Review Article

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Chiral separation and analysis of antifungal drugs by chromatographic and electromigration techniques: Results achieved in 2010–2020

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Abstract: The determination and separation of enantiomers is an interesting and important topic of research in various fields, e.g., biochemistry, food science, pharmaceutical industry, environment, etc. Although these compounds possess identical physicochemical properties, a pair of enantiomers often has different pharmacological, toxicological, and metabolic activities. For this reason, chiral discrimination by using chromatographic and electromigration techniques has become an urgent need in the pharmaceutical field. This review intends to offer the "state of the art" about the separation of chiral antifungal drugs and several related precursors by both liquid and gas chromatography, as well as electromigration methods. This overview is organized into two sections. The first one describes general considerations on chiral antifungal drugs. The second part deals with the main analytical methods for the enantiomeric discrimination of these drugs, including a brief description of chiral selectors and stationary phases. Moreover, many recent applications attesting the great interest of analytical chemists in the field of enantiomeric separation are presented.

Keywords: antifungal drugs, chiral separation, electromigration methods, nano-LC, HPLC, SFC

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1 Introduction

Antifungal substances were discovered almost 80 years ago by D. W. Woolley. Since 1958, chlormidazole was the first specific drug that was commercialized in medical practice for topical application [1].

Fungal infections have emerged in the past 40 years and are becoming a major public health alarming disease. The increasing rate of invasive fungal infections [2,3] is due to the increased incidence of the immune-compromised patient population, including the rising number of acquired immunodeficiency for the advent of HIV, cancer patients with chemotherapy, organ transplant recipients receiving immunosuppressive therapy, on the one hand; the increasing number of invasive devices (catheters, artificial joints, and valves), on the other hand.

The *azoles* compounds are important synthetic medicinal products so named for the presence of an imidazole or triazole moiety in the molecule. They are well-known due to their quite extensive potent activities towards many pathogenic fungi. In addition, some of them exhibit additional antibacterial and antiparasitic properties.

The almost accepted action mode of azole compounds relies on the inhibition of Lanosterol 14α-demethylase (CYP51A1), a key enzyme in the ergosterol biosynthesis. The latter is a fundamental component of the plasma membrane structure and its depletion causes a weakening of the membrane. This is related both to the consequent change in the activity of many membrane-bound enzymes associated with nutrient transport and chitin synthesis and to the accumulation of toxic 14α-methylated sterols in membranes [4,5].

A great part of active substances, belonging to imidazole and triazole derivatives, shows optical isomerism, like more than half of drugs on the market [6].

The enantiomers of the same molecule have identical physical and chemical properties in an achiral environment (e.g., air–water exchange, abiotic absorption, and

transformation), but they enantioselectively interact with biological systems, e.g., enzymes, proteins, receptors, and other chiral molecules [7]. In this frame, the pharmacological activity may also be related to just one enantiomer, being the other one inactive or even toxic. Consequently, enantiomers may have different pharmacokinetic processes as well as therapeutic effects.

Although antifungal drugs, such as imidazole and triazole derivatives, are present as racemic mixtures in medicinal products, a lot of applications demonstrated different biological effects of each enantiomer. For instance, a bacteriostatic test indicated that *R*-(−)-fenticonazole shows the minimum inhibitory concentration (MIC) of the antifungal effects [8] and (+)-(*S*)-fenticonazole exhibited a faster absorption and elimination in female rats than did (−)-(*R*)-fenticonazole [9]. Although the genaconazole is administered as a racemate, only *RR* form is the active enantiomer while *SS*-form is the inactive one. The concentration trend for a time in human serum does not show appreciable differences [10]. *R* enantiomer of azole compounds, such as fluconazole, genaconazole, itraconazole, posaconazole, ravuconazole, voriconazole, and sertaconazole, is two times more active than the *S*-form, which is considered inactive [11]; (−) enantiomer of ketokonazole is more potent against some strains of fungi [12] since, as demonstrated after administration of rac-ketoconazole to rats, the concentration of (+)-ketoconazole in plasma was two-fold higher than that of (−)-ketoconazole [13].

In recent years, due to their excellent antifungal activity and a relatively low environmental persistence, triazole fungicides have also become an important category of fungicides with wide applicability in agriculture. In this respect, this molecules family, considered as pesticides, was introduced for the treatment and protection of plants, such as cereals, soybeans, fruits, and vegetables since the mid-1970s.

As above reported, it is known that most of the triazole fungicides/pesticides used as a pharmaceutical drug are chiral, like approximately 30% of anthropogenic agrochemicals. Moreover, their optical isomers exhibit different bioactivity, toxicity, and degradation in the environment. For instance, the *R*-enantiomer of diniconazole and uniconazole shows stronger fungicidal activities, whereas the *S*-enantiomer has higher plant growth regulating activity [14]; the degradation of the difenoconazole stereoisomers also showed stereoselectivity in vegetables and soil [15].

Besides, when introduced in an environmental medium, the enantiomers of pesticides show different effects on living organisms and they can act as biological markers of environmental toxicity: for example, (2*R*,4*S*)-propiconazole exhibited 2.43–23.47-fold higher fungicidal activity toward *Rhizoctonia solani, Fulvia fulva, Alternaria sonali*, and *Botrytis cinerea* than (2*S*,4*S*)-propiconazole [16]. A similar trend was observed for (2*R*,4*S*)-difenoconazole, whose fungicidal activity against *Botrytis cinerea* is about 24.2 times higher than that of its (2*S*,4*S*)-stereoisomer [15].

Although a different effect of drug enantiomers in pharmaceutical and agrochemical applications is demonstrated, the racemic form is still currently available on the markets of both medical products and plant treatments. Furthermore, it is estimated that about 50–75% of racemic pesticide emissions are unnecessary and cause environmental pollution [17]. To reduce the risks of side effects or unspecific toxicities derived from the administration of the no-active enantiomer, the new trend of the industrial sector is focused on the preparation of enantiomerically pure compounds: in this way, the same effect would be produced by half the dose of the racemic form. In this frame, the development of analytical methodologies for the chiral separation and determination of single-enantiomer forms and their impurities is highly required. In doing this, pharmacokinetics and pharmacodynamics studies (including absorption, distribution, metabolism, and excretion) are needed, for both pharmaceutical and environmental (agrochemical) applications. The determination of a racemic antifungals in biological fluids of humans or animals treated with one dose of drug can be useful to investigate the dose/effect relationship during a therapeutic drug monitoring (TDM): this information can guide the physician in optimizing the appropriate dose.

Through studies of the enantioselective behavior of chiral pesticides in agri-food products or in the environment, the absorption, transformation, and degradation processes in various plants, soil, water, and aquatic life are generally also evaluated. Thus, a better knowledge of individual degradation or toxicological data of each enantiomer could reduce the amount of pesticide avoiding unnecessary stereoisomer and be useful to evaluate the environmental risk and food safety.

This article reports an overview of both recent HPLC and CE (covering the literature since 2010) and an as exhaustive as possible review of GC, SFC, and miniaturized liquid chromatographic applications for chiral separations of antimycotic drugs. The effect of chromatographic/electrophoretic experimental conditions on chiral recognition, theoretical considerations, and new approaches in sample preparation will also be discussed.

2 Chiral separation of antifungal drugs: Optimization of chromatographic and electrophoretic methods, sample preparation, and some selected applications

This section describes several selected articles related to the chiral separation of antifungal drugs such as imidazole and triazole derivatives. Several analytical methods, combining sample preparation procedures with chromatography instrumentation, can face any analytical challenge. Therefore, chromatographic and electromigration analytical methods such as GC, HPLC, SFC, CE, CEC, and CLC, the most used in pharmaceutical, biological, and environmental analysis, have been here considered. Analysis of chiral antifungal compounds used as agricultural fungicides reported from 2011 will be discussed [18], while those related with pharmaceutical formulations and biological samples from 2010 and 2016, respectively, will be considered [1,19]. Applications are grouped based on the methodology used, also critically discussing the experimental condition used, the CSP, and the chiral resolution mechanism involved in the chiral recognition.

2.1 High-performance liquid chromatography

Since HPLC is a quite rapid and nondestructive analysis, able to work in a wide temperature range and with a choice of different detectors, nowadays it is the most widely used technique for chiral separations and achiral analysis of triazole antifungal drugs [18,20].

Although a chiral separation by liquid phase analytical techniques can be carried out by two different experimental approaches, indirect or direct separation methods, the last one is employed in most cases. This strategy involves the bonding or adsorption of a chiral selector (CS) to silica or monolithic support on forming a chiral stationary phase (CSP) that is packed in separation columns. Less frequently, a CS is added to the mobile phase.

Even if in the past a wide variety of CSs have been used to resolve many fungicides [18], recently, most of the work has been carried out utilizing polysaccharide-based CSPs exhibiting optimum recognition capability towards fungicide compounds. In a couple of cases, CSPs modified with a β-cyclodextrin derivative [21] or human serum albumin and α_1-acid glycoprotein column [22] were applied.

Although different types of polysaccharide-based CSPs have been studied, those containing cellulose or amylose derivatives have offered high enantioselectivity toward many compounds and therefore are the most used in this field.

Even if their enantio-recognition capability is determined by the higher-order structure of the natural polymer, when the cellulose- or amylose-based CSPs are modified by introducing some groups (e.g., methylbenzoate, phenylcarbamate, tribenzoates), a higher enantioresolution can be obtained. By the addition onto the polysaccharide skeleton phenyl carbamate moiety of an electron-donating methyl group and/or an electron-withdrawing chloro or fluoro groups, the chiral resolving capability can be extended toward a wide range of chiral molecules, demonstrating the almost universal applicability of this polysaccharide-based CSPs [23,24]. Table 1 shows the list of polysaccharide-based CSPs used for the enantioseparation of imidazole and triazole derivative compounds reported in Tables 2–4.

Table 1: Classification of CSPs polysaccharides-based and commercial trade name

Chiral selector	Abbreviation	Commercial trade name
Amylose tris(3,5-dimethylphenylcarbamate)	ADMPC	Chiralpak® AD, AD-R, AD-RH, IA
Amylose tris(3-chlorophenylcarbamate)		Chiralpack® ID
Amylose tris(3-chloro-4-methylphenylcarbamate)		Chiralpack® IF
Amylose tris(3-chloro-5-methylphenylcarbamate)	ACMPC	Chiralpack IG®
Amylose tris(5-chloro-2-methylphenylcarbamate)		Lux® Amylose-2, Sepapak® 3, LA2
Cellulose tris(3,5-dimethylphenylcarbamate)	CDMPC	Lux® Cellulose-1, Chiralcel® OD-R, OD-R, OD-RH, Chiralpack® IB
Cellulose tris(4-methylbenzoate)		Lux® Cellulose-3, Chiralcel® OJ, OJ-H, OJ-RH
Cellulose tris(3,5-dichlorophenylcarbamate)	CDCPC	Sepapak® 5, SP5, Chiralpack® IC
Cellulose tris(3-chloro-4-methylphenylcarbamate)		Lux® Cellulose-2, Sepapak® 5, LC2
Cellulose tris(4-chloro-3-methylphenylcarbamate)		Lux® Cellulose-4, Sepapak® 4, LC4

