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Recent developments in the high-throughput separation of biologically active chiral compounds via high performance liquid chromatography

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ABSTRACT

Bioactive compounds, including active pharmaceutical ingredients (APIs), are often chiral molecules where stereoisomers have different biological and therapeutic activity. Nevertheless, the preparation of these molecules can lead to racemic or scalemic mixtures (it is not trivial to produce just the optically pure compound). The evaluation of the enantiomeric purity of bioactive compounds, and therefore quality, is indeed of fundamental importance for regulatory scopes. Chiral high performance liquid chromatography (HPLC) is the gold standard technique to separate and to purify enantiomers. This comes from the wide availability of commercial chiral stationary phases (CSPs) and operational modes, which makes the technique extremely versatile. In recent years, the most relevant trend in the field of chiral analytical HPLC has been the development of CSPs suitable for fast or even ultrafast separations, thus favoring the high throughput screening of biologically active chiral compounds. This process has somehow lagged behind compared to achiral HPLC, due to a series of practical and fundamental issues. The experience has shown how in chiral chromatography even very basic concepts, such as the supposed kinetic superiority of core-shell (pellicular) particles over fully porous ones to improve the chromatographic efficiency, cannot be taken for granted. In this review, the most relevant fundamental and practical features that must be taken into consideration to design successful high-throughput, fast enantioseparations will be discussed. Afterwards, the main classes of CSPs and the most relevant, recent (last five-year) high-throughput applications in the field of the separation of chiral bioactive compounds (for pharmaceutical, forensic, food, and omics applications) will be considered.

1. Introduction

Enantiomers, i.e. the two isomers of a chiral molecule differing for the spatial distribution of the substituent groups on the chiral center, possess identical chemico-physical properties, except for the interactions with other chiral species and with the plane polarized light.

Many bioactive compounds and active pharmaceutical ingredients (APIs), either natural or synthetic, have at least one chiral center [1–3].

As such, they may exhibit different pharmacokinetic, toxicological and pharmacological properties, being the biological activity highly stereospecific [4–6] (Fig. 1).

Therefore, the importance of characterizing and assessing the enantiomeric purity of these molecules, through the measure of the enantiomeric excess (defining the abundance of one enantiomer over the other one [7]), represents a key step during drug development/production [8]. Fast, efficient and selective methods are therefore necessary to analyze chiral bioactive molecules at all stages of their production up to their commercialization, which is subordinated to strict safety guidelines imposed by Regulatory Agencies. Chiral high performance liquid chromatography (HPLC) is the most important tool available to researchers for this purpose since solubility, retention and selectivity of enantiomers can be drastically varied by changing the experimental conditions (mobile and stationary phases, temperature). Nowadays, a very large number of chiral selectors have been developed and tested as CSPs. The most important classes of CSPs include polysaccharide-based (cellulose and amylose, but also cyclodextrin and cyclofructan), protein-based (cellobiohydrolase, CBH, etc.), macrocyclic antibiotics, and the so-called Pirkle-type CSPs (that are synthetic CSPs where chiral selectors are small chiral molecules bonded to silica particles) [3].

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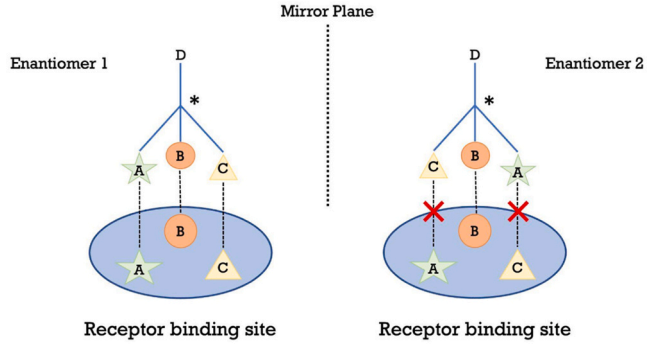

Fig. 1. Different bonding mechanisms between the active site of the biological receptor and the two enantiomers of a pharmaceutical compound. Biological receptors recognize the two species as if they were two very different compounds. The chiral center is indicated with an asterisk.

In the attempt to improve the performance of chromatographic separations, "highly efficient" particles have been designed. They are based on either sub-2 μm fully porous particles (FPPs) or superficially porous particles (SPPs) also referred to as core-shell or pellicular particles [9], usually in the size of 2.7 μm particle diameter or smaller. SPPs contain a solid inner silica core, inaccessible to analytes, which is covered with a porous layer where molecular adsorption and diffusion happen. In achiral chromatography, especially under reversed phase (RP) conditions, SPPs have been proven to permit the achievement of superior efficiency, over their fully porous counterparts (of comparable size), at significantly smaller operating back-pressure [10–14].

In the last decade, the trend towards the preparation of very efficient functionalized particles (either FPPs or SPPs), suitable for fast and ultrafast separations, has interested the field of chiral chromatography as well. This tendency was delayed, compared to achiral RP HPLC, due to a series of fundamental and practical reasons, including the difficulty to adapt traditional techniques of surface modification to small particles, the fact that small particles tend to aggregate during functionalization (with detrimental effects in terms of packed bed homogeneity and therefore column efficiency, see below), the mechanical resistance and long-term stability of particles functionalized with chiral selector at high flow rates/high pressure (needed when performing fast separations), the lack of fundamental studies and understanding of mass transfer and chiral recognition processes on CSPs and, last but not least, the difficulty to efficiently pack chiral particles (often polar) into small-diameter (2.1 mm internal diameter or less) chromatographic columns [15].

The rationale for the use of SPPs in chiral chromatography has been the same as in achiral RP chromatography: by diminishing the porous zone, the intraparticle molecular diffusion path length available to molecules becomes smaller, leading to an improvement in column efficiency. This is due to a decrease of all the terms responsible for band broadening, as explained in Section 2 [11,16]. However, the preparation and use of chiral SPPs has shown how, in chiral chromatography, things are more complicated than expected. For instance, the comparison between chiral FPPs and SPPs, functionalized under identical experimental conditions, has revealed that the specific bonding density of chiral selector (i.e., the amount of selector per square meter of silica) on SPPs is larger than on FPPs (for reasons that are still unknown) [17,18]. While it is acknowledged that the specific bonding density has a major impact on the outcome of a chiral separation by modifying the enantioselectivity of the phase, from a kinetic viewpoint its effect has been largely underestimated even in fundamental studies on CSPs. Analogously, the molecular surface diffusion (which has been proven to be one of the most important mechanisms of mass transfer through porous particles in RP chromatography) was found to be very limited, or even absent (localized adsorption), in chiral separations on CSPs with significant impact on the efficiency [19].

This review starts with a short overview of the fundamentals of chiral liquid chromatography to emphasize on the most critical aspects that must be considered for the achievement of high-throughput separations. After a discussion on the main categories of CSPs, a revision of the most relevant and recently published applications focusing on high-throughput experimentation, bidimensional and fast or even ultrafast separations of bioactive compounds (in food chemistry, forensic applications, omics, APIs) will be presented.

2. Fundamentals

The equation describing the resolution, R_s, between adjacent peaks in chromatography is the well-known Purnell's equation [20,21]:

$$R_s = \frac{\sqrt{N}}{4} \bullet \frac{\alpha - 1}{\alpha} \bullet \frac{k}{k+1} \quad (1)$$

where N is the number of theoretical plates, k the retention factor ($k = (t_R - t_0)/t_0$, being t_R and t_0 the retention and the hold-up time, respectively) and α the selectivity [22]. In the case of chiral chromatography, α represents the so-called enantioselectivity, that is the ability of a CSP to separate enantiomers (expressed as the ratio between the retention factor of the more retained enantiomer over that of the less retained one). The number of theoretical plates per meter (N/m), or the height of the theoretical plate, HETP (H), that is the column length divided by N, are the parameters commonly in use to quantify the efficiency of columns of different length. The higher N (or the smaller H), the more efficient the column. On the other hand, the reduced HETP, h, given as H/d_p (being d_p the particle diameter) is used to compare the performance of columns packed with particles of different geometries/size [23–27].

