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Review

Recent applications of liquid chromatography–mass spectrometry in natural products bioanalysis

Jie Xing, Chunfeng Xie, Hongxiang Lou *

School of Pharmaceutical Sciences, Shandong University, Jinan 250012, PR China

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Abstract

Natural flavonoids, alkaloids, saponins and sesquiterpenoids have been extensively investigated because of their biological and physiological significances, as well as their promising clinical uses. It is necessary to monitor them or their metabolites in biological fluids for both pre-clinical studies and routine clinical uses. The successful hyphenation of LC and MS, which was thought as "the bird wants to marry with fish", has been conducted widely in biological samples analysis. This present paper reviewed the feasibility of LC–MS techniques in the identification and quantification of natural products (flavonoids, alkaloids, saponins and sesquiterpenoids) in biological fluids, dealing with sample preparation, LC techniques, suitability of different MS techniques. Perspective of LC–MS was also discussed to show the potential of this technology. The citations cover the period 2002–2006. We conclude that LC–MS is an extremely powerful tool for the analysis of natural products in biological samples.

Keywords: Liquid chromatography-mass spectrometry; Natural products; Bioanalysis; Flavonoids; Alkaloids; Saponins; Sesquiterpenoids

Contents

Abbreviations: RP-HPLC, reversed phase high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; LC–PDA–MS, liquid chromatography-photodiode array-mass spectrometry; LC–MS/MS, liquid chromatography tandem mass spectrometry; LC–MS^n, liquid chromatography multi-stage mass spectrometry; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; APPI, atmospheric pressure photoionization; TSP, thermospray; FAB, fast-atom bombardment; MALDI-TOF, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; TQ, triple quadrupole; QIT, quadrupole ion trap; Q-TOF, quadrupole time-of-flight; MRM, multiple reaction monitoring; SRM, selective reaction monitoring; SIM, selective ion monitoring; CID, collision induced dissociation; LLE, liquid–liquid extraction; SPE, solid phase extraction; PPT, protein precipitation

* Corresponding author at: School of Pharmaceutical Sciences, Shandong University, 44 West Wenhua Road, Jinan 250012, PR China. Tel.: +86 531 88382012; fax: +86 531 88382019.
E-mail address: louhongxiang@sdu.edu.cn (H. Lou).

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1. Introduction

Almost half of the drugs currently in clinical use derive from natural origin. Natural products are gaining international popularity and under the renaissance as drug candidates [1]. A variety of pharmacological principles are flavonoids [2–7], alkaloids [8–15], saponins [16–19] and sesquiterpenoids [20,21]. Many studies focused on the chemistry and bioactivity of these natural products, which promoted the discovery of drug candidates or lead compounds from natural sources.

However, little is known about their mechanisms of action and even less about their pharmacokinetic properties partly because of lacking specific and sensitive analytical methods [22,23]. Investigators have shown increasing interest in developing and optimizing analytical methods for detection of natural products in biological matrices. These methods mainly include reversed phase high-performance liquid chromatography (RP-HPLC) in combination with UV absorbance [24–27], fluorescence [28,29] and electrochemical detection [30,31]. Furthermore, fluorescence quenching, thin layer chromatography and high performance capillary electrophoresis were also used [12,13]. Since saponins and sesquiterpenoids have no appropriate ultraviolet or fluorescent chromophores, derivatization [32,33], evaporative light scattering detection (ELSD) [34–36] or radioimmunoassay (RIA) techniques [37] have frequently been performed. However, these HPLC methods are still not sensitive or reproducible enough for detecting trace-level parent natural products or their metabolites in biofluids [38–45]. On the other hand, because many alkaloids, flavonoids or sesquiterpenoids are very unstable and decompose fast in human body [46–48], it is required to determine their metabolic fates including structure characterization and quantitative analysis of metabolites. Many terpenoid saponins have a narrow therapeutic index with serious side effects [49,50], which makes it essential to accurately measure them in blood samples from a safety point of view [39,51–55]. Since the introduction of the electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) techniques, liquid chromatography with mass spectrometry (LC–MS) has been widely employed for the bioanalysis of natural products, including their metabolism and pharmacokinetics because of its excellent specificity, speed, and sensitivity [56–65].

This review focused on the current use of LC coupled to tandem MS to determine bioactive natural products and their metabolites in biological fluids. Data were presented on the application of various tandem MS instruments and their capacity to identify/confirm target or non-target compounds. The instrumental setup and the experimental setting, essentials of the successful coupling of LC and MS, sample preparation techniques, and perspectives of LC–MS were also outlined. All these aspects will provide some references in metabolic and pharmacokinetic studies of other natural products in vivo.

2. Extraction and isolation methods

In most cases, biological samples cannot be assayed directly, but require a pretreatment to dispose endogenous proteins, carbohydrates, salts, and lipids. Although the sample pretreatment for LC–MS/MS assays does not need to be as elaborate as other LC assays especially those utilizing UV detection, it remains pivotal to remove matrix components that might contaminate the system or cause ion suppression when high sensitivity is needed [66]. Protein precipitation, solid phase extraction and liquid liquid extraction are the main sample preparation concepts combined with LC–MS/MS to analyze natural products in biofluids.