Table 2: Applications with high-performance liquid chromatography

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Column	Experimental conditions	Remarks	Ref.
Bifonazole, butoconazole, econazole, fenticonazole, flutrimazole, isoconazole, miconazole, sertaconazole, sulconazole, ornidazole, voriconazole	—	—	LC-UV (230 nm)	Per-4-chlorophenylcarbamate-β-CD	4.6 mm ID × 25.0 cm SP: CS immobilized on 5 µm silica particles	MP, *n*-Hex/EtOH or *n*-Hex/IPA or *n*-Hex/EtOH/TEA; injected volume, 20 µL; flow rate, 1.0 mL/min	Comparative study with the commercial 3,5-dimethylphenyl carbamate-β-CD CSP	[21]
Econazole, ketoconazole, miconazole, tebuconazole, propiconazole	Wastewater and sludge sample	1) Lyophilized and homogenized sludge samples 2) SPE (wastewater and sludge sample)	LC-ESI-MS/MS	HAS or AGP	2.0 mm ID × 10.0 cm (HSA) or 4.0 mm ID × 10.0 cm (AGP); SP: CS coated on 5 µm silica particles	(HSA column) MP, isocratic mode ACN/H_2O, containing 10 mM NH_4Ac pH 7; (AGP column) MP, gradient mode ACN/H_2O, containing 10 mM NH_4Ac pH 7; flow rate, 0.25 or 0.3 mL/min; *T*, 25°C	Recovery: 77–102% for wastewater and 71–95% for sludge; LOQ (individual enantiomers): 0.3–10 ng/L for wastewater, 3–29 ng/g for sludge	[22]
Butoconazole, enilconazole, isoconazole, sulconazole, ornidazole, econazole, ketoconazole flutrimazole	—	—	LC-UV (230 nm)	Amylose tris-(3,5-dimethylphenylcarbamate) or Cellulose tris-(3,5-dimethylphenylcarbamate) or Cellulose tris(3.5 dichlorophenylcarbamite) or Amylose tris(3-chlorophenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS immobilized on 5 µm silica particles	Isocratic mode MP, *n*-Hex/EtOH or *n*-Hex/IPA or *n*-Hex/*n*-buOH; injected volume, 20 µL; flow rate: 1.0 mL/min; *T*, 20°C	Mechanism of chiral recognition	[25]
Butoconazole, econazole, enilconazole, fenticonazole, flutrimazole, isoconazole,	—	—	LC-UV (230 nm)	Amylose tris(3,5-dimethylphenylcarbamate) or Cellulose tris(3,5-dimethylphenylcarbamate) or Cellulose tris(3,5-dichlorophenylcarbamate) or	4.6 mm ID × 25.0 cm; SP: CS immobilized on 5 µm silica particles	Isocratic mode MP, *n*-Hex/EtOH/DEA or *n*-Hex/IPA/DEA or *n*-Hex/*n*-buOH/DEA; injected		[26]

(*continued*)

Table 2: (*continued*)

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Column	Experimental conditions	Remarks	Ref.
itraconazole, ketoconazole, miconazole, ornidazole, sertaconazole, sulconazole				Amylose tris(3-chlorophenylcarbamate)		volume, 20 µL; flow rate, 1.0 mL/min; *T*, 20°C		
Diniconazole, epoxiconazole, fenbuconazole, hexaconazole, penconazole, prothioconazole, simeconazole, tebuconazole, tetraconazole, uniconazole	—	—	LC-MS/MS	Cellulose tris(3-chloro-4-methylphenylcarbamate) or Cellulose tris(3,5-dimethylphenylcarbamate) or Cellulose tris(4-methylbenzoate) or Amylose tris(5-chloro-2-methylphenylcarbamate)	2.0 mm ID × 15.0 cm; SP: CS coated on 3 µm silica particles	ACN or MeOH or ACN/H_2O or MeOH/H_2O in Hac; injected volume, 5 µL; flow rate, 0.2 mL/min	Dynamic range, 0.5–500 µg/L; LOD, 0–2–2.5 µg/L	[27]
Bifonazole, econazole, enilconazole, itraconazole, ketoconazole, metomidate, miconazole, ornidazole, sulconazole, terconazole	—	—	LC-UV (220 nm)	Amylose tris(3,5-dimethyl-phenylcarbamate) (ADMP,C) or Amylose tris(5-chloro-2-methylphenylcarbamate) or Cellulose tris(3,5-dimethylphenylcarbamate) or Cellulose tris(3-chloro-4-methylphenylcarbamate) or Cellulose tris-(4-methylbenzoate) or Cellulose tris(4-chloro-3-methylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS coated on 5 µm silica particles	Isocratic mode MP, MeOH, EtOH, ACN, with/without additive, FA, DEA		[28]
Butoconazole, econazole, fenticonazole, isoconazole, ketoconazole, miconazole, sertaconazole, sulconazole			LC-DAD (230 nm)	Cellulose tris(3,5-dichlorophenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS immobilized on 5 µm silica particles	Normal phase, *n*-Hex/EtOH/DEA. Polar organic phase, ACN or MeOH, and DEA. Reversed-phase mode, MeOH/H_2O or ACN/H_2O in NH_4HCO_3		[29]

(*continued*)

Table 2: (*continued*)

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Column	Experimental conditions	Remarks	Ref.
						Flow rate, 0.6–1.0 mL/min; *T*, 25°C		
Econazole, micomazole	—	—	LC-UV (220 nm)	Cellulose tris(3-chloro-4-methylphenylcarbamate) or Cellulose tris(4-chloro-3-methylphenylcarbamate) or Cellulose tris(3,5-dichlorophenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS coated on 5 µm aminopropylsilanized silica particles	ACN/DEA/HAc or ACN/DEA/FA or ACN/DEA/TFA; injected volume, 20.0 µL; flow rate, 1.0 mL/min; *T*, 25°C		[30]
Eniconazole	—	—	LC-UV	Cellulose tris(4-chloro-3-methylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS coated on 5 µm silica particles	MP, *n*-Hex/IPA/DEA; flow rate: 1 mL/min; *T*, 25°C		[31]
Miconazole	—	—	LC-UV (245 nm)	Amylose tris(3-chloro-5-methylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS immobilized on 5 µm silica particles	Normal phase, MP, *n*-Hex/EtOH or *n*-Hex/2-PrOH; flow rate: 1.0 mL/min		[32]
Miconazole, sulconazole	—	—	LC-UV (254 nm)	Amylose tris(3-chloro-5-methylphenylcarbamate)	3 mm ID × 5.0 cm; SP: CS immobilized on 1.6 µm silica particles	MP, ACN; injected volume, 1 µL; flow rate, 0.5 mL/min	*Rs*, 1.49 (Sulconazole); *Rs*, 2.00 (Miconazole)	[33]
Miconazole, tebuconazole	—	—	LC-UV (254 nm)	Amylose tris(3-chlorophenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS immobilized on 5 µm silica particles	MP, *n*-Hex/IPA; injected volume, 10 µL; flow rate, 1.0 mL/min; *T*, 20°C	*Rs*, 2.2 (Miconazole); *Rs*, 4.22 (Tebuconazole)	[34]
Econazole, miconazole	—	—	LC-UV	Cellulose tris(3,5 dichlorophenylcarbamite)	4.6 mm ID × 25.0 cm; SP: CS immobilized on 5 µm silica particles	MP, ACN/H_2O in NH_4HCO_3 pH 9; injected volume, 10 µL; flow rate, 0.7–1.0 mL/min; *T*, 25°C		[35]
Bifonazole, butoconazole, econazole, enilconazole,	—	—	LC-UV	Cellulose tris(4-methylbenzoate)	4.6 m ID × 25.0 cm; SP: CS coated on 10 µm silica particles	Isocratic or gradient mode MP, *n*-Hex/EtOH or *n*-Hex/IPA or		[36]

(*continued*)

Table 2: (*continued*)

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Column	Experimental conditions	Remarks	Ref.
fenticonazole, isoconazole, miconazole, sertaconazole, tioconazole						*n*-Hex/EtOH/ MeOH and/or DEA; injected volume, 10 µL; flow rate: 0.8 mL/min; *T*, 25°C		
Cis-itraconazole(4 Stereoisomers)	—	—	LC-UV (274 nm)	Cellulose tris-(3,5-dimehylphenylcarbamate) or Cellulose tris-(4-methylbenzoate)	4.6 mm ID × 25.0 cm; SP: CS coated on 5 µm silica particles	MP, *n*-Hex/EtOH or MeOH/ACN; injected volume: 20 µL; flow rate, 1.0 mL/min; *T*, 20°C	Stereoisomers, (i) Rs_1, 0.89; Rs_2, 0.81 (normal phase); (ii) Rs_1, 0.45; Rs_2, 1.94 (polar organic phase); (iii) Rs_1, 0; Rs_2, 1.53 (polar organic phase)	[37]
Posaconazole-related stereoisomers	—	—	Chiral–chiral 2D-LC-UV (262 nm)	1D: Cellulose tris (3,5-dimethylphenylcarbamate) and 2D, Cellulose tris(3,5-dichlorophenylcarbamate) or Amylose tris(3-chloro-4-methylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS immobilized on 5 or 3 µm silica particles	Normal phase MP, 1D, *n*-Hex/ EtOH + DEA; 2D, *n*-Hex/Iso-PrOH/ DMC or *n*-Hex/ DMC; flow rate, 0.5 or 1.0 mL/min		[38]
Diniconazole, epoxiconazole, fenbuconazole, hexaconazole, penconazole, tetraconazole	—	—	LC-DAD (220 nm)	Cellulose tris (3,5-dimethylphenylcarbamate)	4.6 mm ID × 25.0 cm (5 µm) or 2.0 mm ID × 15.0 cm (3 µm); SP: CS coated on silica particles	MP, ACN/H_2O or MeOH/H_2O; injected volume, 10 µL; flow rate, 0.3 or 1 mL/min; *T*, 20°C		[39]
Cyproconazole bromuconazole, etaconazole, famoxadone, fenbuconazole, metconazole, simeconazole	—	—	LC.DAD (220 nm)	Amylose tris(5-chloro-2-methylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS coated on 5 µm silica particles	MP, ACN/H_2O or MeOH/H_2O; injected volume, 10 µL; flow rate, 0.8 mL/min; *T*, 20–40°C		[40]
Voriconazole and its impurities (A–E)	Pharmaceutical formulation	Powder in sterile water	LC-UV (256 nm)	Cellulose tris(4-chloro-3-methylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS coated on 5 µm silica particles	MP, ACN/MeOH/ DEA/TFA; injected volume:	Analysis time, 8 min	[41]

(*continued*)

Table 2: (*continued*)

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Column	Experimental conditions	Remarks	Ref.
		and diluted in water and MP				40 µL; flow rate, 1.0 mL/min; *T*, 40°C		
Posaconazole	Drug formulation		LC-UV (262 nm)	Cellulose tris(3,5 dichlorophenylcarbamite)	4.6 mm ID × 25.0 cm; SP: CS immobilized on 5 µm silica particles	MP, IPA/CH_2Cl_2/DEA; injected volume, 10 µL; flow rate, 0.6 mL/min; *T*, 25°C	LOD, 0.06 µg/mL; LOQ, 0.2 µg/mL; dynamic range: 0.2–1.5 µg/mL	[42]
Itraconazole	Human blood plasma	(i) Borate buffer, MTBE were (ii) Mixed, centrifuged for five (iii) The organic layer evaporated by N_2. (iv) Reconstituted in EtOH ethanol	LCxLC-FD (λ_{ex}, 269 nm, λ_{em}, 374 nm)	(*R,S*)-Hydroxypropyl-modified β-CD or Cellulose tris(3,5-dimethylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS coated or immobilized on 5 µm silica particles	Gradient mode MP, Hex/IPA; injected volume, 75 µL; flow rate, 1.5 mL/min; *T*, 40°C	LOD, 1.3 ng/mL (each epimer); LOQ, 25 ng/mL (each epimer)	[43]
Miconazole	Rat plasma	(i) NaOH and extraction solvent, *n*-Hex/isoprOH (ii) Vortex, centrifuged (iii) Evaporated by N_2	LC-MS	Cellulose tris(3,5-dichlorophenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS immobilized on 5 µm silica particles	MP, NH_4HCO_3 in ACN/H_2O; injected volume: 10 µL; flow rate, 0.6 mL/min; *T*, 20°C	Pharmacokinetic study; LOQ: 0.50 ng/mL	[44]
Cis-enantiomers of ketoconazole	Human urine	PT-CFs-µ-SPE	LC-DAD (250 nm)	Amylose tris-(3,5-dimethylphenylcarbamate)	4.6 mm ID × 10.0 cm; SP: CS immobilized on 3 µm silica particles	MP, EtOH; injected volume, 20 µL; flow rate, 1.0 mL/min; *T*, 25°C	Recovery: 100.74–106.90%; dynamic range: 12.5–400 ng/mL (each enantiomer)	[45]
Cis-enantiomers of ketoconazole	Human urine	PT-MIP-µ-SPE	LC-DAD (250 nm)	Amylose tris-(3,5-dimethylphenylcarbamate)		MP, EtOH/H_2O; injected volume,	Recovery: 83.24–84.39%;	[46]

