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Review

Enantiomeric separation of organophosphorus pesticides by high-performance liquid chromatography, gas chromatography and capillary electrophoresis and their applications to environmental fate and toxicity assays☆

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ABSTRACT

In recent years, the continuous evolution of the field of stereochemistry has produced a heightened awareness of the applications of pure enantiomers of agrochemicals. This review describes reports of the enantiomeric separation of commercial organophosphorus pesticides (OPs) and the applications of these methods to research on the enantioselectivity of the toxicity and environmental fate of these compounds. Chiral OPs can be analysed by high-performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE). These different separation techniques for OP enantiomers are briefly discussed, and their applications are presented.

Contents

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1. Introduction

The significance of molecular chirality has been widely recognised in the life sciences [1,2]. Many commercial agrochemicals in current use also have chiral centres and thus consist of enantiomers. Enantiomers of chiral compounds have identical physical–chemical properties and thus appear as a single compound in standard analyses. However, due to their stereoselective interactions in biological systems, enantiomers often behave drastically differently in their toxicity and environmental fate.

Among the most important agrochemicals currently used, organophosphorus pesticides (OPs) are widely employed because of their high activity, ease of use and rapid degradation under natural conditions. They have been widely applied as insecticides, herbicides, acaricides, fungicides, and plant growth regulators for controlling disease and growth. The first commercial OP (bladan) was synthesised in the 1930s by German chemist Gerhard Schrader [3]. Until the early 1960s, all OPs were achiral, except for a few compounds such as EPN, cyanofenphos, iprobenphos and edifenphos. In the late 1960s, chiral centres began to be introduced into OPs, giving rise to such compounds as methamidophos, profenofos and salithion [4]. According to Garrison, 30% of OPs currently used are chiral [5], and only one OP, bialaphos, which is a naturally occurring substance possessing a chiral centre, is commercialised in an optically pure state [4].

Since the 1960s, numerous studies have demonstrated that the enantiomers of chiral OPs generally possess different biological activities. Some results have shown that the preferred configuration could be reversed between and among different organisms or between in vitro and in vivo assays [4]. Therefore, in order to reduce pesticide use and protect the environment from unintended effects, it is greatly advantageous to separate the enantiomers of chiral OPs, to study their biological activities in pure enantiomeric forms, and eventually to market the enantiomer that shows higher biological activity.

Generally, there are two ways to obtain enantiopure standards. The first is enantioselective synthesis, which requires the introduction of one or more new and desired elements of chirality. The other is any of a number of enantiomeric separation techniques, which have become increasingly relevant on a preparative scale. Chromatography techniques such as supercritical fluid chromatography (SFC), high-performance liquid chromatography (HPLC) and gas chromatography (GC), and capillary electrophoresis (CE) have long been the methods of choice in the fields of enantiomeric preparation and analysis. In the current literature, many studies have focused on the development of chiral selectors that have contributed to the great advances in chiral separation by chromatographic and electrophoretic techniques [6–10]. This rapid improvement in separation techniques makes these the most effective and widely employed approaches for preparing single-enantiomer standards and for analysing chemical behaviours of enantiomers in the environment.

In this review, we first classify chiral OPs in terms of differences in their stereogenic centres (such as the number and positions of stereogenic centres). Then an overview of the current state of knowledge of HPLC, GC and CE techniques for the chiral separation of OPs is presented, and preferred conditions for the chiral separation of OPs are also discussed. Finally, information concerning the stereoselective toxicity and degradation of OPs, obtained via chromatographic and electrophoretic separation techniques, is presented. Enantioselectivity in toxicological aspects of chiral OPs has also been reported by Kurihara and Miyamoto; however, specific reviews of the chiral separation of OPs are rare, and most of the information dates to before 1995 [4]. Hence, it appeared advisable to update the progress in this field and review the latest information on the enantiomeric separation of OPs and the stereoselectivity of their toxicity and environmental behaviour.

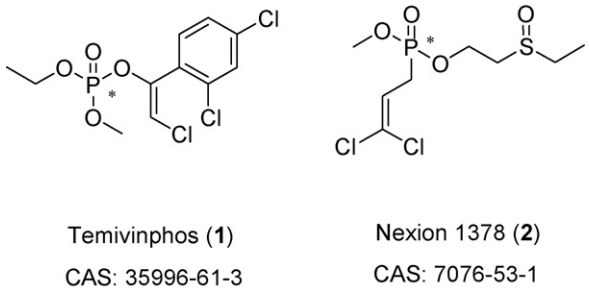


Fig. 1. Chemical structures of chiral phosphates containing asymmetric phosphorus atoms.

2. Classification of OPs based on the position of chiral centre

Chiral OPs generally can be divided into three broad classes: compounds with asymmetric phosphorus centres, asymmetric carbons, or both.[1] There are 52 chiral OPs described in one current handbook of Agricultural Chemicals [11], and these consist of pesticides, herbicides and fungicides. Specifically, they can be classified as follows.

