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Exploring the performance of cellulose tris-3,5-dichlorophenylcarbamate as a stationary phase for the chiral electro-chromatographic separation of azole antifungals

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ABSTRACT

In this study, the enantiorecognition and separation performance of cellulose tris-3,5-dichlorophenylcarbamate (CDCPC) as the chiral selector for the enantioseparation of some chiral antifungal compounds was investigated. The CDCPC was bonded to 5 μm porous silica particles and the stationary phase packed into fused silica capillaries (250 mm x 75 μm I.D.) using the "slurry method". The column was used for the separation of twelve selected azole compounds in their enantiomers by capillary electrochromatography (CEC). To study chiral recognition, the mobile phase was modified changing the organic solvent, water content, buffer type, concentration, and pH. Each change resulted in the enhancement of specific interactions at the expense of others, leading to chiral recognition. Their effects on retention factors, enantiomeric resolution, and peak broadening are critically discussed. The developed methodology was used as an appropriate test bench to evaluate the stability of the stationary phase under different mobile phase conditions, demonstrating a remarkable enantioselective capacity even after several tens of hours of continuous use. The optimized CEC-UV method was very effective in the enantiomeric separation of seven out of twelve azole fungicides, totaling fourteen separated peaks in a single 30 min run analysis.

Introduction

In recent decades, chiral azole antifungal drugs have been separated in their enantiomers using different chromatographic methods. Gas chromatography (GC) hyphenated with ion trap mass spectrometry was employed to quantitatively analyse two simeconazole enantiomers [1]. Supercritical fluid chromatography was successfully utilized for the enantioseparation of many triazole compounds by using amylose or cellulose tris-(3,5-dimethylphenylcarbamate) (CDMPC) as a chiral stationary phase (CSP) [2–4]. High-performance liquid chromatography (HPLC) coupled with UV detector or mass spectrometry was widely used for the enantiomeric separation of antifungal compounds for environmental, agribusiness, and biological applications [5–9].

In addition to above-mentioned chromatographic conventional techniques, enantiomer separation of antifungal drugs has also been achieved with microfluidic techniques, e.g., nano- and capillary-liquid chromatography (nano-LC/CLC) utilizing capillary column of 75–150 μm I.D. The columns contained chiral selectors (CS) bonded/adsorbed on the wall by in-situ-polymerization [5]. Recently, capillaries packed with a CSP-silica modified with cellulose 3,5-dichlorophenylcarbamate (CDCPC) were also used [10].

Among other microfluidic techniques, the electromigration ones are considered powerful tools for chiral analysis because they can offer a high separation efficiency and can be a green alternative to HPLC and GC due to the limited use of organic solvents. In this respect, the chiral separation of imidazole and triazole derivatives has been performed by capillary electrophoresis (CE) modifying the background electrolyte (BGE) with derivatized cyclodextrins [5]. Data about the use of electromigration methods for the analysis of pollutants also including some enantiomers have recently been reported [11].

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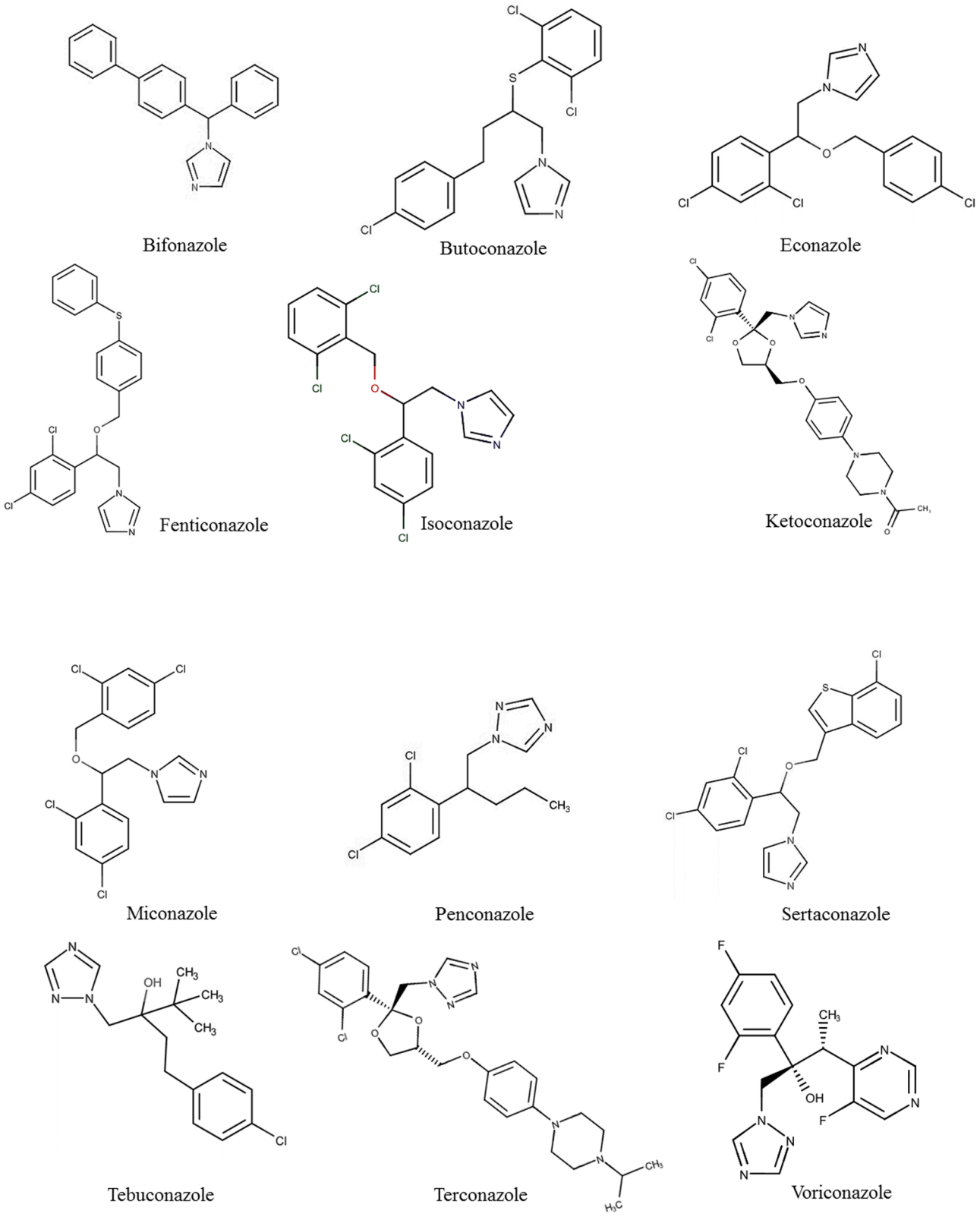

Fig. 1. Chemical structure of the studied fungicides.