It is well known that, for a given column, the efficiency varies with the flow rate. The van Deemter (VD) equation describes this dependence. In adimensional coordinates, i.e. reduced HETP against reduced velocity, the VD equation is written:

$$h = a(\nu) + \frac{b}{\nu} + (c_s + c_{ads})\nu + h_{heat} \quad (2)$$

where ν is the reduced interstitial velocity, defined as [14]:

$$\nu = \frac{u_e d_p}{D_m} \quad (3)$$

being u_e the interstitial velocity and D_m the bulk molecular diffusion coefficient. The interstitial velocity represents the velocity of the eluent through the void space between packed particles. Since this is the only space, inside a chromatographic column, where the stream of mobile phase flows (there is no flow through the pores of packing particles), this is the right velocity to be considered in efficiency studies.

The minimum of the VD curve is employed to define the optimal velocity, ν_{opt}, to which corresponds the lowest plate height, $h_{\min}$, achievable with a given column under specific experimental conditions. Usually, a h equal to 2 is considered to represent the lower limit achievable with modern columns.

Eq. 1 shows that the plate height, and therefore the efficiency, depends on the different sources of non-equilibrium inside the column, namely: eddy diffusion (a term of VD equation), longitudinal diffusion (b), mass transfer resistance across the stationary phase (c_s), adsorption-desorption kinetics (c_{ads}) and, when it comes to fast separations on columns packed with very fine particles, frictional heating (h_{heat}) that originates from the energy dissipated by the stream of mobile phase flowing through the packed bed under the effect of the very high back-pressure applied to the system to generate large flow rates [24].

The different terms of the VD can be experimentally evaluated and interpreted by using proper models of adsorption/diffusion in porous media.

Eddy dispersion keeps into account the sources of band broadening

related to flow unevenness into the column. Even though, in first approximation and in many textbooks, this term is considered independent of the flow rate, it is well-known that it changes by changing the flow rate to reach a plateau at relatively large flows [24].

Molecular diffusion describes band broadening in absence of flow and, as such, it prevails at low flow rates, when natural diffusion of analytes through the packed bed is the dominant mechanism of band broadening. The *b*-term can be evaluated with stop-flow (also referred to as "peak parking") measurements [28]. Its expression is:

$$b = 2(1 + k_1)\frac{D_{eff}}{D_m} \tag{4}$$

where k_1 represents the zone retention factor, $k_1 = (t_R - t_e)/t_e$. The zone retention factor is conceptually identical to the more commonly employed retention factor, but it refers to the interstitial time, t_e, i.e. the time spent by molecules in the interstitial volume, instead of the hold-up time including also the time spent by molecules in the stagnant mobile phase inside pores. D_{eff} represents the effective diffusion coefficient, accounting for both diffusion in the bulk mobile phase (with molecular coefficient diffusion, D_m) and through the particles (with specific intraparticle diffusivity) [29]. Very often, under the experimental conditions employed in chiral chromatography, the diffusion of molecules on the stationary phase is very reduced or even absent (localized adsorption). Therefore, surface diffusion cannot act to release gradient concentrations generated by flow velocity variations in the direction of flow, as well as perpendicularly to it, contributing to increased eddy dispersion [30–32]. This effect becomes increasingly more relevant at higher retention.

The slope of the VD equation at high flow rates is controlled by the mass transfer between mobile and stationary phase, the latter in principle also including the contribution by the adsorption-desorption kinetics. This term is usually neglected in achiral chromatography since the adsorption-desorption kinetics is fast, unless very large molecules are considered. On the other hand, the possibility of a slow chiral recognition between chiral molecules and chiral selectors has been recognized in chiral chromatography, so that the adsorption-desorption term is usually considered among band broadening sources [15,19]. Even the most advanced models of chromatography do not allow to directly evaluate c_{ads}. On the other hand, the information on adsorption-desorption kinetics available from other, independent studies (e.g, NMR investigation) is difficult to adapt to chromatographic data, at the point that this is one of the most intriguing theoretical, open challenges.

The description of adsorption thermodynamics in chiral chromatography usually requires the use of heterogeneous adsorption models, i.e. models able to account for (at least) two different kinds of interactions, referred to as selective and nonselective [33,34]. Selective interactions are enantiomer-specific and are at the basis of enantioselectivity. On nonselective sites, on the opposite, both enantiomers behave identically. Nonselective interactions contribute to retention but not to enantioselectivity [17]. The simplest equation describing the accumulation, due to convection and diffusion, of an analyte between the mobile and stationary phase, in an infinitely narrow slice ∂z of a column and in absence of chemical reactions and by assuming a quasi-instantaneous equilibrium between mobile and stationary phase, is the differential Mass Balance Equation (MBE), reported below in the form of the so called equilibrium-dispersive model of chromatography [23]:

$$\frac{\partial C_i}{\partial t} + F\frac{\partial q_i}{\partial t} + u\frac{\partial C_i}{\partial z} = D_{a,i}\frac{\partial^2 C_i}{\partial z} \tag{5}$$

where C_i represents the concentration of the *i-th* species ($i = 1, 2$ for the first and the second enantiomer, respectively) in the mobile phase, u is the mobile phase linear velocity, F is the phase ratio (i.e. the ratio between the volume of stationary and mobile phases), $D_{a,i}$ is an apparent dispersion coefficient accounting for the effect of all band broadening sources and, finally, $q_i = f(C_1, C_2)$ is the competitive adsorption isotherm, where the concentration on the stationary phase of one enantiomer depends on the concentration in the mobile phase not only of itself, but also of the second enantiomer (in competition, indeed, with the first one for adsorption). The most employed competitive model for chiral separation is the so-called Bi-Langmuir isotherm. This isotherm can be considered as an extension of the traditional Langmuir isotherm [17], where it is assumed the presence on the stationary phase of two different adsorption sites characterized by two different binding constants b_i [21]. Thus, chiral sites (*sel*) can enantioselectively recognize the enantiomers whereas, on nonselective ones (*nsel*), enantiomers are identically retained:

$$q_i = \frac{q^s_{sel} b_{sel,i} c_i}{1 + b_{sel,1}c_1 + b_{sel,2}c_2} + \frac{q^s_{nsel} b_{nsel} c_i}{1 + b_{nsel}(c_1 + c_2)} \tag{6}$$

q^s_{sel} and q^s_{nsel} represent the saturation capacities for selective and nonselective sites (equal for both enantiomers on each site). The resolution of Eq. 5, once a proper model of adsorption has been defined (e.g., Eq. 6), is at the basis of the so-called inverse method of isotherm determination that represents one of the most useful techniques for gathering the adsorption binding constants. The inverse method relies on the comparison between the shapes of chromatographic peaks measured under nonlinear conditions and those simulated by the numerical resolution of the mass balance equation when an isotherm model (with an initial set of isotherm parameters) has been chosen. Minimization of the distance between experimental and simulated peaks, by optimizing isotherm parameters, allows to obtain the adsorption isotherm [35]. Through the adsorption isotherms, the binding constant between enantiomers and CSPs can be measured under the experimental conditions at which the experiment is performed.