(*continued*)

Table 2: (*continued*)

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Column	Experimental conditions	Remarks	Ref.
					4.6 mm ID × 10.0 cm; SP: CS immobilized on 3 µm silica particles	20 µL; flow rate, 1.0 mL/min; *T*, 25°C	dynamic range: 6.25–650.00 ng/mL (each enantiomer)	
Butoconazole, econazole, fenticonazole, isoconazole, ketoconazole, miconazole, sertaconazole,	Environmental water	m-G-Fe_3O_4-SPE	LC-MS/MS	Cellulose tris(4-methylbenzoate)	4.6 mm ID × 15.0 cm; SP: CS coated on 5 µm silica particles	Gradient mode MP, NH_4Ac in ACN/H_2O; injected volume, 10 µL flow rate, 0.5 mL/min; *T*, 20°C	LOD: 0.11–0.32 ng/L; LOQ: 0.28–0.67 ng/L; recovery: 70–92%	[47]
Four stereoisomers of propiconazole	Soil grape	QuEChERS-SPE	LC-UV (220 nm)	Cellulose tris(3,5-dimethylphenylcarbamate)	4.6 mm ID × 55.0 cm; SP: CS coated on 5 µm silica particles	MP, Hex/EtOH; injected volume, 50 µL; flow rate, 0.6 mL/min; *T*, 25°C	LOD: <0.02 mg/kg; LOQ: <0.05 mg/kg; mean recovery: 89.6–99.8%	[48]
Triticonazole	Soil, cucumber, tomato, pear, cabbage apple	QuEChERS	LC-UV (260 nm)	Cellulose tris(3-chloro-4-methylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS coated on 3 µm silica particles	MP, ACN/H_2O; injected volume: 20 µL; flow rate, 0.7 mL/min	LOD, 0.0012–0.0031 mg/kg; LOQ, 0.0036–0.0091 mg/kg; recovery: 84.1–103.2%	[49]
Hexaconazole, tebuconazole, triticonazole,	Fruits and vegetables (including cucumbers, grapes, carrots, gourds, lettuce, pears, apples, potatoes, soybeans, and rape willows)	QuEChERS	LC-MS	cellulose tris(3,5-dichlorophenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS immobilized on 5 µm silica particles (via *thiol-ene* addition reaction)	Gradient mode MP, A: 0.01% FA-B: ACN; injected volume, 10 µL; flow rate, 0.4 mL/min; *T*, 20°C	Dynamic range, 0.1–100; LOD, 0.22–0.61 µg/kg; LOQ, 0.67–2.01 µg/kg	[50]
Chiral metabolites of fenbuconazole (RH-9129, RH-9130)	Soil Water (Irrigation Canal)	QuEChERS (soil) SPE (water)	LC-MS/MS	Cellulose tris(3,5-dimethylphenylcarbamate)	4.6 mm ID × 15.0 cm; SP: CS coated on 5 µm silica particles	MP, NH_4Ac in ACN/H_2O; injected volume, 10 µL; flow rate, 0.5 mL/min; *T*, 25°C	Dynamic range, 1.0–125 g/L; LOD: <0.8 g/kg; LOQ: about 2.5 g/kg	[51]

(*continued*)

Table 2: (*continued*)

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Column	Experimental conditions	Remarks	Ref.
Metconazole	Soil flour	QuEChERS	LC-UV (220 nm)	Cellulose tris(3,5-dimethylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS coated on 5 µm silica particles	MP, *n*-Hex/EtOH; injected volume, 10 µL; flow rate, 1.0 mL/min; *T*, 25°C	Soil, LOD, 0.24 mg/kg; LOQ, 0.97 mg/kg; Flour, LOD, 0.90 mg/kg; LOQ, 1.87 mg/kg; recovery: 94.98–104.89%	[52]
Diniconazole, epoxiconazole, fenbuconazole, hexaconazole, tetraconazole	Soil Water	QuEChERS SPE	LC-MS/MS	Cellulose tris(3,5-dimethylphenylcarbamate)	4.6 mm ID × 15.0 cm; SP: CS coated on 5 µm silica particles	MP, ACN/H_2O in NH_4Ac; injected volume, 10 µL; flow rate, 0.45 mL/min; *T*, 25°C	Recovery: 76.4–108.1% (soil), 81.2–106.5% (water); dynamic range, 1.0–125 µg/L; LOD, than 1.0 µg/kg or µg/L; LOQ, 3.0 µg/kg or µg/L	[53]
Fembuconazole	Strawberry	QuEChERS	LC-MS/MS	Cellulose tris(3,5-dimethylphenylcarbamate)	2.0 mm ID × 15.0 cm; SP: CS coated on 3 µm silica particles	MP, FA in ACN/H_2O; injected volume, 5 µL; flow rate, 0.2 mL/min; *T*, 25°C	Dynamic range, 1–500 µg/L; LOD, 0.6/kg; LOQ, 2 µg/kg	[54]
Cyproconazole (four stereoisomers)	Cucumber Soil	QuEChERS	LC-MS	Cellulose tris(3,5-dimethylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS coated on 5 µm silica particles	MP, ACN/H_2O; injected volume, 10.0 µL; flow rate, 0.5 mL/min; *T*, 20°C	LOD, 1.0–1.50 µg/kg; LOQ, 1.25–2.0 µg/kg; recovery, 86.79–92.47%	[55]
Difeconazole (four stereoisomers)	Cucumber and tomato soil	QUECHERS method	LC-UV (220 nm)	Cellulose tris(4-methylbenzoate)	4.6 mm ID × 25.0 cm; SP: CS immobilized on 5 µm silica particles	MP, *n*-Hex/EtOH; flow rate, 0.8 mL/min; *T*, 20°C		[15]
Chiral metabolites of fenbuconazole (RH-9129, RH-9130)	Soil	QuEChERS	LC-MS/MS	Cellulose tris((3,5-dimethylphenyl)carbamate)	4.6 mm ID × 15.0 cm; SP: CS coated on 5 µm silica particles	MP, NH_4Ac in ACN/H_2O; injected volume, 10 µL; flow rate, 0.5 mL/min; *T*, 25°C		[56]
Difenoconazole	Cucumber, tomato, and soil matrix	SLE, SPE, QuEChERS	LC-UV (220 nm)-	Cellulose tris(4-methylbenzoate)	4.6 mm ID × 25.0 cm; SP: CS coated on 5 µm silica particles	MP, *n*-Hex/EtOH; injected volume: 50 µL; flow rate,	Recovery, 81.65–94.52%; dynamic range,	[57]

(*continued*)

Table 2: (*continued*)

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Column	Experimental conditions	Remarks	Ref.
						0.8 mL/min; *T*, 20°C	0.5–50 µg/mL; LOQ, 0.04–0.1 µg/mL	
Hexaconazole, penconazole, tebuconazole	Ground and surface water	DLLME ACN (dispersive solvent); $CHCl_3$ (extraction solvent)	LC-DAD (220, 230, 426 nm)	Cellulose tris-(3,5-dichlorophenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS coated on 5 µm silica particles	MP, Petroleum ether/EtOH or IPA, *n*-Hex/EtOH or IPA; injected volume, 10 µL; flow rate, 1.0 mL/min; *T*, 20°C	Dynamic range, 30–1,500 µg/L; recovery, 88.7–103.7%; LOD, 8.5–29.0 µg/L	[58]
Cyproconazole	Algae *Chlorella pyrenoidosa*	LPE, (acetic ether) and UAE	LC-UV (222 nm)-MS/MS	Cellulose tris(3,5-dimethylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS coated on 5 µm silica particles	MP, ACN/H_2O; injected volume, 10.0 µL; flow rate, 0.5 mL/min; *T*, 25°C	Recovery, 75–120%; LOD, 0.1 µg/L; LOQ, 0.3 µg/L	[59]
Cyproconazole	*T*adpole samples (*Rana nigromaculata*)	LPE, (acetic ether and *n*-Hex) and UAE	LC-MS/MS	Cellulose tris(3,5-dimethylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP: CS coated on 5 µm silica particles	MP, ACN/H_2O; flow rate, 0.5 mL/min; *T*, 25°C	Dynamic range, 0.01–10 mg/L; LOD, 0.1 µg/L	[60]

Table 2 reports selected applications performed by LC related to the chiral separation of triazole antifungal drugs published during the selected period (2010–2020).

In this period, the attention was paid mainly on polysaccharide-based CSPs where the CS was coated or immobilized on silica particles. As can be observed, CSs such as cellulose tris(3,5-dimethylphenylcarbamate) [25–28,37–39,43,48, 51–55,57,58,60], cellulose tris(3,5-dichlorophenylcarbamate) [25,26,29,30,35,38,42,44,49], cellulose tris(4-chloro-3-methylphenylcarbamate) [28,30,31,41], cellulose tris(3-chloro-4-methylphenylcarbamate) [27,28,30,49], cellulose tris-(4-methylbenzoate) [27,28,36,37,47,56,58], amylose tris-(3,5-dimethylphenylcarbamate) [25,26,28,45,46], amylose tris (3-chlorophenylcarbamate) [24,32], amylose tris(5-chloro-2-methylphenylcarbamate) [27,28,40], amylose tris(3-chloro-5-methylphenylcarbamate) [32], and amylose tris(3-chloro-4-methylphenylcarbamate) [33,38] have been applied to the enantiomeric separation of antifungal compounds in environmental, agri-food, and biological applications, but above all to analytical standards. In this regard, many authors have focused their effort on the field of a basic research approach. Their experimental works highlighted the versatility of these stationary phases and how their structure and especially the substituents on the CS can influence the enantioresolution in relationship to the LC-conditions.

A study dedicated to understanding the chiral recognition mechanism of these chiral compounds was addressed by Zhu et al. [25] also using the molecular docking software. Here, four immobilized polysaccharide-based CSPs were compared studying the effect of the CS type on the enantioselectivity of azole analytes in NP conditions. Theoretical information about the binding energies and the conformations of CSP-solute complexes were obtained with this approach. From this and HPLC experiments, the differences in binding energies between the complexes formed by (*R*)- and (*S*)-isomers were determined by multiple intermolecular interactions.

From these studies, it was supposed that the chiral recognition mechanism was based on the simultaneous presence of Cl-atoms, O/S-atoms, imidazole groups, and phenyl groups in their azole structure. These groups contributed to hydrogen bonding, π–π, and hydrophobic interactions with the polysaccharide backbone (the hexatomic ring and its O-atom) and/or side chain (phenyl group, NH, C═O, and Cl). Specifically, the simulation results showed that: (i) phenyl groups of carbamate side chains on CSP could have π–π interactions with the phenyl group of analytes; (ii) the imidazole group of the azole analyte was seldom the main chiral recognition site, but it was often found to indicate the polymer cavity; (iii) the hydrogen bond between the O/S-atoms was interacting with the chiral center and the NH group of the carbamate moiety. Besides, the HPLC results revealed that chlorinated phenylcarbamate of cellulose offered better separation for most of the target antifungals. The theoretical results, reported in ref. [23], highlighted additional aspects about the chiral discrimination: (i) the hydrophobic interactions with the hexatomic ring or the Cl-atom in the polymer and Cl-atom present in the analyte; (ii) the interactions between the electronegative atom (O, S, or F-atom) of the enantiomer and the electronegative atom (O and Cl-atom) in the CS.

This trend was confirmed by other researchers who compared four CSPs-polysaccharide derivatives (silica-immobilized [26] or silica-coated [27,28,30]) by using different chromatographic experimental conditions.

