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Review paper

Benzodiazepines in complex biological matrices: Recent updates on pretreatment and detection methods

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ABSTRACT

Benzodiazepines (BDZs) are used in clinics for anxiolysis, anticonvulsants, sedative hypnosis, and muscle relaxation. They have high consumptions worldwide because of their easy availability and potential addiction. They are often used for suicide or criminal practices such as abduction and drug-facilitated sexual assault. The pharmacological effects of using small doses of BDZs and their detections from complex biological matrices are challenging. Efficient pretreatment methods followed by accurate and sensitive detections are necessary. Herein, pretreatment methods for the extraction, enrichment, and preconcentration of BDZs as well as the strategies for their screening, identification, and quantitation developed in the past five years have been reviewed. Moreover, recent advances in various methods are summarized. Characteristics and advantages of each method are encompassed. Future directions of the pretreatment and detection methods for BDZs are also reviewed.

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1. Introduction

Benzodiazepines (BDZs) include diazepam, flurazepam, clonazepam, oxazepam, chlor-diazepine, and triazolam, etc. They have been used as hypnotics, antidepressants, and tranquilizers. BDZs are also known as psychoactive drugs owing to their mechanism of inhibiting the central nervous system. BDZs structures are composed of benzene ring fused with seven-membered nitrogen-containing heterocycles. 1,4-BDZs are widely used antianxiety and anticonvulsants in clinics.

In recent years, mental disorders have been on rise, which has led to the widespread use of BDZs. Data suggests that in 2016, there were approximately 1 billion people who suffered from mental disorders or drug abuse [1]. In Europe, the trend of BDZs usage has increased, and various ways have been devised to control the use and reduce drug prevalence [2–5]. In September 2020, the United States Food and Drug Administration passed a document requiring boxed warnings for all BDZs to "address the serious risks of abuse, addiction, physical dependence, and withdrawal reactions" [6]. Studies have shown that the use of BDZs is related to the severity of many diseases and even an increase in mortality [7]. This may be due to interactions between BDZs and other substances such as opioids, alcohol, and others [8].

According to the data from United States Substance Abuse and Mental Health Services Administration, 29.7 million people (11.2% of the population) used BDZs in 2015 [9]. A recent study shows that, in 2020, there were 23.7% more BDZ overdoses per 100,000 emergency department visits than in 2019. In one year, the death toll of BDZs increased by 42.9% (from 1004 to 1435). The death toll of prescribed BDZs increased by 21.8% (from 921 to 1122). The death toll of illegal BDZs increased by 519.6% (from 51 to 316) [10]. A study of a drug receiving among the elderly population in 10 different countries and territories in Asia revealed that 44.3% received BDZs [11]. In Japan, the most common hypnotic drugs in patients are BZDs (59.7%) [12], which are the most commonly prescribed drugs in Korea [13]. In China, the use of BDZs accounted for 27.3% of patients aged 65–79 and 38.5% of patients aged over 80 [14]. It was also found that the use of BDZs significantly increased the risk of injurious falls [15] and hip fractures [16] among older people. The

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use of BDZs by adults in the United States and the number of deaths caused by illegal use of BDZs were higher than previously reported, and misuse accounted for about 20% of total usage [17]. Clinical cases and data demonstrate the prevalence of BDZs use and the threat to life caused by substance abuse exacerbated by unclear regulatory limits [18]. BDZs exist in complex matrix environments with tiny content, which requires sample preparations to preconcentrate the target substance and avoid inferences from others. Moreover, rapid, sensitive, and selective detecting procedures are necessary in clinical and forensic cases.

Since 2017, two reviews on the pretreatment and detection methods of BDZs have been published [19,20]. In 2019, Qriouet et al. [19] reported pretreatment methods such as liquid-liquid extraction (LLE) and solid phase extraction (SPE), and detection methods including liquid chromatography (LC), electroanalytical, nuclear magnetic resonance, etc. Honeychurch [20] described developments in the design and application of electrochemical approaches for determining BDZs from the 1960s to 2019. In the past five years, both pretreatment and determination techniques have been developed. It is thus imperative to overview the implementation of advanced techniques for the pretreatment and detection of BDZs.

In the present work, literature since 2017 is critically reviewed on advances in pretreatment techniques and detection methods of BDZs in diverse sample matrices, as shown in Fig. S1. The objectives are to: 1) provide a summary of pretreatments (such as LLE, SPE, and other microextraction methods) and analysis methods (such as LC-based methods, sensors, etc.); 2) search the progress on BDZs pretreatment techniques regarding innovations (automation, miniaturization, etc.), solvents (ionic liquids (ILs), deep eutectic solvents (DESs), supramolecular solvents (SUPRASs), etc.), and materials (metal organic frameworks (MOFs), molecular imprinted polymers (MIPs), graphene oxide (GO), etc.); 3) emphasize high-resolution mass spectrometry (HRMS) (time-of-flight mass spectrometry (TOF MS), Orbitrap MS, etc.) and ion sources (direct analysis in real-time (DART), matrix-assisted laser desorption/ionization (MALDI), surface-assisted laser desorption/ionization (SALDI), etc.) for BDZs detection; 4) compare and evaluate the advantages and disadvantages of preprocessing procedures for BDZs such as liquid phase microextraction (LPME), solid phase microextraction (SPME), the quick, easy, cheap, effective, rugged, and safe (QuEChERS) and matrix solid phase dispersion (MSPD), etc.; 5) discuss future trends in BDZs pretreatments and detections, and provide coherent resources to apply these methods for BDZs analysis.

2. Pretreatment methods

2.1. Traditional pretreatment methods for BDZs

Commonly used sample pretreatment methods for BDZs include protein precipitation [21,22], filtration [22–25], enzymatic hydrolysis [26–29], direct solid-liquid extraction [23,30,31] and others. Techniques like ultrasonic-assisted extraction [31,32] and microwave-assisted extraction [33,34] have been developed to improve extraction efficiency regarding low concentrations and shortening the extraction times.

Derivatization of target analytes is necessary before gas chromatography (GC) for analyzing thermally unstable or non-volatile BDZs [35,36]. Common derivatization methods for determining BDZs include silanization [35–37], acylation [38], and alkylation [38]. *N*-methyl-*N*-(*tert*-butyl-dimethylsilyl)-trifluoroacetamide [35–37] is a derivatization reagent to promote sensitivity and stability over other silanization reagents. Double derivatization enhances the sensitivity and selectivity of GC for detecting substances compared with single derivatization, thus improving the resolution and peak shape [36].

Dried blood spots (DBS) sampling is used for pretreating whole blood samples. The progress in automatic DBS extraction makes pretreatment easier and faster [39]. Filter paper and blood spot matrix to be analyzed may cause undesired effects on the DBS method. Extracted blood spot (EBS) overcomes this problem. It uses biocompatible SPME coating to replace the filter paper used in previous DBS procedures for achieving preconcentration, clean-up, and extraction of analytes in a single step. Mirnaghi and Pawliszyn [40] introduced an automatic EBS procedure for extracting diazepam from human whole blood with the recovery of 96%–102% in extraction time of <0.5 min per sample.

For biological samples like exhaled breath, special processing devices are required to clean and enrich them. Beck et al. [41] developed a sampling procedure and optimized by polymer particle filter having a mouthpiece for the sampling of 13 target drugs, including diazepam and oxazepam. The sampling time of the optimized method was reduced to 3 min without pumping. The recovery of target drugs was 93%–116%.

2.2. Development of LLE methods for BDZs

LLE is a pretreatment method of extraction and separation based on different solubility of target analytes in immiscible extraction solvents and sample solutions. Common extraction solvents are ethyl acetate [42], 1-chlorobutane [43], butyl acetate [44], etc. The extraction by mixed solvents is better than that of single solvent, such as *n*-hexane-ethyl acetate [45], chloroform-isopropanol [46], and dichloromethane-*n*-heptane-isopropanol [47].

BDZs are difficult to separate from aqueous and organic phases. The introduction of salting-out agent assists in BDZs extraction. Salting out assisted liquid-liquid extraction (SALLE) improves extraction efficiency in the pretreatment procedure. The miscibility of extraction solvent and water is reduced by adding sodium chloride to the extraction solvent, which increases recovery and extraction efficiency. Soltanmohammadi et al. [48] compared the effect of the SALLE method on diazepam recovery by adding different concentrations of salt solutions. The recoveries increased with the increase in salt amount and reached 82%–90% at the selected 30% (*m*/*V*) salt concentration. The implementation of SALLE reduces the consumption of organic solvents. Bidny et al. [49] developed the SALLE method for extracting and purifying more than 185 drugs and metabolites, including BDZs, from whole blood. After optimizations, both satisfactory recoveries (between 80% and 100%) and reduced cost of organic solvent (3 mL of acetonitrile) have been obtained.

Supported liquid extraction (SLE) is a sample separation and purification method with inert porous filler for adsorption over the large surface area, which removes phospholipids and proteins from blood and protects chromatographic columns and mass spectrometric instruments. SLE improves extraction efficiency, sample recovery, and accuracy of analytical methods. Arnhard et al. [50] compared the extraction capacity of 35 BDZs from urine by SPE and SLE. Recoveries of most analytes adopting SLE were higher after the optimization of extraction conditions, including pH, extractant, and SLE column type. SLE with two steps process (loading and eluting) takes little time for its easier operation. Sauve et al. [51] utilized SLE for extracting BDZs from antemortem and post-mortem whole blood with the analysis time of 8 min, including equilibration.

The extraction process of conventional LLE methods requires a long time. LLE with low-temperature partitioning (LLE-LTP) is

developed to overcome this drawback. As a low-cost extraction method, LLE-LTP achieves extraction and separation of targets by immersing sample/solvent mixture in liquid nitrogen, which reduces the extraction time and improves extraction efficiency. de Paula et al. [52] achieved rapid extraction of five BDZs from beverages by the LLE-LTP method. Manual stirring to cool the sample to a low temperature with liquid nitrogen freeze aqueous phase, which reduced sample preparation time (took 50 s), and recoveries were 80%–110%. LLE-LTP consumes fewer organic reagents and is environment-friendly. Magalhães et al. [53] established the LLE-LTP method for rapid extraction of BDZs from human urine. Only 0.5 mL of acetonitrile was consumed for each sample. After optimization, the recovery ranged from 72.4% to 100.4%. The extraction process of 200 samples took less than 2 h.

Cloud point extraction (CPE), an LLE technique, uses surfactants for extracting target analytes. It is being applied to the analysis of targets in various fields. This method uses a small amount of volatile organic solvents and is environment friendly. Tabrizi et al. [54] used 1.5 mL of non-ionic surfactant having properties of non-flammability and non-volatility in the CPE process to extract oxazepam from human urine. Recoveries of 87%–94% were obtained under optimized parameters affecting extraction efficiency, such as an equilibrium temperature of 40–45 °C.

Many kinds of solvents are applied in LLE. SUPRASs overcome the drawbacks of traditional organic solvents, such as toxicity and a polluted environment. The vapor pressure and viscosity of SUPRASs can be ignored compared with traditional extractants. There is little loss of organic solvents of low boiling points when the sample is stirred and subsequently yields high extraction efficiency. Liu et al. [55] developed the LLE method based on SUPRASs for the extraction and purification of nine BDZs from human urine and blood. The volume ratio of tetrahydrofuran and 1-hexanol, which created SUPRASs, have been optimized to get recoveries of 81.12%–102.76% for urine and 80.74%–95.84% for blood.

2.3. LPME

LPME has received attention with the development of microextractions. LPME overcomes deficiencies of traditional LLE by using micro- or nano-quantities of organic solvents for extraction, achieved through device and process miniaturization. LPME used for BDZs include single-drop microextraction (SDME), hollow fiber liquid-phase microextraction (HF-LPME), dispersive liquid-liquid microextraction (DLLME), etc., as shown in Table S1 [36,48,56–79].

2.3.1. SDME

The earliest form of LPME is SDME which includes direct-immersed SDME (DI-SDME) and head space SDME. DI-SDME extracts analytes from solution by the organic solvents suspended in a chromatographic micro-sampler needle or Teflon rod end. The process is simple in operation and inexpensive (Fig. 1A) [80]. Jin et al. [56] used SDME to extract triazolam from urine. Extraction parameters such as solvent, time, temperature, and shaking speed were compared and optimized. When the extractant volume was increased from 200 to 400 μL, the chromatographic peak area increased by 4664.8, while it only increased by 42.5 when the extractant volume increased from 400 to 600 μL, indicating that extractant volume had an effect on extraction results. Recoveries ranged from 88% to 90% under optimal conditions.

2.3.2. HF-LPME

In SDME, the organic droplets suspended on a micro injector needle are easy to fall off during stirring. HF-LPME has been developed to overcome this drawback. The technique uses porous hollow fiber as support for organic extractants, and the target is extracted first from the sample solution to the hollow fiber membrane and then received by the inner cavity phase. Compared with SDME, HF-LPME has good stability with immobilizing and protecting organic solvents and consumes little amount of organic solvent. The fiber is cheap and disposable.