2.1. Protein precipitation (PPT)

Protein precipitation is the simplest means of sample pretreatment. Due to the selectivity of MS detectors, it is thought that sample pretreatment for LC–MS/MS assays is redundant. In fact, the largely polar and hydrophilic character of some natural products makes it difficult to extract analytes from plasma with organic solvents, and PPT technique was therefore often used [67,68]. The results showed the deproteinization by acetonitrile gave a good resolution and high recovery. Based on the reasons above, many natural products in biological fluids were extracted by this method [69–76]. It is also worth noting that precipitation of proteins with acids may catalyze the hydrolysis of some conjugates such as glucuronides and sulfates [23].

2.2. Solid phase extraction (SPE)

In the bioanalysis of natural products, solid phase extraction is a frequently used technique for sample pretreatment. Compared with the PPT procedure, the SPE method reduces the serum background greatly. SPE is chosen for the extraction and purification of analytes due to its high selectivity, speed of extraction, the potential for automation, and the fact that much lower volumes of organic solvents are required than those for liquid-liquid extraction [8,77]. Sample preparation using reversed-phase SPE was widely used for flavonoids, alkaloids, saponins and sesquiterpenoids [47,48,52,78].

In some cases, it is necessary to acidify or alkalify the biological fluids containing analytes before transferring samples to the SPE column [79–81]. SPE can be performed off-line manually, semi-automated, or on-line. Biological fluids such as urine

and serum can be processed using automated SPE in a 96-well format for high-throughput quantification of analytes [82]. The limit of detection is several times lower for the online method than that for the off-line method. Similarly, the reproducibility is generally better than that for the off-line method [83,84].

It is known that the efficiency of SPE depends on the type of sorbents, the sample volume and pH, the content of organic modifier and the volume of elution solvent [85]. Due to the wide range of cartridges and solvents that can be employed, SPE is a versatile technique. SPE cartridges that have been evaluated include: SH, GP, C_{18}, C_8, C_2 and Oasis MCX mixed-mode cartridges. The Oasis HLB is advantageous for natural polyphenolic compounds, alkaloids, saponins and sesquiterpenoids [48,51,86–93].

2.3. Liquid–liquid extraction (LLE)

Liquid–liquid extraction is especially suited for lipophilic compounds. Flavonoids in biofluids were usually extracted by ethyl acetate after acidification [94–96], whereas alkaloids were usually extracted by chloroform or ether after alkalification [97–103]. Saponins and sesquiterpenoids were mainly extracted by *n*-butanol, methylene chloride, ether or ethyl acetate [104–108].

The selection of sample pretreatment techniques for each biofluid depends on expected analyte concentrations and required detection limits. For urine, the expected high concentration of metabolites allows for a simple dilution of sample prior to analysis. Acetonitrile protein precipitation provides sufficient pre-concentration and protein removal for quantitative analysis of analytes in biofluids. Matrix suppression data indicate that SPE or LLE is required prior to LC–MS bioanalysis [107]. In other instances, the supernatant observed after protein precipitating could be subjected to SPE procedure, or biological fluids were subjected to both SPE and LLE procedures [43,53,81,108,109].

3. LC–MS bioassays

Suitable interfaces and MS scan techniques for the bioanalysis of natural products or their metabolites are presented in this section. The optimizing of the experimental LC–MS conditions and bioanalysis method validation are also outlined.

3.1. Interfacing systems

The different LC–MS interfaces that have been used to determine natural products and their metabolites by tandem MS share one common characteristic: they were designed to provide a soft-ionization process that leads to a mass spectrum with only a few ions. The atmospheric-pressure ionization (API) techniques, electrospray, and atmospheric-pressure chemical ionization, are the first choice [42,45,47,98,104]. These two interfaces show greater ionization stability, and more sensitivity than other interfaces, such as thermospray (TSP) [110].

FAB is another useful soft ionization MS technique for molecular weight determination. However, the low *m/z* region is crowded with signals resulting from the matrix and these matrix signals are not very reproducible [111]. In the MALDI technique, sample ions are usually analyzed by a time-of-flight (TOF) mass analyzer. MALDI-TOF-MS has advantages over other methods, including high speed of analysis, good sensitivity, and good tolerance toward contaminants [112]. Although this MS interface has not been applied to natural products bioanalysis, its ability to determine mass with an accuracy approaching or better than 1 part per million may ensure the correct identification of their metabolites recovered from biofluids. The recently introduced method of atmospheric pressure photoionization (APPI) has expanded the applicability of API techniques towards less polar compounds. Comparison of ESI, APCI, and APPI in the identification of analytes in biofluids has been studied [113]. There are significant analytical benefits with ESI at low (<1 μL/min) flow rates, such as nanospray (10 nL/min or less) [14].

3.2. MS scan techniques

Single-stage MS can be used in combination with UV detection to facilitate the identification of interests in biological samples with the help of standards and reference data [38]. However, there are still several analytical shortcomings deriving from the characteristic mass spectrum of some analytes, which often gives only a molecule adduct or a weak fragment ion [42,54]. The matrix components enhance or suppress the detector response and yield great variances between the relative abundance of different ions in the spectra [110]. The matrix analyzed and the sample preparation procedure should be taken into consideration together with the chromatographic and spectrumscopic selectivity. For the identification of unknowns, tandem mass spectrometry (MS/MS or MS^n) is often used.

Many combinations of tandem MS have been tried. The triple quadrupole (TQ), hybrid quadrupole-time of flight (Q-TOF) and quadrupole ion trap (QIT) mass spectrometers are the most successful examples [110].