Under normal phase (NP) mode, the best chiral resolution, for most azole compounds, was obtained with mobile phases containing *n*-hexane/isopropanol mixtures. However, other alcohols (ethanol or butan-2-ol) and an ammine additive (i.e., DEA) were also investigated. Under these conditions, chlorinated cellulose phenyl carbamate provided, in general, better enantiomeric separation compared to cellulose tris(3,5-dimethylphenylcarbamate) and higher recognition capability than the other polysaccharide-based CSPs [26]. On the contrary, a less marked difference was obtained with two CSP-amylose-based, chlorinated, and non-chlorinated derivatives, which showed similar enantioseparation ability in terms of the number of separated analytes.

Reversed-phase chiral chromatography was also studied for the separation of various triazole fungicide enantiomers. Using mixtures of 0.1% (v/v) aqueous formic acid solution in methanol or acetonitrile at different proportions as mobile phase, 21 fungicides were resolved in their enantiomers [27]. As observed in NP-mode, also in RP-mode, polysaccharide-based CSP showed a larger recognition capability than the amylose-based CSP, especially when electron-withdrawing groups were introduced on the phenyl carbamate moieties.

This work also emphasized the suitable chromatographic conditions for MS coupling by using the ESI interface. Therefore, the proposed method may be useful for identification and determination at low concentrations of this class of compounds in biological, food, and environmental samples.

Chiral versatility is flanked by robustness at slightly harsh conditions. In this respect, antimycotic drugs were also studied on polysaccharide-based chiral columns with polar organic mobile phases with some additives such as FA [28,30], DEA [28–30], TFA [30], and buffered solutions [29].

Chiral recognition ability in polar organic solvent chromatography was faced in a systematic comparative

Table 3: Supercritical fluid chromatography applications

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Column	Experimental conditions	Remarks	Ref.
Ketocokazole, itraconazole	—	—	SFC-UV (254 nm)	Amylose(6-(*R*-phenylethylcarbamate)-2,3-(3,5-dimethylphenyl carbamate))	4.6 mm ID × 25.0 cm; SP, CS coated on silica particles	MP, CO_2/MeOH/EtOH; flow rate, 2.5 mL/min; *T*, 30°C		[66]
Cyproconazole, diniconazole, hexaconazole, propiconazole, tebuconazole, tetraconazole	—	—	SFC-DAD (220 nm)	Amylose(3,5-dimethylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP, CS coated on 10 µm silica particles	MP, CO_2/EtOH or MeOH or IPA + 0.1% (v/v) TEA or TFA; injected volume, 20 µL; flow rate, 2–4 mL/min; *T*, 35°C		[67]
Econazole, miconazole, itraconazole, sulconazole	—	—	SFC-DAD (225, 254 nm)	Amylose(3,5-dimethylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP, CS coated on 10 µm silica particles	MP, CO_2/MeOH, EtOH, IPA, ACN; injected volume, 20 µL; flow rate, 2 mL/min; *T*, 35°C		[68]
Bifonazole, econazole, flusilazole, ketoconazole, hexaconazole, miconazole, sulconazole, tetraconazole	—	—	SFC-DAD	Amylose tris(3,5-dimethylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP, CS coated on 10 µm silica particles	MP, CO_2/EtOH or MeOH or IPA or ACN; injected volume, 20 µL; flow rate, 2 mL/min; *T*, 35°C		[69]
Bifonazole	—	—	SFC-DAD (225 nm)	Amylose(3,5-dimethylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP, CS coated on 10 µm silica particles	MP, CO_2/MeOH, EtOH, IPA, ACN; injected volume, 20 µL; flow rate, 2 mL/min; *T*, 35°C		[70]
Ketoconazole	—	—	SFC-DAD	Amylose(3,5-dimethylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP, CS coated on 10 µm silica particles	MP, CO_2/EtOH, 0.1% (v/v) TFA, 0.1% (v/v) TEA; injected volume, 5 µL; flow rate, 3 mL/min; *T*, 35°C	Analysis time: < 7 min; *Rs*: 4.29	[71]
Ketoconazole	—	—	SFC-DAD	Amylose(3,5-dimethylphenylcarbamate) or cellulose(3,5-dimethylphenylcarbamate)	4.6 mm ID × 25.0 cm; SP, CS coated on 10 µm silica particles	MP, CO_2/EtOH, 0.1% (v/v) TFA, 0.1% (v/v) TEA or CO_2/IPA; injected volume, 5 µL; flow rate, 3 mL/min; *T*, 35°C		[72]
Triticolazole	—	—	SFC-DAD (254 nm)	Cellulose tris(3,5-dimethylphenylcarbamate)	4.6 mm ID × 15.0 cm; SP, CS	MP, CO_2/EtOH or MeOH or IPA or n-prOH or 1-buOH; injected volume,		[73]

(*continued*)

Table 3: (*continued*)

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Column	Experimental conditions	Remarks	Ref.
					coated on 5 µm silica particles	10 µL; flow rate, 2.5 mL/ min; *T*, 35°C		
Econazole, ketoconazole, miconazole, sulconazole	—	—	SFC-Q-Tof2-UV (220 nm)	Amylose(3,5-dimethylphenylcarbamate) or cellulose(3,5-dimethylphenylcarbamate) or cellulose tris[4 methylbenzoate]	4.6 mm ID × 25.0 cm; SP, CS coated on 5 µm silica particles	MP, CO_2/EtOH + 0.1% (v/v) IPA; injected volume, 10 µL; flow rate, 2.5 mL/min; *T*, 35°C		[74]
Chiral metabolites of fenbuconazole (RH-9129, RH-9130)	Tomato, cucumber, apple, peach, rice, wheat, soil	QuEChERS	SFC-MS/MS	Amylose tris-(3,5-dimethylphenylcarbamate)	3.0 mm ID × 150.0 mm; SP, CS coated on 2.5 µm silica particles	Gradient elution MP, CO_2/EtOH; injected volume, 1 µL; flow rate, 0.4 mL/min; post-column, MS-solvent: 0.1% (v/v), HFo/MeOH	Dynamic range: 2.5–250 µg/L; $R^2 \geq$ 0.9963; Recovery: 76.3–104.6%; LOQ: 0.13–3.31 µg/kg	[75]
Pyrisoxazole stereoisomers	Cucumber, tomato, soil	QuEChERS	SFC-MS/MS	Amylose tris(3,5-dimethylphenylcarbamate)	4.6 mm ID × 15.0 cm; SP, CS immobilized on 3 µm silica particles	MP, CO_2/MeOH; injected volume, 2 µL; flow rate, 2 mL/min; *T*, 35°C	LOD: 0.03–0.15 µg/ kg; LOQ: 0.09–0.48 µg/kg	[76]

study on cellulose-based CSPs containing three chlorine [30].

This experimental work was focused on CSPs with cellulose tris(3-chloro-4-methylphenylcarbamate), cellulose tris(4-chloro-3-methylphenylcarbamate), and cellulose tris(3,5-dichlorophenylcarbamate) as the CSs silica-coated, studying the effect of different acidic additives, namely TFA, AcA, or FA, in ACN-based mobile phases on the chiral resolution.

In this work [30], the authors remarked on the importance of electron-donating and electron-withdrawing groups introduced in the phenyl carbamate moieties in the chiral recognition. Among the studied CSPs, the one containing cellulose tris(3,5-dichlorophenylcarbamate) offered higher enationresolution and higher retention factor when econazole and miconazole were analyzed. On the contrary of TFA, baseline enantiomers separation was obtained when the MP contained an appropriate concentration of FA and AcA.

These CSP types were also studied by other authors [27] eluting in RP mode, while amylose tris(3-chloro-5-methylphenylcarbamate)-immobilized silica particles were applied in NP condition for the chiral separation of some antifungal drugs [32]. Recently, normal, polar organic, and reversed-phase were compared for the chiral resolution of miconazole, econazole, isoconazole, sulconazole, butoconazole, fenticonazole, sertaconazole, and ketoconazole with a CSP-silica-based containing immobilized cellulose tris(3,5-dichlorophenylcarbamate) [29].

Although it is not a typical strategy in chiral separations, a gradient elution mode was used for chiral separation of different imidazole derivatives employing a CSP-cellulose tris(4-methylbenzoate) (silica-coated) and NP conditions [36]. In isocratic mode, the use of mixtures *n*-hexane/alcohol (2-propanol, EtOH, MeOH) allowed a satisfactory enantioseparation of the target compounds. However, for long retention times (>60 min), asymmetric and broaden peaks were observed. The application of four gradient elution programs, combining *n*-hexane/EtOH and EtOH/MeOH or *n*-hexane and EtOH, reduced below 37 min the retention time of eight substances and below 65 min the retention time of fenticonazole enantiomers.

An interesting chromatographic approach for the separation of twelve posaconazole-related stereoisomers in a single run was proposed by Xu et al. [38]. The chiral–chiral two-dimensional liquid chromatography (2D-LC) system was developed based on multiple heart-cutting 2D-HPLC systems. A six-port and a plug-in ten-port two-position switching valves were joined in a suitable configuration where the CSP-cellulose tris(3,5-dimethylphenylcarbamate) was used as a 1D separation column, while cellulose tris(3,5-dichlorophenylcarbamate) and amylose tris(3-chloro-4-methylphenylcarbamate) were used in parallel for the 2D. Once the mobile phase compositions for each dimension and cutting time were adjusted, the system resulted to be a powerful tool for the separation of stereoisomers of chiral drugs containing multiple asymmetric centers.

The reversal enantiomer elution order (EEO) could be a useful solution to problems related to the separation and quantification of a certain enantiomer which occurs at trace levels in a mixture containing high concentrations of its antipode. This is particularly necessary for practical applications especially in pharmaceutical and/or clinical analysis. This topic has been widely studied by Chankevetadze's group employing polysaccharide silica-based CSP for other compounds [60–64]. EEO was observed by employing different CS types [62,64], modifying mobile phase [62,64], changing the column temperature [61–64], etc.

This approach was also investigated in analyzing antimycotic enantiomers [28]. In this respect, a reversal of EEO of terconazole on cellulose tris(3,5-dimethylphenylcarbamate) and amylose tris(3,5-dimethylphenylcarbamate) by using MeOH as a mobile phase was observed. The reason was ascribed to the different polysaccharide backbone, organized as polymer D-glucopyranose unit linked through α 1,4-linkages in the case of amylose, while β-1,4-linkages in the case of cellulose. Reversal of EEO of terconazole was observed comparing cellulose tris (3,5-dimethylphenylcarbamate) and cellulose tris(4-methylbenzoate) in the same experimental conditions.

Identification and quantitation of impurities in a pharmaceutical formulation is a current topic in the pharmaceutical industry. Considering that many drugs are administered as a single enantiomer, the presence of the enantiomeric impurity in the preparation could affect the drug efficacy and safety.

It is known that the eutomer is pharmacologically more active than its antipode (distomer).

In this respect, voriconazole enantiomers were baseline separated in less than 10 min under RP conditions using a CSP-cellulose tris(4-chloro-3-methylphenylcarbamate) [41].