HF-LPME can be divided into two-phase and three-phase microextractions. In two-phase HF-LPME, both pores and cavities of hollow fibers are filled by organic solvents, thus achieving a one-step equilibrium between water samples and organic solvents. In three-phase HF-LPME, a non-polar solvent (usually dodecane) is immobilized inside hollow fibers pores, providing a supported liquid membrane (SLM), and another *n*-dodecane immiscible organic solvent is located inside the cavity of the hollow fiber. The purification by three-phase microextraction mode is more prominent, and extraction efficiency is higher. Nazaripour et al. [59] compared the effects of two-phase and three-phase HF-LPME on oxazepam and lorazepam extractions from urine. For two-phase HF-LPME, 1-octanol served as the acceptor phase. For three-phase HF-LPME, acetonitrile was the acceptor phase in hollow fiber lumen and *n*-dodecane containing 7.5% (*m*/*V*) trioctylphosphine oxide as SLM which provided recoveries of 88.0%–102.0%. Three-phase HF-LPME exhibited high enrichment and low consumption of organic solvents. Nazaripour et al. [59], after optimizations, showed that three-phase HF-LPME had better enrichment with a preconcentration factor of 101–257, while the preconcentration factor of two-phase HF-LPME was 70–180.

New extractants improve the extraction efficiency of HF-LPME. Rezaei et al. [58] extracted BDZs from water, fruit juice, plasma, and urine using HF-LPME based on SUPRASs. SUPRASs were synthesized by agglomerating decanoic acid water vesicles under the action of tetrabutylammonium and optimized the extraction parameters. Results showed that the pH of the sample solution was an important factor affecting the stability of the condensed phase and the extraction efficiency. When pH was less than 5.0, especially in acidic conditions (pH 3.0), the signal of BZDs decreased. When the sample solution pH increased from 5.0 to 9.0, the peak area increased, but with further increase in pH, the peak area decreased. It was inferred that pH 9.0 was suitable for extraction. The recoveries ranged from 90.0% to 98.8% under optimal conditions. An automatic instrument based on HF-LPME improved the operation repeatability and reduced extraction time. Nazaripour et al. [59] utilized automated HF-LPME apparatus to extract oxazepam and lorazepam from human urine and blood, which simplified the operation steps, shortened extraction time (took 30 min), and increased extraction efficiency. The recoveries ranged from 88.0% to 102.0%.

New types of microextraction methods have been developed based on HF-LPME. Parallel artificial liquid membrane extraction (PALME) is easy to operate and is green microextraction. The analyte is extracted from an aqueous donor solution (sample), passes through organic SLM, and enters an aqueous receptor solution (extractant). Extensive sample cleaning is achieved by preventing the transfer of charged compounds and macromolecules, such as proteins and phospholipids, through SLM. Using 96 channel pipette, it achieves semi-automation, meets the needs of high flux and high sensitivity, and has a low cost. The price is 1/5–1/10 of current extractions such as SPE and SLE. Vårdal et al. [81] employed PALME for extracting BDZs from whole blood. After optimizations, the extraction recovery was higher than 52%, and consumption of organic solvents was kept to a minimum (each sample <5 μL).

Electro-membrane extraction (EME) is a microextraction proposed by Pedersen-Bjergaard [82] in 2006. The method applies a sustained electric field at both ends of SLM (Fig. 1B) [83]. Electric field forces drive the target analyte to traverse the liquid membrane for extraction from the sample into receptor solution, which is in

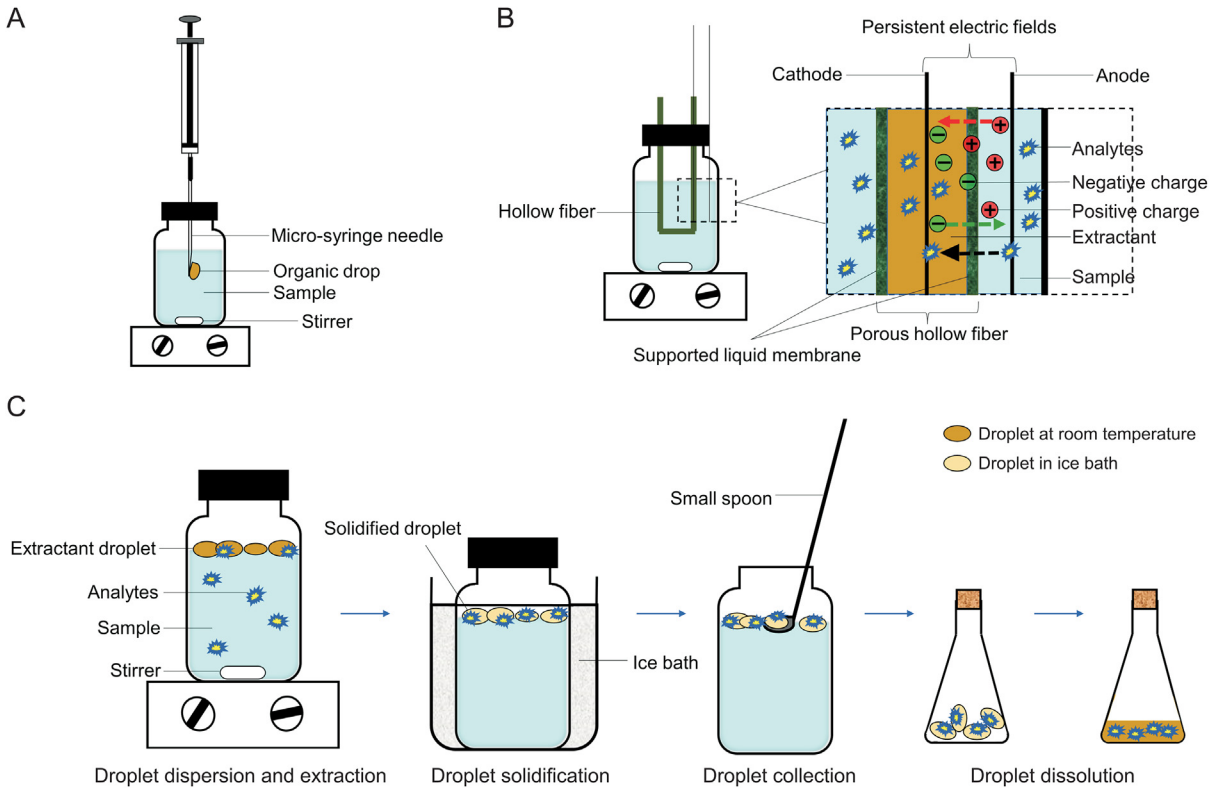

Fig. 1. Flow charts of different liquid phase microextraction (LPME) technologies. (A) The single-drop microextraction (SDME) procedure [80], (B) the electro-membrane extraction (EME) procedure [83], and (C) the solidified floating organic droplet microextraction (SFODME) procedure [85]. Reprinted from Refs. [80,83,85] with permission.

contrast to HF-LPME/PALME with mass transfer facilitated by passive diffusion of pH gradients. The extraction time reduces and extraction efficiency improves owing to electrokinetic migration caused by an additional electric field. Vårdal et al. [76] utilized EME in 96-well format to extract nine BDZs from plasma. The extraction process was completed in 15 min. Recoveries ranged from 38% to 74%. The effect of electric field force on 7-aminoclonazepam and alprazolam was obvious. Recoveries of two substances in separate EME went up from 0% to 80% when voltage was supplied. Organic solvents consumed in EME were 100 μL of formic acid as donor solution, 100 μL of trifluoroacetic acid as acceptor solution, and 3 μL of 2-nitrophenyl octyl ether as SLM.

A new type of EME is developed called pulsed electro-membrane extraction (PEME) to avoid the system instability caused by the increase of voltage under a direct current power supply in an ordinary EME process. PEME improves the stability of the extraction system by reducing the thickness of the electrical double layer on both sides of SLM. The driving force of PEME is the pulsed voltage. PEME further improves the extraction recovery in a short extraction time. Ara et al. [77] compared the extraction efficiency of HF-LPME and PEME for olanzapine from human plasma and urine. The preconcentration factors of drugs from urine and plasma by PEME were higher than those by HF-LPME (59–78 vs. 43–65). After optimization of extraction parameters such as ionic strength, SLM composition, and pulse duration, recoveries of 88.5%–103.1% by PEME were obtained in contrast to 87.5%–93.2% by HF-LPME. The optimal extraction time was set as 30 min for PEME, which was half of that of HF-LPME. Moreover, PEME could omit the step of removing protein and thus simplify the pretreatment process of the complex matrix due to the elimination of binding between analyte and protein under action potential.

Stir bar microextraction (SBME) is another type of micro-extraction developed from HF-LPME. In this method, the extractant is sealed in short porous hollow fiber to make a solvent bar to extract the target analyte. Compared with ordinary HF-LPME, SBME simplifies extraction devices without using microinjectors. SBME has been improved to enhance extraction recoveries and accuracy. Sheikh et al. [78] established dual solvent SBME with different acceptor phases to extract clozapine and lorazepam from human plasma. The extraction process took 30 min, and extraction recoveries of clozapine and lorazepam reached 95.4% and 74.3% with enrichment factors 343 and 263. They fastened the solvent bars with a staple pin as a stirrer to immerge bars in the donor solution instead of floating on the donor solution like ordinary SBME. High contact rates between sample solution and solvent bars contributed to intra- and inter-day reproducibility with a relative standard deviation (RSD) of 1.8%–5.5%.

2.3.3. DLLME

2.3.3.1. Conventional DLLME method. DLLME is a pretreatment method where the extractant forms tiny dispersed organic droplets in the presence of dispersant. The target analyte is extracted to the organic phase in the homogeneous system of emulsion formed by an aqueous sample, dispersant, and extractant. Compared with conventional LLE, the dispersant increases the contact area between the sample solution and the extractant for improving extraction efficiency and enrichment capacity in a short processing time. Oledzka et al. [62] developed the DLLME method to simultaneously extract eight BDZs from human urine. Ethanol and dichloromethane were the dispersant and extractant, respectively. After optimization, the extraction process was completed in 3 min, and recoveries were above 90.65%. DLLME is environment-friendly and cost-effective due to the low consumption of organic solvents and high throughput. Fisichella et al. [69] used 100 μL of chloroform and 250 μL of methanol as extractant and dispersant solutions during DLLME for extracting 70 kinds of analytes, including BDZs,

with recoveries of 21%–99%. Wielens Becker et al. [66] compared the cost of extracting diazepam from environmental water by DLLME and SPE, and a lower cost of DLLME (0.43 USD) was achieved compared with SPE (13.44 USD).

2.3.3.2. Advances in extraction solvents for DLLME. Green chemistry focuses on alternative environmentally benign extraction solvents. ILs are a class of green solvents having low toxicity, volatility and flammability. ILs-based DLLME (IL-DLLME) with low consumption of organic solvents has also been used to extract target analytes from environmental matrices. De Boeck et al. [63] showed the feasibility of IL-DLLME for extracting multiple BDZs from whole blood. [BMIm][PF_6] was chosen as extractant after comparing three ILs ([HMIm][PF_6], [BMIm][PF_6], and [OMIm][PF_6]) with recoveries of 24.7%–127.2% under optimized conditions.

Magnetic ionic liquids (MILs) are functionalized ILs responding to external magnetic fields due to paramagnetic components in their cationic or anionic moieties. In DLLME, MIL-based extractants containing target analytes are retrieved by an external magnet without time-consuming centrifugation which shortens the extraction time. da Silva et al. [84] fabricated MIL of [$P_{6,6,6,14}^{+}$]$_2$[$MnCl_4^{2-}$] for extracting 15 contaminants, including diazepine, from river water in 3 min with a total sample pretreatment time of 5 min. Recoveries of 75%–119% were achieved. The use of low quantities of MIL extractants and organic solvents in DLLME makes the process environment-friendly. The organic solvent proposed by da Silva et al. [84] was 5 μL of mixture of methanol and acetonitrile (1:1, *V*/*V*) as the dispersant.

Another extraction solvent consistent with green chemistry is DES. Unlike ILs, DESs are composed of hydrogen donor and hydrogen receptor parts through hydrogen bonds, which supports the compatibility with GC or LC. DESs are environmentally benign compared with ionic solvents as they can be made from materials like primary natural products with lower costs. DESs have lower toxicity and higher biodegradability. Soltanmohammadi et al. [48] developed microextraction with DES-based DLLME to extract diazepam from urine. DES was synthesized by mixing choline chloride as a hydrogen bond acceptor and three hydrogen bond donors in the best proportion. The recovery of diazepam was 82%–90%, with complete extraction in less than 20 min.