Among the electromigration techniques, capillary electrochromatography (CEC) offers the best features of CZE (high separation efficiency) and HPLC (high selectivity). CEC has also been applied to the enantioseparation of fungicides, e.g., econazole and ketoconazole. The fused silica capillary wall was modified with graphene oxide (GO) and maltodextrin (MD) as the CS and it was applied to electromigration technique such as the open-tubular CEC (OT-CEC) [12]. In addition, other cellulose-based CSPs such as cellulose tris(3,5-dichloro phenylcarbamate) or cellulose tris(3-chloro-4-methylphenylcar bamate) and (cellulose tris(4-chloro-3-methylphenylcarbamate) were packed into fused silica capillaries and applied to the resolution of some fungicide enantiomers by CEC and/or nano-LC [13,14].

In this study, the CSP silica-based immobilized-CDCPC (i-CDCPC) was packed in 100 μm fused silica capillaries and the enantioseparation capability of the CSP toward twelve racemic fungicides (Fig. 1) was investigated by CEC. The effect of mobile phase composition on the chiral resolution of the target compounds was studied considering key chromatographic parameters including retention factor (*k*), enantiomeric resolution (*Rs*), and chromatographic efficiency (*N*). Optimal experimental conditions have been identified through a suitable compromise between maximum enantioseparation and the shortest analysis time.

Experimental

Chemicals and materials

Ethanol (EtOH), 1-propanol (1-PrOH), 2-propanol (2-PrOH), ammonia solution (30% *w/w*), glacial acetic acid (99% *w/w*), formic acid (99% *w/w*) were purchased from Carlo Erba (Rodano, Milan, Italy) while ammonium hydrogen carbonate ($NH_4HCO_3 \geq 99.0\%$ *w/w*) was obtained from Aldrich-Fluka-Sigma S.r.l. (Milan, Italy). Acetonitrile of HPLC grade (ACN), methanol (MeOH), acetone, and HPLC ultrapure water (filtered through 0.2 μm and packaged under nitrogen) were from VWR (International PBI S.r.l. Milan, Italy). All chemicals were of analytical reagent RS grade and used without further purification.

Standand racemic mixtures (purity ≥ 97.0%) of bifonazole (1-(p,α-diphenylbenzyl)imidazole) (CAS, 60628- 96-8), butoconazole ((±)−1-[4-(4-chlorophenyl)−2-[(2,6-dichlorophenyl)thio]butyl]−1H-imidazole mononitrate) (CAS, 65277-42-1), econazole (1-(2-((4-chlorophenyl)methoxy)−2-(2,4-dichlorophenyl)ethyl)−1H-imidazole) (CAS, 27220-47-9), fenticonazole 1-(2-(2,4-dichlorophenyl)−2-((4-(phenylthio)benzyl)oxy)ethyl)−1H-imidazole (CAS, 72479-26-6), ketoconazole ((±)-cis-1-acetyl-4-(4-[(2-[2,4-dichlorophenyl]−2-[1H-imidazol-1-ylmethyl]−1,3-dioxolan-4-yl)-ethoxy]phenyl)piperazine) (CAS65277-42-1), isoconazole (1- [2-(2,4-dichlorophenyl)−2-[(2,6-dichlorophenyl) methoxy]ethyl]−1H-imidazole) (CAS, 27523- 40-6), miconazole ((±)−1-[2-(2,4-dichlorobenzyloxy)−2-(2,4-dichlorophenyl)ethyl]−1H-imidazole) (CAS, 22916–47–8), penconazole (1-[2-(2,4-diclorofenil) pentyl]−1,2,4-triazole) (CAS, 66246-88-6) sertaconazole (1-(2-((7-chlorobenzo[β]thiophen-3-yl)methoxy)−2-(2,4-dichlorophenyl)ethyl)−1H-imidazole) (CAS, 99592-32-2), tebuconazole (1-(4-chlorophenyl)−4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol) (CAS, 107534-96-3) terconazole(1-(4-((2-(2,4-dichlorophenyl)−2–1H-1,2,4-triazol-1-ylmethyl)−1,3-dioxolan-4-yl)methoxy)phenyl)−4-(1-methylethyl)piperazine) (CAS, 67915-31-5) and voriconazole (2R,3S-2-(2,4-difluorophenyl)−3-(5-fluoropyrimidin-4-yl)−1-(1H-1,2,4-triazol-1-yl)butan-2-ol) (CAS, 137234-62-9) having purity ≥ 97.0% were purchased from Aldrich-Fluka-Sigma S.r.l. (Milan, Italy).

To prepare the stock solutions (1 mg/mL), the weighted powder of each analyte was dissolved in MeOH in a 2 mL polypropylene microcentrifuge tube.

The working solutions (at 100 μg/mL) were obtained by diluting each stock solution in ACN/H_2O 50/50 *(v/v)* or MeOH/ H_2O 50/50 *(v/v)* or EtOH/MeOH 50/50 *(v/v)*. All standard solutions were stored at −18 °C.

50 mL of stock buffer solutions at a concentration of 500 mM were set every week as follows: formic acid or acetic acid, after dilution with ultrapure water, were titrated with 5 M ammonia solution to prepare ammonium formate or ammonium acetate buffer at the desired pH; ammonium hydrogen carbonate, at pH 9–11 was obtained dissolving the weighed amount of the powder ultrapure water and titrated with an ammonia solution (approx. 5 M).

The 10 mL glass volumetric flask of polar organic mobile phases was prepared everyday by dissolving the proper volume of buffer solution in ACN/water, MeOH/water ACN/MeOH/water mixture.

Instrumentation

A Crison Basic pH 20 (Crison Instruments SA, Barcelona, Spain) equipped with the combined electrode and a temperature sensor was used for accurate pH buffer measurement by three-point calibration with the appropriate certified buffer solutions at pH 4.01, 7.00, and 9.21.

An ultrasonic bath (model FS 100b Decon, Hove, UK) was helpful in sonicating mobile phases, dissolving analytes, and making homogeneous the slurry of stationary phase and packing bed into a capillary column during the packing process.

A Stereozoom 4 optical microscope (Bausch & Lomb, Rochester, New York, USA) was used to observe the column during the packing procedure and check its status.

An HPLC pump (Perkin Elmer Series 10, Palo Alto, CA, USA) was used for packing the column and for its equilibration with a mobile phase when deemed appropriate.

A silica capillary (Polymicro Technologies™, Silsden, UK), with 375 μm O.D. and 75 μm I.D., was utilized to prepare capillary columns for CEC experiments.