The method was successfully applied to evaluate the chiral stability of the commercial ophthalmic solution containing the single enantiomer of voriconazole and to determine both the other undesired voriconazole enantiomer and some impurities. A similar approach was applied for the purity control of posaconazole utilizing NP elution and a different CSP column (cellulose tris(3,5-dichlorophenylcarbamate)); the method was validated obtaining LOD and LOQ of enantiomer values 0.06 and

Table 4: Applications in nano/capillary liquid chromatography

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Column	Experimental conditions	Remarks	Ref.
Diniconazole, terbuconazole	—	—	Nano-LC-UV (219 nm)	β-CD	Monolithic column, 150 µm ID × 25.0 cm; SP, β-CD methacrylate-*co*-EGDMA	MP, 0.1% (v/v) TFA in MeOH/H_2O; injected volume 0.2 µL; flow rate, 0.3 µL/min	*Rs*, 2.5(terbucomazole), 1.26(diniconazole)	[78]
Miconazole	—	—	Nano-LC-UV (240 nm)	β-CD	Silica monolithic column, 100 µm ID × 15.0 cm; SP, TMOS-MTMS	MP, 0.1% (v/v) TFA in MeOH/H_2O; injected volume, 0.2 µL; flow rate, 0.3 µL/min	*Rs*, 1.26	[79]
Sulconazole, miconazole	—	—	Nano-LC-UV (219 nm)	CALB	Monolithic column, 150 µm ID × 25.0 cm; SP, GMA-*co*-EGDMA	MP, 0.1% (v/v) TFA in MeOH/H_2O; injected volume, 0.2 µL; flow rate, 0.3 µL/min	*Rs*, 1.22–1.52	[80]
Hexaconazole, miconazole	—	—	Capillary-LC-UV (219 nm)	Colistine sulfate	Monolithic column, 150 µm ID × 25.0 cm; SP, GMA-*co*-EGDMA	MP, MeOH/H_2O; flow rate, 1 µL/min	*Rs*, 1.2–1.6	[81]
Hexacolazole, micolazole, sulconazole	—	—	Nano-LC-UV (19 nm)	Amylose phenylcarbamate derivative	Monolithic column, 150 µm ID × 25.0 cm; SP, GMA-*co*-EGDMA	MP, MeOH/H_2O or *n*-Hex/IPA with or without TFA; injected volume, 0.2 µL; flow rate, 1 µL/min	*Rs*, lower than 1.7	[82]
Miconazole, sulconazole	—	—	Nano-LC-UV (219, 240 nm)	SWCNTs	Monolithic column,150 µm ID × 20.0 cm; SP, BuMA-*co*-EMDA-*co*-SPMA or GMA-*co*-EDMA	MP, 0.1% (v/v) TFA in MeOH/H_2O; injected volume, 0.5 µL; flow rate, 0.3 µL/min	*Rs*, 1.47–1.57	[83]

0.2 µg/mL, respectively. The method was applied to the analysis of a pharmaceutical formulation; the impurity of posaconazole enantiomer (distomer) was determined after spiking the sample at 0.1% concentration level.

A chiral analytical method can be also applied to pharmacodynamics and pharmacokinetics studies of two enantiomeric forms. The chromatographic separation system consisting of a two-chiral column (CSP-(*R*,*S*)-hydroxypropyl-modified β-CD and CSP-cellulose tris(3,5-dimethylphenylcarbamate)) in series was applied to develop a highly specific analytical method for chiral separation, determination, and quantification of itraconazole in human plasma samples [43]. After 6 h of oral administration of a single 200-mg dose of itraconazole, the analysis of plasma showed a 3.4-fold difference between the concentrations of the epimer mixtures. The results, reported in Figure 1, confirm how the pharmacodynamics for two enantiomers could be different, therefore the chiral analysis can be extremely useful in TDM studies.

Similar behavior was observed in a human urine sample from a volunteer after oral administration of rac-KTZ *cis*-enantiomers exhibiting different profiles of cumulative urinary excretion for each enantiomer [45]. In this application, after optimization of chiral separation on the CSP-amylose tris-(3,5-dimethylphenylcarbamate) column in less than 10 min, the authors focused their study on the development of an SPE device. The extraction of the racemic mixture of ketoconazole from human urine was achieved by using pipette tip-based cigarette filters (obtained from a local market) for micro-solid-phase extraction (PT-CFs-m-SPE) [45] and imprinted polymer micro-solid-phase extraction (PT-MIP-m-SPE) [46]. Once the various parameters, such as washing solvent, eluent type and volume, pH, material quantity, sample volume, and ionic strength, were systematically optimized, the extraction efficiency was about 100% of the recovery.

Another interesting application field is the determination of drugs in wastewaters. In fact, since the excreted pharmaceuticals and/or their metabolites can be found in these types of waters, their analysis is of paramount importance.

An LC-MS/MS method for the determination of seven chiral imidazole antifungal drugs in river water, as well as in influent and effluent wastewaters collected at a wastewater treatment plant, was proposed [47]. An easy, high speed, and low-cost magnetic solid-phase extraction (MSPE) was used. Graphene-modified with Fe_3O_4 nanoparticles (G-Fe_3O_4) offered a recovery in the range of 70–92% and LODs and LOQs values in the range of 0.11–0.32 and 0.28–0.67 ng/L, respectively.

Alongside pharmacological and veterinary applications, fungicides are applied in agriculture against

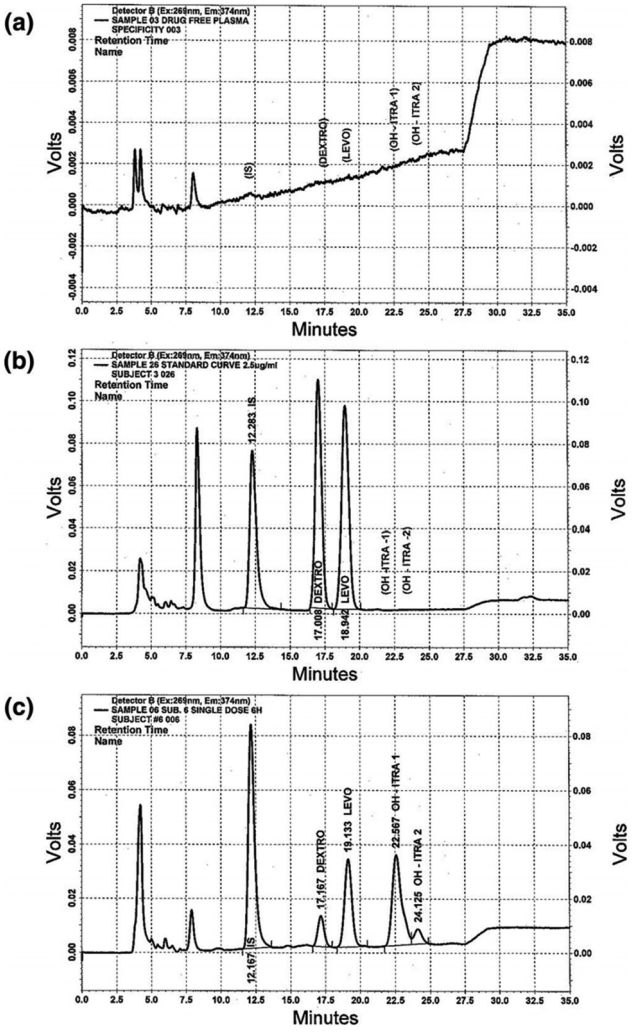

Figure 1: HPLC chromatograms: (a) drug-free plasma, (b) plasma sample spiked with itraconazole at 2.5 µg/mL, (c) human plasma sample collected 6 h after the administration of a 200 mg dose of itraconazole. Reprinted from ref. [43].

infections, promoting better agricultural production and product quality. These agrochemicals are considered pesticides that could be dangerous for health. Therefore, it is necessary to monitor their presence in agri-food products and in the environment. In addition, the chiral analytical method can investigate the environmental stereochemistry of triazole fungicides in food matrices. The second part of Table 2 reported some recent experimental works where stereoselective degradation or dissipation process study was discussed to comprehensively understand the behavior of each enantiomer, in the environment, to minimize any future risk for humans, animals, and the whole biosphere. A great part of works is dealing with chiral LC coupled with UV or MS detector for the determination and quantification of fungicide enantiomers

extracted and purified by QuEChERS method from vegetable, fruit, and environmental matrices [48–57], while hexaconazole, tebuconazole, and penconazole were extracted from ground and surface water by using the rapid and environmentally friendly dispersive liquid–liquid microextraction [58].

The chiral methodology was also applied to investigate the probable enantioselective degradation/dissipation of pesticides in agri-food and environmental samples. Generally, it was assumed that the degradation of the enantiomers in plants follows approximately a pseudo-first-order kinetics trend and half-life can be evaluated. Based on these considerations, studies of the fembuconazole degradation in strawberries have found less than 6 days half-life for each enantiomer and constant enantiomeric fraction values with no differences of the enantioselective degradation behavior [54]. On the contrary, by studying the concentrations of four cyproconazole stereoisomers in cucumber with a chiral column CSP-cellulose tris(3,5-dimethylphenylcarbamate) based in LC-MS system, a different bioactivity was observed. The strongest fungicidal activity was observed for (2*S*,3*S*)-(−)-cyproconazole, while its degradation was slower than that of (2*R*,3*S*)-(+)-cyproconazole [55]. Similar behavior was found for the stereoselective degradation of difenoconazole in vegetables. (2*S*,4*S*)-Stereoisomer exhibited the highest-toxic and the lowest-bioactive, contrary to what was observed for (2*R*,4*S*)-difenoconazole. Moreover, experiments under aerobic or anaerobic conditions showed that (2*R*,4*R*)- and (2*R*,4*S*)-difenoconazole were preferentially degraded in the soil. These implications suggest that the use of pure (2*R*,4*S*)-difenoconazole instead of the commercial stereoisomer mix might likely be a better approach for higher bioactivity reducing environmental pollution [15].

Some investigations were addressed to study fungicides as pesticides released in lakes, rivers, coastal seawater, etc. In this respect, the enantioselective toxicity, elimination, and bioaccumulation of cyproconazole in aquatic organisms such as Algae *Chlorella pyrenoidosa* [59] and tadpole (*Rana nigromaculata*) were studied [60]. The results revealed an enantioselective ecotoxicity, digestion, and uptake of chiral pesticide cyproconazole on ecological indicators. This fact suggests that more attention to environmental analysis at the chiral level investigation should be paid.

2.2 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is a useful analytical technique for resolving and purifying racemic mixtures on CSPs. SFC is presented as a sustainable chromatographic technique because of the use of nontoxic mobile phases, and it has many advantages compared to the traditional liquid chromatography. Unlike the most widely used organic solvents, carbon dioxide (CO_2) is a nonreactive, environmentally friendly, and cheap solvent characterized by low viscosity and high diffusivity; moreover, it is considered safe by FDA and EFSA. As a result, high flow rates can be used, without losing resolution nor efficiency, for improving the separation of chiral enantiomers, shortening the analysis time (providing a 3 to 5-fold reduction), and reducing waste products.

Generally, since super/subcritical CO_2 has a low polarity, most analytes cannot elute. For this reason, the mobile phase is combined with carbon dioxide and alcohol-type modifiers, such as methanol, ethanol, and isopropanol, and in some cases ACN. Occasionally, to obtain better peak shapes, triethylamine (TEA), TFA, and other additives may also be added.

An overview of SFC applications for chiral separations of pharmaceutical compounds including antimycotic drugs was previously published [65].

As it is shown in Table 3, the chiral separation of imidazole derivatives was achieved by SFC employing LC column packed with polysaccharide-based CSPs.

To our best knowledge, the first enantiomeric separation of a fungicide was reported by Thienpont et al. [66]. An LC column, packed with CSP-amylose(6-(*R*-phenyl ethyl carbamate)-2,3-(3,5-dimethylphenylcarbamate))-based, was used for the enantioseparation of ketoconazole and itraconazole. By changing some experimental parameters, such as pressure and polar modifier content, the mobile phase was adjusted as a mixture of CO_2, methanol–ethanol, and octanoic acid. These conditions were suitable to achieve the baseline chiral separation of itraconazole.

The potential of CSP-amylose tris-(3,5-dimethylphenylcarbamate) based in SFC was confirmed for the enantioseparation of a large list of triazole compounds [67–70].

In general, this class of compounds was eluted when the CO_2-mobile phase was mixed with organic alcohol or acetonitrile. However, the latter (at high concentration) caused strong retention and, therefore, longer time analysis (60 min) and poor resolution. As reported, only the enantiomers of miconazole and econazole were baseline-resolved in less than 20 min, while those of bifonazole in less than 40 min [69].

Better chiral separations were achieved when super/subcritical CO_2 was mixed with an alcohol-type organic modifier: methanol for diniconazole and four stereoisomers of cyproconazole, 2-propanol for

tetraconazole and four stereoisomers of propiconazole, and ethanol for hexaconazole [67]. Occasionally, small additive contents, such as 0.1% (v/v) TEA and TFA in the mobile phase, had a positive effect on retention and peak shape. In these conditions, a high resolution of ketoconazole was obtained in 7 min [72].