3. Stationary phases: Chiral selectors and particles innovations

In this section, the most employed CSPs for the separation of bioactive molecules will be shortly reviewed. They include polysaccharide-, protein-, macrocyclic antibiotics-based CSPs and Pirkle type CSPs.

3.1. Polysaccharide-based CSPs

The most diffused and versatile class of CSPs is that of polysaccharide-based resins, including cellulose and amylose biopolymers. These are naturally chiral and easily available materials, which makes them appealing for a broad range of chromatographic applications. Cellulose is a linear biopolymer made of hundreds to thousands of units of D-(+)-glucose bonded by β-1,4-glycosidic bonds and forming a chain folded through hydrogen bonds [36]; it shows strong chiral recognition properties and high loading capacity [2,37]. In the most recent design, polysaccharide selector does not simply coat the silica matrix, but it is chemically bonded to it. This process makes the stationary phase more resistant to acidic or basic pH and to an extended range of organic solvents, allowing it to be used in normal-phase (NP), RP, HILIC etc, simply by changing the experimental conditions. Usually, NP is the mode of choice because chiral recognition is based on π–π, dipole–dipole, and hydrogen bonding interactions, and they are considered more efficient under NP conditions. Nevertheless, the organic solvents employed (such as hexane) are often not compatible with mass spectrometry (MS) detectors. Therefore, if the chromatographic analysis is to be coupled to MS, polar organic mode (POM) can be a valid alternative, since it employs acetonitrile and alcohols in mixture, favoring LC-MS compatibility [38].

Polysaccharide-based CSPs can also be derivatized to improve enantioselectivity. An established brand of immobilized polysaccharide CSPs is Chiralpak, a group of amylose- or cellulose-based resins by Chiral

Technologies Europe (Illkirch, France). Their peculiarity is that they are functionalized with phenyl carbamate groups bearing chloride or methyl substituents in different positions (Fig. 2). These modifications lead to different selectivities, making these columns complementary to each other. Moreover, thanks to the immobilization of the chiral selector, they are highly stable to solvents and backpressure, which ensures their stability over time.

Another relevant type of saccharide selectors is represented by the class of cyclodextrins. Cyclodextrins are cyclic oligosaccharides made of a number of glucose subunits up to 13, typically 6–8 for chromatographic applications. Depending on the number of glucoses bonded together, cyclodextrins are identified as α-, β-, γ-cyclodextrins for 6, 7 or 8 glucopyranose units, respectively. As an example, the performance of a hydroxypropyl-β-cyclodextrin (Hp-β-CD) dynamically coated stationary phase was recently investigated towards the enantioseparation of nonsteroidal anti-inflammatory drugs, including ketoprofen, naproxen and ibuprofen [40] in RP LC. For all these drugs, the S-enantiomer presents a pain-killer activity whereas the D-form is inactive. Thus, the enantiomeric excess of the enantiomers must be carefully evaluated. Both hydrophobic and steric interactions were found to be accountable for the separation mechanism. The same selector was used by Armstrong's group in HILIC conditions, bonded to SPPs and FPPs, to compare the performance of different particle geometries. In this particular case, the efficiency was found to be higher for SPPs, which also gave the best selectivities for small polar molecules. Also, chiral SPPs were employed for the fast separations (with fast meaning in less than 1 min [41]) of 5 beta-blockers [42].

Cyclofructans also belong to the class of polysaccharide CSPs [1,43]. Cyclofructans are macrocyclic oligosaccharides, consisting of six or more β-(2,1)-D-fructofuranose units, whose hydroxyl groups can be derivatized with different substituents to provide different selectivities.

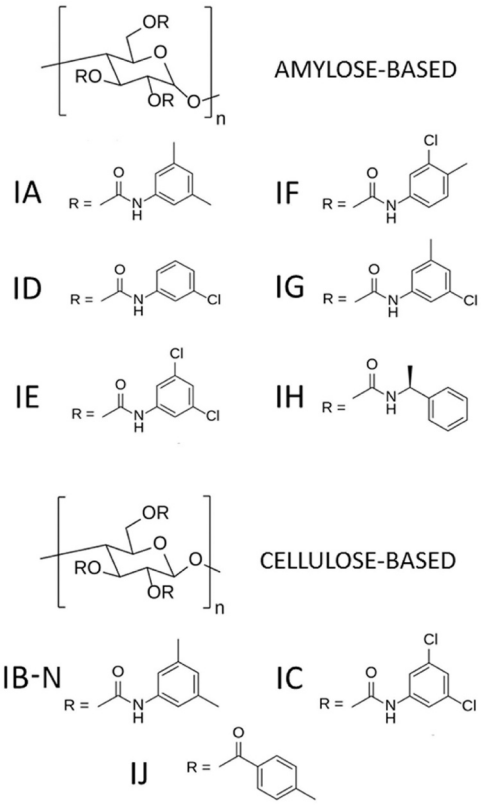

Fig. 2. Different Chiralpak polysaccharide-based selectors, derivatized with chloride- or methyl-substituted phenyl carbamate. Each derivatization or modification is represented by an acronym (IA, ID, etc.).
Reproduced with permission from De Luca et al. [39].

The preparation reported in Spudeit et al. [44], where cyclofructan was modified with an isopropyl group, represents the first chemically bound core-shell chiral stationary phase (see Fig. 3). This selector was tested both in NP and in polar organic mode to separate pairs of enantiomers of different small molecules, such as amlodipine, a calcium channel blocker drug used to treat high blood pressure, and fipronil, an insecticide acting on the insect central nervous system. The comparison between SPPs and FPPs has shown that the optimal flow rate for the column packed with SPPs was twice as large as that measured for the column prepared with FPPs (0.5 vs. 1 mL/min, respectively), which implies shorter analysis time.

Agathokleous and coworkers investigated the differences between a cellulose-, a cyclodextrin- and a cyclofructan-based selectors for the resolution of enantiomeric pairs of pharmaceuticals, including antihistamines, painkillers, thyroid hormones and anti-inflammatory drugs [36]. Among the columns tested in that work, cyclofructan selectors provided better enantioselectivities toward a greater range of analytes, while the cyclodextrin column gave the worst results in terms of enantioselectivity.

3.2. Protein-based CSPs

For this class of CSPs, the chiral selector of choice is a proteinaceous species immobilized on a silica support. An example is the enzyme Cellobiohydrolase (CBH), which is commercialized for example as Chiralpak CBH column, by Daicel Corporation. This stationary phase can be used to separate enantiomers of basic drugs containing basic nitrogen atoms and other chemical groups involved in hydrogen bonds, such as alcohols, carbonyl, esters, ethers, amides etc. Protein-bonded CSPs are used almost exclusively in RP-LC mode [38].

A Chiralpak CBH stationary phase was recently compared to a polysaccharide one modified with 3,5-dimethylphenyl carbamate (Lux Cellulose-1 from Phenomenex), for the separation of enantiomers of three β-blockers, one antacid and four cathinones (a group of new psychoactive drugs); the first column was used in RP-LC conditions while the second one in polar organic elution mode. Despite different conditions having been changed and investigated (organic modifier, pH, concentration of counterions etc.), the best results were obtained with the cellobiohydrolase selector [38]. According to the authors, the CBH CSP has been found to be especially useful to separate basic drugs. The enantiorecognition process with CBH columns is based on the onset of different interactions: firstly, on ionic interactions between carboxylic acid residues on the protein and basic analytes, and secondly on hydrogen bonding and hydrophobic interactions, as it was similarly theorized also by Scriba [45] and Millot [46]. On the other side, with the cellulose-based CSPs, chiral recognition is mainly due to the formation of hydrogen bonding, dipole-dipole interactions and π-π interactions, and sterically favored by chiral cavities within the helical cellulose chain [45,46].