The CEC experiments were performed with the Agilent ^{3D}CE system (Agilent Technologies, Waldbronn, Germany), equipped with a diode-array UV detector and an autosampler. The detector was set at 205 nm with a rise time of 0.5 s and 20 Hz while an air thermostat system controlled the column temperature (20 °C). A Chemstation software (Rev. A.09.01, Agilent Technologies) controlled the instrument, data collection, and reprocessing.

Chiral stationary phase and capillary column packing procedure

The CDCPC, immobilized on 5 μm silica particles, was used for CEC experiments. This material was provided by Enantiosep GmbH (Münster, Germany).

A previously described procedure, based on the slurry packing method, was used for packing the CSP into the capillary columns [15].

Based on our experience, to obtain a homogeneous slurry, ACN was selected as the slurry medium with the optimum CSP concentration of about 50 mg/mL. The packed bed homogeneity was ensured by high backpressure during preparation, 30–35 MPa, and continued use of the ultrasonic bath achieving stable CSP suspension into both pre-column and silica capillary. In addition, the stationary phase was blocked into the capillary using semi-permeable frits sintered silica. These were obtained at maximum pressure in an 80/20 ACN/distilled water (v/v) mixture and by heating a capillary portion for 7 s at 650–700 °C utilizing a lab-made heating wire system. Packed capillary length 25.0 cm, effective length 26.5 cm.

Capillary electrochromatographic separation of fungicide enantiomers

The packed capillary was firstly flushed with the mobile phase using an HPLC pump at 10 MPa, then placed into the CE cartridge and finally into the instrument. A voltage ramp from +5 to +25 kV for 30 min for conditioning the column.

At the end of the working day, the two capillary ends were submerged into the vials containing MeOH/water 90:10 (v/v). Both vials

Table 1
Effect of pH on retention factor (k_1),enantioselectivity (α) and enantioresolution (Rs) of studied azole antifungal drugs. CEC conditions: 100 μm I.D. packed with i-CDCPCCP (5 μm) Lpack = 25 cm, Leff = 26.5 cm; mobile phase, 5 mM buffer (pH 3.0–10.0) in ACN/water (95:5, v/v); injection: 10 bar x 0.5 min; applied voltage:+20 kV; column temperature: 20 °C; detection wavelength: 205 nm. For other experimental conditions, see the text.

		$pKa^{(*)}$	pH							
			3.0	4.0	5.0	6.0	7.0	7.9	9.0	10.0
T_{eof} (min)			2.4	2.4	2.5	2.7	2.5	2.6	2.5	2.4
	k_1		–	3.4	3.1	2.5	3.3	2.0	1.8	2.4
Bifonazole	α	6.36	–	–	–	1.04	1.04	1.05	1.05	1.05
	Rs		–	–	–	0.6	0.7	0.6	0.6	0.7
	k_1		–	5.1	4.4	4.3	4.8	4.7	4.8	5.0
Butoconazole	α	6.51	–	1.16	1.16	1.17	1.17	1.17	1.15	1.16
	Rs		–	0.9	2.8	2.9	3.0	2.8	2.7	2.8
	k_1		–	3.8	3.7	3.6	3.9	3.7	3.6	3.4
Econazole	α	6.48	–	0.92	1.14	1.14	1.15	1.14	1.17	1.16
	Rs		–	1.6	2.5	2.3	2.6	2.7	2.5	2.6
	k_1		–	–	5.0	4.7	4.9	4.5	4.8	4.9
Fenticonazole	α	6.48	–	–	1.23	1.23	1.22	1.20	1.23	1.24
	Rs		–	–	2.7	2.4	2.8	2.7	2.7	2.8
	k_1		5.4	5.6	5.3	4.5	5.1	3.8	3.6	4.5
Isoconazole	α	6.48	1.15	1.16	1.14	1.15	1.16	1.14	1.15	1.15
	Rs		1.3	1.1	2.5	2.3	2.6	2.5	2.3	2.5
	k_1		6.1	5.9	5.3	4.5	4.8	3.7	3.6	4.2
Ketoconazole	α	6.42	–	–	1.14	1.15	1.15	1.16	1.14	1.17
	Rs		–	–	2.5	2.4	2.6	2.4	2.4	2.5
	k_1		4.0	4.5	5.2	4.8	5.2	4.4	4.1	4.0
Miconazole	α	6.48	1.15	1.16	1.15	1.18	1.18	1.19	1.18	1.19
	Rs		1.9	1.8	2.3	2.4	2.5	2.4	2.8	2.8
	k_1		–	–	–	1.4	1.6	1.7	1.7	1.8
Penconazole	α	1.51	–	–	–	1.16	1.16	1.18	1.18	1.17
	Rs		–	–	–	1.7	1.8	1.9	2.2	2.1
	k_1		5.1	5.5	6.0	5.0	5.7	5.7	4.7	3.9
Sertaconazole	α	6.48	1.10	1.13	1.11	1.11	1.13	1.12	1.11	1.12
	Rs		1.8	2.0	2.1	1.8	2.0	1.9	1.8	2.1
	k_1		–	–	–	0.8	1.1	1.2	1.8	2.1
Tebuconazole	α	5.00	–	–	–	1.12	1.10	1.08	1.17	1.14
	Rs		–	–	–	0.9	1.0	1.3	1.2	1.3
	k_1		–	–	12.4	11.6	12.6	12.4	12.1	12.4
Terconazole	α	8.45	–	–	1.06	1.08	1.04	1.11	1.14	1.11
	Rs		–	–	1.3	1.2	1.1	2.4	2.5	2.1
	k_1		5.6	5.1	5.1	4.9	5.1	5.2	4.6	5.2
Voriconazole	α	12.70	–	–	1.15	1.14	1.15	1.17	1.17	1.12
	Rs		–	–	2.2	2.5	2.2	2.4	2.5	2.4

(*) MarvinSketch 19.24.0 (hpp://www.chexon.com).

were pressurized at 10 bar during the experiments. The individual racemic fungicide mixtures were hydrodynamically injected, applying pressure of 10 bar for 0.5 min at the anodic end. The standard separation voltage was 25 kV, while a linear voltage ramp (+25 to +30 kV in 15 min) was applied with the selected mixture.

Results and discussion

Effect of pH and buffer concentration of the mobile phase on the enantioseparation of the studied fungicides

Effect of the mobile phase pH on the enantioresolution of the studied fungicides

Based on previously published data about the enantiomeric separation of antifungal compounds, utilizing columns containing CSPs polysaccharide-based, by nano-LC and CEC [10,14], polar organic-water mobile phases were selected in this study. It is known that, in CEC, compounds move toward the detector influenced by their electrophoretic mobility and electroosmotic flow. Therefore, the selection of a suitable buffer system affects various aspects of the chromatographic separation, e.g., the degree of protonation/deprotonation of free silanols onto the stationary phase and/or on the capillary wall, the charge of the chiral selector and analytes, etc. In this study, a series of antifungal compounds, including eight imidazole derivatives and four triazole derivatives were selected as model compounds.