Quadrupole mass filter has now become one of the most widely used mass spectrometers because of its ease of handling, small size, and relatively low cost. The versatility of tandem MS allows one to employ various selective screening strategies (i.e., full-scan, neutral-loss, precursor-ion, and product-ion scan modes and reaction-monitoring experiments). TQ instruments are especially useful in group-specific detection of metabolites. For example, phase II metabolites, such as glucuronides and sulfates, can be selectively detected by using positive ion ESI and neutral loss scan of 176 and 80 u, respectively [114]. In negative ion ESI, sulfate conjugates produce abundant product ions at *m/z* 80 (SO_3^-) and *m/z* 97 (HSO_4^-), and glucuronides give ions at *m/z* 175 (deprotonated glucuronide moiety) and *m/z* 113 (fragment of glucuronide moiety), providing specific marker ions for the selective detection of sulfates and glucuronides in the precursor ion mode [115]. Multiple reaction monitoring (MRM) provides the high sensitivity required in quantitative analysis [13,42,116,117]. The sensitivity of the full-scan mode may not always suffice the metabolic study. The use of ion trap (IT) and time-of-flight mass spectrometers

has increased [77,108,118,119], which provide high full-scan sensitivity.

The main advantage of ion-trap instruments is the possibility to perform MS^n experiments, which provides some structural information of flavonoids or saponins [108,120]. Therefore, the coupled LC–MS^n method is an initial choice for the structure elucidation of drug metabolites. Metabolites can be identified through comparing their chromatographic retention times, changes in observed mass and tandem MS spectra with those of the parent drug even without standards for each metabolite [118,121]. Some newly developed ion-trap styles such as orbitrap and LTQ are gaining more sight in the drug discovery study [122,123].

The recently introduced API-TOF mass spectrometry can provide high-resolution analysis and the elemental compositions of metabolites with a mass accuracy better than 10 ppm. The quadrupole-TOF mass spectrometer (Q-TOF) provides high sensitivity for the determination of metabolites [23]. However, it has a relatively poor dynamic linear range for quantitative analysis, compared to that of quadrupole instruments [65].

3.3. Optimizing of chromatographic mass spectrometric conditions

Reversed-phase liquid chromatography (RPLC) is usually utilized for the separation of analytes in tandem MS for its robustness and ease of method development.

The selectivity offered by MS scan mode, such as SRM, makes HPLC separation unnecessary. However, if the analytes are eluted too quickly, coelution may occur with low amounts of interference or artifact peaks from the matrixes, which can be detected at the same SRM transitions. By separating the analytes chromatographically, a further element of selectivity is added in the form of different retention times for each analyte [124].

In RPLC, most stationary phases are based on silica that has been chemically modified with octadecyl (C_{18} or ODS) or octyl (C_8 or ODS). Using a short HPLC column can reduce the chromatographic time and facilitate the high throughput analysis [45,125].

Another important problem for MS is the so-called ion suppression effect, which can reduce the ionization of analytes. Both ESI and APCI show matrix effects, and ESI is much more susceptible than APCI. Sample preparations could reduce (clean-up) or magnify (pre-concentrate) matrix effects [126]. The synergistic effect of ionization type, sample preparation techniques and the bio-fluid on the presence of matrix effect in quantitative liquid chromatography (LC–MS/MS) analysis has been reported [107].

The selection of mobile phase is a critical factor in achieving good chromatographic behavior and appropriate ionization [93]. Low-surface tension and a low-dielectric constant of the solvent promote ion evaporation, which favors the ionization process. By using a low concentration of formic acid or ammonium acetate, lower than 10 and 100 mM for ESI and APCI, respectively, mobile phases are kept at low pH, which can prevent peak tailing and enhance the positive ionization of flavonoids, alkaloids, saponins and sesquiterpenoids [42,66,75]. Acid modifier is important to suppress the interactions of these groups with residual traces of metals in the stationary phase that are detrimental to peak shape. In negative ion mode, ammonium formate and ammonium chloride are often used as modifiers to improve the shape peak and to enhance the MS response of analytes [76,90,127].

The column effluent flow-rate is considered to be important for the sensitivity of LC–API-MS methods. Though most mass spectrometers can nowadays operate at flow-rates up to 2 mL/min for ESI and 4 mL/min for APCI, lower flow-rates are often chosen because these instruments can work better and less maintenance is required. The choice of an appropriate eluent flow-rate in LC also depends on the dimension of the LC column. Flow rates between 20 and 1000 μL/min are commonly applied with packed LC columns, the diameters of which range from 1 to 4.6 mm. [8,128]

There are several MS instrumental parameters that have drastic influence on the ionization efficiency. The optimization of the MS parameters includes the adjustment of typical interface parameters such as the ionization voltage in ESI and the discharge-needle current in APCI, respectively, and the pressure of the spraying/nebulizing potential. Using TQ MS/MS, the collision energy and the pressure of collision gas are other MS parameters to be optimized. Many reports indicate the need to optimize the tandem MS conditions [98,117]. There is not a clear rule on how to select the optimum value for those parameters because they depend on the specific design of any individual interface. They should be determined experimentally by evaluating the sensitivity and the fragmentation of each analyte that is infused from a standard solution by a syringe pump and mixed with the mobile phase by means of a T-piece. Conditions for fragment formation are manually optimized and the most abundant fragment ions are usually chosen for the SRM or MRM transition.