3.3. Macrocyclic antibiotics-based CSPs

Another important class of chiral selectors is that of macrocyclic antibiotics, introduced by Armstrong and coworkers more than 30 years ago [47], when they described the use of vancomycin, thiostrepton and rifamycin B as chiral selector. Antibiotics can be glycopeptides, peptides, polyols, etc., containing amine, hydroxyl, and carboxylic functionalities, and can be acidic, basic or neutral. They usually present high enantioselectivity and they are suitable for both NP and RP applications, even though they appear stable for a longer time if run in NP conditions [37]. On these CSPs, the chiral recognition depends on different mechanisms, including π-π interactions, hydrogen bonding, and specific interactions in hydrophobic pockets [47].

Probably, the most employed macrocyclic antibiotics-based CSP is teicoplanin (see Fig. 4(a)), a tetracyclic glycopeptide widely studied for several different applications such as for the separation of amino acids

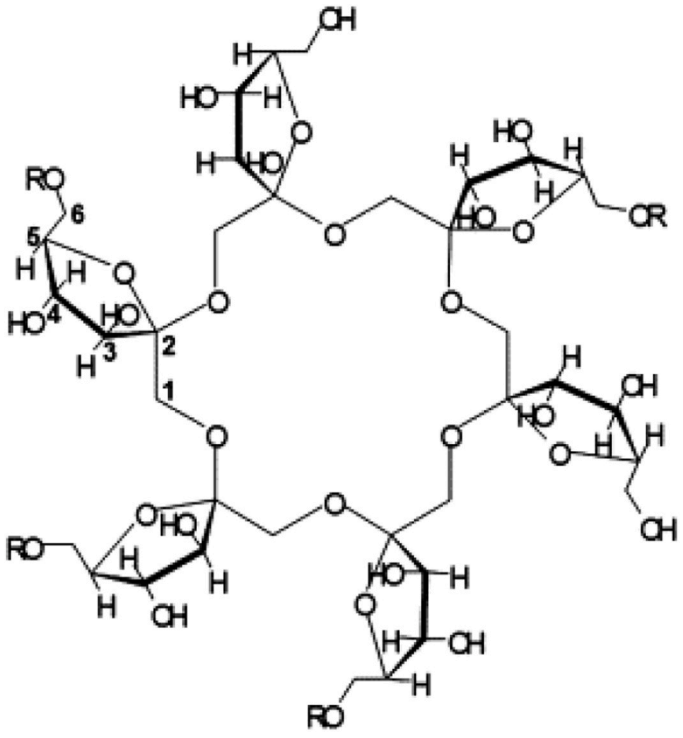

Fig. 3. Chemical structure of isopropyl cyclofructan 6.
Reproduced with permission from Spudeit et al. [44].

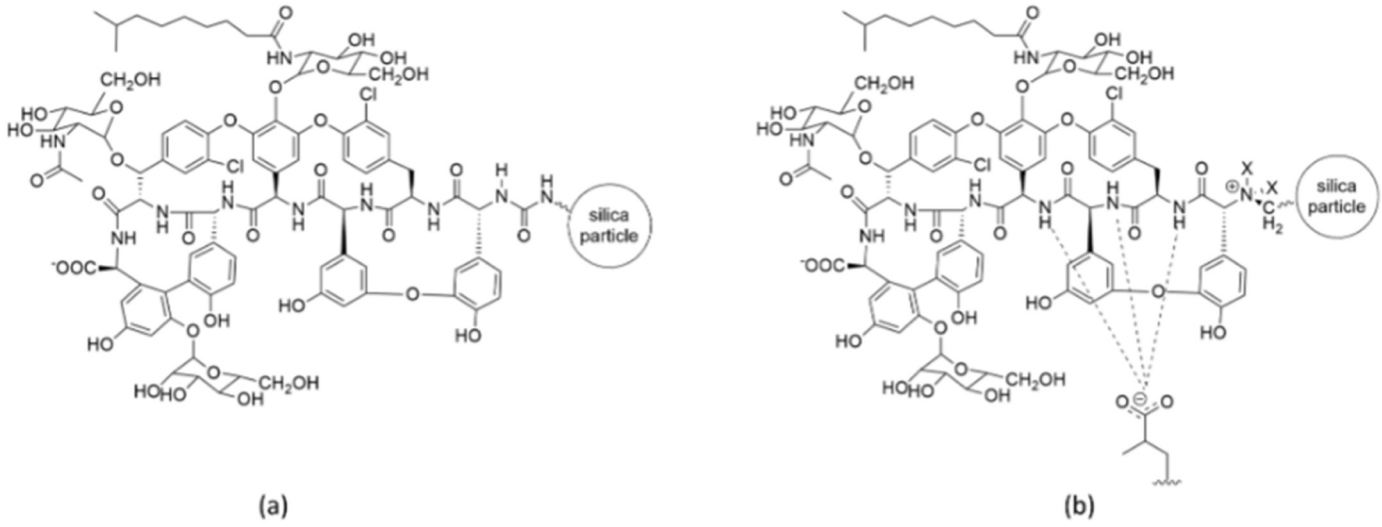

Fig. 4. Schematic representation of the chemical structures of commercial teicoplanin CSP (a) and the zwitterionic-teicoplanin version (b), for which also possible hydrogen bond interactions between carboxylate group and the CSP are reported.
Reproduced with permission from Felletti et al. [21].

(phenylalanine, tyrosine, tryptophan) contained in food supplements [1, 48] or the quantification of small APIs [49].

Interesting applications of vancomycin and rifamycin for the separation and quantification of chiral APIs are reported in [37,50,51].

Vancomycin, for instance, was tested for the separation of fluorinated APIs from their desfluorinated but structurally similar impurities [52]. Authors' aim was to compare vancomycin, teicoplanin, cyclofructan, and hydroxypropyl-β-cyclodextrin bonded on 2.7 μm SPPs with each other and with a commercially available Chirobiotic V column (Supelco) made of FPPs with 5 μm particle diameter. Improvement in the peak shape and resolution was observed with the vancomycin SPP column compared to the Chirobiotic V column, while selectivities were comparable.

Recently, a modified version of commercial teicoplanin CSP has been introduced [53], namely zwitterionic-teicoplanin. The phase was designed with the purpose of overcoming some limitations typical of the commercial teicoplanin CSP, namely the Donnan's exclusion of anions observed on this CSP at neutral or slightly acid pH. The repulsion is provoked by the presence of repulsive interactions between negatively charged molecules and the stationary phase, that at these pH values, also brings a negative charge due to the deprotonation of the carboxylic unit (see Fig. 4(a)). In the zwitterionic-teicoplanin CSP (see Fig. 4(b)), the

negative charge on the carboxylic unit is, on the opposite, neutralized by the positive charge on the quaternary nitrogen employed to link the chiral selector to the silica particle.

The zwitterionic CSP has been characterized in detail in terms of kinetics and thermodynamics by some of the authors of this review. In particular, it was shown that the behavior of the CSP dramatically changes by changing the organic modifier employed in organic-aqueous mobile phases [21]. By investigating both the adsorption behavior of molecules with different chemical properties and the preferential adsorption of the mobile phase components on the stationary phase (excess isotherms), it was explained why methanol allows to improve the enantioselectivity of the CSP, while acetonitrile permits to achieve extraordinarily high efficiency and fast separations. This means that, with this SP, it is possible to shift from high selectivity to high efficiency features and vice versa simply changing the organic solvent.