2.3.3.3. Advances in extraction patterns. Improved DLLME techniques taking auxiliary measures have been developed. Ultrasound-assisted DLLME (UA-DLLME) has been applied due to the rapid and simple procedure. Compared with DLLME, UA-DLLME accelerates mass transfer between extractant and sample solutions through ultrasonic vibrations for achieving thorough and homogenous extraction with favorable recoveries and shorter extraction time. Piergiovanni et al. [70] compared DLLME and UA-DLLME for extracting eight BDZs in beverages and found that UA-DLLME had a better recovery of 25.15% from tonic water. Extraction was ultrasonically completed in 1 min using UA-DLLME, which was less than DLLME requiring manual operation. Compared with DLLME, it leaves out the use of dispersant under ultrasonic vibrations. The extractant loss is minimized, which promotes higher extraction efficiency. The organic solvent consumption is reduced. Meng et al. [72] consumed 168 μL of ethyl acetate as the extractant in UA-DLLME without any dispersant, and recoveries of target four BDZs from urine were 81.4%–91.3%.

Ultrasound-assisted low-density solvent DLLME (UA-LDS-DLLME) is an emerging method. Based on ultrasound assistance, UA-LDS-DLLME uses a solvent of lower density than aqueous medium as an extractant, which enables easier analytes collection after centrifugation to improve extraction efficiency. Meng et al. [72] used ethyl acetate as an extractant to achieve the extraction of four BDZs from urine with an ultrasonic bath in 5.5 min. Recoveries of analytes were 73.8%–85.5% under optimized conditions. Consumption of small solvent amounts (150 μL) made it environment-friendly.

Solidified floating organic droplet microextraction (SFODME) is a method for easy separation and collection of extractants from an aqueous sample medium (Fig. 1C) [85]. The extraction solvent uses low-density solvent with low toxicity, and its melting point is close to room temperature. After centrifugation, small droplets of dispersed extraction solvent float on the surface of the aqueous sample solution. Floating drops of organic solvent are solidified after a short ice bath, which makes their collection easier and improves extraction efficiency. Farsimadan et al. [73] applied optimal SFODME to extract diazepam from urine and serum with recoveries of 93.00%–104.10%.

Emulsification microextraction, such as ultrasound-assisted emulsification microextraction (UAEME), is another sample pretreatment method based on DLLME, which preconcentrates target analytes from complex matrices. Surfactants are chosen as amphoteric emulsifiers to accelerate the dispersion of extraction solvent in an aqueous sample solution by emulsifying the organic solvent under ultrasound irradiation. Goudarzi et al. [74] developed UAEME combined with SFODME to preconcentrate nitrazepam and midazolam from human serum. The extraction recoveries for target analytes were >91.0%. Moreover, water-immiscible extractants were more soluble in the aqueous phase due to the amphoteric effect of surfactants, without using a toxic dispersant. Goudarzi et al. [75] applied UAEME to extract alprazolam and chlordiazepoxide from human serum with recoveries of >93.0%. Only 50 μL of extractant and 2 μL of emulsifier were consumed in the process.

Extraction combining back extraction (BE) and DLLME has also been applied to extract BDZs, called DLLME-BE. After regular DLLME, the extraction solvent containing the target is separated from the aqueous solution and then mixed with an immiscible organic solvent to transfer the target to this extraction solvent. Interfering impurities are less likely to transfer to a second organic solvent, which provides better clean-up compared with three-phase HF-LPME. Based on retaining the advantages of DLLME, DLLME-BE improves the enrichment factor of targets in a short extraction time. Ghambarian et al. [68] utilized DLLME-BE to extract four BDZs from urine and plasma in an extraction time of 7.5 min, which was less compared with HF-LPME, UA-DLLME, etc. Recoveries of 45.0%–88.6% and enrichment factors of 225–497, except for alprazolam, were obtained. The use of acetonitrile as the second organic solvent was compatible with GC detection for improving the method's sensitivity.

Air-assisted liquid-liquid microextraction (AALLME) is a simple and fast microextraction consuming low quantities of organic solvent. AALLME has a similar principle to that of DLLME, except that it does not require the addition of a dispersant. The extraction is completed in a short time by repeated suction and release of a mixture of extractant and sample solvents, using a syringe with a needle to achieve rapid dispersion. This improves the mass transfer rate because of the large contact area and increases the extraction efficiency. Ghadi et al. [61] extracted three BDZs from fruit juice, human urine, and tablets by AALLME. The extraction time of <1 min was less than that of other extraction methods. Extraction recoveries of these BDZs were 82.1%–97.1% under optimal conditions.

2.3.4. Others

2.3.4.1. Switchable solvents based homogenous liquid-liquid microextraction (SS-HLLME). HLLME differs from SDME, DLLME, and HF-LPME. Extractants of HLLME are hydrophilic solvents miscible with water. The interface between an aqueous phase and the organic

phase is large. Mass transfer equibalance is established quickly with the appearance of phase separation, and thus, extraction of target analytes is synchronous. SS-HLLME has been developed and applied in pretreatments. Convertible solvents have different affinities with water through different forms of conversions. SS-HLLME simplifies operation steps and improves extraction recoveries and shortens extraction time. Shahraki et al. [79] used SS-HLLME with *N*,*N*-dipropylamine as the extraction solvent to extract nitrazepam from human urine. Solvent pH was adjusted by adding hydrochloric acid and sodium hydroxide to control the conversion of hydrophilic and hydrophobic forms of extraction solvent. After optimization, the extraction recoveries of nitrazepam from human urine were 87.00%–91.02%. The whole extraction took 2 min using 100 μL of extractant and met the requirements of green chemistry.

2.4. SPE

SPE combines extraction column and LC to separate, purify, and concentrate samples from complex matrices. The types of commercial columns used for BDZs include C_{18}, hydrophilic-lipophilic balance (HLB), mixed-mode cation exchanger, etc. Mixed-mode columns are used for the simultaneous extraction of multiple substances, including BDZs [35,86]. Kaartama et al. [86] applied Strata-X-C mixed-mode SPE cartridges for extracting midazolam from rabbit plasma with recoveries of 82.1%–109.7%. SPE in pretreating BDZs is summarized in Table S2 [35,37,50,67,86–100].

The 96-well plate-based SPE increases sample throughput and reproducibility with shorter time and less laborious steps. Turner et al. [92] used water-wettable SPE adsorbents in a 96-well plate for extracting BDZs from human urine, which simplified the extraction by omitting conditioning and equilibration, and avoided the time-consuming transfer step after hydrolysis. The recoveries were 76%–102.5%.

2.4.1. Improved solid phase extraction

2.4.1.1. Advances in adsorbent materials. Recently, various advanced materials have been developed and applied in multiple fields (Fig. 2) [101–103], for example, as novel adsorbents for optimizing sample pretreatment to deal with complex sample backgrounds and achieve higher extraction efficiencies. Nanomaterials as alternative SPE adsorbents hold promise due to exposed surface area with more accessible active sites compared to micro-sized adsorbents. Esmaeili-Shahri et al. [104] fabricated Fe_3O_4@SiO_2 core-shell nanocomposite for SPE of five BDZs from wastewater and human hair with a recovery of 84.9%–90.5%. Low-packing amounts of nano-adsorbents consumed little eluents, which aligns to green chemistry. An et al. [105] extracted six BDZs from urine using polystyrene nanofibers as SPE sorbent in 5 min, which was shorter than LLE and ordinary SPE. Only 20 mg of adsorbent and 1.22 mL of organic solvent were used in the extraction process, with recoveries of 90.4%–113.3%.

GO is a carbon material with a high surface area and abundant functional groups. SPE based on GO provides superior purification because of strong adsorption characteristics. He et al. [106] fabricated an adsorbent using GO for SPE of 10 BDZs from livestock urine with the recovery of 74.6%–95.2%. Nanocomposites based on modified GO were applied with better extraction efficiency. Plastiras et al. [107] selected a nanocomposite of GO with magnetic chitosan (GO-Chm) as an adsorbent for extracting BDZs from surface water. The recoveries of two target analytes ranged from 93.6% to 112.9% under optimized conditions.

MOFs are hybrid porous nanomaterials synthesized by metal ions and organic ligands through coordination junctions. They have been developed as a new type of adsorbents due to their unique characteristics. MOFs can be combined with other substances because of their tunable structures for promoting selective adsorption of target analytes and achieving better extraction efficiency. They have high porosity, large surface area and active binding sites. Du et al. developed a new type of composites as adsorbents by diatomite, and MOF material called zeolitic imidazolate framework-8 (ZIF-8@Dt-COOH), and extracted three BDZs from urine with average recoveries of 80.0%–98.7% (Fig. 2A) [101]. The commercial adsorbents (HLB and C_8/cation resin) exhibited recoveries of 68.5%–97.6% and 65.3%–114.3%, respectively.

MIPs are another kind of adsorbent that are highly cross-linked three-dimensional networks resulting from the polymerization of functional monomers and cross-linked monomers in the presence of template molecules. After polymer formation, the template molecule is dissociated, leaving a cavity that can selectively capture targets similar to the shape, size, and function of the template molecule in chemical structure. Compared with non-imprinted polymer (NIP), MIPs-based SPE achieves better extraction efficiency. Hasanah et al. [108] fabricated MIPs microspheres through precipitation polymerization with homogeneous and smaller pore sizes using diazepam as template molecules for the selective extraction of diazepam from blood serum. The recoveries were 104.63%–106.63% for MIPs, while 20.88%–21.68% for NIPs. MIPs provide purification efficiency and reduce matrix effects compared with traditional adsorbents. Varenne et al. [109] found that extraction using the HLB column showed more complex peaks than the MIPs-based method via LC-MS, while recoveries were comparable.

Monolithic adsorbent columns have been developed for sample pretreatments owing to their abundant pore structures and biocompatibility. Monolithic columns provide better extraction efficiency, and are in situ fabricated with high porosity and large surface area. Zhao et al. [110] prepared a reusable poly (*N*,*N*-dimethylaminoethyl methacrylate-*co*-ethylene glycol dimethacrylate) monolithic column and extracted four BDZs from human urine without dilution and filtration steps. This avoided latent pollution and simplified the extraction steps. Recoveries of targets were 83.7%–103%. In contrast to commercial adsorbents, the adsorption efficiency of the prepared monolith was comparable to or higher than C_{18}, cationic-exchange, and HLB with lower interferent intensities.

SPE methods based on modifications of adsorbents have progressed. Surfactant-assisted SPE has been optimized for sample pretreatments through surface modification of adsorbent with surface active agents. The formation of hemimicelles or micelles mixed aggregates on the adsorbent surface by surfactants contributes to the retention of target analytes, further improving the extraction efficiency. Esmaeili-Shahri et al. [104] modified cationic surfactant cetyltrimethylammonium bromide on composite adsorbents for increasing the solubility of five BDZs and promoted the formation of mesopores. The peak areas of analytes increased with the addition of surfactants, and recoveries were 84.90%–90.50% under optimized conditions.

2.4.1.2. Magnetic solid phase extraction (MSPE). MSPE is an alternative sample pretreatment method for complex matrices. MSPE has high extraction efficiency and purification effect. Plastiras et al. [107] synthesized GO-Chm for extracting alprazolam and flunitrazepam from surface water with recoveries of 93.6%–112.9% under optimized conditions. Compared with traditional SPE, magnetic adsorbents are conveniently collected from the mixture by an external magnet in MSPE, avoiding tedious centrifugation and filtration. He et al. [106] fabricated magnetic graphene (Fe_3O_4-G) for extracting 10 BDZs from urine with recoveries of 74.6%–95.2%, and three extractions took 15 min.