The chiral discrimination via reversible partitioning mechanism with the chiral selector (CS), as in the case of CSP-polysaccharides, is highly complex and relies on both intermolecular forces and the geometry of the chiral molecule. In separation science, experimental factors that can influence the interaction between analyte and CS include controlling the mobile phase composition, i.e., pH, ionic strength, organic phase content, and the type of solvent (whether protic or aprotic) strongly influence the retention and enantioresolution of the studied compounds.

To study these aspects, buffered aqueous solutions were prepared at different pH levels using ammonium formate (pH 3), ammonium acetate (pH 6–7), and ammonium bicarbonate (pH 8–10), all at the final concentration of 5 mM. CEC analyses were carried out using isocratic elution, with a mobile phase composed of acetonitrile/buffered water (95:5, v/v).

Table 1 reports comparative results demonstrating the effect of pH on the enantiomeric resolution of the studied antifungal drugs.

As can be viewed, an increase in the pH leads to a general increase of the enantioresolution factor $k_1\,\alpha$ up to pH 8–9, while at pH 10, the values are statistically unchanged.

While the phenylcarbamate group of the CSP remains uncharged within the studied pH range, it is likely that a strong electrostatic interaction that could be occurs between the analyte and the chiral selector it was a negligible effect. The amide group may facilitate the formation of hydrogen bonds with the analyte, thereby orienting each enantiomer within the chiral site of the CSP. Consequently, pH changing, by influencing the charge of analytes, could either promote or inhibit the formation of secondary CS interactions, and then a possible

Table 2
Effect of buffer concertation on retention factor (k_1), selectivity (α), enantioresolution (Rs), and peak efficiency (N_1/m) of studied azole antifungal drugs. CEC conditions: 100 μm I.D. packed with i-CDCPC (5 μm) Lpack = 25 cm, Leff = 26.5 cm; mobile phase, 5 mM ammonium bicarbonate pH 9.0 in ACN/water (95:5, v/v). For other experimental conditions, see the text.

		Buffer concentration (mM)		
		2.5	5.0	7.5
T_{eof} (min)		2.6	2.5	2.4
Bifonazole	k_1	1.4	1.8	1.9
	α	1.05	1.05	1.05
	Rs	<0.5	<0.5	<0.5
	N_1/m	–	–	–
Butoconazole	k_1	2.7	4.8	3.2
	α	1.19	1.15	1.19
	Rs	2.0	2.7	2.4
	N_1/m	23,305	28,067	27,080
Econazole	k_1	2.2	3.6	2.5
	α	1.19	1.17	1.17
	Rs	1.8	2.5	1.8
	N_1/m	25,345	31,550	29,463
Fenticonazole	k_1	3.1	4.8	3.0
	α	1.19	1.23	1.19
	Rs	2.1	2.7	2.1
	N_1/m	24,658	30,563	26,234
Isoconazole	k_1	2.8	3.6	3.6
	α	1.16	1.15	1.16
	Rs	1.7	2.3	1.9
	N_1/m	25,327	28,844	26,354
Ketoconazole	k_1	2.8	3.6	3.6
	α	1.16	1.14	1.16
	Rs	1.9	2.4	2.0
	N_1/m	26,456	28,814	27,354
Miconazole	k_1	3.1	4.1	3.9
	α	1.19	1.18	1.19
	Rs	1.9	2.8	2.4
	N_1/m	25,846	29,182	28,542
Penconazole	k_1	0.7	1.7	1.4
	α	1.21	1.18	1.16
	Rs	1.7	2.2	2.0
	N_1/m	38,532	42,059	41,354
Sertaconazole	k_1	3.2	4.7	4.1
	α	1.13	1.11	1.13
	Rs	1.6	1.8	1.7
	N_1/m	25,733	28,183	27,463
Tebuconazole	k_1	1.0	1.8	1.0
	α	1.10	1.17	1.12
	Rs	0.9	1.2	1.1
	N_1/m	–	–	–
Terconazole	k_1	12.4	12.1	12.0
	α	1.16	1.14	1.10
	Rs	2.5	2.5	2.0
	N_1/m	16,844	19,925	17,533
Voriconazole	k_1	4.2	4.6	5.3
	α	1.16	1.17	1.15
	Rs	1.7	2.5	1.9
	N_1/m	26,842	29,184	27,357

Table 3
Effect of water content on retention factor (k_1), selectivity (α), enantioresolution (Rs), and peak efficiency (N_1/m) of studied azole antifungal drugs. CEC conditions: CEC conditions: mobile phase, 5 mM ammonium bicarbonate pH 9.0 in ACN/water. For other experimental conditions, see the text.

		water content (%, v/v)			
		5	10	15	20
t_{eof} (min)		2.5	2.8	2.9	3.2
Bifonazole	k_1	1.8	2.1	2.3	2.5
	α	1.05	1.05	1.70	1.08
	Rs	0.6	0.6	0.5	0.5
	N_1/m	–	–	–	–
Butoconazole	k_1	4.8	4.4	4.5	5.1
	α	1.15	1.15	1.13	1.11
	Rs	2.7	2.2	1.9	1.8
	N_1/m	28,067	17,009	13,365	12,213
Econazole	k_1	3.6	3.2	3.3	4.1
	α	1.17	1.13	1.13	1.10
	Rs	2.5	1.7	1.8	1.6
	N_1/m	31,550	19,013	17,869	11,988
Fenticonazole	k_1	4.8	4.0	3.8	4.1
	α	1.23	1.11	1.13	1.11
	Rs	2.7	2.1	2.2	2.0
	N_1/m	30,563	19,017	16,275	13,905
Isoconazole	k_1	3.6	3.7	4.0	4.2
	α	1.15	1.13	1.12	1.11
	Rs	2.3	1.9	2.0	1.6
	N_1/m	28,844	21,349	19,670	12,718
Ketoconazole	k_1	3.6	4.0	4.3	4.5
	α	1.14	1.13	1.11	1.12
	Rs	2.4	1.8	1.7	1.8
	N_1/m	28,814	16,417	14,637	11,427
Miconazole	k_1	4.1	4.3	4.7	5.0
	α	1.18	1.15	1.14	1.13
	Rs	2.8	2.2	2.4	2.4
	N_1/m	29,182	23,377	17,895	16,621
Penconazole	k_1	1.7	1.3	1.5	2.5
	α	1.18	1.18	1.16	1.15
	Rs	2.2	1.8	1.7	1.4
	N_1/m	42,059	33,235	27,812	10,629
Sertaconazole	k_1	4.7	5.9	6.0	6.2
	α	1.11	1.11	1.13	1.10
	Rs	1.8	1.6	1.6	1.4
	N_1/m	28,183	21,007	15,498	13,523
Tebuconazole	k_1	1.8	1.3	1.4	1.8
	α	1.17	1.04	1.04	1.05
	Rs	1.2	0.8	0.8	0.6
	N_1/m	–	–	–	–
Terconazole	k_1	12.1	12.9	13.2	13.5
	α	1.14	1.08	1.05	1.11
	Rs	2.5	2.2	2.9	2.8
	N_1/m	19,925	14,016	12,832	10,223
Voriconazole	k_1	4.6	5.4	5.6	5.8
	α	1.17	1.12	1.13	1.12
	Rs	2.5	1.6	1.9	1.7
	N_1/m	29,184	15,686	14,190	12,712