3.4. Pirkle-type stationary phases

The so-called Pirkle- or brush-type stationary phases include a wide class of synthetic CSPs where the chiral selector is a small chiral molecule. Some examples are phenylglycine, leucine, quinine and their derivatives [2]. The first example of these CSPs was given by Pirkle, Welch and Lamm in 1992 [54], who described a highly reproducible synthetic procedure for their preparation later successfully applied to particles of different geometries and size (even very fine particles). A very relevant example of Pirkle-type selector is the Whelk-O1 made of 1-(3,5-Dinitrobenzamido)- 1,2,3,4,-tetrahydrophenanthrene (Fig. 5), which can separate successfully a wide range of analytes families, including amides, carbamates, ketones, carboxylic acids, aromatic species etc, especially in NP conditions [55]. Ismail et al., for instance, compared, under NP conditions, the performance of a 2.6 μm SPPs column with two columns packed with 2.5 μm and 1.8 μm FPPs, all having Whelk-O1 as chiral selector. Despite the fully porous nature of the particles, the sub-2 μm ones were found to give better efficiencies than the 2.6 μm SPPs, whose efficiency was instead similar to the 2.5 μm FPPs. The authors ascribed the reason to a combination of a slower adsorption–desorption kinetics and to a larger eddy dispersion characterizing the SPPs, where the specific bonding density of chiral selector was larger [56]. A very interesting feature of synthetic CSPs is that they can be prepared in the two opposite configurations. For instance, in the case of the Whelk-O1 selector (that possesses two chiral centers, Fig. 5) the CSP can be prepared either in the S,S- or in R,R-configuration. Based on the reciprocal principle of selectand–selector-systems [57], columns of opposite configuration allow to invert the elution order of enantiomers. Clearly, the two columns must be equivalent to each other as dimensions, packing, etc. [58].

This approach, also referred to as "Inverted Chirality Columns Approach" (ICCA), has been proven to be particularly useful for characterizing complex mixtures where several enantiomeric compounds are present (even if in traces) by observing their exchange of position in the chromatograms obtained on the two columns of opposite configuration. Another interesting application of the ICCA is when only one enantiomer is available as reference. This technique was applied to some APIs such as camptothecins [58,59] and to natural products. Mazzoccanti and coworkers, for instance, have recently used ICCA to assess enantiomeric excess of cannabinoids contained in therapeutic hemp [60]. Identifying all the peaks was a particularly difficult task, since minor cannabinoids or racemates are not always available as reference materials. With the ICCA approach, they managed to quantify the minor enantiomer of (-)-Δ^9-THC, which was partially covered by the major tailed enantiomer with one column, while it was better resolved with the other column with opposite configuration.

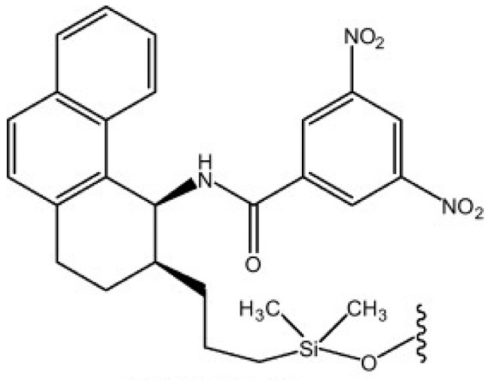

Fig. 5. Structure of Whelk-O1 chiral selector. Two chiral centers are present. Modified with permission from Asnin et al. [61].

4. Recent developments in high-throughput separations of chiral bioactive compounds

The information in the following paragraphs is based on the last six-year literature (2017–2023). In this period, several interesting works have been published showing the potential of novel CSPs prepared with both very fine, highly efficient particles but also traditional ones to achieve high-throughput, fast enantioseparations. The most important field is that of impurity profiling and characterization of chiral APIs, but there are also other emerging applications in which the fast determination of enantiomers is increasingly needed, as it will be discussed. For the sake of convenience, this section has been divided into specific paragraphs focused on the different types of applications.

4.1. Separation of chiral APIs and impurity characterization

The identification of impurities is one of the most important requirements for the quality and safety of drug products. Regulatory Agencies impose specific thresholds for the presence of impurities into a pharmaceutical product, from which the need to quantify and characterize them. The most challenging impurities to be removed are the product-related ones, i.e. species with similar chemo-physical properties to the target, which represent critical quality attributes (CQAs). In many cases, these species are structural isomers of the target API or, even, their enantiomer. The presence of these impurities can interfere with the pharmacological activity of a drug, as it was demonstrated, e.g., almost 60 years ago by the sad case of thalidomide [62].

The increasing number of samples to be analyzed in pharmaceutical industries requires ever faster separation approaches, to cope with the necessity of high-throughput enantiopurity assays [63,64]. Some researchers such as Armstrong, Welch, Regalado and their coworkers have considerably contributed to this field in the last years, by developing fast, high-throughput approaches for the separation of very common chiral APIs, such as ibuprofen, warfarin, omeprazole and some of their derivatives [65,66]. These experiments were conducted not only by employing high-efficiency macrocyclic antibiotic-based CSPs made on 2.7 μm SPPs and sub-2 μm FPPs but also with conventional coated and immobilized polysaccharide-based CSPs made on 2.5 and 3 μm FPPs, showing that enantioseparations in less than 1 min were possible in most cases.

Another important class of chiral molecules is that of amino acids. Many works have been published on the high-throughput separation of these compounds [67–70], which are increasingly studied for therapeutic purposes. This is due not only to their biological functions, but also because they are the building blocks to produce peptide-based drugs (a very promising class of biopharmaceuticals). Indeed, peptides are usually produced via solid-phase synthesis by employing N-α-FMOC (fluorenylmethoxycarbonyl) proteinogenic and non-proteinogenic amino acids. Some of the authors of this work have recently demonstrated that zwitterionic teicoplanin CSPs allowed to achieve high-throughput separations of these compounds, no matter their nature

(neutral nonpolar, neutral polar, acidic or basic) [67], also thanks to the absence of Donnan's exclusion.

Furthermore, the authors found that if this CSP is made on 2.7 μm SPPs with large pores (160 Å), extraordinary efficiency can be reached, almost comparable to that achievable with 2.0 μm SPPs with smaller pores (90 Å), for the separation of N-protected amino acids (including N-α-FMOC) and other APIs (such as Ketorolac), as it is shown in Fig. 6. In addition to very high efficiency, the wide pore CSP was characterized by very large enantioselectivity and small retention times, which make it suitable for high-throughput applications [68].

Other noteworthy works are focused on the development of methods for the fast screening of less common therapeutic agents, such as imeglimin, which is used to treat type-2 diabetes [71]. A sensitive chiral liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed to achieve the separation of imeglimin enantiomers in less than 5 min by employing a 100 × 4.6 mm column packed with 3 μm FPPs functionalized with a polysaccharide-based CSP and a flow rate of 0.5 mL/min. Also, methyldopa enantiomers have been separated in less than 5 min with a 100 × 4.6 mm teicoplanin aglycone CSP made on 2.7 μm SPPs operated at a flow rate of 1 mL/min [72]. The S-α-enantiomer of methyldopa is used as an antihypertensive agent mainly for the treatment of gestational hypertension.