When the column temperature was increased, the fluid viscosity and density decreased, but no obvious effect on the retention factor was observed [69]. In the case of the chiral azoles studied, the temperature effect was different depending on the type of organic modifier. When a high percentage of organic modifier was needed for the sulconazole separation (15–30%), the retention factor decreased with a temperature increase. On the contrary, the retention factor of both hexaconazole and econazole was increased when 5% ethanol or isopropanol [69] and 15% isopropanol, respectively, were present in the mobile phase [68].

Some experimental works discussed the effect of temperature on selectivity concerning the van't Hoff equation. The study of the temperature effect revealed different results considering each analyte and the organic modifier used. In this respect, the selectivity of bifonazole was increased rising the temperature when methanol was used, while decreased with ethanol or propan-2-ol [70]. A slight increase of the selectivity with the temperature was observed in the case of sulconazole with propan-2-ol, but the opposite effect occurred for miconazole [68].

Considering the thermodynamic viewpoint, the entropic and enthalpic terms were estimated from van't Hoff plot. The isoenantioselective temperature T_{iso} (where the enthalpic and entropic contributions are balanced and peak coelution occurs) was calculated. Different temperatures were observed for each studied antifungal imidazoles at different organic modifiers in the mobile phase [68]. In addition, this experimental work showed that the chiral separation can be enthalpy-driven or entropy-driven in relationship to isoenantioselective temperature value. In this regard, Bernal et al. also investigated the temperature effect on the chiral separation of ketoconazole [71]. When methanol was added to the mobile phase, the increase of temperature from 35 to 60°C led to a reduction of chiral separation until enantiomeric coelution at 40°C (isoelution temperature). A reversal of elution order and an increase of *Rs* were observed above the isoelution temperature, where the separation is theoretically entropy-driven [72].

Ketoconazole and triticolazole enantiomers were separated by using CSP-cellulose tris(3,5-dimethyl phenyl carbamate)-based column [72,73]. In particular, the enantioseparation of ketoconazole was also investigated in a study comparing cellulose and amylose CSP polysaccharide-based derivatized with 3,5-dimethylphenylcarbamate [72]. The effect of four different organic modifiers (methanol, ACN, 2-propanol, and ethanol) on chromatographic parameters was studied. It was concluded that the polysaccharide structure can influence the choice of the mobile phase. When amylose-based CSP was used, the decrease of the modifier polarity resulted in a decrease of retention contrary to the results obtained with cellulose-based CSP.

SFC, like LC, can be easily coupled to MS [74–76]. In this regard, an interesting SFC-MS interface was reported coupling a Q-TOF 2 hybrid mass spectrometer, equipped with ESI interface, with the SFC system. This device was modified with additional makeup liquid to provide an easy MS coupling. By using this configuration, a mixture containing four chiral antimycotic drugs (miconazole, econazole, sulconazole, and ketoconazole) was resolved in their enantiomers in one run, as reported in Figure 2 [74].

Moreover, the low viscosity of the supercritical fluid and the high flowrate provided a very short delay time and no significant band broadening effect when long-distance between the outlet column and the detector was used. Although a 100 μm ID × 10 m was used to join UV and MS detector, no significant differences in peak shape and peak width were observed in the UV and TIC chromatogram comparison.

When triazole fungicides are applied in agricultural activities, they cannot be considered only as pharmaceutical drugs but also as pesticides. Due to their high hazards for human health and their potential role in groundwater contamination, EU guidelines declare strict limits for these compounds. For this reason, it is needed to identify and quantify their presence in environmental and agri-food matrices. A method for the simultaneous detection and quantification of fenbuconazole and its chiral metabolites in fruit, vegetable, and soil has been reported [75]. In addition, four stereoisomers of pyrisoxazole in cucumber, tomato, and soil have been analyzed [76]. CSP-amylose tris-(3,5-dimethylphenylcarbamate)-based assured baseline resolution in less than 3–5 min. High selectivity was obtained by utilizing an MS coupling, which also guaranteed high sensitivity and accurate quantification thanks to the MS fragmentation method.

2.3 Miniaturized liquid chromatography and electromigration techniques

Even though HPLC and more recently SFC are chromatographic separation techniques widely used in chiral

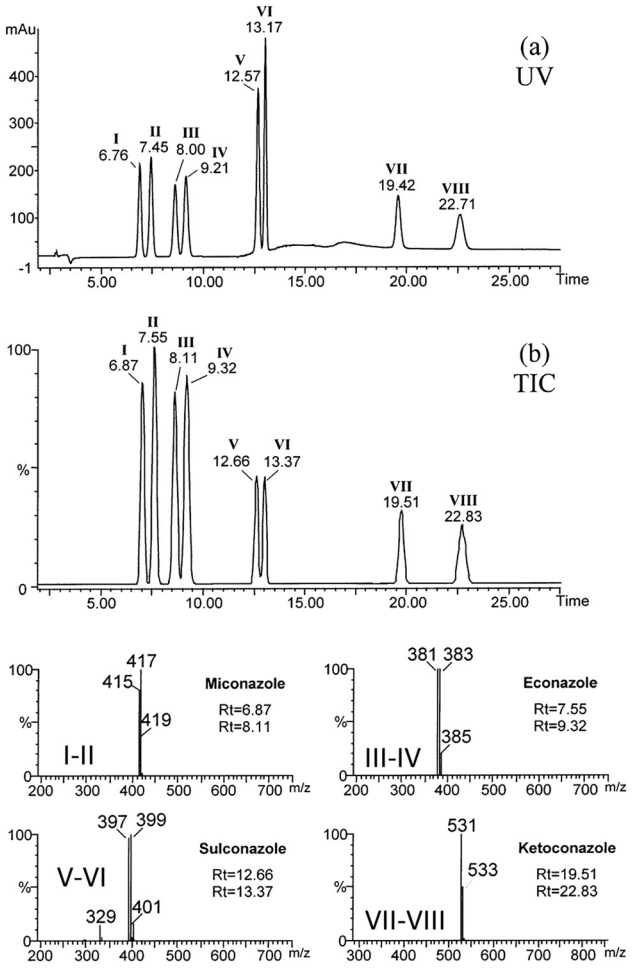

Figure 2: SFC chiral separation of four antimycotic drugs mixture by using LC-column packed with CSP- Amylose (3,5-dimethylphenylcarbamate)-based. (a) UV and (b) TIC chromatograms. (I–VIII) Mass spectra of target compounds. Adapted from ref. [74].

separation of antifungal drugs as imidazole and triazole derivatives, alternative miniaturized analytical methodologies have gained importance. Their great potential consists in high selectivity, low injected sample volumes, short analysis time, and low analysis costs [77]. In addition, because of the low volumes of mobile phases applied with electromigration (CE and CEC) and chromatographic techniques (nano-LC and CLC), these methodologies can be considered eco-friendly systems.

In Tables 4 and 5 are listed selected experimental works in chiral separation of antifungal drugs by using nano-LC/CLC and CE/CEC, respectively. It is worth noting that, as previously described for HPLC and SFC, the studies and research carried out have been focused on developing new CSPs and their potential for enantiomers analysis.

2.3.1 Nano/capillary liquid chromatography

As reported in Table 4, all articles performed nano/CLC separation by using chiral monolithic columns. In this respect, the potential of different CSs into 100–150 µm ID monolithic columns manufactured by *in situ* polymerization procedure was evaluated.

The recognition ability of β-CD was investigated on the separation of the enantiomers of different classes of pharmaceuticals and antifungal drugs. A novel β-CD-based CSP, 2,3,6-tris(phenyl carbamoyl)-β-CD methacrylate-coethylene glycol dimethacrylate monolithic capillary column was prepared via copolymerization in a *one-pot* or post-modification procedure [78]. β-CD was also immobilized as β-cyclodextrin phenyl carbamate-based silica monolithic column achieving acceptable resolution of miconazole [79].

Via copolymerization of a binary monomer mixture consisting of glycidyl methacrylate (GMA) and ethylene glycol dimethyl acrylate (EGDMA), a 150 µm ID-fused capillary was used for novel affinity monolithic capillary columns functionalized with Candida antarctica lipase B (CALB) [80] or with a macrocyclic antibiotic, namely colistin sulfate [81]. The prepared capillary columns were investigated for the enantioselective nano-LC separation of some antifungal drugs (sulconazole, siconazole, hexaconazole) obtaining enantiomeric resolution factors in the range of 1.2–1.6.

The same authors proposed a different chiral monolithic column preparation based on CS encapsulation into polymer-based monolithic backbones. CS amylose 2,3(3,5-dimethylphenylcarbamate)-6-ethylphenylcarbamate was entrapped into GMA-*co*-EGDMA reporting resolutions lower than 1.7 in normal and reverse conditions [82].

Organized in nanoscale dimension in intrinsic helicity, the single-walled carbon nanotubes (SWCNTs) exhibit chiral recognition capacity. This interesting property was investigated in novel CSP based on SWCNTs encapsulated into different polymer-based monolithic backbones [82]. The highest enantioselectivity was achieved under reversed-phase conditions for organic aqueous mobile phase methanol-based emphasizing a low environmental impact trend.

2.3.2 Capillary electromigration methods

In chiral analysis, CE is considered a powerful alternative compared to HPLC and GC, due to its high chromatographic efficiency and minimum consumption of solvents

Table 5: Applications using electromigration methods

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Capillary	Experimental conditions	Remarks	Ref.
Econazole	Culture medium (*Spirodela polyrhiza*)	—	CE-DAD (200 nm)	Sulfated-β-CD/	50 µm ID × 58.5 cm	BGE, 25 mM phosphate buffer (pH 3.0)/1.5% *S*-β-CD; injection, 50 mbar × 10 s; V, −20 kV; *T*, 30°C	Dynamic range, 1–30 mg/L; LOD, 0.7–0.8 mg/L; LOQ, 2.2–2.6 mg/L	[84]
Econazole	Culture medium (*Daphnia magna*)	—	CE-DAD (200 nm)	Sulfated-β-CD/	50 µm ID × 58.5 cm	BGE, 25 mM phosphate buffer (pH 3.0)/1.5% *S*-β-CD; injection, 50 mbar × 10 s; V, −20 `kV; *T*, 30°C	Dynamic range, 1–30 mg/L; LOD, 1.0–1.1 mg/L; LOQ, 3.3–3.6 mg/L	[85]
Econazole	Wastewater	SPE (PMO-TESB-1)	CE-DAD (200 nm)	Sulfated-β-CD/	50 µm ID × 58.5 cm	BGE, 25 mM phosphate buffer (pH 3.0) and 2% sulfated-β-CD; V, −20 kV; injection, 50 mbar × 10 s; *T*, 20°C	LOD, 5.9–6.6 and 19.5–21.9 µg/L; recovery. 58.5–72.4%	[86]
Four enantiomers of propiconazole	Water-soil slurry	Centrifuged-filtered GC-MS, extracted with methyl *tert*-butyl ether (GC-MS)	MEKC-UV (190 nm) GC-MS	HP-γ-CD β-Cyclodextrin (GC)	CE, 75 µm ID × 57.0 cm; GC, chiral capillary column, (30 m × 0.25 mm × 0.25 µm); coating, 20% (w/w) *tert*-butyldimethylsilyl-β-cyclodextrin	CE, BGE: 30 mM HP-γ-CD, 75 mM SDS in 25 mM phosphate buffer (pH 7); hydrodynamic injection, 10 s; V, 30 kV; *T*, 23°C. GC, carrier gas, He; injection, 1 µL; flow rate, 1.5 mL/min	Biotransformation: aerobic conditions over five months	[87]
Fluconazole, ketoconazole, itraconazole, sertaconazole	—	—	CE-UV (230 nm)	SBE-β-CD	50 µm ID × 31.2 cm	BGE, 50 mM phosphate-triethanolamine buffer, pH 2.5 and BSE-β-CD; injection, 50 mbar × 10 s; V, +30.0 kV; *T*, 25°C		[88]
Iodiconazole	—	—	CE-DAD (200 nm)	HP-γ-CD	50 µm ID × 64.5 cm	BGE, 30 mM NaH_2PO_4 buffer pH 2, 10 mM HP-γ-CD; injection, 50 mbar × 3 s; V, +20 kV; *T*, 20°C		[89,90]
Econazole, isoconazole, miconazole, sulconazole	—	—	NACE-UV (230 nm)	HMAS-β-CD	50 µm ID × 48.5 cm	BGE, 10 mM ammonium camphorSO_3^- and 20 mM HMAS-β-CD/in MeOH acidified with 0.75 M FA; injection, 50 mbar × 3 s; V, +25 kV; *T*, 15°C		[91]
Enilconazole	—	—	CE-UV (200–220 nm)	β-CD or HDMS-β-CD	50 µm ID × 40.0 cm	BGE, 50 mM KH_2PO_4 pH 2; injection, 5 mbar × 5 s; I, 150 µA; *T*, 20°C	Enantiomer migration order	[31]