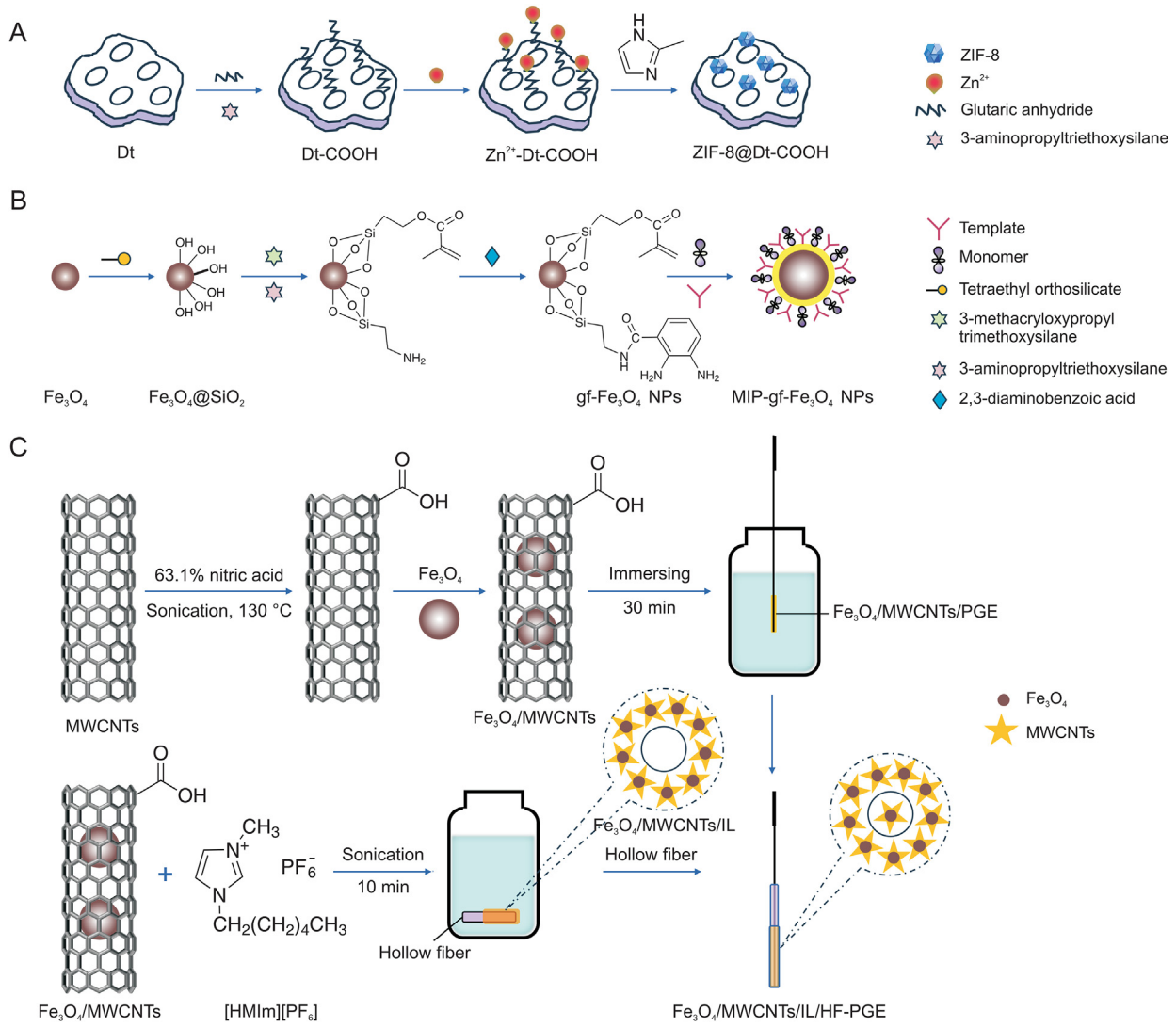

Fig. 2. Schematic representations of synthesis methods of different new materials in benzodiazepines (BDZs) pretreatment and detection process. The schematic representation for the preparation of (A) diatomite-supported zeolitic imidazolate framework-8 sorbent (ZIF-8@Dt-COOH) [101], (B) molecular imprinted polymer-based graft-functional Fe_3O_4 nanoparticles (MIP-gf-Fe_3O_4 NPs) [102], and (C) ionic liquid and magnetic multi-walled carbon nanotube modified hollow fiber pencil graphite electrode (Fe_3O_4/MWCNTs/IL/HF-PGE) [103]. Dt: diatomite; ZIF-8: zeolitic imidazolate framework-8; gf-Fe_3O_4 NPs: graft-functional Fe_3O_4 nanoparticles; MIP: molecular imprinted polymer; MWCNTs: multi-walled carbon nanotubes; PGE: pencil graphite electrode; IL: ionic liquid; HF: hollow fiber. Reprinted from Refs. [101–103] with permission.

2.4.1.3. Advances in SPE extraction patterns. Automatic SPE has been developed for sample pretreatments as an on-line method. SPE operations by automatic robots can save manpower and reduce human operational errors. This further improves the traceability of analysis results and increases sample throughput. Jiang et al. [100] used online automated SPE for extracting diazepam and its metabolites from the human oral fluid. The extraction and detection were completed in 21 min with a recovery of 65.1%–80.8% under optimized conditions.

Column-switching solid phase extraction (CS-SPE) is an automated SPE technique in which two chromatographic columns are linked by an on-off valve. Target analytes are enriched on the trapping column while impurity components are eluted, and analytes are then separated on the switched analytical column. CS-SPE is time-saving with less labor and high throughput. Lee et al. [88] applied the on-line column-switching system to automatically extract and analyze 31 BDZs from human plasma with a recovery of 83%–95%. The extraction and analysis process cost 8 min.

2.4.2. Micro solid phase extraction (μ-SPE)

The μ-SPE is an extraction at a miniaturized level. Micro-extraction by packed syringe (MEPS) is the μ-SPE, fulfilling the green chemistry concept. MEPS achieves higher extraction efficiency with minimal amounts of organic solvent and sample. This is thus eco-benign and applicable to locations such as crime scenes. Magrini et al. [111] used modified MEPS for extracting five BDZs from an alcoholic beverage. The recoveries ranged from 70.7% to 74.1% with little sample volumes (<10 μL) and organic solvents (<500 μL). Adsorbents have been developed for μ-SPE. Rahbar et al. [112] prepared green hydrogel fiber with a calcium/copper alginate framework modified by CuO nanoparticles as μ-SPE adsorbent to extract diazepam and oxazepam from human serum. The recoveries ranged from 82.1% to 109.7% due to the large surface area and higher loading efficiency after modification. The high selectivity and rapid mass transfer of μ-SPE shorten extraction time. Vejar-Vivar et al. [113] developed the coupling of the MEPS syringe with an electrospray ionization (ESI) source to extract and detect seven BDZs from postmortem samples. It consumed about 12 min

for each sample.

2.5. QuEChERS

The sample pretreatment method, QuEChERS, is derived from words describing its characteristics, i.e., quick, easy, cheap, effective, rugged and safe. Removal of water after extraction and adsorption of impurities reduces the matrix interference and improves the extraction efficiency. Huang et al. [114] first treated urine with acetonitrile and NaCl in sequence, and added $MgSO_4$ and primary secondary amine after vortex and centrifugation, with recoveries of 81.0%–100.5% for 11 BDZs. da Silva et al. [115] investigated the effects of salting out (step 1) and dispersive extraction conditions (step 2) in QuEChERS for 15 compounds, including four BDZs from blood. Extraction recovery of 80.3%–87.7% and matrix effect of −1.9% to 2.4% were obtained by using 500 μL of acetonitrile and 100 mg of salt mixture of magnesium sulfate, sodium chloride, and sodium citrate dihydrate (4:1:1, *m*/*m*/*m*), without step 2, followed by another 60-s vortex and centrifugation. QuEChERS does not require tedious operating procedures and is of low cost, needs shorter time, and has less sample volume compared with SPE or LLE. Famiglini et al. [116] utilized QuEChERS to effectively extract eight benzodiazepines from only 0.5 mL of milk-based alcoholic drinks, which could satisfy analytical requirements for very small samples originating from crime scenes. Recoveries were obtained at a lower concentration in the range of 39.24%–66.24%, except for bromazepam (8.89%). Kaki et al. [117] developed QuEChERS using 500 mg of composite, QuEChERS salt and 1 mL of acetonitrile, which are lower than those used by LLE and SPE. Recoveries of midazolam from 0.2 mL of human plasma were 92.7%–117.0%. The extraction process was completed in 30 min.

Diverse materials have been developed for dispersive solid phase extraction (DSPE) to target BDZs, such as nanomaterials with good dispersion, improving the mass transfer and extraction efficiency. Yu et al. [118] applied QuEChERS using Fe_3O_4 magnetic nanoparticles as an adsorbent for purifying and enriching BDZs from blood, and the recoveries ranged from 88.2% to 113.2%. MOF-based adsorbents are promising materials for DSPE. Li et al. [119] synthesized a chemically stable and reusable composite of zeolite imidazole framework@hydroxyapatite (ZIF-8@HAP) as the DSPE adsorbent for extracting three BDZs from urine. The surface area of the prepared composite was nearly 44 folds compared with that of single HAP, contributing to a high recovery of 88.7%–102.0%. Adsorbents with considerable surface area allow sufficient contact between adsorbent and analyte in a short adsorption time of 2 min with low consumption of adsorbents (60 mg). Biomaterial-based adsorbents have also been applied in DSPE with high extraction efficiency because of unique and diverse functional groups, biocompatibility, biodegradability, renewability, and non-toxicity in comparison to traditional sorbents. Samadi et al. [120] used 35 mg of crab shell powder as an adsorbent to extract and separate three BDZs from biological substrates with 36.0%–95.6% recoveries.

2.6. SPME

SPME has been used for sample pretreatments to cater to the shortfalls of SPE, as shown in Table S3 [40,60,102,121–142]. The principle of SPME is based on the equilibrium partitioning of substances to be separated between stationary and aqueous phases rather than the extraction of substances measured in their entirety. Properties of extraction coatings for SPME play a role in extraction efficiency. In addition to commercial extraction coatings such as polydimethylsiloxane (PDMS), divinylbenzene (DVB), polyacrylate, etc., mixed-mode extraction coatings like C_{18}-strong cation exchanger coating [121] are used to extract analytes of different polarities.

Various extraction fibers have been developed for SPME to overcome the disadvantages of traditional adsorbents. The nature of the packed stationary phase determines the adsorption in SPME. MIPs have excellent properties as adsorbents. Recently, MIPs have been applied in SPME, improving selectivity and extraction efficiency. Abrão et al. [122] used diazepam as a template to synthesize hybrid restricted access MIP (RAMIP) fiber modified with bovine serum albumin layer for extracting BDZs from human serum. RAMIP fiber exhibited 98% rejection capacity for proteins, which was higher than 91% that of MIP fibers without restricted modification. The extraction process took 20 min. The production cost per fiber based on MIP was 0.55 USD which was low compared with traditional commercial fibers.

Compared with traditional SPME, polymer monolith microextraction (PMME) performed on columns with specific structures is less costly and has a simple preparation process. It is developed to improve selectivity and adsorption efficiency. Yao et al. [124] synthesized poly (methacrylic acid-ethylene glycol dimethacrylate-*N*-vinylcarbazole) monolithic column for PMME of three BDZs from beer and urine. High porosity and a strong affinity with target analytes due to π−π interactions between media and targets resulted in recoveries of 83.3%–94.7% for beer and 81.4%–93.3% for urine.

Modifications to monolithic columns further enhance the extraction performance. Graphene with optimal adsorption characteristics has also been studied. Strong π−π stacking interactions between monolith and graphene improve the chemical stability of monolith column. Porous structures with large specific surface areas and abundant π-electrons are the reasons for higher extraction efficiency for BDZs. Yao et al. [125] synthesized graphene-modified monolithic capillary column based on poly (*N*-vinylcarbazole-divinylbenzene) to extract and enrich trace BDZs from urine and hair with recoveries of 78.6%–85.6% and 87.2%–94.3%, respectively.

The modification of fiber coatings improves the physicochemical stability of materials used for long periods and extraction efficiency. GO is used as an adsorbent owing to the large surface area and oxygen-containing functional groups, having a number of adsorption sites for higher extraction efficiency. Alizadeh et al. [123] synthesized ZnO-GO nanocomposite as a coating of fused silica fibers for SPME of diazepam and oxazepam from human urine with recoveries of 94%–105%. A limit of detection (LOD) of 0.5–1.0 μg/L was obtained by the proposed SPME with ZnO-GO nanocomposite coating, while the SPME obtained an LOD of 5.0–8.0 μg/L with PDMS/DVB coating under the same detection conditions.

The modified fiber coating is more stable compared with traditional fiber coatings. The extraction efficiency barely decreased by the adsorbent prepared by Alizadeh et al. [123] after 50 successive extractions. Mirnaghi et al. [141] prepared biocompatible C_{18}-polyacrylonitrile (PAN) coating as an extraction phase for extracting BDZs and compared the extraction recovery and coating stability prepared with varying morphologies by different preparation methods. The coating volume of sprayed C_{18}-PAN 96-blades increased 9.4-fold compared to dipped C_{18}-PAN rod fibers, along with a 3.6-fold increase in nordiazepam recovery. Extraction recoveries were reproducible over 70 extractions.

Immunoaffinity SPME as an extraction method improves selectivity for target analytes by immobilizing specific antibodies onto the support. The specific recognition and adsorption guarantee extraction efficiency. Evans-Nguyen et al. [138] used an anti-benzodiazepine immunoaffinity nanogold with a high surface area for extracting diazepam and alprazolam from human plasma. The

modification of SPME reduced the effects of interfering substances and enhanced the signal-to-noise ratio (S/N) of spectra.

2.6.1. Headspace solid phase microextraction (HS-SPME)

HS-SPME is an improved SPME for extracting volatile and semi-volatile analytes (Fig. 3A) [143]. This modification helps avoid contaminations by high molecular and non-volatile substances in sample matrices such as human secretions or urine. Barati et al. [127] prepared nanostructured poly-pyrrole-dodecylbenzenel-sulfonate (PPy-DBS) fiber with the porous structure for HS-SPME to extract alprazolam from plasma. The extraction recovery reached 93%.

The equilibration time of HS-SPME was less than that of direct extraction under the same sample mixing conditions for volatile components. Nakhodchi et al. [126] used PPy-DBS fibers for extracting midazolam from human plasma with a recovery of 91%–95%. The extraction and detection process were completed in 25 min.