Finally, 19 over 27 Cobalt bis(dicarbollide) (COSAN) metallacarboranes derivatives were separated on a semisynthetic hydroxypropyl β-cyclodextrin-based column (100 ×4.6 mm, 2.7 μm SPPs) in less than 10 min [73]. These compounds are used as pharmacophores in the preparation of biologically active hybrid organic—inorganic compounds or to improve the pharmacological properties of antisense oligonucleotides, nucleosides, and DNA intercalators. However, even if COSAN are considered an emerging class of compounds in medicinal chemistry, the impact of their chirality on biological activity is still partially unknown.

4.2. Metabolomics

Metabolomics is the scientific study of metabolites and of their chemical processes related to the cell metabolism. Analytical profiling techniques are needed to determine and measure large numbers of metabolites present in biological samples. These studies can be carried out according to two distinct methodologies: in untargeted metabolomics, a comprehensive analysis of all the measurable analytes in the sample (including chemical unknowns) is performed, while in targeted approaches only a defined group of metabolites is considered [74].

In general, however, metabolomics relies on the use of analytical techniques capable of gathering a high amount of information. For this reason, LC coupled with mass spectrometry (LC-MS) represents one of the most widely used platforms for this purpose. The data obtained are then interpreted through advanced statistical modeling.

When studying complex biological systems, a large number of samples is required to reach an acceptable statistical power, which are most often characterized by a very high degree of heterogeneity and variability. In addition, biological responses (and thus the number and type of metabolites) depend on many genetic and environmental factors. However, since metabolic profiling is increasingly required and applied in different fields (including medicine, drug development, nutrition, etc), there is also an increasing need to develop high-throughput and fast separation methods for the determination of analytes, including the emerging field of chiral metabolites (for which, very often, total analysis times can be in the order of 30 min or even more) [75].

In the last few years, many authors have worked on the development of faster LC-MS methods for the separation of chiral metabolites. For instance, two different approaches have been proposed to separate (S)-(+)- and (R)-(-)-Ibuprofen enantiomers in biological samples in a few minutes. Choi et al. developed a tandem LC-MS/MS method using a cellulose-based column (150 ×4.6 mm, 3 μm FPPs) through which the ibuprofen enantiomers were separated in 10 min at a flow rate of 0.4 mL/min [76]. Xiao and coworkers demonstrated that it is possible to separate these enantiomers in less than 5 min by operating a cellulose based column (150 ×2.0 mm, 3 μm FPPs) at a flow rate of 0.2 mL/ min [77]. Both methods were applied to investigate the pharmacokinetics of ibuprofen enantiomers in dog and in Chinese preterm neonates' plasma samples, respectively.

Other authors have reported on the high-throughput separation of chiral amino acids through LC-MS/MS. Crown ether CSPs are particularly suitable for these applications, indeed with these kinds of CSPs it was possible to separate 41 amino acids and their isomers without derivatization. The method has been then applied to identify D-amino acids in colostral, transitional, and mature preterm human milk [78]. In addition, by using either a crown ether-based CSP or a zwitterionic cinchona alkaloid one, Nakano and coworkers reported that it was possible to separate more than 100 non-proteinogenic amino acids and their metabolites in biological samples [75].

A very challenging application involving chirality of amino acids is that focused on the development of a chiral LC-MS/MS methods for the determination of epimeric peptides in β-amyloid (Aβ), which is considered to be involved in the evolution of Alzheimer's disease. Recent studies have demonstrated that there are substantial differences in Aβ between normal elderly people and patients affected by Alzheimer's diseases, who showed a higher amount of Aβ epimers due to racemized and isomerized residues of aspartic acid (Asp) and serine (Ser). Peptide epimers are diastereomers and, therefore, can be separated on achiral reversed phase columns; however, recent studies have reported that CSPs allow a better resolution of these compounds [79,80]. Indeed, all the 20 isobaric Aβ peptide epimers containing Asp, isoAsp, and Ser

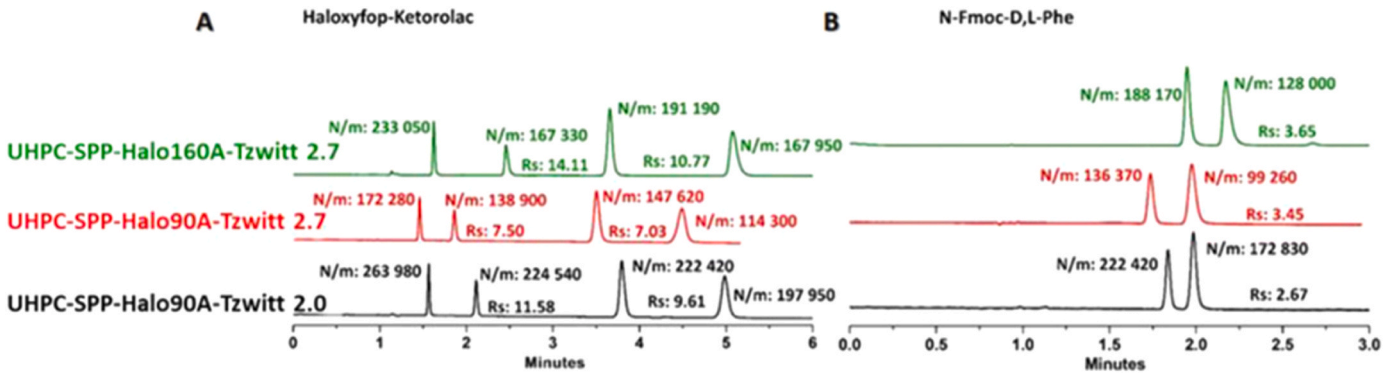

Fig. 6. Separation of A) Haloxyfop + Ketorolac and B) N-Fmoc-D,L-phenilalanine on three columns (100 ×4.6 mm) packed with 2.7 μm SPPs 160 Å (green), 2.7 μm SPPs 90 Å (red), 2.0 μm SPPs 90 Å (black). Mobile phase: acetonitrile/water 85:15%(v/v) + ammonium formate 15 mM, flow rate: 1 mL/min, temperature: 35 °C, UV detection at 214 nm.
Modified with permissions from Ismail et al. [68].

isomers have been baseline separated by employing a quinine-based CSPs [81], representing the first comprehensive approach to detect and quantify these important metabolites. Stereoisomeric forms (enantiomers and diastereomers) of some peptides have been separated through the employment of quinine-based CSPs also in a work by Ianni et al. [82]; the aim was to propose generic enantio- and diastereoselective screening conditions for peptide analysis in polar organic mode, with suitable ionic additives [82].

Finally, Lämmerhofer and coworkers have developed a LC-MS/MS approach for the enantioseparation of oxylipins, which are oxidation products generated by oxidative stress, enzymatic reactions or autoxidation of polyunsaturated fatty acids (PUFAs). Oxylipins have a very important role in living organisms, and they act as signaling molecules. Indeed, some of them are involved in the progression of thrombo-inflammation, while others play a key role in metabolic disorders, cardiovascular diseases, or cancer [83]. Their biological function is strictly dependent on their stereochemistry, since only one of their enantiomeric forms is active. However, in order to get unbiased conclusions into biochemical processes, it is necessary to perform enantioselective assays of these metabolites. The separation of 19 chiral oxylipins was achieved by using a sub-2 μm amylose-based CSP with analysis times lower than 5 min for each enantiomeric pairs at a flow rate of 0.3 mL/min [84].

4.3. Forensics and toxicology applications

Another field in which there is an urgent need for high-throughput enantioseparations is that of forensic and toxicology applications. Many drugs and novel psychoactive substances (NPS) are, indeed, chiral and, most of the times, only one of the two enantiomers has side effects, while the biological activity of the other could greatly differ [85,86]. State, local or federal laboratories are pushing towards the development of high-throughput analytical methods as the number of samples to be analyzed is constantly increasing. In addition, in many cases jurisdictional requirements are satisfied only if a large number of samples on the entire seizure is analyzed.