(*continued*)

Table 5: (*continued*)

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Capillary	Experimental conditions	Remarks	Ref.
(+)-2*R*,4*S* and (−)-2*S*,4*R* ketoconazole enantiomers	—	—	CE-DAD (200 nm)	OHP-β-CD	50 µm ID × 64.5 cm or 50 µm ID × 40.0 cm	BGE, 100 mM NaH_2PO_4 pH 3.00 and 0.5–70 mM OHP-β-CD 100 mM H_3PO_4 pH 3.00 with TEA and 1.64–13 mM OHP-β-CD; injection, 50 mbar × 2 s or 30 kV × 8 s; V: +30 kV or +25 kV; *T*, 20°C	Migration order inversion study	[92]
Econazole	Formulated cream sample	(i) CH_2Cl_2, sonication (ii) Diol-SPE	MEKC-DAD	HP-γ-CD	50 µm ID × 64.5 cm	BGE, 40 mM HP-γ-CD, 40 mM SDS in 20 mM phosphate buffer, pH 8; injection, 3 kV × 3 s; V, +30 kV; *T*, 25°C	LOD, 3.6–4.3 mg/L	[93]
Tioconazole, isoconazole, fenticonazole	Human urine Cream formation	SPE	MEKC-DAD (200 nm)	DM-β-CD or HP-γ-CD	50 µm ID × 64.5 cm	BGE, 35 mM HP-γ-CD, 10 mM DM-β-CD, 35 mM phosphate buffer (pH 7.0), 50 mM SDS, 15% (v/v) ACN; injection, 3 kV × 3 s; V, +27 kV; *T*, 30°C.	Recovery, 93.6–106.2%	[94]
Ketoconazole (four stereoisomers)	—	—	MEKC -DAD (200 nm)	TM-β-CD	50 m ID × 64.5 cm	BGE, 10 mM phosphate buffer, pH 2.5, 5 mM SDS, 20 mM TM-β-CD in 1.0% MeOH (v/v); injection, 50 mbar × 5 s; V, +25 kV; *T*, 25°C		[95]
Hexaconazole, penconazole	—	—	MEKC-DAD (200 nm)	HP-γ-CD	50 µm ID × 64.5 cm	BGE, 40 mM HP-γ-CD, 50 mM SDS in 25 mM phosphate buffer (pH 3); injection, 50 mbar × 70 s; V, −25 kV; *T*, 35°C	Online pre-concentration: sweeping-CD-MEKC; LOD: 0.1–0.2 mg/L	[96]
Econazole, miconazole	—	—	CE-UV (201 nm)	Glu-β-CD	50 µm ID × 50.0 cm	BGE, 120 mM NaH_2PO_4 pH 2.5 and 0.5 mM Glu-β-CD; injection, 0.5 psi × 5 s; V, −20 kV; *T*, 30°C		[97]
Econazole, ketoconazole,	—	—	CE-UV	HP-β-CD/DTAC	50 µm ID × 49.0 cm polyacrylamide coated capillary	BGE, 30 mM HP-β-CD, 30 mM		[98]

(*continued*)

Table 5: (*continued*)

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Capillary	Experimental conditions	Remarks	Ref.
itraconazole, miconazole,						NaH_2PO_4/H_3PO_4, 20 mM DTAC; injection, 0.5 psi × 5 s; V, ± 20 kV		
Ketoconazole, sulconazole, econazole	—	—	CE-DAD (237 nm)	$[TMLV]^+$ $[Tf_2N]^-$/ HP-β-CD	50 µm ID × 50.0 cm	BGE, 40 mM NaH_2PO_4 pH 3 in 40% MeOH and 20 mM $[TMLV]^+$ $[Tf_2N]^-$; injection, 50 mbar × 5 s; V, +25 kV; *T*, 20°C		[99]
Ketoconazole, sulconazole	—	—	CE-DAD (205 nm)	$[BMIm]^+$ $[BLHvB]^-$/ dextrin	50 µm ID × 50.0 cm	BGE, 50 mM tris-H_3PO_4 pH 2.7 in buffer solution 2.0% (w/v) dextrin and 30 mM $[BMIm]^+$ $[BSMB]^-$; injection, 50 mbar × 5 s; V, +22 kV; *T*, 20°C		[100]
Ecoconazole, ketoconazole, voricoazole	—	—	CE-DAD (215, 220 nm)	TMA-d-PAN/ maltodextrin	50 µm ID × 50.0 cm	BGE, 60 mM tris-H_3PO_4 pH 3 and 3% (w/v) maltodextrin/50 mM TMA-d-PAN; injection, 50 mbar × 2 s; *V*, +20–28 kV; *T*, 20°C		[101]
Sulconazole	—	—	CE-DAD (230 nm)	Chondroitin sulfate D or Chondroitin sulfate E	50 µm ID × 50.0 cm	BGE, 20 mM tris/H_3PO_4 buffer solution pH, 2.0–4.0 containing 5.0% CSE or CSD; injection, 50 mbar × 5 s; *V*, +15 kV; *T*, 15°C		[102]
Sulconazole	—	—	CE-DAD (220 nm)	CSC/glycogen	50 µm ID × 50.0 cm	BGE, 40 mM Na_2HPO_4 pH 3.0 and 2.0% CSC/3.0% glycogen; injection, 50 mbar × 4 s; *V*, −20 kV; *T*, 20°C		[103]

and reagents. Besides, this technique could be a valid approach to study new and/or expensive CSs.

In CE, the CS is usually added to the background electrolyte (BGE). As can be seen in Table 5, the enantiomeric separation of antifungal drugs as imidazole and triazole derivatives was achieved mainly by using CDs [31,84–103]. Marina and coworkers reported the separation of the two enantiomers of econazole from environmental matrices by using sulfated-β-CD as a CS with an enantioresolution factor of 6.5 and 7.5 min of analysis time. Stability and toxicity studies of racemates and enantiomers under abiotic and biotic conditions on *Spirodela polyrhiza* [84] and *Daphnia magna* [85] were evaluated. These results constitute an interesting approach to estimate the stability, undertaking drug biodegradation and bioaccumulation studies in the aquatic ecosystem. Econazole was also spiked in wastewater samples and extracted by periodic mesoporous organosilica-1,4-bis (trimethoxysilylethyl)benzene as sorbent material in the SPE procedure, obtaining recovery values ranging between 58.5 and 72.4% [86]. Similar study was focused to soil pollution and, subsequently, groundwater due to a propicolazole and its four enantiomers. Baseline enatioresolution was achieved by using MEKC and employing 2-hydroxypropyl γ-cyclodextrin. This study was addressed to biotransformation of propiconazole analyzing its rate of loss from the spiked water phase of two different soil-water slurries, followed under aerobic conditions over five months [87].

Different CD derivatives were applied with success for chiral separations of triazole antifungal agents. Sulfobutyl ether-β-CD (SBE-β-CD) was used for ketoconazole, itraconazole, fluconazole, and sertaconazole [86], while hydroxypropyl-β-CD (HP-β-CD) was employed for iodiconazole [89] and heptakis(2-*O*-methyl-3-oacetyl-6-*O*-sulfo)-β-CD (HMAS-β-CD) in nonaqueous capillary electrophoresis (NACE).

As in HPLC, SFC the enantiomer migration order (EMO) is an interesting aspect also in CE chiral separations and it is useful for one enantiomer quantification at trace levels in the sample with an excess of its antipode. EMO was observed on econazole when β-CD or the sulfated heptakis(2-*O*-methyl-3,6-di-*O*-sulfo)-β-CD (HMDS-β-CD) were used as the CSs [29]. In addition, this phenomenon was also studied by Thormann et al. [92]. At low pH, when the concentration of (2-hydroxypropyl)-β-cyclodextrin (OHP-β-CD) was changed in the range of 0.5–70 mM, the migration order change of (+)-2*R*,4*S* and (−)-2*S*,4*R* ketoconazole enantiomers was observed. The authors also compared the results with the ones obtained applying the dynamic computer simulation data approach.

The usual approach to increase the chiral recognition of CD, especially when uncharged enantiomers are analyzed, is the use of sodium dodecyl sulfate (SDS) with running BGE. This approach is known as CD-micellar electrokinetic chromatography mode (CD-MEKC). Here, the migration of each enantiomer is determined by competitive distributions into the aqueous buffer solution, CD, and micelles. This multimodal electromigration mode allowed the baseline enantioseparation of econazole and it was successfully applied to a cream sample by using SPE-HP-γ-CD-MEKC [93] and tioconazole, isoconazole, and fenticonazole in human urine sample and commercial cream formulation by SPE-HP-γ-CD/DM-β-CD-MEKC [94]. The presence of SDS below its CMC value with TM-β-CD in the BGE greatly influenced the resolution and selectivity of chiral enantiomers of ketoconazole, allowing a baseline separation of all its four stereoisomers [95]. Furthermore, the presence of SDS in the running BGE was helpful for three chiral triazole fungicides but especially for online sample preconcentration technique, namely *sweeping*, improving detection limit of 28–41-fold compared to the one attained by HP-β-CD-MEKC [96]. When enantiomers still were not completely separated with CDs or derivatized CDs, ionic liquids were also used in addition [98–100]. In this regard, DTAC in combination with HP-β-CD resulted effective for the separation of azole antifungal enantiomers [98]. Figure 3 shows the comparative results, where the interactions between DTAC and HP-β-CD could influence the formation of inclusion complex by increasing the enantio-recognition capability. In the same way, the synergistic enantioseparation effect with CIL (chiral ionic liquids) and CS was investigated by preparing a chiral BGE which contained $[TMLV]^+$ $[Tf2N]^-$/HP-β-CD [99].

The potential of a mixture of $[BMIm]^+$ $[BLHvB]^-$/dextrin was also studied by observing the chiral resolution of ketoconazole, while the chiral resolution of sulconazole was improved more than 2–3 time [100].

Finally, linear oligo-polysaccharides providing a wide range of enantioselectivity in CE represent a valid alternative to CD, due to their water solubility and weak UV absorption. Even if some enantiomeric separations have been achieved with neutral polysaccharides such as dextrin, as previously described, recently the attention has focused on the use of anionic polysaccharides. Chondroitin sulfates, which are negatively charged polysaccharides, were used as CS or in combination with glycogen for chiral separation of sulconazole [98–103].