discriminating factors for chiral recognition.

At low pH (pH=3.0), only sertaconazole were baseline resolved (Rs>1.5), while miconazole and isoconazole exhibited an Rs= 1.9 and 1.3, respectively. All other compounds were poorly or not separated in their enantiomers. An increase of the mobile phase pH caused an improved enantioresolution for all studied compounds.

For bifonazole, butoconazole, econazole, fenticonazole, ketoconazole, and terconazole, a progressive increase in chiral resolution was observed as they were in the non-charged state. However, other analytes exhibited different behaviors. At pH 3, despite the protonation of the imidazole nitrogen in isoconazole, miconazole (an isoconazole isomer), and sertaconazole, chiral discrimination was still observed. Conversely, nearly neutral analytes within the pH range, such as penconazole, tebuconazole, and voriconazole, showed enantioresolution only up to pH 6–7. These results suggest that pH-dependent hydrogen bonding also promotes the interaction with the chiral selector.

Concerning the CSP cellulose (3,5-dichlorophenylcarbamate), the introduction of electron-withdrawing halogen groups onto the phenyl moieties markedly modified/increased the carbamate residues' polarities.

Generally, the CSP amylose or cellulose-based has the helical carbon backbone with phenylcarbamate moieties as side chains. In this high-ordered secondary structure, precisely the phenylcarbamate groups form grooves and ravines containing both polar and hydrophobic regions where a lot of noncovalent interactions, such as hydrophobic, hydrogen bonding, π-π, and dipole-type interactions, are responsible for accommodating and discriminating enantiomers [16–18].

Apart from hydrogen bonding, which involves molecular groups containing oxygen, nitrogen, and sulfur, the interaction of aromatic groups bearing highly electronegative halogen atoms contributes to enantiomeric discrimination too.

Table 4
Effect of MeOH (in ACN/MeOH ratio) content on retention factor (k_1), selectivity (α), enantioresolution (Rs), and peak efficiency (N_1/m) of studied azole antifungal drugs. CEC conditions: mobile phase, 5 mM ammonium bicarbonate pH 9.0, 95/5 (v/v) organic phase/water. For other experimental conditions, see the text.

		Methanol content (%, v/v)(*)							
		0	10	20	30	40	50	60	70
t_{eof} (min)		2.5	2.7	2.7	3.3	3.8	4.7	6.1	7.4
	k_1	1.84	1.43	1.23	1.06	1.01	0.91	0.99	1.20
Bifonazole	α	1.05	1.05	1.05	1.04	1.04	1.05	1.05	1.06
	Rs	0.6	0.6	0.6	0.5	0.5	0.6	0.6	0.6
	N_1/m	–	–	–	–	–	–	–	–
	k_1	4.8	2.4	2.0	1.7	1.6	1.5	1.6	2.0
Butoconazole	α	1.15	1.20	1.21	1.22	1.23	1.23	1.25	1.28
	Rs	2.7	2.6	2.8	2.9	3.1	3.2	3.6	3.9
	N_1/m	28,067	24,590	29,154	33,235	35,845	38,474	38,779	37,495
	k_1	3.6	1.9	1.5	1.2	1.2	1.1	1.3	1.5
Econazole	α	1.17	1.15	1.13	1.11	1.10	1.11	1.10	1.11
	Rs	2.5	1.9	1.7	1.4	1.4	1.2	1.4	1.4
	N_1/m	31,550	26,767	32,593	38,699	42,968	–	39,532	37,541
	k_1	4.8	2.7	2.1	1.8	1.7	1.6	1.9	2.0
Fenticonazole	α	1.23	1.16	1.14	1.12	1.11	1.12	1.10	1.10
	Rs	2.7	2.1	1.9	1.7	1.6	1.6	1.5	1.6
	N_1/m	30,563	27,560	29,312	31,274	34,369	31,736	33,515	35,634
	k_1	3.6	2.6	2.1	1.7	1.6	1.5	1.7	1.8
Isoconazole	α	1.15	1.14	1.13	1.11	1.11	1.11	1.10	1.10
	Rs	2.3	1.9	1.9	1.6	1.5	1.4	1.5	1.4
	N_1/m	28,844	25,965	30,087	34,005	37,181	34,761	37,079	34,302
	k_1	3.6	5.7	5.1	5.0	4.5	4.5	5.1	5.5
Ketoconazole	α	1.14	1.18	1.17	1.19	1.20	1.20	1.17	1.18
	Rs	2.4	2.7	2.9	2.9	3.4	3.0	2.9	3.0
	N_1/m	28,814	25,743	26,942	27,331	25,089	23,514	30,839	27,035
	k_1	4.1	3.1	2.3	2.0	1.7	1.7	2.0	2.3
Miconazole	α	1.18	1.16	1.14	1.12	1.12	1.11	1.11	1.10
	Rs	2.8	2.2	1.7	1.7	1.6	1.6	1.5	1.5
	N_1/m	29,182	25,930	26,803	33,509	33,650	33,536	31,563	33,683
	k_1	1.7	1.0	0.9	0.8	0.8	0.7	0.7	0.8
Penconazole	α	1.18	1.20	1.23	1.25	1.26	1.26	1.28	1.27
	Rs	2.2	2.6	2.6	2.7	2.6	2.7	2.9	2.9
	N_1/m	42,059	46,749	43,596	41,713	40,345	43,915	45,038	40,728
	k_1	4.7	3.1	2.4	2.0	1.8	1.7	1.8	2.2
Sertaconazole	α	1.11	1.12	1.12	1.10	1.10	1.10	1.09	1.09
	Rs	1.8	1.7	1.5	1.6	1.3	1.4	1.4	1.4
	N_1/m	28,183	23,943	25,557	38,995	32,921	32,327	38,440	32,786
	k_1	1.8	0.7	0.5	0.5	0.4	0.4	0.4	0.5
Tebuconazole	α	1.17	1.10	1.09	1.12	1.05	1.03	1.04	1.05
	Rs	1.2	0.9	0.6	0.4	0.4	0.4	0.4	0.4
	N_1/m	–	–	–	–	–	–	–	–
	k_1	12.1	6.2	4.3	3.6	3.3	3.5	4.2	5.6
Terconazole	α	1.14	1.14	1.14	1.12	1.12	1.14	1.12	1.13
	Rs	2.5	1.8	2.0	2.0	1.7	2.0	1.9	2.1
	N_1/m	19,925	17,672	24,977	26,928	25,413	25,132	25,839	23,308
	k_1	4.6	2.7	2.1	1.7	1.6	1.5	1.6	1.9
Voriconazole	α	1.17	1.15	1.14	1.11	1.10	1.11	1.10	1.09
	Rs	2.5	2.3	1.8	1.1	1.0	0.9	1.5	1.4
	N_1/m	29,184	29,368	30,386	–	–	–	32,453	32,627