3.4. Validation

Following development of a bioanalytical LC–MS/MS assay, and before implementation into clinical pharmacological studies or routine uses, it needs to be validated. Validation is essential to ensure the accuracy and precision of the acquired data. In 2001, the Food and Drug Administration (FDA) published guidelines for the validation of bioanalytical assays, which were considered to be the standard for validation parameter requirements [129]. The guidelines generally apply to bioanalytical procedures such as LC based assays (including LC–MS and LC–MS/MS). Good science and effective medical care demand inexpensive validated methods with high throughput, which are capable of simultaneously analyzing multiple drugs in various matrices. The analytical methodology should be validated in terms of precision, accuracy, limit of detection, limit of quantification, specificity, linearity and range, ruggedness and robustness. They describe which parameters should be assessed, how they should be assessed, and the requirements that should be met. In addition, the guidelines specifically describe the need to ensure the lack of matrix effects for LC–MS/MS assays.

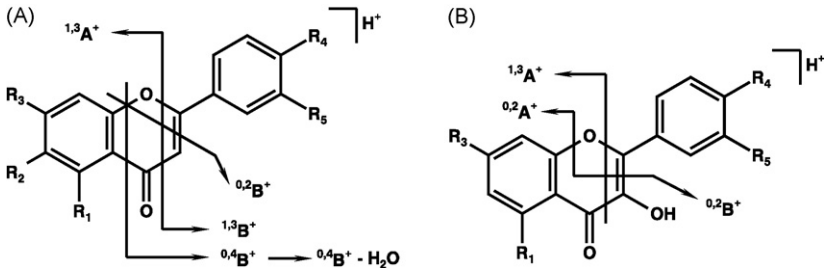

Fig. 1. Nomenclature and diagnostic product ions of protonated (A) flavones and (B) flavonols formed under low-energy CID [138].

4. Applications

Many papers have been published on LC–MS determination of natural products and their metabolites in blood, plasma, serum, urine, bile or feces. However, only those covering drugs of interest in clinical pharmacology are mentioned below. An overview of the LC–MS methods for the above natural products is presented in Tables 1–4.

4.1. Flavonoids

The extent and the form of absorption of flavonoids, after oral administration, are importantly unsolved problems in studying their potential effects [116,130–132]. A series of reviews on the absorption, metabolism, and bioactivity of flavonoids have been published [133–135]. Glucuronidation, sulfation and methylation were the main metabolic pathways of flavonoids [47,136,137].

Many metabolic studies of flavonoids have been reported by LC–MS technique. A review on the application of MS methods for the determination of flavonoids in biological samples has been published in 2004 [133]. Depending on the structure, flavonoid glycosides undergo collision-induced cleavage of the *O*-glycosidic bond producing deprotonated aglycone product ions. Some neutral diagnostic losses and specific retro Diels–Alder fragments were obtained [138]. Nomenclature and diagnostic product ions of protonated flavones and flavonols formed under low-energy CID are shown in Fig. 1. Based on the MS fragmentation characteristics, flavonoid metabolites can be supposed [132,139,140]. These metabolites were mainly analyzed by ESI-MS/MS. For example, the metabolism of anthocyanin has been studied with this interface [47,78]. The authors found five anthocyanin metabolites in urine: three monoglucuronides of pelargonidin, one sulfoconjugate of pelargonidin and pelargonidin itself. Another study on the identification of anthocyanin metabolites was conducted by a TQ mass spectrometer. In MRM mode, several conjugated metabolites were identified. O'Leary et al. demonstrated that quercetin-3-glucuronide can be further metabolized following the pathways of methylation of the catechol functional group and hydrolysis of the glucuronide by endogenous β-glucuronidase followed by sulfation to quercetin-3′-sulfate [141]. Though many metabolic studies of flavonoids have been reported by TQ instruments (in MRM, precursor ion or product ion mode) [140,142], the IT mass spectrometer was more widely used [136,143,144]. For example, detailed investigations on the identification of flavonoid metabolites after the consumption of onions were published by Mullen et al. [145,146]. The analysis was performed by LC-ion trap mass spectrometry and 23 metabolites of quercetin were identified, which is shown in Fig. 2. They included methylation of the aglycone and the formation of mono-, diglucuronides and sulfate conjugates. A *m*/*z* value of 176 or 80 for the substitution group indicated a glucuronide or sulfate residue. Regioselectivity often occurs in the glucuronidation process. However, the exact glucuronidation sites could not be confirmed only by MS technique, and NMR data of reference standard are needed. Based on the MS and NMR data, an isomer of scutellarin was identified [130]. In some instances, TOF was used to obtain the exact mass of flavonoid metabolites [137,147]. Due to the good response on UV and MS detector, flavonoid metabolites were often identified by liquid chromatography photodiode array mass spectrometry (LC–PDA–MS) assay [145,148]. Being phenolic compounds, flavonoid metabolites can be detected in both positive and negative ion mode, which is excellent in the structural analysis [149].

