Ophiopogonis* [106]). The LC–MS approaches utilized are manifold (e.g. LC–ESI-TOFMS, LC–APCI-MS or LC–ESI-MS), but they do not differ much from already described ones.

4. Conclusions

This manuscript reviews recent LC–MS applications for the analysis of natural products (alkaloids, flavonoids, terpenes and others) in medicinal plants, commercial products or biological fluids. Based on the currently available literature this approach appears to be the method of choice for diverse analytical issues in this field of research. Whether it is the identification of unknown compounds by high-resolution MS or MS^n, the confirmation of known substances by single quadrupole systems, the determination of trace amounts by triple quadrupole or time of flight systems, or the use of a mass spectrometer for fingerprinting studies. A variety of established (ESI, APCI) and innovative (APPI) ionization techniques further broadens the spectrum of possible applications and detectable analytes.

In respect to chromatographic separations no cessation is obvious as well. The increasing use of sub 2 μm stationary phases and (consequently) of highly efficient pumps (e.g. UPLC) dramatically improves separation speed and sensitivity without sacrificing resolution. As disadvantages of LC–MS sometimes observed matrix effects, ion suppression, a diminished linear range compared to other detectors like UV, and the high acquisition costs have to be mentioned. Apart from that the desirable features certainly prevail, so that the role of LC–MS as analytical key technique will further be strengthened in the future.

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