2.6.2. Thin-film solid phase microextraction (TF-SPME)

TF-SPME is applied for sample extraction and preconcentration. The flat film coated with liquid organic solvent is immersed in sample solution for extraction, and analytes are then desorbed for assay. The coating is optimized to meet the extraction needs of more analytes by overcoming the disadvantage of commercial SPME coating (C_{18}, PDMS/DVB). Sobczak et al. [130] evaluated the extraction efficacy of 12 coatings for extracting 48 prohibited substances, including alprazolam, clonazepam and flunitrazepam. The optimization results showed that mixed coating containing C_8 + CN (1:1) particles exhibited the best extraction for all analytes due to π–π and dipole–dipole interactions. Improvements to the coatings were also made to achieve extraction efficiency and reduce the cost. Mirnaghi and Pawliszyn [40] developed ultraviolet (UV)-dried PAN-cover C_{18}-PAN coating as a modified coating of TF-SPME for blood spot sampling to clean and extract diazepam. This modification improved the whole blood biocompatibility of coating for long-term use while being reusable (30 times). The recoveries ranged from 96% to 102%.

The extraction time can be further reduced, and efficiency is improved with modification of TF-SPME procedure. Mirabelli et al. [129] extracted diazepam from beverages and biological fluids using TF-SPME with high stability in ultrasound. The extraction time was 5 min. Automated TF-SPME is also used to save extraction time and improve sample throughput. Mirnaghi et al. [142] used automatic 96-blade TF-SPME to extract BDZs from human serum. The high throughput and automated procedure enabled the analysis in 1.5 min per sample.

2.6.3. Bar sorptive extraction

Stir bar sorptive extraction (SBSE) enriches and separates target analytes from the complex matrix (Fig. 3B) [144]. Adsorption of analytes through the coating of the glass tube with an internal magnetic core allows extraction to be completed while stirring itself. Compared with traditional SPME, SBSE with larger stationary phase volumes achieves better extraction efficiency. To overcome the limitations of poor selectivity and low adsorption efficiency of traditional coatings such as hydrophobic PDMS, various stir bar coatings have been developed, including MIPs [102], monolithic materials [135], and nano/magnetic materials [102]. Li et al. synthesized molecularly imprinted stir bar coating for enriching clonazepam from herbal health foods, contributing 1.15-fold and 6.26-fold higher adsorption of clonazepam than that of nitrazepam and midazolam, respectively (Fig. 2B) [102]. The recovery of target clonazepam was 89.8%–103.3%.

Auxiliary methods are applied to the desorption step to overcome the drawbacks of long desorption times of conventional SBSE and the consumption of large amounts of organic solvents

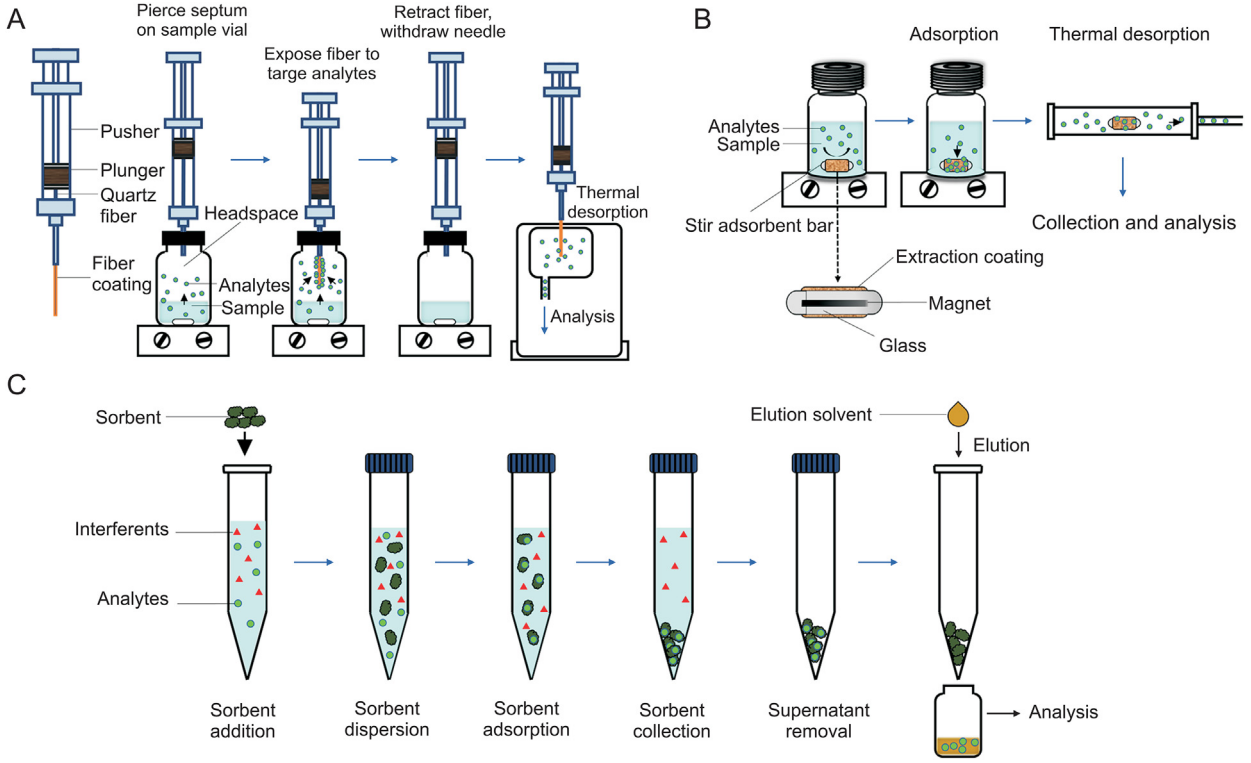

Fig. 3. Flow charts of different solid phase microextraction (SPME) technologies. (A) The headspace solid phase microextraction (HS-SPME) procedure [143], (B) the stir bar sorptive extraction (SBSE) procedure [144], and (C) the dispersive solid phase microextraction (dSPME) procedure [145]. Reprinted from Refs. [143–145] with permission.

for desorption. Torabizadeh et al. [135] used an ultrasound-assisted method to accelerate the desorption of diazepam and nordazepam in 10 min. The use of organic solvents for desorption can be minimized to 250 μL of methanol which is environmentally benign.

Recently, extraction devices based on bar adsorption have been developed to meet sample pretreat demands. High throughput bar adsorptive microextraction (HT-BAμE) is efficient sample pretreatment for achieving high-throughput extraction with fewer analytical steps, labor and processing time. Ahmad et al. [136] developed miniaturized static HT-BAμE equipment for extracting eight BDZs from biological samples with a recovery of 33.0%–104.5%. This enabled the simultaneous adsorption and desorption of 100 samples. The average processing time per sample was 2 min, comparable or even shorter than other pretreatment methods. Trace amounts of organic solvents (100 μL) were used for the desorption of analytes for each sample in HT-BAμE.

2.6.4. In-tube SPME

In-tube SPME is a miniaturized extraction where extraction is achieved by capillary coating or filling of stationary phase in the capillary. Compared with traditional SPME, in-tube SPME achieves robust extraction efficiency in a shorter time. Yao et al. [128] synthesized monolithic polymer column in the silanized capillary by in situ thermal initiated polymerization for extracting six BDZs from beer and urine. The in-tube SPME process took 30 min with the recovery of 80.4%–94.2% and 79.6%–95.2%, respectively. Miniaturized in-tube SPME required minimal amounts of solvent. Only 80 μL of methanol was used for dilution in the in-tube SPME process [128].

2.6.5. Dispersive solid phase microextraction (dSPME)

The dSPME is an efficient method for sample extraction which uses dispersed nanomaterials as adsorbents (Fig. 3C) [145]. It facilitates rapid mass transfer using lower amounts of organic solvents and sorbents in minimal extraction time with high adsorption efficiency. Shiri et al. [131] prepared magnetic nanoparticles (Fe_3O_4@SiO_2@Kit-6@NH_2) for dSPME of clonazepam from biological fluids and wastewaters with the recovery of 94.6%–100.7%. The optimized adsorption time was 6 min. Only 25 mg of nanoparticles as adsorbent and 30 μL of methanol as eluting solvent was consumed in dSPME. The application of auxiliary technology such as vortex-assisted dSPME [131], and ultrasonic-assisted dSPME [132] make the dispersion of adsorbent more rapid and uniform, thus shortening the sample pretreatment time.

The dSPME has been developed for optimizing the extraction. Pipette-tip micro-solid phase extraction (PT-μSPE) has been applied for on-site sample pretreatment as a simple and rapid method. As miniaturized SPE, the amounts of adsorbent, solvent, and sample used in the process are minimized. Amini et al. [133] used 5 mg of adsorbent with 750 μL of sample for extracting BDZs by PT-μSPE with recoveries of 92.4%–100.3%. Only 250 μL of organic solvent was used for the dilution. The convenient and rapid procedure of PT-μSPE reduced extraction time to 2 min. Nanocomposites are also used as alternative adsorbents of PT-μSPE owing to the high surface area with porous structures, which improve extraction efficiency compared to traditional adsorbents. Amini et al. [133] prepared polyacrylonitrile/MIL-53(Fe) nanofiber as the adsorbent for PT-μSPE of oxazepam and nitrazepam from wastewater and biological fluids. The recoveries were from 92.4% to 100.3%.

Fabric phase sorptive extraction (FPSE) has been used for enriching target analytes from the complex matrix. A characteristic adsorbent-coated support is soft fabric with abundant pore structures and permeability. Compared with traditional SPE, FPSE medium is introduced directly into the sample matrix without any pretreatment, clean-up, or solvent evaporation. Samanidou et al. [137] extracted four BDZs (alprazolam, diazepam, lorazepam, and bromazepam) from human serum by optimized FPSE with the recovery of 92.5%–107% in equilibration time of 20 min. The biodegradable and reusable adsorption medium and minimum consumption of organic solvents make the FPSE procedure environment-friendly and of low cost. Samanidou et al. [137] compared the extraction performance of three FPSE adsorbents, of which sol-gel poly(ethylene glycol) performed the best. Only 500 μL of CH_3CN:CH_3OH (50:50, *V*/*V*) was consumed for elution in FPSE.

2.6.6. Automatic SPME

To improve the extraction efficiency and save time, automated SPME with high-throughput and rapid analysis is used for extraction and enrichment of target analytes from the complex matrix. Automatic SPME reduces extraction equilibrium time with high precision. Mirnaghi et al. [141] applied an automatic 96-blade SPME for extracting four BDZs from human plasma. The robotic autosampler enabled the automated analysis of 96 samples in less than 1.7 min per sample. Recoveries of 74%–97% were obtained.

Since conventional automated devices are bulky and expensive, miniaturized automatic extraction devices with low cost are suitable for extracting small amounts of samples. Miniaturized automatic extraction device does not require large sample volumes or long extraction/desorption time. Ghiasikhou et al. [140] developed a capillary gap sampler for automatic SPME of three BDZs from human plasma and direct delivery of extracts to MS. Rapid and reproducible sampling and the extraction process were performed by a robotic arm in a few minutes with small sample volumes (<40 μL).

2.6.7. In vivo SPME

In addition to in vitro extraction and enrichment, SPME can also be applied to in vivo analysis of certain ingredients directly in animals. Compared with SPME, this in vivo study can better make the dynamic analysis of the target analyte during the physiological process with minimal injury to the organism and without blood extraction. The analysis time and steps can be reduced and simplified. Lord et al. [139] proposed in vivo SPME for extracting BDZs from the peripheral vasculature of beagle dogs. The extraction was performed by inserting fine surgical steel wool coated with PPy as adsorbent into Beagle Dog peripheral blood vessels. The in vivo sampling process took as little as 1 min, and whole process, including instrumental analysis, was completed in 30 min.

2.7. MSPD

MSPD is a simple and rapid sample pretreatment method. SPE material functionalized with C_{18} is ground with a sample to give a mixture in a semi-dry state and packed as filler. The elution of target analytes is achieved by washing the column with different solvents. A high extraction efficiency is guaranteed in a simple and convenient procedure. Qu et al. [146] utilized MSPD to extract 13 sedative drugs, including BDZs, from meat with recoveries of 77.4%–100.2%. This indicated better extraction and purification than LLE (61.8%–108.4%) and SPE (47.3%–95.2%). Compared with traditional sample pretreatments, MSPD can simplify the sample processing process by eliminating the need for operating steps such as tissue homogenization, sedimentation, centrifugation, pH adjustment, and sample transfer, which also avoid the loss of sample and reduce analysis time. Qu et al. [146] achieved extraction

of target analytes using optimal MSPD in an extraction time of 15 min, shorter than LLE (3 h) and SPE (2.5 h).