HPLC combined with ESI and APCI interfaces were both used for the quantitative analysis of flavonoids and their

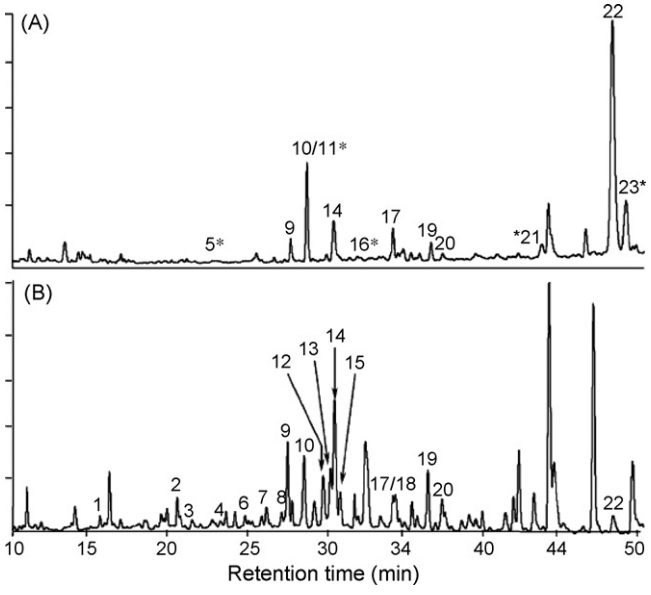

Fig. 2. HPLC–DAD–MS spectrum of quercetin metabolites in (A) a plasma extract and (B) urine obtained from human after the consumption of onions [145].

Table 1
Methods for the determination of representative flavonoids and their metabolites in biofluids by LC–MS

Flavonoids	Samples	Extraction techniquies	Protocol	Mass analyzer	Scan mode	LLOQ or LLOD (ng/mL or μM*)	References
Anthocyanin	Urine	SPE	Sep-Pak C_{18}	TQ	+; ESI; MRM	–	[47,78]
Baicalein, baicalin	Plasma	LLE	Ethyl acetate	TQ	+; ESI; SRM	10	[119]
CTN986	Serum	SPE	Bakerbond C_{18}	TQ	+; ESI; MRM	2	[66]
Glabridin	Plasma	SPE	Sep-Pak C_8	TQ	−; ESI; MRM	0.1	[129]
Hesperidin, naringin	Serum	SPE	Oasis HLB	TQ	−; APCI; SRM	2	[153]
Scutellarin	Plasma	SPE	–	TQ	+; ESI; SRM	0.2	[84]
Lithospermate B	Serum	LLE	Ethyl acetate	TQ	−; ESI; SRM	8	[152]
Baicalin	Plasma	PPT	Methanol-hydrochloric acid	IT	+; ESI; SRM	100	[67]
Baicalin	Urine, bile	Centrifugation	–	IT	−; ESI; MS^n	–	[120]
Anthocyanin	Plasma	SPE	Sep-Pak C_{18}	TQ	+; ESI; MRM	–	[109]
Anthocyanin	Tissue	PPT + SPE	Methanol + Sep-Pak C_{18}	TQ	+; ESI; MRM	–	[142]
Anthocyanin	Plasma	SPE	Sep-Pak C_{18}	Single-Q	+; ESI; SIM	0.14*	[151]
Anthocyanin	Urine	SPE	Sep-Pak C_{18}	TQ	+; ESI; MS/MS	0.001*	[140]
Cyanidin-3-glucuronide	Plasma	SPE	Sep-Pak C_{18}	Q-TOF	+; ESI; MS/MS	–	[137]
Daidzein, genistein	Urine	SPE	Bond Elut C_{18}	Single-Q	+; ESI; SIM	–	[150]
Delphinidin	Plasma	SPE	Sep-Pak C_{18}	TOF	+; ESI; Full scan	–	[147]
Koparin	Urine	LLE	Eethyl acetate	IT	−; ESI; MS^n	–	[144]
Pytoestrogen	Urine	LLE	Ethyl ether	IT	−; ESI; SRM	–	[148]
Puerarin	Plasma	PPT	Acetonitrile	TQ	−; ESI; MRM	20.8	[116]
Quercetin	Plasma, urine	PPT	Acetone	TQ	−; ESI; MRM	0.5, 1	[111]
Quercetin-4′-glucoside	Plasma	PPT + LLE	Acetone + ethyl acetate	IT	−; ESI; MS^n	–	[139]
Wogonin	Plasma	LLE	Hexane-ether	IT, TQ	+; ESI, APCI; MS^n, SRM	0.25	[62]
Polyphenols	Urine	LLE	Ethyl acetate	TQ	−; ESI; MRM	0.01–1*	[57]

Table 2
Methods for the determination of representative alkaloids and their metabolites in biofluids by LC–MS