Recent developments have shown that it is possible to separate, in small groups simultaneously, almost 40 chiral NPS with different chemistry (belonging to the classes of pyrovalerones, benzofurans, phenydine and phenidates) on a native vancomycin column (50 ×2.1 mm, 2.7 μm SPPs) with total analysis times for each enantiomeric pair below 5 min at a flow rate of 0.5 mL/min [87]. Fig. 7 shows

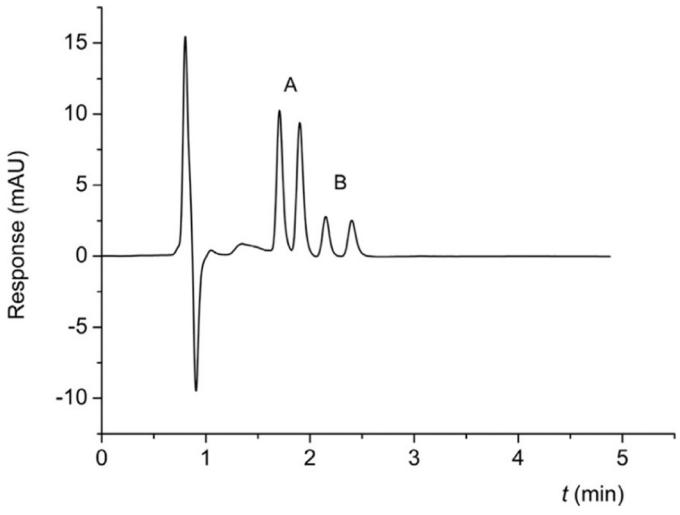

Fig. 7. Chromatogram showing the simultaneous separation of PV8 (A) and MPDV (B) enantiomers on the native vancomycin column. Mobile phase: methanol/acetic acid/triethylamine 100/0.1/0.05%(v/v/v), flow rate: 0.5 mL/min, ambient temperature, UV detection at 220 nm.
Reproduced with permissions from Folprechtová et al. [87].

the simultaneous separation of two different chiral NPS, namely 1-Phenyl-2-(pyrrolidin-1-yl)-heptan-1-one (PV8) and 3,4-Methylenedioxy--pyrovalerone (MPDV).

The same CSP was also employed, together with other three chiral adsorbents (namely, modified vancomycin glycopeptide, hydroxypropyl-cyclodextrin and isopropyl-cyclofructan) bonded to 2.7 μm SPPs, for a comprehensive screening aimed at separating 150 pharmacology and toxicology related chiral amines. Among these compounds it is worth mentioning amphetamine, cathinone, 3,4-methylenedioxymethamphetamine (MDMA), ketamine and methadone as well as some of their derivatives. It was found that the hydroxypropyl-cyclodextrin CSP outperformed the others for its capacity of resolving the largest number of stimulants. This column was also employed to achieve a baseline separation of 18 racemic controlled drugs in a single LC-MS run in less than 30 min [88].

In addition, Liu and coworkers developed a sensitive LC-MS/MS method for the separation of fourteen chiral β2-antagonists in animal derived foods by employing a vancomycin-based CSP [89]. Separation times were under the order of 10 min. β2-antagonists are illicitly added to animal feed to increase growth rate but it has been found that excessive concentrations of these compounds could cause serious consequences to human health, including tachycardia, headaches, nausea and even death. The authors reported that for most of the β2-antagonists the R-enantiomer was more abundant than the corresponding S- one in pork, beef, and lamb, except for cimaterol, for which the distribution was the opposite.

4.4. Food analysis

The number of samples to be analyzed in the food industry is constantly increasing, especially due to the necessity of strictly determining the quality and safety of food products before commercialization.

One of the most important applications is, indeed, that focused on the separation of pesticides. These compounds are commonly used in agriculture to keep crops healthy and prevent them from the development of diseases, therefore contributing to improving the harvesting productivity. However, the massive use of these compounds could be responsible for the presence of pesticide residues in food samples as well as in the environment, with a potential risk for human health. Many pesticides are chiral and the two enantiomers may be metabolized in different ways [90] or they could have distinct biological activity on the target. The European Food Safety Authority (EFSA) reports that if the toxicity between the enantiomers is higher than one order of magnitude and the concentration of the more toxic enantiomers is relevant, the risk assessment should be defined according to this species [91]. This is why the development of high-throughput, fast enantioseparations is necessary in this field. Recently, it has been shown that it is possible to simultaneously separate multiple triazole fungicides in fruits and vegetables (including apples, bananas, strawberries and tomatoes) by means of a β-cyclodextrin-bonded CSP [92]. The six pairs of chiral fungicides (such as hexaconazole, diniconazole and other compounds) have been successfully separated through the developed LC-MS/MS method, by allowing for the high-throughput analysis of a total of 90 fruit and vegetable samples in a short time.

Another class of chiral molecules for which the demand of chiral profiling in food is constantly increasing is that of amino acids. Indeed, these compounds (especially D-amino acids) play a beneficial key role in some diseases such as schizophrenia, renal dysfunction and amyotrophic lateral sclerosis (ALS) [93] and they are also added on purpose to dietary supplements [94]. Also, they have been recently found in different foodstuffs [95–97], and, in particular, in fermented food. They are thought to be derived from the starting materials or to be a consequence of microbial fermentation processes [98]. For this reason, many researchers are searching for high-throughput screening methods to separate these chiral compounds in food matrices. A recent work has

reported about the separation of all underivatized amino acids in 15 min on a crown ether chiral column (150 ×3.0 mm, 5 μm) at a flow rate of 0.4 mL/min. The developed LC-MS method was then applied to different food matrices (black vinegar, kimchi and yogurt) for the high-throughput determination of D-amino acids. D-amino acids contained in Japanese fermented products including vinegar, cheese and nam pla (a seasoning), have been analysed also through 2D-LC-MS/MS, by exploiting a reversed-phase column in first dimension (C18) and a chiral stationary phase in second dimension (made in-house). Good selectivities are required in both dimensions, especially since the reversed-phase separation was employed to get rid of interfering unknown compounds [99].

Often, the separation of amino acids results difficult since they are not easily retained and separated in RPLC because of their polarity, and therefore they need derivatization procedures. Multimodal stationary phases can overcome this limit: particularly, zwitterionic Chiralpak ZWIX(+) and Chiralpak ZWIX(-) represent excellent options due to their possibility to separate hydrophobic, polar, acidic and basic amino acids. With the aim of determining the amino acid content in food supplements, Raimbault and coworkers tested those columns with uncommon additives dissolved in the mobile phase, such as Methanesulfonic acid (MSA), in HPLC and in SFC. MSA proved able to resolve 12 free amino acid pairs out of 19 with good peak shape, including the isobaric Leucine and Isoleucine [100].

Achiral-chiral 2D-LC has been employed by Varfaj et al. for the analysis of branched-chain amino acids: providing enantioselectivity in the second dimension allows to remove the derivatization steps generally required during chromatographic separations of these compounds, contributing to make the process greener [101].