(*) The content of organic phase was 95 % (v/v) as ACN/MeOH ratio.

However, many factors such as the interactions between molecular groups shown in π-π interaction that allow molecular orientation into the chiral cavity of CS may affect the chiral separation. At the same time, large aromatic groups, as in the bifonazole structure, could be an important steric encumbrance for CS. In this regard, this analyte shows the lowest chiral resolution probably due to both the lack of halogen substituents and an important steric effect of chiral carbon.

Based on the findings, it was observed that the chiral resolutions were significantly enhanced in a weakly basic environment. Specifically, when utilizing a mobile phase prepared with an ammonium bicarbonate buffer at pH 9, remarkable improvements were observed in peak shapes, efficiencies, and electroosmotic flow, leading to an incread enantioresolution compared to different buffer types [19]. Similar results were obtained in HPLC and nano-LC for the separation of some fungicide enantiomers using a cellulose-based Chiralpak IC CSP and the i-CDCPC, respectively [10,20]. Therefore, a mobile phase with the buffer at pH 9 was selected for further experiments.

Effect of buffer concentration on the fungicide enantioresolution

The mobile phase ACN/water (95:5, v/v) was supplemented with 5 mM of bicarbonate buffer at concentrations in the range of 2.5–7.0 mM for CEC experiments. Table 2 reports the effect of the buffer concentration on some chromatographic parameters.

As can be observed, the increase of the buffer concentration, and thus the ionic strength of the mobile phase, led to slightly higher retention factors and analysis times (data not shown). The results at 2.5 and 5 mM suggest the involvement of an ion exchange mechanism that, reducing chromatographic band broadening, entails a consequent improvement of enantioresolution contrary to what was observed in nano-LC [8]. A further increase in the buffer concentration at 7.5 mM caused a decrease in the enantioresolution factors. While in pressure-driven chromatographic techniques, increasing the ionic strength can be a strategy to improve peak shape and efficiency, in CEC, this effect could overlap with the Joule heating effect, leading to the opposite outcome as it was actually observed. Based on the obtained results and analysis of the

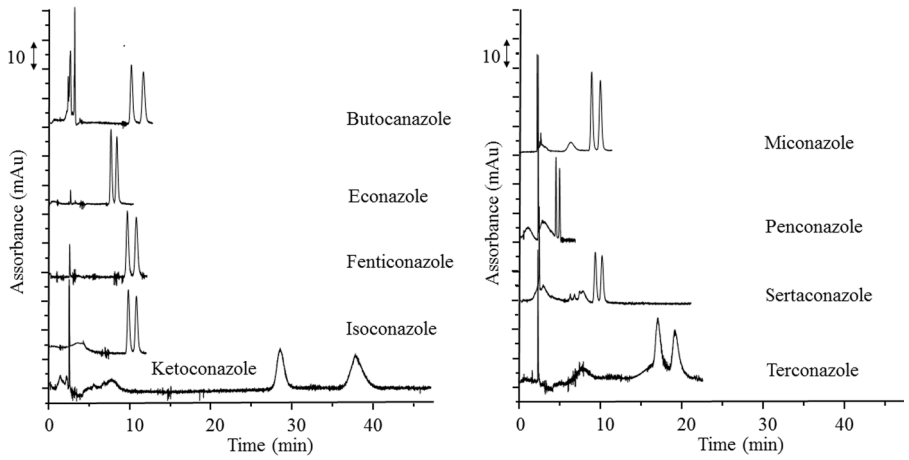

Fig. 2. CEC chiral separation of the studied antifungal drugs. Sample, 100 μg/mL in 50/50 MeOH/water; mobile phase, 5 mM ammonium bicarbonate (pH 9) in 10/85/5 (v/v/v) MeOH/ACN/H_2O); Inj: 10 bar x 0.3–0.5 min; applied Voltage: +25 kV; Detection, 205 nm. 10 bars pressure on both vials. For other experimental conditions, see text.

electrochromatograms, a buffered mobile phase of 5.0 mM at pH 9 was selected for further studies. The highest efficiency (N/m) for all studied fungicide enantiomers was observed at 5 mM buffer concentration in the mobile phase.

Effect water content and organic modifier in the mobile phase

The effect of the water content in the mobile phase on chromatographic parameters for CEC enantiomers separation was studied in the range of 5–20%, v/v keeping constant the buffer concentration (5 mM at pH 9.0). Table 3 shows the obtained data.

As can be noted, a higher water percentage at the expense of ACN caused a decrease of the electroosmotic flow (EOF) due to the increase of the mobile phase viscosity. Additionally, a general rise in the retention factor was observed. This is likely related to a reversed-phase interaction mechanism between the analytes and the stationary phase.

Although the analytes exhibited higher retention on the column, the greatest enantioresolution factors were obtained at 5%, v/v of water. The increase of the amount of water caused an increase of the current within the range of 0.8–2.5 μA along with the broadening of the chromatographic band (reduction in theoretical plates) and, consequently, lower *Rs*. Based on these results, the 95/5 ACN/water ratio was selected as the best compromise among *Rs*, analysis time, and efficiency.

A further study was carried out modifying the type of the organic modifier by introducing a protic solvent. Methanol was added to the mobile phase reducing the content of ACN. A mixture of ACN/MeOH in the range 95/0 to 25/75 (v/v) was used to study its effect on enantiomeric separation. Table 4 reports some electrochromatographic data modifying the mobile phase with MeOH.