Alkaloids	Samples	Extraction techniquies	Protocol	Mass analyzer	Scan mode	LLOQ (ng/mL)	References
Aconitine, hypaconitine	Plasma	SPE	Bond Elut HF	TOF	+; ESI; SIM	10	[77]
Morphine, codein	Urine	SPE	Strata-X-C	IT	+; ESI; SRM	25	[8]
Vincristine	Plasma	SPE	Bond Elut C_2	Single Q	+; ESI; SIM	10	[81]
Methylehedrine	Plasma	LLE	Hexane-dichlormethane-isopropanol	TQ	+; APCI; SRM	0.1	[9]
Oxymatrine	Plasma	LLE	Chloroform	Single Q	+; ESI; SIM	5	[98]
Bulleyaconitine	Plasma	LLE	Ether	IT	+; ESI; MRM	0.12	[99]
Colchicine	Plasma	LLE	Dichlormethane	–	+; ESI; SIM	0.5	[97]
Capsaicin, nonivamide	Blood, tissue	LLE	–	–	+; ESI; MRM	1	[103]
Aconitum alkaloid	Urine	SPE	C_{18}	IT	+; ESI; MS^n	–	[46]
Dehydrocavidine	Plasma, urine	PPT	Methanol	TQ	+; ESI; MRM	1, 10	[13]
Amphetamine	Blood	SPE	Oasis MCX	–	+; ESI; SIM	–	[125]
Atropine	Uine	SPE	ODS-18	Q-Trap	+; ESI; MS^n	–	[121]
Berberine, palmatine	Plasma	LLE	Ethyl ether	Single-Q	+; ESI; SIM	0.31	[154]
SN-38	Plasma, tissue	PPT	Acetonitrile-acetic acid	TQ	+; ESI; SRM	0.5, 1	[75]
SN-38	Plasma	PPT	Acetonitrile-acetic acid	TQ	+; ESI; MRM	0.05	[10]
9-nitro-camptothecin	Bile, urine	SPE	Sep-Pak C_{18}	IT, Q-TOF	+; ESI; MS^n	–	[118]
Galantamine	Plasma	LLE	Toluene	TQ	+; ESI; SRM	1	[15]
Heroin	Urine	SPE	C_{18}	TQ	+; ESI; MRM	0.1	[60]
Oxymatrine	Plasma	PPT	Methanol	TQ	+; ESI; SRM	1	[12]
Morphine	Plasma	PPT	Acetonitrile	TQ	+; ESI; MRM	0.5	[128]
Morphine	Plasma	SPE	Oasis HLB	TQ	+; ESI; SRM	0.5	[92]
Morphine	Plasma	SPE	Oasis MCX	Single-Q	+; ESI; SIM	0.5	[156]
Piperine	Urine	PPT	Acetonitrile	IT	+; ESI; MS^n	–	[11]
Pseudoephedrine	Plasma	SPE	Oasis HLB	TQ	+; ESI; MRM	2	[93]
Pseudoephedrine	Plasma	PPT	Methanol	IT	+; ESI; MS^n	5	[68]
Cocaine, morphine	Plasma	LLE	Methyl-butyl ether	TQ	+; Nanospray; MRM	–	[14]

Table 3
Methods for the determination of representative saponins and their metabolites in biofluids by LC–MS

Saponins	Samples	Extraction techniquies	Protocol	Mass analyzer	Scan mode	LLOQ (ng/mL)	References
Astragaloside-IV	Plasma	LLE	*n*-Butanol	Single-Q	−; ESI; SIM	2	[54]
Astragaloside-IV	Plasma, urine	SPE	Oasis	TQ	+; ESI; SIM	10	[91,162]
Astragaloside-IV	Plasma	PPT	Acetonitrile	TQ	+; ESI; MRM	1	[158]
Butulinic acid	Plasma	PPT	Acetonitrile + methanol	Single-Q	−; ESI; SIM	0.2	[74]
Cucurbitacin I	Plasma	PPT + LLE	Acetonitrile + dichlormethane	Single-Q	−; ESI; SIM	10	[106]
Ginsenoside Rb1	Plasma; urine	LLE	*n*-Butanol	Q-TOF	−; +; ESI; SIM	–	[104]
Ginsenoside Rh2	Plasma; urine	LLE	*n*-Butanol	Q-TOF	+; ESI; SIM	133	[65]
Ginsenoside	Plasma; urine	SPE; LLE	Bond Elut C_{18}; *n*-butanol	IT	+; ESI; MS^n	–	[108]
Glycyrrhetic acid	Plasma	SPE	Oasis MCX	Single-Q	−; ESI; SIM	0.1	[44]
Glycyrrhizin	Plasma	LLE; SPE	Dichlormethane; Florisil	TQ	+; ESI; MRM	10	[52]
Oleandrin	Serum; urine	LLE	Ethyl acetate	Q-Trap	+; ESI; MS^n	1	[160]
Oleanolic acid	Plasma	SPE	Oasis HLB	TQ	−; ESI; SRM	0.02	[39]
Ginsenoside Rg1, Rh1	Plasma	PPT	Acetonitrile	Single-Q	−; ESI; SIM	1.56	[90]
Ginsenoside Rb1, Rg1	Plasma	SPE	Oasis HLB	Single-Q	−; ESI; SIM	10	[68]
Ginsenoside Rg3, Rh2	Plasma	PPT; LLE	Methanol; *n*-butanol	Single-Q	−; ESI; SIM	2–10	[159]
Ginsenoside Rg3	Plasma, urine	LLE	Ethyl acetate	Q-TOF	−; ESI; SIM	30	[63]
Ginsenoside Rg3	Plasma	LLE	Diethyl ether	TQ	−; ESI; MRM	0.5	[117]
Soyasaponin I	Feces	SPE	Oasis HLB	TQ	+; ESI; Full-scan	–	[43]
Triptolide	Plasma	LLE	Hexane-dichlormethane-isopropanol	Single-Q	+; ESI; SIM	0.8	[51]
Ursolic acid	Plasma	SPE	–	Single-Q	−; APCI; SIM	10	[105]
Digoxin	Plasma	PPT	Acetonitrile	TQ	+; ESI; MRM	0.1	[45]
Dioscin	Plasma	PPT	Acetonitrile	TQ	−; ESI; MRM	1	[163]

metabolites in biofluids. They were quantified directly or determined after enzymatic hydrolysis with authentic standards. The detection by ESI or APCI was carried out in the positive or negative ion mode—the data could be collected in SIM [150,151], SRM or MRM [66,84,129,152,153] mode. Typically the limit of quantification with LC–MS/MS varied between 0.01 and 100 ng/mL, which was often sufficient in the quantitative analysis of flavonoids and their metabolites [23]. Compounds can be determined by LC/MS/MS without complete separation due to its specific and sensitive characteristics. Therefore, LC/MS/MS is a high throughput analysis method. A study on the quantitation of polyphenols in biofluids has been carried out. The authors simultaneously quantified 15 polyphenols and related compounds in human urine using an LC–ESI-MS method with an analytical run time of only 6 min [57].