2.8. Summary of pretreatment methods

In summary, the improvement of pretreatment methods of BDZs revolves around three aspects. First, the concept of green chemistry by using solvents such as ILs, DESs, etc., in LLE and LPME has gained increasing attention. Second, simple, rapid, and inexpensive pretreatments methods are to be developed. Nanomaterial-based adsorbents and magnetic adsorbents are applied in SPE, QuEChERS, and SPME, enhancing mass transfer. The addition of ancillary techniques, such as salting out, low temperature, ultrasound, and vortex, accelerates the dispersion and improve extraction speed. Extraction devices and miniaturized extraction methods are developed to simplify the extraction and improve extraction efficiency. Third, the improvement of selectivity toward target analytes is of concern. Adsorbents with high selectivity, such as MIPs, as well as coatings with immunoaffinity, are developed. New green adsorbents and solvents with higher extraction performance, selectivity, convenience, rapidity, miniaturization, and automation are designed to improve the pretreatment methods of BDZs. A comparison of the advantages and disadvantages of different pretreatment methods for BDZs is shown in Table S4.

3. Determination methods

Efficient analytical protocols have been proposed and applied for varieties of targets or unknown compounds, which have been described in the following sections.

3.1. LC

The detection and analysis of analytes of interest are managed by separating them from the complex matrix. LC is a widely used analytical technique for separations based on the selectivity of components in the mixture for the stationary and mobile phases. The application of the LC method for BDZs is shown in Table S5 [21–24,27–30,32,34,39–42,44–47,49,51,58–61,63,65,69–71, 73–78,81,87–93,95–102,104–107,109–112,115,119–122,125,128, 130–133,135–137,141,142,146–161].

3.1.1. Optimization of chromatographic conditions

The properties of chromatographic columns affect the retention behavior of target substances. High performance liquid chromatography (HPLC) columns are packed with silica gel of particle size 2.7–5 μm. They are divided into: normal phase and reversed phase (RP) columns. RP columns such as C_{18} [91,109], C_8 [93,151], and C_6H_5 [136] are used for separating non-polar BDZs.

Ultra-high performance liquid chromatography (UHPLC) uses small particle sizes (1.7–2.5 μm). UHPLC has column efficiency, sensitivity, and speed, with minimum organic solvent consumption. Behnoush et al. [46] compared the performance of HPLC (250 mm × 4.6 mm, 5 μm) and UHPLC (100 mm × 3 mm, 3 μm) for seven BDZs from postmortem samples with a runtime of 40 min and 15 min, respectively. The retention times of target analytes by HPLC were longer than those by UHPLC, approximately 3–7 times. LODs of 0.01–0.07 μg/mL and 0.001–0.004 μg/mL were obtained for HPLC and UHPLC, respectively. Only 21.5 mL of mobile phase at a flow rate of 0.7 mL/min was consumed in UHPLC, saving half of the solvent compared with that in HPLC.

For RPLC, methanol-water [101,110,147,148] and acetonitrile-water [70,107] are the common mobile phase compositions that often work in gradient elution mode to improve sensitivity and achieve good separation of components with large property differences. Additives such as buffer salts [102,105], acetic acid [125,136], and formic acid [28,70,111], and others [61,78] are added to mobile phase as modifiers to improve peak shape, raise separation degree, and increase method sensitivity.

Hydrophilic interaction chromatography (HILIC) separates polar and hydrophilic substances. Unlike normal phase LC, strong hydrophilic stationary and mobile phases consist of low aqueous/high organic solvents in HILIC. They provide good retention and separation selectivity and improve the solubility of polar analytes in the mobile phase. The low back pressure of HILIC allows higher flow rates in longer columns with small particle sizes, which reduces time and improves efficiency. Yuan et al. [87] applied HILIC to detect diazepam and estazolam from human plasma with a chromatographic run time of <5 min.

Micro-fluidic liquid chromatography (micro-LC) has been developed for sensitivity, ease of use, and high throughputs. Compared with traditional LC systems, micro-LC with microflow rates provides incomparable sensitivity of a 10-folds increase while maintaining high throughputs. Jiang et al. [162] used a micro-LC system with a microflow rate of 3 μL/min for separating and detecting 11 BDZs from hair with low LODs and limits of quantitation (LOQs) of 0.008–0.03 and 0.025–0.125 pg/mg, respectively, 2–10 times lower for all target analytes than traditional LC with flow rate 0.5 mL/min. Micro-LC systems are durable, and their microfluidic flow control provides reproducibility in retention times, while a small dead volume of flow path enables precise analysis. The microfluidic separation uses pico-liter to nano-liter samples and reagents, enabling separation and detection in seconds to tens of seconds with efficiencies 1–2 orders higher than conventional assays. The reduced sample requirements and savings of nearly 90% in solvent usage lead to environment friendliness and economical operation. Jiang et al. [163] used micro-LC for analyzing five trace BDZs from 2 mg of hair sample and less than 40 μL of mobile phase (A: methanol:acetonitrile (3:1, *V*/*V*) containing 0.1% (*V*/*V*) formic acid; B: water containing 0.1% (*V*/*V*) formic acid) was consumed. LODs of 0.001–0.08 pg/mg and LOQs of 0.0125–0.25 pg/mg were obtained. Results showed 30–70 folds improvement in sensitivity compared to published articles.

3.1.2. LC-MS

LC-single quadruple MS (LC-Q-MS) is the common LC-MS with the quantitative ability and high sensitivity. The detection system provides specificity and accuracy, reducing false negative and false positive rates in immunoassays. Q-MS has, however, the disadvantage of poor qualitative ability due to fewer fragments of information. Triple quadrupole MS (QQQ MS) with cascade function is developed to make up for this defect. This is composed of three quadrupole rods in series where the first quadrupole (Q1) and the third quadrupole (Q3) serve as mass filters and the second quadrupole (Q2) as an ion collision cell. Except for the general product ion scanning function, QQQ is also capable of selective reaction monitoring (SRM), multi-reaction monitoring, parent ion scanning, and neutral loss, which can be helpful in structural studies of distinct groups. Mazzarino et al. [159] utilized multi-targeted QQQ MS in SRM acquisition mode for screening and detection of 38 drugs, including 16 BDZs and their metabolites in human urine with lower LOD of 5 ng/mL. QQQ MS has improvements in resolution. Ares-Fuentes et al. [160] used LC-QQQ MS for qualitative and quantitative analysis of five designer BDZs in plasma with precursor ion (mass to charge ratio (*m*/*z*)) of 353.76 for clonazolam. LC-Q-MS developed by Karampela et al. [154] provided molecular ions (*m*/*z*) of 285 for diazepam and 388 for flurazepam.

Ion trap MS (IT MS) is used by storing ions outside the trap and changing the electric field to push ions out of the trap at different *m*/*z* for detection. Compared with QQQ MS, IT MS is less expensive.

The resolution of IT MS is several times higher than that of Q-MS with localized high-resolution mode. Based on stronger qualitative analysis capabilities than QQQ MS, IT MS gives structural information about the molecule and provides high sensitivity with low LODs. Licata et al. [157] used IT MS for the screening and identification of 50 compounds, including 15 BDZs and their metabolites, in 234 real hair samples from headache patients. LODs and LOQs of 2.0–10.0 pg/mg and 10.0–20.0 pg/mg were obtained, respectively. IT MS also has stability and provides reproducible analytical results. Sun et al. [156] detected eight BDZs in blood using IT MS with intra-day precision (RSD%) of less than 3.2% and inter-day precision (RSD %) of less than 5.1% for each compound. LODs were lower than 0.3 ng/mL.

Depending on electrode shape, IT MS can be divided into two types, three-dimensional IT MS and linear ion trap MS (LIT-MS) [164]. The structure of LIT-MS is similar to that of QMS and consists of two sets of hyperbolic-shaped stage rods and two plates at both ends, with narrow slits open on one of the class rods. Compared with three-dimensional IT MS, ions are trapped in the line segment in the axial direction of the stage rod rather than concentrated at one point in the lit, which improves capture efficiency and weakens the space charge effect [164]. LIT-MS has thus improved sensitivity and reproducibility. Hu et al. [158] applied LIT-MS for detecting eight BDZs in blood with LODs and LOQs of 0.01–0.10 and 0.10–0.25 ng/mL, respectively. RSDs were 4.3%–11%.

Compared with low-resolution mass spectrometers described above, HRMS overcome the disadvantage of poor qualitative ability with higher resolution and more accurate qualitative results. There are also advantages concerning different kinds of HRMS. TOF MS is the common HRMS with fast scanning speed and high sensitivity. Tomková et al. [44] detected 10 designer BDZs from human serum in 6 min with LODs and LOQs of 0.10–0.15 and 0.33–0.50 ng/mL, respectively. TOF MS accurately discriminates ions with strong resolving power and qualitative ability. Nozawa et al. [165] distinguished etizolam and triazolam based on their exact masses of 343.0778 and 343.0511 using high-resolution TOF MS. LODs of eight target BDZs in human blood ranged from 0.07 to 2 ng/mL.

Orbitrap MS is another HRMS with multiple advantages. Compared with TOF MS, Orbitrap MS overcomes the disadvantages of the large size and complexity of maintenance operations. Orbitrap MS has high resolution to the nearest five decimal places for molecular weights. Yao et al. [125] obtained exact masses for six BDZs according to the ion chromatogram of 321.01921, 326.08548, 295.07450, 282.08732, 300.08982, and 309.09015, respectively. Wu et al. [166] used Orbitrap MS to determine trace amounts of flunitrazepam from beverage samples. LODs were 0.2 and 1.0 ng/L for ginger ale and Arizona tea samples, respectively, four orders lower than other reported methods.

Orbitrap MS generates full scan data over a wide mass range providing a range of quantitative and qualitative analyses. Vincenti et al. [71] used Orbitrap MS with a full scan range between 50 and 800 m/z for determining 12 BDZs residues from drink and food paraphernalia surfaces on the crime scene. Pettersson Bergstrand et al. [28] achieved the detection of 28 designer BDZs from human urine with a scanning m/z range of 100–650.

Ion mobility spectrometry (IMS) is an atmospheric pressure separation and detection technique, also considered as special MS without chromatographic separation, based on the mobility of gas-phase ions in weak electric fields. Charged analytes migrate in drift tubes with different characteristics and speeds (drift velocities) to achieve the separation of ions and their qualitative analysis, like chromatographic retention times. In addition to traditional ^{63}Ni foil radioactive ionization [167] as the ionization source in IMS, a radioactivity-free corona discharge source [126,127,134] with improved safety and ionization efficiency has been employed. Compared with prevalent chromatographic and mass spectrometric methods, IMS is portable and less expensive without consuming organic solvents, which is thus eco-friendly and suitable for on-site trace analyte analysis. BDZs can have a sensitive response in positive ion mode owing to BDZs with high gaseous basicity and higher proton affinity [167]. Nakhodchi et al. [126] used IMS to detect midazolam from human plasma with an LOD of 52 μg/L. IMS provides reliable analytical results in a few milliseconds due to high response speed. The total run time of IMS is 5–25 ms [168], less than that of chromatographic procedures. Sun et al. [134] achieved rapid screening of five BDZs in dietary supplements by IMS in short drift time of 11.38 ms.

3.1.3. Different ion sources

Mass spectrometers first ionize the test compounds, so ion source optimization plays a role in mass spectrometric methods. The common ion sources are ESI and atmospheric pressure chemical ionization (APCI). A combination of ESI and APCI as a multimode ionization source has also been used. Compared to the traditionally used single ion source, multimode ionization source with multifunction and high throughput provides sensitivity for each analyte present in a sample in a single run, which is applicable to analytes with wide polar and volatile range. Galaon et al. [32] compared the difference in sensitivity of mass spectrometric methods using ESI-only, APCI-only, and multimode ESI-APCI ionization under specific chromatographic conditions. The mixed-mode ion source of ESI-APCI exhibited the highest sensitivity, which brought an average of 35% and 350% detector response increase compared to ESI-only ionization and APCI-only ionization, respectively.

The developments of MS in recent years have been fueled by various versatile ionization techniques. Desorption electrospray ionization (DESI) is an ambient mass spectrometric ionization that incorporates both ESI and desorption functions. The sample is deposited on the carrier surface by solvent and detected by a mass analyzer after ionization and desorption of charged spray. DESI enables direct, rapid, and nearly cross-contamination free analysis of samples under atmospheric pressure conditions with little or even no sample pretreatment procedures. D'Aloise et al. [169] used DESI-MS to rapidly quantify flunitrazepam from alcoholic beverages without any extraction or derivatization and obtained an LOQ of 3 μg/mL. DESI-MS also has the potential for high-throughput analysis. D'Aloise et al. [169] achieved the analysis of six samples in 6 min without any sample carry-over observed.

MALDI, as a soft ionization technique, has been used for analyzing non-volatile and thermally labile macromolecular samples. At the same time, there are relatively few applications for small organic molecules like BDZs. Compared with traditional ion sources, MALDI can ionize some difficult samples with high sensitivity to give intact ionization products in a simple mass spectrum without obvious fragments. Nozawa et al. [165] utilized MALDI to achieve ionization and detection of eight BDZs from human blood with LOD as low as 0.07–2 ng/mL. MALDI is easier to operate with rapid analysis speed than LC or GC. The preparation process of MALDI only takes less than 5 min, and analysis of each sample can be completed in 3 min by the irradiation of several lasers without a complex preadjusted process of LC or GC.