Introducing a protic organic modifier in the presence of acetonitrile can produce an effect already observed in nano-LC during the enantiomeric separation of flavanone derivatives and antifungal compounds [10,15]. As expected, the EOF decreased by increasing the MeOH content. The retention factor for all studied fungicides also varied following a U-shaped profile. A minimum value of k_1 was observed for intermediate MeOH/ACN mixtures. At the same time, it was higher when the mobile phase was rich in MeOH or ACN, indicating a greater affinity for the stationary phase.

An improved chromatographic efficiency is usually observed with increasing MeOH content. In this work, unexpectedly, the effect of reduced chromatographic band dispersion with higher MeOH content only increased chiral resolution for some studied compounds

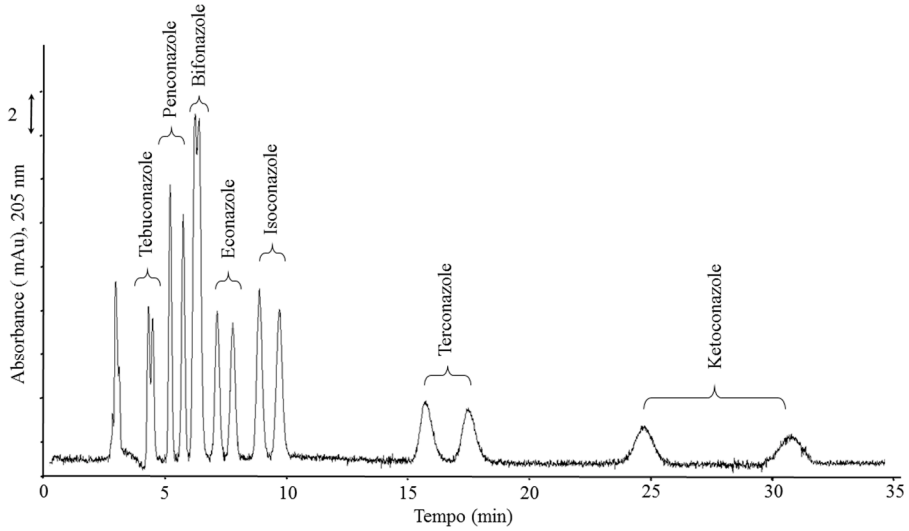

Fig. 3. Electrochromatogram of the chiral separation of seven azole antifungal enantiomers. Experimental conditions: sample concentration, 100 μg/mL in 50/50 MeOH/EtOH; detector, 205 nm; injection, electrokinetic 10 kV, 10 s; $T = 20$ °C; applied voltage: +25 kV, $t = 15$–20 min gradient mode (1 kV/min); $t = 20$ min, $V = 30$ kV. For other experimental conditions, see Fig. 2 and text.

(butoconazole, ketoconazole, and penconazole). As shown in Table 4, most of the analytes exhibited the opposite effect. This trend could be attributed to the presence of hydrogen bonding, which promotes stereospecific interactions between the analytes and the stationary phase likely favored by an aprotic solvent. On the other hand, the small effect observed for bifonazole, terconazole and the increase in *Rs* for butoconazole, ketoconazole, and penconazole are likely related to their molecular structure and could be worth of further study.

Fig. 2 reports the electrochromatograms of some selected racemic fungicides obtained by CEC.

Fig. 3 reports the CEC enantioseparation of a sample mixture containing selected racemic fungicides. In this analysis, to reduce the separation time, a gradient voltage was applied achieving the chiral separation in less than 30 min. In addition, the sample mixture was prepared by dissolving the stock standard solutions in MeOH/EtOH, (*v/v*) solution that resulted more effective than other solvents (higher efficiency and enantioresolution) due to the stacking effect of sample band.

The robustness of the CEC-UV-based methodology and the stability of the stationary phase were evaluated on a mixture of seven antifungal analytes by performing a method repeatability study. Under the final experimental conditions, the mixture was injected six times each ($n = 6$) for three consecutive days ($n = 18$), assessing the variance in retention times and peak areas of the selected target analytes penconazole, econazole, isoconazole, terconazole, and ketoconazole.

After hundreds of injections and tens of hours of operations under various stress conditions, the capillary column exhibited relative standard deviation (RSD) values for retention times in the range of 3.5–6.3 % for the same day and 5.9–7.1 % for different days. Additionally, RSD values for intra- and inter-day precision of peak areas were found to be in the range of 5.9–7.1 % and 6.9–9.8 %, respectively.

Conclusion

This experimental work focuses on the development of an analytical method for the chiral separation of selected racemic mixtures of imidazole and triazole derivatives using CEC coupled with a spectrophotometric detector. A laboratory-packed capillary column 100 μm I.D., packed with a polysaccharide-based CSP, enabled the separation of 12 racemic mixtures, 10 out of which reached the baseline separation.

The chiral recognition was investigated changing the mobile phase composition, including organic and aqueous phase content, type and pH buffer, and concentration; the effects of such modifications on retention factors, enantioresolution, and band broadening peak were thoroughly evaluated.

The developed method was used as an appropriate test bench to assess the stability of the stationary phase under various mobile phase conditions; exceptional enantioselective capabilities were exhibited even after prolonged and continuous usage. In addition, the optimized CEC-UV method demonstrated remarkable effectiveness in the chiral separation of seven out of twelve chiral azole fungicides, separating fourteen peaks within a single 30-minute run analysis.

Authors' contribution

Massimo Giuseppe De Cesaris: formal analysis. Giovanni D'Orazio: investigation, writing, supervision. Chiara Fanali: investigation, writing, editing. Alessandra Gentili: Review & editing, supervision. Salvatore Fanali: Review & editing, supervision, project administration.

CRediT authorship contribution statement

Massimo Giuseppe De Cesaris: Formal analysis. **Giovanni D'Orazio:** . **Chiara Fanali:** Writing – review & editing, Investigation, Formal analysis. **Alessandra Gentili:** Supervision, Formal analysis. **Salvatore Fanali:** Writing – review & editing, Supervision, Methodology.

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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References

[1] J. Li, F. Dong, J. Xu, X. Liu, Y. Li, W. Shan, Y. Zheng, Enantioselective determination of triazole fungicide simeconazole in vegetables, fruits, and cereals using modified QuEChERS (quick, easy, cheap, effective, rugged and safe) coupled to gas chromatography/tandem mass spectrometry, Anal. Chim. Acta 702 (2011) 127–135, https://doi.org/10.1016/j.aca.2011.06.034.

[2] Z.Zheng Y.Tao, Y. Yu, J. Xu, X. Liu, X. Wu, F. Dong, Y. Zheng, Supercritical fluid chromatography-tandem mass spectrometry-assisted methodology for rapid enantiomeric analysis of fenbuconazole and its chiral metabolites in fruits, vegetables, cereals, and soil, Food Chem. 241 (2018) 32–39, https://doi.org/10.1016/j.foodchem.2017.08.038.