4.2. Alkaloids

Due to the versatile structure properties, alkaloids show different metabolic and pharmacokinetic characteristics in vivo. Many alkaloids, such as Aconitum alkaloids decomposed rapidly and it was difficult to detect them in body fluid. Oxymatrine could rapidly reduce to more absorbable matrine by intestinal bacteria [12,154]. Scopolamine has a rapid gastrointestinal absorption [155]. The metabolism of natural alkaloids has been investigated in detail [46,53,121]. Glucuronidation may be the main route of some alkaloids metabolism, such as morphine [156] and berberine [22]. Many alkaloids also undergo extensive metabolism, such as 9-nitro-camptothecin [118].

For the basic property of alkaloids in structure, the determination of alkaloids in biofluids was exclusively by LC–MS in the positive ion mode. The metabolites were in general separated

Table 4
Methods for the determination of representative sesquiterpenoids and their metabolites in biofluids by LC–MS

Flavonoids	Samples	Extraction techniquies	Protocol	Mass analyzer	Scan mode	LLOQ (ng/mL)	References
Artemether	Plasma	LLE	Chlorobutane-isooctane	TQ	+; APCI; SIM	5	[41]
Dihydroartemisinin	Urine	–	–	TQ	+; ESI; SRM	–	[64]
Arteether	Plasma	LLE	Hexane-ethyl acetate	TQ	+; ESI; SIM	4.4	[165]
Artesunate	Plasma	SPE	Oasis HLB	Single-Q	+; APCI; SIM	1	[48]
Artemisinin	Plasma	LLE	Ethyl ether	TQ	+; ESI; MRM	1	[42]
Artemisinin	Plasma	PPT	Acetonitrile	TQ	+; ESI; MRM	1.4	[40]

and assayed by liquid chromatography-ion trap mass spectrometry (LC–MS^n) and further identified by comparison of their mass spectra and chromatographic behaviors with reference substances. By this technique, aconitine was found to biotransform into at least six metabolites in rat liver microsomal incubates or other biofluids [53]. A sensitive and specific LC–MS^n for atropine was reported and 11 metabolites in rat urine were identified [121]. The use of accurate mass measurements in metabolite detection has increased significantly since the introduction of API-TOF mass spectrometers. Li et al. [118] reported that seven metabolites of 9-nitro-20(s)-camptothecin (9-NC) were found in rats with the ion trap and accurate mass TOF mass spectrometers. The new nanospray technique has been applied to the quantification of the glucuronide metabolite of cocaine and morphine in rat plasma [14]. This method only requires a limited number of samples, and the efficient LC/MS/MS workflow is preserved. Another interesting hyphenated technique for metabolite identification and structure characterization is LC–NMR–MS. This combination provides more reliable structure elucidation of metabolites than any of the spectroscopic methods alone. A new major urinary metabolite of piperine was detected and identified with the help of LC–NMR–ESI-MS in rat urine [11].

Quantitation of alkaloids in biofluids was mainly carried out by ESI and APCI in the positive ion mode—the data were collected in SRM or MRM mode. Typically the limit of quantitation with LC/MS/MS varied between 0.01 and 100 ng/mL, which was sufficient for the quantitative analysis. LC–ESI-MS/MS analysis for the quantification of morphine, codeine, morphine-3-β-D-glucuronide, morphine-6-β-D-glucuronide, and codeine-6-β-D-glucuronide in human urine has been studied [8], and the reconstructed SRM chromatogram of the analytes is shown in Fig. 3.

4.3. Saponins (triterpenoid and steroidal saponin)

The stepwise cleavage of sugar moieties appears to be the major metabolic pathway of triterpenoid saponins for oral administration, indicating that they may be metabolized quickly through the rat gastrointestinal tract [52,157]. The absolute bioavailability of some triterpenoid saponins is low. For example, the oral bioavailability of Rg1 is about 1.33% [65].

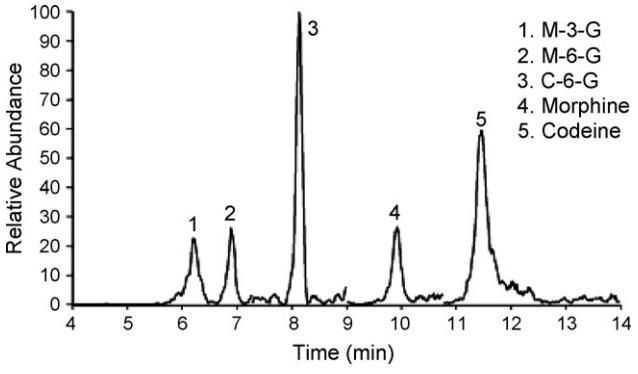

Fig. 3. Reconstructed SRM chromatogram of the analytes of interest (morphine, codeine and their conjugates) obtained from the injection of urine fortified at 70 ng/mL [8].