4.5. 2D-LC

Reaching a good resolution between analytes peaks contained in multicomponent mixtures through a single chromatographic step can be a challenging task. Orthogonal separations can be obtained through two-dimensional liquid chromatography (2D-LC), a powerful tool to increase resolution, especially between peaks of closely related compounds. A first reason is that in the two dimensions two different selectivities are employed, to allow the separation in the second dimension (^{2}D) of species not resolved by the first dimension (^{1}D). A second reason is that two different detectors can be employed for the two dimensions, such as DAD in the first dimension and MS in the second one to enable peak identification also of unknown species [102]. A requisite of bidimensional liquid chromatography is that the separation in ^{2}D must be very fast with respect to the one in ^{1}D. Chiral chromatography can be used in one of the two dimensions: particularly, since after the introduction of core-shell and small particles, chiral chromatography runs have become ultrafast, it results a suitable method for the second dimension, which is preferable in order to isolate and separate the enantiomeric pair from other species. This approach has been used for the analysis of different bioactive molecules, ranging from amino acids, drug metabolites in human body samples and pharmaceutical impurities [103–109].

Regalado and coworkers [102] have recently designed an automated online 2D-LC set-up capable of screening multiple columns in both dimensions, without the need of manual intervention, to simplify the research of the best experimental conditions and the method development. Accelerating and automating the screening steps is of pivotal importance especially for the pharmaceutical industry. In that work, the authors combined online heart-cutting achiral RPLC in ^{1}D with achiral or chiral RPLC in ^{2}D for the separation and analysis of an API and its impurities. The reversed-phase columns used were C18 of different brands, C8, CN, phenyl and PFP, whereas the chiral columns tested in ^{2}D were a vancomycin-based CSP as well as different cellulose-based adsorbents with different substituents. They demonstrated that, after the RPLC separation in ^{1}D, it was possible to obtain baseline separation of achiral components coeluting in achiral conditions (^{1}D) by using a polysaccharide column in ^{2}D (see Fig. 8). This result highlights the importance of screening also CSPs to improve the separation of closely related achiral compounds.

Lynen et al. [110] combined temperature-responsive (^{1}D) and reversed-phase chiral LC (^{2}D) to improve enantioselectivity in the separation of a number of pharmaceuticals. Using temperature-responsive polymers as SP in ^{1}D allows the use of purely aqueous mixtures as mobile phases. This facilitates the refocusing of solutes at the outlet of ^{1}D, a critical point in the design of 2D-LC procedures since often the elution strength of the solvent flowing from column-1 causes band broadening or even breakthrough of the recycled peak. Using purely aqueous mixtures in ^{1}D, instead, completely avoids these unwanted effects. This can be obtained with temperature-responsive polymers (N-propylacrylamide in this case), where the elution is promoted by a change in temperature instead of the organic modifier content. This setup was tested for the separation of samples of racemic pharmaceuticals with different polarity, such as secobarbital, hexobarbital, oxazepam and ibuprofen. All the samples analyzed were baseline separated with at least one of the CSPs tested (vancomycin, teicoplanin and cellulose-based CSPs).

Sometimes it happens that the target product has more than one chiral center, leading to an increased number of stereoisomers (2^n, where n is the number of stereocenters) and, consequently, to a more challenging method development for their separation. Baseline separation of so many species profoundly similar to each other can be reached with very long analysis times, even more than one hour, and after significant method development effort [111,112]. In achiral ^{1}D, unwanted achiral species are removed and diastereoisomers are separated and sent into the chiral ^{2}D. To meet the need for a versatile method able to resolve enantiomers and diastereomers of a given molecule, a 2-dimensional *multicolumn* LC approach (2D-mLC) has been developed recently [113]. In this setup, multiple CSPs are first screened in the second dimension to find the best conditions to separate species with multiple chiral centers, then for each ^{1}D fraction the best stationary phase is chosen. This means that, along the same run, a different CSP can be used to separate each peak deriving from the first dimension, allowing for an increased selectivity and orthogonality. The procedure has been

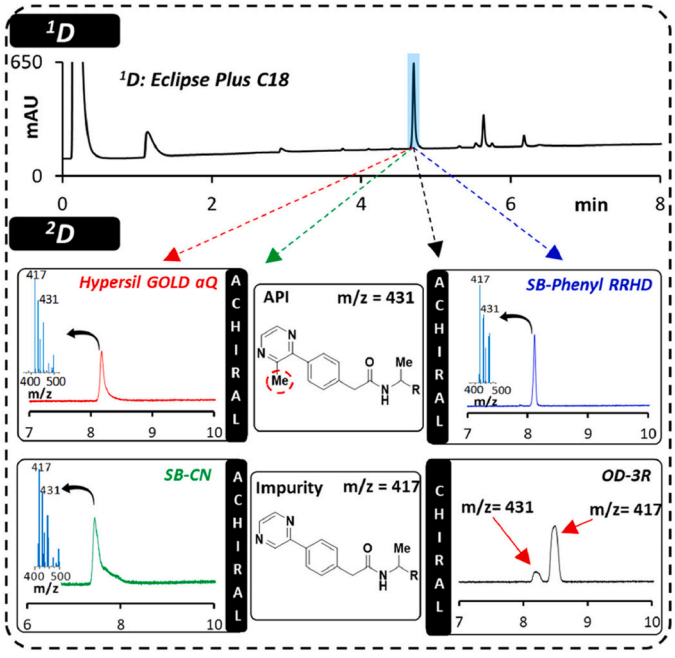

Fig. 8. Separation of an API from its closely related impurity by using achiral RPLC in 1D and chiral or achiral SP in 2D. Only the chiral column separates the two analytes at the baseline.
Reproduced with permission from Wang et al. [102].

successfully completed for three samples of pharmaceuticals with three chiral centers each using a C18 (^{1}D) and different polysaccharide columns (^{2}D).

In conclusion, 2D-LC has not yet completely established for routine analysis, especially at industrial level, but it is foreseeable that in the near future it will be more and more involved in everyday analysis thanks to its resolution power and to the possibility to quickly screen different operating conditions (e.g. stationary and mobile phases).

5. Future trends

High-throughput, fast enantioseparations can be now achieved for several classes of chiral compounds, with retention times that could be even comparable to those of sensors. This is possible thanks to the latest improvements in the preparation of CSPs made on high-efficiency particle formats. However, many recent studies have demonstrated that also with columns packed with traditional adsorbents made on 3 or 5 μm FPPs, if operated under optimized conditions, it is possible to achieve enantioseparations in the range of a few minutes. This is mainly due to two factors: the increased understanding of chiral separations from both a kinetic and a thermodynamic viewpoint and the extraordinary advancements not only in the functionalization of chiral adsorbents with different chemical groups and therefore different enantioselectivity, but also in the technology of packing (chiral) chromatographic columns. However, the need for high-throughput, fast enantioseparations will be increasingly urgent in the next future, especially in those fields where these assays are necessary from an industrial/commercial point of view (pharmaceutical and food industries, for instance) as well as for forensic analysis and omics approaches. In addition, the widespread use of 2D-LC will continuously require the development of fast enantioseparations in the second dimension in, e.g., achiral×chiral platforms.

Besides continuous research into the improvement of high-throughput LC approaches, it can be envisaged that also supercritical fluid chromatography (SFC) will be increasingly explored for this purpose. This is due to the fact that the employment of supercritical fluids as the mobile phase promotes faster mass transfer kinetics as compared to LC, making SFC a potentially relevant alternative for liquid chiral separations characterized by slow mass transfer [114]. A field where chiral SFC is expected to become a reference technique is the enantioseparation of lipids, which are highly soluble in supercritical fluid CO_2. Recent works have shown that SFC coupled to MS has a great potential towards high-throughput lipidomic analysis and that it could be the ideal analytical method for clinical laboratories where thousands of samples are routinely processed [115,116].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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