[3] L. Toribio, M.J. del Nozal, J.L. Bernal, C. Alonso, J.J. Jiménez, Enantiomeric separation of several antimycotic azole drugs using supercritical fluid chromatography, J. Chromatogr. A 1144 (2007) 255–261, https://doi.org/10.1016/j.chroma.2007.01.047.

[4] M. Garzotti, M. Hamdan, Supercritical fluid chromatography coupled to electrospray mass spectrometry: a powerful tool for the analysis of chiral mixtures, J. Chromatogr. B 770 (2002) 53–61, https://doi.org/10.1016/S1570-0232(01)00582-7.

[5] G. D'Orazio, C. Fanali, C. Dal Bosco, A. Gentili, S. Fanali, Chiral separation and analysis of antifungal drugs by chromatographic and electromigration techniques: results achieved in 2010-2020, Rev. Anal. Chem. 40 (2021) 220–252, https://doi.org/10.1515/revac-2021-0136.

[6] X. Jing, H. Xue, X. Sang, X. Wang, L. Jia, Magnetic deep eutectic solvent-based dispersive liquid-liquid microextraction for enantioselectively determining chiral mefentrifluconazole in cereal samples via ultra-high-performance liquid chromatography, Food Chem. 391 (2022) 133220, https://doi.org/10.1016/j.foodchem.2022.133220.

[7] H. Jiang, X. Huang, H. Xue, M. Wang, Y. Qi, L. Jia, X. Jing, Switchable deep eutectic solvent-based homogenous liquid-liquid microextraction combined with high-performance liquid chromatography-diode-array detection for the determination of the chiral fungicide mefentrifluconazole in water, fruit juice, and fermented liquor, Chirality 34 (2022) 968–976, https://doi.org/10.1002/chir.23445.

[8] E. Lucci, L. Antonelli, M. Gherardi, C. Fanali, S. Fanali, A. Scipioni, P. Lupattelli, A. Gentili, B. Chankvetadze, A liquid chromatography-mass spectrometry method for the enantioselective multiresidue determination of nine chiral agrochemicals in urine using an enrichment procedure based on graphitized carbon black, Anal. Bioanal. Chem. (2023), https://doi.org/10.1007/s00216-023-05098-4.

[9] E. Lucci, C. Dal Bosco, L. Antonelli, C. Fanali, S. Fanali, A. Gentili, B. Chankvetadze, Enantioselective high-performance liquid chromatographic separations to study occurrence and fate of chiral pesticides in soil, water, and agricultural products, J. Chromatogr. A 1685 (2022) 463595, https://doi.org/10.1016/j.chroma.2022.463595.

[10] C. Dal Bosco, F. Bonoli, A. Gentili, C. Fanali, G. D'Orazio, Chiral nano-liquid chromatography and dispersive liquid-liquid microextraction applied to the analysis of antifungal drugs in milk, Molecules 26 (2021) 7094, https://doi.org/10.3390/molecules26237094.

[11] C. Fanali, S.Della Posta, Capillary electromigration techniques applied to pollutant analysis- a review, J. Chromatogr. Open 4 (2023) 100101, https://doi.org/10.1016/j.jcoa.2023.100101.

[12] M. Ma, Y. Xi, Y. Du, J. Yang, X. Ma, C. Chen, Maltodextrin-modified graphene oxide for improved enantiomeric separation of six basic chiral drugs by open-tubular capillary electrochromatography, Mikrochim. Acta. 187 (2019) 55, https://doi.org/10.1007/s00604-019-4037-x.

[13] B. Chankvetadze, I. Kartozia, J. Breitkreutz, Y. Okamoto, G. Blaschke, Effect of organic solvent, electrolyte salt and a loading of cellulose tris (3,5-dichlorophenylcarbamate) on silica gel on enantioseparation characteristics in capillary electrochromatography, Electrophoresis 22 (2001) 3327–3334, https://doi.org/10.1002/1522-2683(200109)22:15<3327:AID−ELPS3327>3.0.CO;2-J.

[14] V. Perez-Fernandez, E. Dominguez-Vega, B. Chankvetadze, A.L. Crego, M. A. Garcia, M.L. Marina, Evaluation of new cellulose-based chiral stationary phases Sepapak-2 and Sepapak-4 for the enantiomeric separation of pesticides by nano liquid chromatography and capillary electrochromatography, J. Chromatogr. A 1234 (2012) 22–31, https://doi.org/10.1016/j.chroma.2012.01.035.

[15] G. D'Orazio, C. Fanali, M. Karchkhadze, B. Chankvetadze, S. Fanali, Enantiomeric separation of some chiral analytes using amylose 3,5-dimethylphenylcarbamate covalently immobilized on silica by nano-liquid chromatography and capillary electrochromatography, J. Chromatogr. A 1520 (2017) 127–134, https://doi.org/10.1016/j.chroma.2017.09.017.

[16] B. Chankvetadze, E. Yasima, Y. Okamoto, Dimethyl-, dichloroand chloromethyl-phenylcarbamate derivatives of amylose as chiral stationary phases for high performance liquid chromatography, J. Chromatogr. A 694 (1995) 101–109, https://doi.org/10.1016/0021-9673(94)00729-S.
[17] B. Chankvetadze, E. Yashima, Y. Okamoto, Chloromethylphenylcarbamate derivatives of cellulose as chiral stationary phases for high-performance liquid chromatography, J. Chromatogr. A 670 (1994) 39–49, https://doi.org/10.1016/0021-9673(94)80278-5.
[18] B. Zhu, F. Zhao, J. Yu, Z. Wang, Y. Song, Q. Li, Chiral separation and a molecular modeling study of eight azole antifungals on the cellulose tris(3,5-dichlorophenylcarbamate) chiral stationary phase, New J. Chem. 42 (2018) 13421–13429, https://doi.org/10.1039/C8NJ01845F.
[19] S. Salido-Fortuna, C. Dal Bosco, A. Gentili, M. Castro-Puyana, M.L. Marina, G. D'Orazio, S. Fanali, Enantiomeric analysis of drugs in water samples by using liquid-liquid microextraction and nano-liquid chromatography, Electrophoresis 44 (2023) 1177–1186, https://doi.org/10.1002/elps.202300025.
[20] J. Zhang, J. Sun, Y. Liu, J. Yu, X. Guo, Immobilized cellulose-based Chiralpak IC chiral stationary phase for enantioseparation of eight imidazole antifungal drugs in normal-phase, polar organic phase and reversed-phase conditions using high performance liquid chromatography, Chromatographia 82 (2019) 649–660, https://doi.org/10.1007/s10337-019-03688-y.