LC–MS is selective and sensitive enough to carry out the pharmacokinetic study of saponins [158–160]. Generally, ion sensitivities for the saponins were greater in the negative ion mode, while more structural information on ginsenosides was obtained in the positive ion mode. Therefore, both modes have been used for saponin bioanalysis [161]. Identification of ginsenosides and their hydrolysis products in the systemic circulation in man was usually performed by ion trap LC–MS [108]. A total of nine metabolites of ginsenoside Rb1 were detected in urine and feces samples collected after intravenous and oral administration. Oxygenation and the removal of glucose residues were found to be the major metabolic pathways for ginsenoside Rg3 with the aid of Q-TOF [63]. In another study, no deglycosylated products, such as Rh2 and protopanaxadiol, were detected in plasma after administration of ginsenoside Rg3 with the help of a TQ mass spectrometer [117].

The quantitation data for saponins were often collected in SIM or in MRM mode. Typically the limit of quantification with LC/MS/MS varied between 0.02 and 133 ng/mL, which was often sufficient in the quantitative bioanalysis. Due to the low MS response of saponins, adduct ions were usually used for quantitative analysis. In the positive-ion mode, mainly $[M+H]^+$, $[M+NH_4]^+$, $[M+N_a]^+$, and $[M+K]^+$ ions were observed in their ESI mass spectra. In general, the $[M+Cl]^-$ ions at m/z 819 in negative mode and $[M+Na]^+$ ions at m/z 807 in positive mode were chosen for quantitation of astragaloside-IV in SIM scan [54,162]. The $[M+H]^+$ ions of triptolide at m/z 456, the $[M-H]^-$ ions of glycyrrhetic acid at m/z 469.5, the $[M+Cl]^-$ ions of ginsenoside Rg1 at m/z 835.5 and Rh1 at m/z 673.75 were applied [44,51,90]. Ginsenoside Rg3 and its deglycosylated metabolites Rh2 and ppd were also detected in SIM scan mode [158,163]. The quantification was also conducted for astragaloside-IV, dioscin, ginsenoside Rg3 using the following mass transitions in MRM mode: m/z 785.5 → 143.2, m/z 867.5 → 721.5 and m/z 783.8 → 160.8. Digoxin yielded predominantly ammonium adduct and the transition of $[M+NH_4]^+$ ions at m/z 798.6 → 651.6 was used for quantification [45].

4.4. Sesquiterpenoids

The sesquiterpenoid compounds described here were mainly artemisinin and its derivatives, such as artemether, dihydroartemisinin (DHA), arteether, and artesunate (ARTS). Artemisinin exhibited remarkable time-dependent pharmacokinetics and auto-induction metabolism [164]. Artemisinin derivatives were often rapidly converted to their active metabolite DHA in vivo, which was responsible for the antimalarial action. Liquid chromatography coupled with mass spectrometry (LC/MS), using different modes of ionization, has been reported for the detection of artemisinin and its derivatives [40–42].

With a positive ion interface, protonated molecules $[M+H]^+$ of ARTS and DHA were sometimes not seen in abundance, and a fragment of the parent and metabolite molecules was observed having a mass of m/z 221 both for ARTS and DHA. Positive ions were measured using extracted ion chromatogram mode (SIM)

[48]. In another study, the ions at *m/z* 267 ($[M+H–CH_3OH]^+$ and $[M+H–OH]^+$) both for artemether and DHA were used to the quantification of artemether in rat plasma [41]. The $[M+K]^+$ ions at *m/z* 352 for arteether and *m/z* 323 for DHA were used to simultaneous estimation of α, β-arteether and its metabolite DHA, in rat plasma [165]. Artemisinin in rat plasma was studied in MRM mode using the mass transition of protonated ions $[M+NH_4]^+$ at *m/z* 300.4 → *m/z* 209.4 [42], or of protonated ions $[M+H]^+$ at *m/z* 283.2 → *m/z* 247.2 [166].

5. Conclusions and perspectives

Qualitative and quantitative analysis of components at low level was the barrier in the study of active components of TCMs in biological fluids. The only use of chromatography was sometimes time-consuming and not sensitive and specific enough. In the present study, the highly selective and sensitive method LC–ESI-MS was developed and validated for the identification and quantitation of low concentrations of natural products or their metabolites in biofluids.

Further developments of LC–MS may be expected with regards to miniaturization, e.g. the coupling of micro- and/or nano-LC, to tandem MS instruments: this should facilitate the analysis of minute samples. For the sake of high throughput analysis, we have recently observed the emergence of ultra-performance liquid chromatography (UPLC) coupled to mass spectrometry as an alternative to traditional high-performance liquid chromatography techniques. The strengths of UPLC technology promote the ability to separate and identify drug compounds with significant gains in resolution and sensitivity, and marked reductions in the overall analysis time. Automated on-line column extraction or column switching techniques have also been applied for this purpose.

The future for chromatographic analysis of biological samples is multi-methods, and several different analytes can be determined simultaneously in a single run. This is feasible by the use of LC–MS-NMR, which makes qualitative and quantitative determination of compounds or their metabolites possible even at very low concentration.

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