The dispersion of test samples by using a solid matrix is the main characteristic of MALDI. Thus, the choice of the matrix has an impact on the analysis of samples. An ideal matrix has strong electronic absorption at the adopted laser wavelength, better vacuum stability, and lower vapor pressure, as well as better miscibility of analytes in the solid state. Nozawa et al. [165] used α-cyano-4-hydroxy cinnamic acid (CHCA) as the matrix for ionization and detected 7-aminoflunitrazepam (a metabolite of

flunitrazepam). LOD of 0.7 ng/mL was obtained under conditions optimized for matrix and solvent components, which was 1000 folds lower than that of previous reports using 2,5-dihydroxybenzoic acid (DHB; 2.5 μg/mL) or DHB conjugated magnetic nanoparticles as matrices (0.25 μg/mL). Nunes et al. [170] evaluated the effect of five matrices (CHCA, 2′,4′,6′-trihydroxyacetophenone monohydrate, DHB, 2,5-dihydroxybenzoic acid plus 2-hydroxy-5-methoxybenzoic acid, and sinapinic acid) on the ionization and analysis of BDZs. The saturated concentration of CHCA was selected as the optimal matrix owing to its response to all target BDZs. Low LODs for alprazolam and diazepam in river water were 5.0 and 2.5 μg/L, respectively, and 40 ng/g in sediment samples for both drugs.

The organic matrix used in MALDI causes background interference at the low mass end of the mass spectrum due to self-ionization, hindering the detection of small molecular species (less than 700 Da). Hence SALDI based on an inorganic nanomaterial matrix is developed. Compared with MALDI, SALDI is suitable for analyzing small molecules like BDZs with minimal interferences in low mass range with high sensitivity and good stability. Guinan et al. [171] compared the effect of detecting flunitrazepam using three silicon-based substrates (desorption ionization on porous silicon, nanostructure-initiator mass spectrometry (NIMS) and nano-assisted laser desorption ionization) in SALDI process. NIMS produced higher signal intensities for target flunitrazepam (S/N values with standard deviation (SD): 34.6 ± 11.5) compared with the other two matrix (S/N values with SD: 10.1 ± 2.9 and 11.8 ± 2.7), suggesting better sensitivity for BDZs detection. It was confirmed that three matrices showed good detections after six months of storage according to the average S/N for flunitrazepam.

Surface-enhanced laser desorption/ionization is developed based on MALDI to facilitate selective enrichment and capture of analytes and increase mass spectrometric potency for complex background substrates. The sample medium is subjected to surface modifications to obtain a surface with specific recognition and affinity capture for target analytes. The analytes are selectively captured and enriched on the carrier surface, followed by MS analysis with high sensitivity. Lowe et al. [172] developed porous silicon (pSi)-based laser desorption/ionization mass spectrometry method by modifying BDZ antibody on the pSi surface for the selective detection of BDZs. The antibody-modified pSi surface with targeted selectivity showed higher sensitivity with S/N of more than three times for diazepam, which was superior to previous reports on immunoaffinity LDI-MS.

DART is a technique developed after ESI and APCI. At normal temperature and pressure, the excited state atoms generated by electrical discharge of carrier gas, such as helium or nitrogen, directly desorb and ionize the compounds in the sample before detecting by MS in a direct, fast, unscathed, and contactless DART-MS process. In contrast to LC-MS based on ESI, DART-MS has improved sensitivity and does not need longer sample preparations and chromatographic separations. This enables rapid and high-throughput sample analysis within seconds, improving the quantitative and qualitative analysis capabilities of large batches of samples. Mirnaghi and Pawliszyn [40] used DART to identify and quantify BDZ diazepam from blood and completed the DART process in 30 s. LOD and LOQ of diazepam were 0.3 and 1.0 μg/mL, respectively.

Thermal desorption direct analysis in real-time (TD-DART) is developed to increase the sample reproducibility. In contrast to DART-MS, TD-DART-MS includes a thermal desorption device independent of the DART ionization source and a glass T-joint which allows duplicate sample introduction by wiping and desorption. The high sensitivity of nanogram levels is achieved. Jones et al. [173] used TD-DART-MS for the rapid detection of 19 BDZs with LODs of 0.10–1.00 ng for 14 compounds studied. SD of the average S/N ratio was in the range of 3.0–30.4. The excellent qualitative ability of TD-DART-MS in complex background matrix for BDZs was obtained. However, there was signal suppression due to competitive ionization, which can contribute to the screening of trace BDZs from bulk samples or wiped samples.

Dielectric barrier discharge ionization (DBDI) is an ambient ionization technique formed by non-equilibrium state gas discharge with an insulating medium inserted into the discharge space based on the plasma discharge mechanism. Sample molecules are introduced into the mass spectrometer after desorption and ionization by low-temperature plasma between two electrodes isolated by the insulating medium. Compared with DART, DBDI is structurally simpler with lower cost due to stable cryogenic plasma at atmospheric pressure without the need for vacuum devices as well as spray solvents required for DESI. DBDI provides sensitive detection of small molecules. Mirabelli et al. [129] applied a DBDI source connected to MS for determining diazepam in eight illicit drugs from beverages and biological fluids. There was an improvement in sensitivity owing to the loss-free transmission of ions with low LODs in the range of 3–300 pg/mL, which was superior to traditional ESI-MS.

Paper spray (PS) ionization is an emerging ambient ionization technique coupled with various types of MS detectors. It is used for rapid screening and accurate determination of target analytes. The typical device of PS-MS is composed of a paper substrate with a tip, conductive clamp, voltage feeding device, and spray-forming solvents. The high pressure loads the sample onto the paper matrix to ionize for producing a spray, which transfers charged analytes to the mass spectrometer. Compared with other techniques, PS-MS is a simple analytical method with high specificity and sensitivity based on direct ionization of MS without complicated sample pretreatment and chromatographic procedure. Borden et al. [174] utilized PS-MS to detect and determine 49 drugs, including six BDZs (diazepam, alprazolam, flunitrazepam, etizolam, oxazepam, and temazepam). LODs for six BDZs were 3.2–14.0 ng/mL, and lower limits of quantitation (LLOQs) were 9.7–44.0 ng/mL. This was a good quantitative ability of PS-MS for on-site drug detection. The simplified and fast operation without any time-consuming preprocessing allows reduction of the sample analysis cycle. Mass spectrometric results can be generated in 2 min. PS-MS is an environmentally benign method due to the consumption of small amounts of organic solvents in a dilute and jet manner. de Paula et al. [52] developed the PS-MS method for the analysis of five BDZs in four alcoholic beverages with LODs of 0.05 mg/L, consuming 10 μL of methanol in the process of PS-MS, which was lower than other analytical methods. A wide linear range was obtained between 0.05 and 10 mg/L with the determination coefficient (R^2) of 0.9991.

To improve the sensitivity, stability, and robustness of the MS system, the ionKey/MS is developed, which is applicable in a variety of fields. The ionkey/MS consists of a Xevo TQ-S mass spectrometer, ACQUITY UPLC M-Class, ionKey source, and iKey separation unit, which guarantee the system's separation capability with improved sensitivity. Jiang et al. [162] applied ionKey/MS for the quantification of 11 BDZs in hair samples with LODs and LOQs of 0.008–0.03 and 0.025–0.125 pg/mg, respectively. Compared with that of traditional LC-MS/MS, LODs of ionKey/MS were 2–10 times lower for all target analytes and were comparable to conventional methods in terms of accuracy, precision, matrix effect, linearity, etc.. Jiang et al. [163] used ionKey/MS for the analysis and detection of five trace BDZs from hair samples and obtained LODs and LOQs of 0.001–0.08 and 0.0125–0.25 pg/mg. The results showed 30–70 folds improvement in sensitivity compared to published articles. To improve the detection efficiency, reduce experimental errors

caused by human manipulation, and improve the method reproducibility, automated LC-MS have also been applied to the rapid analysis of target compounds. Peter et al. [155] used an automated LC-MS system for the identification and semi-quantitative detection of 58 BDZs, including BDZs from human serum with LODs of 5–10 ng/mL.

3.1.4. LC coupled with other detectors

Due to the high cost of MS instrumentation, HPLC tandem UV detection or diode array detection or photodiode array (PDA) is used for the detection and analysis of BDZs with acceptable sensitivity. The common wavelength set for detecting BDZs is 220–260 nm. Samanidou et al. [137] achieved the detection of diazepam, lorazepam, alprazolam, and bromazepam in serum with PDA detector monitoring at 240 nm and obtained LOD and LOQ of 0.01 and 0.03 ng/μL, respectively.

Light-emitting diodes (LED) without a monochromator has merits as a light source such as low price, low energy consumption and small emission bandwidth. Since most analytes are absorbed in deep UV (<300 nm), applying optical detection in the visible range using LEDs in the chromatographic analysis is limited. To solve this problem, LED based on a modified emitting device in deep UV range has been developed. LED can obtain low LODs due to its stable high output with low noise levels. da Silveira Petruci et al. [149] developed LC-deep-UV LED with an emission band at 235 nm for detecting three BDZs. The deep-UV LED detector performed excellently for a range of compounds. LODs of 0.8–1.3 mg/L were obtained for target BDZs.

An electrochemical detector (ED) coupled to HPLC is used for analyzing BDZs due to its high selectivity and sensitivity. The use of a working electrode with low background current and high electrode stability is conducive to getting better sensitivity and reproducibility. Martins et al. [152] developed the HPLC-ED method using boron-doped diamond as the working electrode for detecting three BDZs from tablet dosage with RSDs of 0.4%–4.9%. LODs between 0.5 μg/mL and 2.0 μg/mL were obtained. The electrochemical detector is time-saving without complex sample pretreatment compared to other analytical methods. Simple dissolution and filtration of tablet samples were needed before LC-ED detection [152], and the elution of three analytes was complete in 18.9 min. Dual electrochemical detection coupled to LC (LC-DED) provides the merit that an electrochemical product is more prone to redox reactions than the parent compound with less interference, which facilitates to lower background currents and higher sensitivity. Honeychurch et al. [153] developed LC-DED in a dual reductive mode for detecting flunitrazepam in coffee, and an LOD of 20 ng/mL was obtained, which was lower than the previous LC-ED method. A linear relationship was obtained in the range of 0.5–100 μg/mL ($R^2 = 0.9981$).

3.2. GC

3.2.1. Optimization of chromatographic conditions

GC is another strategy for separation and analysis, which uses gas as a carrier phase. GC is suitable for qualitative and quantitative analyses of volatile organic compounds with low boiling points and good thermal stability. Unlike LC, GC columns are fused-silica capillary columns that are divided into non-polar, weak polar, medium polar, and strong polar according to polarity. For BDZs with weak polarity, weak polar and non-polar columns such as HP-5 [68,124], HP-5MS [37,55,118,175], DB-5MS [72,129], Rxis-5Sil MS [117], etc. are commonly used. Kaki et al. [117] achieved good separation of two compounds, including midazolam, in human plasma using Rxi-5Sil MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) in GC. A low LOD and LLOQ of midazolam were obtained as 2.5 and 10 ng/mL, respectively. To improve the resolution and peak capacity of one-dimensional GC, two-dimensional GC (GC × GC) is developed, which connects two columns differing in their separation mechanisms and are independent of each other in a tandem manner, packed with a modulator in the middle. All the distillates after separation by the first column are released to the second column in periodic pulses for continued separation, after concentrated aggregation within the modulator, which improves the separation. Bravo et al. [94] developed a dual column (two DB-5MS capillary columns (50 m × 0.2 mm, 0.33 μm film thickness)) GC system for detecting four BDZs from blood and plasma. LODs were 0.14–0.95 ng/mL for whole blood and 0.13–0.93 ng/mL for plasma, respectively.

3.2.2. Development of GC-MS

GC can couple different detectors, including MS, flame ionization detector (FID), electron capture detector (ECD), etc. GC-MS has been used for the determination of BDZs from various matrices. Geng and Zou [57] employed GC-MS for the separation and detection of three BDZs from blood with LOD and LOQ of 0.006–0.010 and 0.018–0.030 mg/L, respectively. Sample components reach equilibrium between the mobile and stationary phases due to fast mass transfer, which reduces the time of the GC-MS process. de Bairros et al. [36] used GC-MS for the simultaneous detection of 11 BDZs from urine with a total time of 11.33 min. LOD values were 0.1–15 ng/mL.

To further enhance the qualitative ability of GC-MS with single quadruple, GC-MS/MS has been developed and optimized for the determination of BDZs in various matrices with higher sensitivity and specificity. Banaszkiewicz et al. [176] developed GC-QQQ MS for the identification and determination of 10 BDZs from human blood. An excellent sensitivity like LC-MS/MS without derivatization was obtained with LODs and LOQs of 0.02–0.53 and 1–2 ng/mL, respectively.