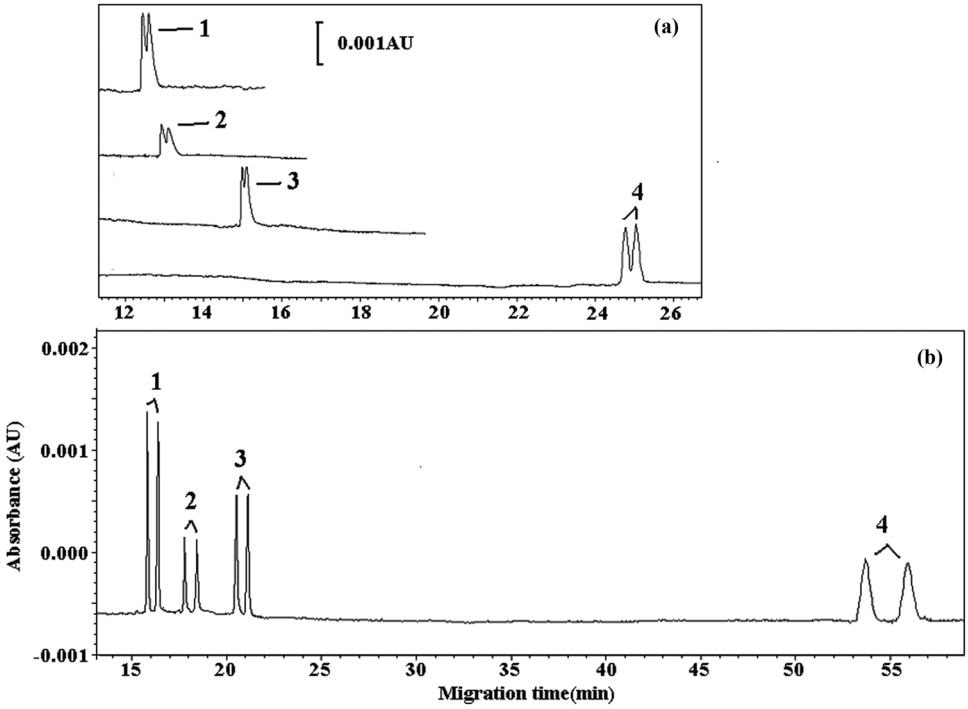

Figure 3: Electropherograms of four azole antifungal enantiomers: (1) miconazole; (2) econazole; (3) ketoconazole; (4) itraconazole. (a) BGE with HP-β-CD, (b) BGE with HP-β-CD/DTAC (CIL). Reprinted from ref. [98].

2.4 Miscellaneous

Table 6 reports the work done with other techniques, CEC and GC, applied to the chiral separation of triazole fungicides.

For exploring the potential of cellulose derivative CS, a 100 µm ID-fused silica capillary was packed with CSP-cellulose-based, where cellulose tris(3,5-dichlorophenylcarbamate) was coated onto silica particle material. By using the polar organic mobile phase, the enantiomeric separation of miconazole was studied by comparing CEC and µ-LC results [104]. Figure 4 reports the enantioseparation of miconazole in both CEC and µ-LC modes. Here the theoretical plate numbers in CEC were 1.6–1.8 times higher compared with those obtained by µ-LC. These results showed the great potential of CEC, a hybrid technique that combines the high efficiency due to the flat electrosmotic flow profile of CE mode and the high selectivity due to the stationary phase of the LC capillary column.

Gas chromatography coupled to ion trap mass spectrometry was used for quantitative analysis of two enantiomers of simeconazole [105] in food matrices and propiconazole in soil–water slurry [87]. The enantiomers were resolved by using a chiral capillary column coated with *tert*-butyldimethylsilyl-β-cyclodextrin in less than 15 min. GC-MS system was combined with a clean-up/enrichment procedure based on the modification of QuEChERS. By introducing graphitized carbon black/primary secondary amine (GCB/PSA) in solid-phase extraction, LOD values lower than maximum residue levels (MRLs) established in Japan were observed [105].

3 Concluding remarks and future trends

This review article reports a collection of works developed from 2010 to present in the field of the enantiomeric separation of antifungal drugs as imidazole and triazole derivatives.

Many analytical methodologies have been developed for the determination of a wide variety of fungicides. HPLC, CE, SFC, and miniaturized LC were the most applied methodologies. On the contrary, to our best knowledge, only in one case GC and CEC were employed for this topic.

From the selected works, it can be concluded that, although several types of CSPs are available, most of the applications in HPLC and SFC made use mainly of

Table 6: Miscellaneous applications

Analytes	Matrix	Sample preparation procedure	Mode/ detection	Chiral selector	Column	Experimental conditions	Remarks	Ref.
Miconazole	—	—	CEC/CLC-UV (214 nm)	Cellulose tris(3.5 dichlorophenylcarbamate) (CDCPC)	Packed column, 100 µm ID × 24.0 cm; SP, CS 25% (w/w) coated on silica particles (5 µm, 100 nm)	MP, NH_4Ac in $MeOH/H_2O$ CEC Injected volume, 10 bar; flow rate: 1 µL/min	Higher resolution and peak efficiency in CEC mode	[104]
Simeconazole	Cucumber, tomato, apple, pear, wheat, and rice	QUECHERS – SPE (GCB/PSA)	GC-ITMS	β-Cyclodextrin	chiral capillary column, (30 m × 0.25 mm × 0.25 µm), coating, 20% (w/w) *tert*-butyldimethylsilyl-β-cyclodextrin	Carrier gas, He; injected volume, 1 µL; flow rate: 1.0 mL/min	LOD: 0.4–0.9 µg/kg	[105]
Four enantiomers of propiconazole	Water-soil slurry	Centrifuged-filtered	MEKC-UV (190 nm)	HP-γ-CD	CE, 75 µm ID × 57.0 cm	CE, BGE, 30 mM HP-γ-CD, 75 mM SDS in 25 mM phosphate buffer (pH 7); hydrodynamic injection, 10 s; *V*, 30 kV; *T*, 23°C	Biotransformation: aerobic conditions over five months	[87]
		GC-MS, extracted with methyl *tert*-butyl ether (GC-MS)	GC-MS	β-Cyclodextrin (GC)	GC, chiral capillary column, (30 m × 0.25 mm × 0.25 µm); coating: 20% (w/w) *tert*-butyldimethylsilyl-β-cyclodextrin	GC, carrier gas, He; injected volume, 1 µL; flow rate, 1.5 mL/min		

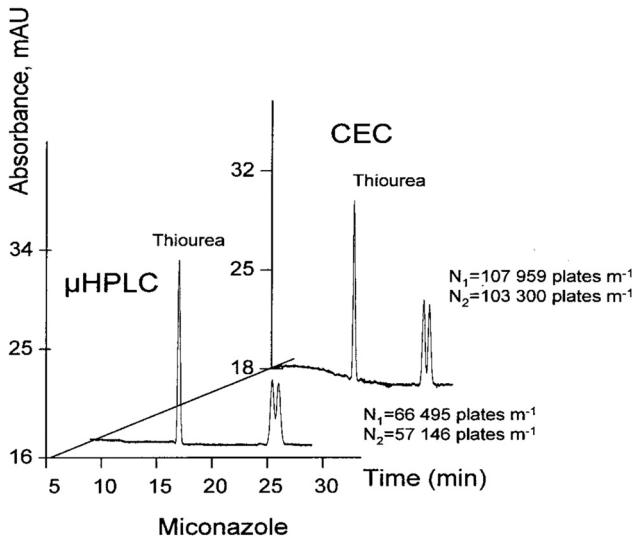

Figure 4: Enantioseparation of miconazole in CEC and CLC mode by using 100 μm ID × 24 cm capillary column packed with CSP-cellulose tris(3,5 dichloro phenyl carbamite)-based. Reprinted from ref. [104].

polysaccharide types, while in CE, CD types were the most used.

Data analysis revealed that the spectrometric detector is the preferred choice in both HPLC and SFC. In addition, the MS coupling was selected for trace level determination of azole antifungal drugs in pharmaceutical, biological, and agri-food applications.

The growing interest in this class of chiral molecules does not seem to correspond to an adequate development of analytical methodologies devoted to environmental and human health protection. A considerable number of works published since 2010 are focused to investigate the potential of CSs in relationship to chromatographic conditions, while a poor list of LC, SFC, and CE articles is devoted to real sample applications and even none to miniaturized LC.

The list also suggests that only slight effort has been devoted to the development of methodologies that follow the sustainability trend and the principles of green analytical chemistry [106].

Although SFC is gaining popularity in this topic for its chromatographic performance and eco-friendly approach, the effort for improving its sensitivity has not been so marked as the one spent for alternative methodologies, such as miniaturized separation techniques.

Even if electromigration techniques, such as CE and CEC, can be considered the green trend of separation science, the difficulty to achieve adequate sensitivity and an easy coupling with MS limits their applicability to real samples.

Finally, among the miniaturized techniques, nano-LC/CLC plays a privileged role compared to electromigration techniques because they combine robustness and applicability in routine analysis. These techniques are useful in different analytical fields, thanks to some interesting features derived from the downscaling effects, such as better chromatographic performance and higher sensitivity due to the reduced dilution effect and a very stable and easy-MS-coupling. Nevertheless, the scarce number of recent works in chiral separation of azole compounds regarding these techniques is very unusual and not justified.

We believe that LC micro-fluidic techniques are, nowadays, the highest expression of sustainable chemistry, and an interesting solution for future chiral approaches. Due to the low consumption of stationary phase and CS, Nano-LC/CLC can be a miniaturized test bench for new CSs, which can then be used for the development of large-scale preparative analytical methodologies in the pharmaceutical field.

Abbreviations

1-butOH	butan-1-ol
AGP	α1-acid glycoprotein
β-CD	beta-cyclodextrin
BGE	background electrolyte
$[BMIm]^+$	1-butyl-3-methylimidazolium(T-4)-
$[BLHvB]^-$	bis[(2*S*)-2-(hydroxy-*O*)-3-methyl-butanoato-*O*]borate
BuMA	butylmethacrylate
CALB	*Candida antarctica* lipase B
CD-MEKC	CD-micellar electrokinetic chromatography
CIL	chiral ionic liquids
CLC	capillary liquid chromatography
CS	chiral selector
CSC	chondroitin sulfate C
CSD	chondroitin sulfate D
CSE	chondroitin sulfate E
CSP	chiral stationary phase
DAD	diode array detector
DEA	diethylamine
DLLME	dispersive liquid–liquid microextraction
DMC	dichloromethane
DM-β-CD	heptakis(2,6-di-*O*-methyl)-β-cyclodextrin
DTAC	decyl trimethyl ammonium chloride

EDMA	ethylene glycol dimethacrylate
EGDMA	ethylene glycol dimethacrylate
FA	formic acid
GCB	graphitized carbon black
Glu-β-CD	glutamic acid-β-CD
GMA	glycidyl methacrylate
EMO	enantiomer migration order
Hac	acetic acid
HDMS-β-CD	sulfated heptakis(2-*O*-methyl-3,6-di-*O*-sulfo)-β-CD
HMAS-β-CD	heptakis(2-*O*-methyl-3-oacetyl-6-*O*-sulfo)-β-CD
HP-β-CD	2-hydroxypropyl-β-CD
HP-γ-CD	hydroxypropyl-γ-CD
HSA	human serum albumin
ID	internal diameter
Inj	injection
Injected volume	injection volume
IPA	2-propanol
ITMS	ion trap mass spectrometry
LC	liquid chromatography
LPE	liquid phase extraction
n-Hex	*n*-hexane
n-prOH	propan-1-ol
m-G-Fe_3O_4-SPE	magnetic graphene modified by Fe_3O_4 nanoparticles solid-phase SPE
MP	mobile phase
MTBE	methyl *tert*-butyl ether
MTMS	methyltrimethoxysilane
NACE	nonaqueous capillary electrophoresis
NH_4Ac	ammonium acetate
OHP-β-CD	hydroxypropyl-β-CD
PMO-TESB-1	periodic mesoporous organosilicas-1,4-bis(trimethoxysilylethyl)benzene 1
PSA	primary secondary amine
PT-CFs-μ-SPE	pipette tip-based cigarette filters for micro-SPE
PT-MIP-μ-SPE	molecularly imprinted polymer micro-solid-phase extraction
Q-Tof2	hybrid mass spectrometer
QuEChERS	quick, easy, cheap, effective, rugged, and safe
Rs	enantioresolution
SBE-β-CD	sulfobutyl ether-β-CD
SDS	sodium dodecyl sulfate
SFC	supercritical fluid chromatography
SPE	solid-phase extraction
TEA	trimethylamine
TMA-d-PAN	tertramethylammonium-d-pantothenate
TMβCD	heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin
$[TMLV]^+$ $[Tf2N]^-$	*N*,*N*,*N*-trimethyl-l-valinol-bis(trifluoromethanesulfon)imide
SP	stationary phase
SPMA	sulfopropyl methacrylate
SWCNTs	single-walled carbon nanotubes
TFA	trifluoroacetic acid
TMOS	tetramethoxysilane
UAE	ultrasound assisted extraction

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