HRMS can also be coupled with GC to improve analytical efficiency and detection sensitivity. Arnhard et al. [50] applied GC-TOF MS for quantifying target BDZs and screening unknown drugs from urine. LODs and LOQs were obtained in the range of 0.4–15.3 and 10–50 ng/mL, respectively. GC-TOF MS used 9.5 min of run time for identifying BDZs from a self-assembled mass spectra library with sufficient (more than 10) data points obtained for one peak.

The ionization modes for GC-MS are electron impact ionization (EI) and chemical ionization (CI), which can be used for non-selective ionization and selective ionization of compounds, respectively. The negative ion chemical ionization (NICI) based MS is more selective and sensitive owing to increased S/N and less background interference compared with positive ion CI source or EI source [177], especially for the detection of negatively charged drugs like BDZs [178]. Karlonas et al. [35] used GC-NICI-MS for the simultaneous detection of 15 BDZs from whole blood with LOD and LOQ of 0.24–0.62 and 0.72–1.89 ng/mL, respectively.

3.2.3. GC coupled with other detectors

In addition to MS, GC is coupled to detectors such as FID [48,123], ECD [124], and nitrogen phosphorus detector (NPD) [94] to provide sensitive and rapid response. Ghobadi et al. [67] utilized GC-FID for determining three ultra-trace amounts of BDZs from water, juice, and urine, consuming a total of 11 min. LODs were 0.02–0.2 μg/L and lower than other methods, such as 0.8–2.8 μg/L for GC-MS [50]. To further improve the detectability of trace amounts of target analytes, the application of ECD and micro-ECD (μECD) has been extended. Compared with GC-ECD, GC-μECD provides higher sensitivity for halogenated chemical analysis [179]. Ghambarian et al. [68] developed GC-μECD for detecting four halogenated BDZs (diazepam, midazolam, alprazolam, and

flurazepam) from water, plasma, and urine with superior sensitivity and lower LOD of 0.005–1.0 μg/L than that of Q-MS or TOF MS. Furthermore, GC with dual detector has been developed and applied for better sensitivity than single detector or MS as an alternative method with lower cost than GC-MS. Bravo et al. [94] utilized the GC-μECD/NPD system for determining four BDZs from blood and plasma, and higher sensitivity than other reports were obtained with lower LOD of 0.04–0.95 ng/mL.

3.3. Sensing methods

Various types of sensors based on the principle of electrochemistry are being used through the development of electronic detections. Compared with other detection methods, such as LC-MS and GC-MS, electrochemical sensors provide sensitivity at a lower cost and do not require expensive instruments and complicated operations. Smith et al. [180] used a screen-printed graphite electrode sensor for detecting flunitrazepam from two beverage samples with an LOD of 0.47 μg/mL, and no sample preparation process was required. As a rapid and simple procedure, electrochemical sensors reduce time due to fast response and high selectivity. Hassan et al. [181] proposed point of care sensor modified with conducting polymer poly(3-octylthiophene) for rapidly determining diazepam in biological fluids with low LOD of 0.126 nmol/mL and in response time of 11 ± 2 s. Electrochemical sensors are stable with a long lifetime. The portable, disposable, and cost-effective sensor proposed by Hassan et al. [181] exhibited stability with a lifetime of three months.

Nanomaterials with unique properties have been used in electrochemical sensors. They act as the modifiers of electrodes due to small electrode size and larger surface area caused by nanostructures which include graphene [182], polyaniline/graphene oxide [31], multi-walled carbon nanotubes [183], poly dopamine-poly folic acid (P(DA-FA)) [184], silver nanofibers/ionic liquid nanocomposite [185], etc. They enhance sensitivity and specificity with strong electrochemical signals. Vahidifar et al. fabricated a pencil graphite electrode modified by multi-walled carbon nanoparticles as a working electrode for determining trace lorazepam from water, urine, and hair by differential pulse voltammetry (Fig. 2C) [103]. The results showed that, in contrast to un-modified electrodes, the disposable modified electrode with high stability and less background interference improved the electron transfer rate on the electrode surface and increased the signal response, which resulted in higher sensitivity with LODs and LOQs of 0.003 and 0.06 μM, respectively. Ashrafi et al. [184] used a glassy carbon electrode modified by P(DA-FA) nanocomposites as an electrochemical sensor for detecting five BDZs from human plasma. Modification amplified the signals with higher electronic transmission rates and a lower LOQ of 0.025 μM.

Electrode modification is essential for improving the selectivity of sensors, such as optimal ionophores of membrane sensors. Hassan et al. [181] compared the potential responses of three calixarene supramolecules when used as ionophores for sensing diazepam to identify optimal ionophore calix[4]arene with the highest affinity for diazepam that improved the sensor specificity. The proposed sensor provided an LOD of 1.2×10^{-7} mol/L. MIPs have also been applied as selective electrochemical sensors due to specific recognition properties. Panahi et al. [186] developed a sensor using MIP nanoparticles as recognition elements for detecting midazolam from urine and injection samples with LOD and LOQ of 1.77×10^{-10} and 5.89×10^{-10} M, respectively.

It is noted that multiple BDZs in a sample cause overlap of electrical signal peaks, limiting the application of voltammetry for the simultaneous determination of multiple drugs to some extent, which can be solved by an array of sensors based on the principle of the electronic tongue. The electronic tongue allows the multisensor system to provide multidimensional information about targets. Herrera-Chacón et al. [187] developed an integrated sensor array with six various metal nanoparticles modified graphite epoxy electrodes as working electrodes for the simultaneous detection of three BDZs without the presence of interfering or overlapping peaks. LOD levels of 4.6–6.0 μM were obtained.

In addition to traditional metal, glass or carbon electrodes, paper-based analytical devices have also been applied in electrochemical detection as carriers for immobilizing sensing elements. Narang et al. [188] developed a paper-based device modified by methylene blue and urchin-like Ag@Pd shell nano-hybrids for the electro-sensing of alprazolam from urine. The amplified electric signals by the modification resulted in a low LOD of 0.025 ng/L.

Optical sensors are based on determining the optical attributes of the target and converting the optical signal to an electronic signal in a detection system [189]. Different from electrochemical sensors, optical sensors have the advantages of non-contact, non-destructive, and hardly disturbed detection, which provide good detection performance for BDZs. Fluorescence monitoring has become a popular optical technique due to the low LODs attainable and the simplicity of the format [190]. Yen et al. [191] prepared hydrophobic carbon dots (hC-dots) probe using D-phenylalanine as a hydrothermal route for sensing and quantification of nimetazepam in beverages based on analyte-induced fluorescence quenching of hC-dots at 430 nm when excited at 365 nm, which provided low LOD of 2.14 μg/mL. The results indicated that detection by selective sensor probe was not disturbed by other common illicit drugs like alprazolam and ketamine. MIPs have been developed as recognition elements for sensing target analytes due to their specific recognition and selective binding functions. Machicote et al. [192] developed a fluorescence recognition system for flow light sensing by synthesized MIPs using hydrolysates of oxazepam as template molecules in a non-covalent blotting method for the indirect detection of oxazepam from drug preparations. LOD and LOQ of 11.2 and 34.2 μg/mL, respectively, were obtained.

Biosensors have been applied as an alternative in the pharmaceutical analysis due to their simplicity, low cost, and sensitivity in contrast to traditional analytical methods. Ashrafi et al. [193] developed a sensitive catalytic platform with amplified sensing signal based on the synergistic effect of biocompatible chitosan and silver nanoparticle-nitrogen doped graphene quantum dots nanoink with good conductivity for determining five BDZs in human plasma. LLOQ of 3.8–61.8 μM was obtained without sample pretreatment. Enzyme-linked immunosorbent biosensors based on antigen-antibody reaction have better specificity and sensitivity for target analytes, avoiding the influence of optical properties and dimness, which was suitable for on-site and continuous detection. Xu et al. [194] developed a thermometric biosensor based on heat production from an enzyme label measured by an enzyme thermistor for detecting diazepam in various beverages. The detection performance of the thermometric biosensor including LOD of 33.71 ng/mL was comparable to that of HPLC-based measurement.

3.4. Capillary electrophoresis (CE)

CE is a liquid-phase separation technique that uses capillary as separation channel and high-voltage direct current electric field as driving force, which provides good separation in a short time. Excellent resolution, sensitivity, and separation performance of analytes can be achieved by making improvements to the capillary column coating, and the modifier added to the buffer. Švidrnoch et al. [43] developed successive multiple ionic-polymer layer

coated capillaries to separate and detect nine BDZs from serum with LODs of 1.5–15.0 ng/mL. The nine BDZs achieved complete separation with a shorter separation time (about 6 min) than that of fused silica capillary (nearly 7 min).

To improve the sensitivity of CE, field-amplified sample stacking (FASS) is used for assistance with CE as an online preconcentration technique. FASS utilizes differences in ionic mobility of analytes in different conductivity regions with two buffers of differing conductivities to achieve analyte packing and signal amplification in capillaries. Oledzka et al. [62] applied the combination of FASS with CE to enhance sensitivity for eight BDZs from urine. LOD of 20–30 ng/mL was obtained, exhibiting 50 to 100-fold decrease in contrast to CE without FASS.

3.5. Other methods

In addition to the above determination methods, there are other analytical methods used to detect BDZs, including high-performance thin-layer chromatography [150], spectroscopic methods [195], electrochemical methods [79,196], immunometric methods [197], and others [198]. Some spectroscopic detection techniques for BDZs involve spectrophotometry [199], Raman [195], chemiluminescence (CL) [200], and others [175]. Surface-enhanced Raman spectroscopy (SERS) based on Raman scattering effects increases quantitative detection [201] of trace BDZs in short time [202]. Platforms such as portable mobile devices [203] and others [204] improve the affinity of SERS for BDZs in complex matrices and the reliability of rapid on-site detection. CL method [200], with its improved technique [205], is also applied for the sensitive detection of BDZs by simple equipment at a fast signal detection speed. In terms of electrochemical methods, voltammetry provides high sensitivity without expensive apparatus compared with MS [79], and more electrodes with good electrochemical performance and stability are being developed as alternative options to achieve superior performance [196]. Enzyme-linked immunosorbent assay [197] and other immunometric methods [24] have also been used for the rapid screening and detection of BDZs.

3.6. Summary of detection methods

A systematic review of a series of widely applied methods for BDZs detection is presented. Focus is placed on advances and advantages of chromatography-MS techniques. The application of chromatographic methods and their characteristics for the analytical detection of BDZs, such as HILIC, are presented. The emphasis is also placed on advances in MS, focusing on the applications, including prevalent methods such as IT MS, TOF MS, and Orbitrap MS, and new MS procedures in BDZs detection. The advantages and disadvantages of various MS techniques are shown in Table S6. The development and progress of ion sources are discussed to improve the sensitivity and resolution of mass spectrometers. Besides, the detection methods based on new types of sensors are covered as well as CE, spectroscopic methods, etc. The sensing methods and the modification and improvement of electrodes based on new materials are encompassed. It is found that LC-MS/MS holds a position in the quantitative detection of BDZs due to analytical performance. However, the availability of chromatography-MS methods is limited by the high price of instruments and their repairs. Therefore, inexpensive and sensitive detection procedures with simple operation steps need to be developed as alternative detection methods.

4. Conclusions and prospects

The widespread use of BDZs in criminal practices such as suicide, abduction and cases of drug-facilitated sexual assault has attracted attention owing to the risk of abuse and addiction. BDZs separations and determinations are thus studied. This is a comprehensive review of current methods for BDZs pretreatment and detection. Various pretreatments have achieved efficient extraction, enrichment, and concentration of BDZs with simple and rapid procedures. Among them, routine LLE and SPE procedures are the most used. Some progress based on new technologies (automation, miniaturization, etc.), new solvents (ILs, DES, SUPRASs, etc.), and new materials (MOFs, MIPs, GO, etc.) have improved the processes with higher extraction efficiency. LPME and SPME provide new selections for the pretreatment of BDZs using fewer organic solvents adapted to the requirements of miniaturization of modern analytical science and a more friendly environment. Preprocessing procedures such as QuEChERS and MSPD have rapid and simple analytical features with high purification and extraction efficiency. Reduction of solvent consumption, sampling volume, analysis time, and operating cost are future trends in terms of pretreatment methods.

For the detection, the methods reviewed allow simple, accurate, rapid, and reproducible determination of BDZs in various sample matrices. LC-MS/MS is the popular method for qualitative and quantitative detection of BDZs with high sensitivity and applicability. The use of column packing with small particle sizes of UHPLC improves the separation and increases analytical speed. The development and use of HRMS (TOF MS, Orbitrap MS, etc.) and various ion sources (DART, MALDI, SALDI, etc.) have increased the method sensitivity. HPLC coupled with conventional detectors for the detection of BDZs also has applications due to simpler operation and lower cost than MS. Although GC-MS often requires derivatization before detection, it has also been used to obtain highly sensitive, precise, accurate, and reliable analytical results. Besides, some new types of sensors and spectrometric methods also have a role in sensitive BDZs determination. Rapid, simple, and cheap analytical processes are always the pursuing targets for researchers. Automated and miniaturized in situ detection hold promise for analyzing BDZs.

CRediT author statement

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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