2.1. Chiral phosphorus centres

According to the diverse substituted groups, OPs with only one chiral centre on a phosphorus atom can be divided into several subclasses, such as phosphates (temivinphos (**1**) and Nexion (**2**)), phosphonates and phosphonothioates (EPN (**3**), EPBP (**4**), leptophos (**5**), trichloronate (**6**), fonofos (**7**), cyanofenphos (**8**), inezin (**9**) and fosamine (**10**)), phosphoro(di)thiolates (prothiofos (**11**), sulprofos (**12**), profenofos (**13**), pyraclofos (**14**), Bopardil RM60 (**15**), diphenprophos (**16**), tebupirimfos (**17**), conen (**18**) and cereton B (**19**)), phosphoramidothioates (methamidophos (**20**), acephate (**21**), fenamiphos (**22**), crufomate (**23**), isocarbophos (**24**), isofenphos (**25**), isofenphos-methyl (**26**), propetamphos (**27**), Dow-ET15 (**28**), amidothionate (**29**) and amiprophos-methyl (**30**)), and cyclic phosphates (salithion (**31**)). The structures of these compounds are presented in Figs. 1–5.

2.2. Chiral carbon centres

Chiral OPs with a carbon chiral centre include dialifos (**32**), malaoxon (**33**), malathion (**34**), phenthoate (**35**), phenthoate-ethyl (**36**), crotoxyphos (**37**), chlorethoxyfos (**38**), trichlorfon (**39**), naled (**40**), oxydeprofos (**41**), vamidothion (**42**), mephosfolan (**43**), butonate (**44**) and aphos (**45**). Their structures are presented in Fig. 6.

2.3. Chiral centres on both phosphorus and carbon atoms

Some chiral OPs contain one or more asymmetric centres each on both a phosphorus atom and a carbon atom. This class of OPs includes chloramidophos (**46**), methyl-chloramidophos (MCP) (**47**), isomalathion (**48**), fosthiazate (**49**), glufosinate-ammonium (**50**), crmart (**51**) and bialaphos (**52**). The structures of these compounds are shown in Fig. 7.

[1] A small number are compounds with asymmetric sulphur atoms, such as fensulfothion.

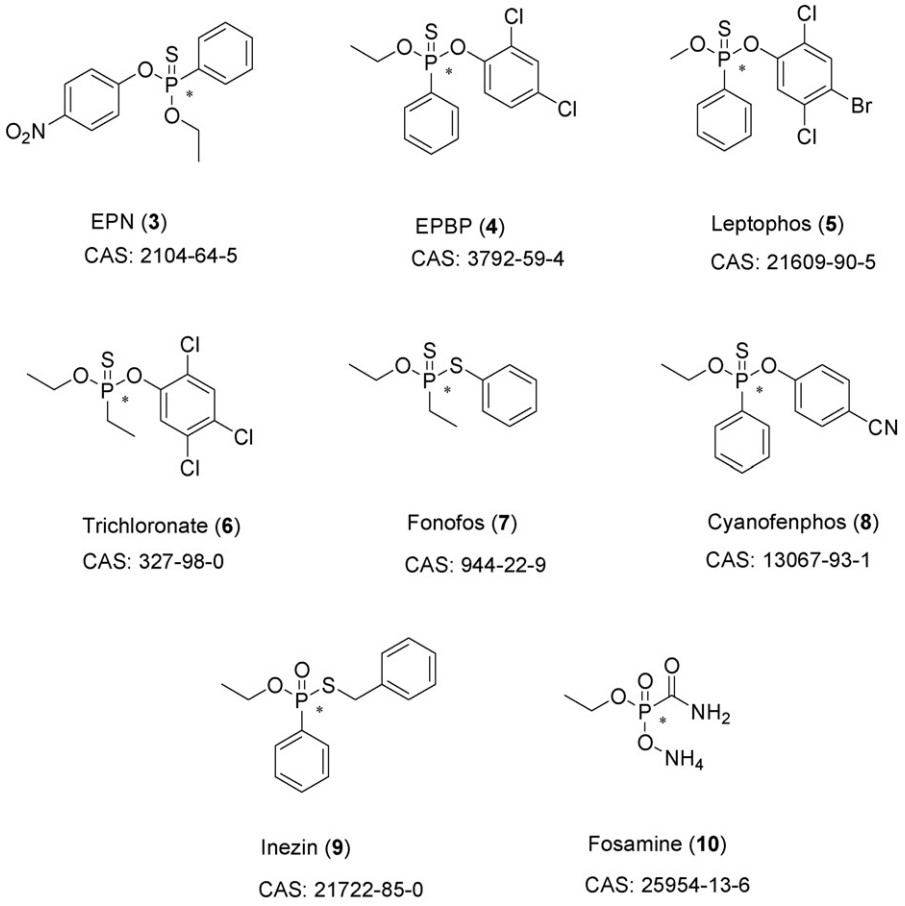


Fig. 2. Chemical structures of chiral phosphonates and phosphonothioates containing asymmetric phosphorus atoms.

3. Chromatographic and electrophoretic separation methods for OPs

Chiral recognition, in chromatographic terms, means preferential interaction of one enantiomer of a substance with one enantiomer of a second substance [12]. It implies the existence of a transient complex formed selectively in a mixture of several species. The most commonly used chromatographic separation methods are HPLC and GC. In addition, based on differences in charge, size and hydrophobicity, electrophoretic separation methods are also efficient in chiral separations [13]. The enantiomeric separation of OPs with asymmetric centres at either phosphorus atoms or carbon atoms by these chromatographic and electrophoretic techniques has long been known.

3.1. One asymmetric centre

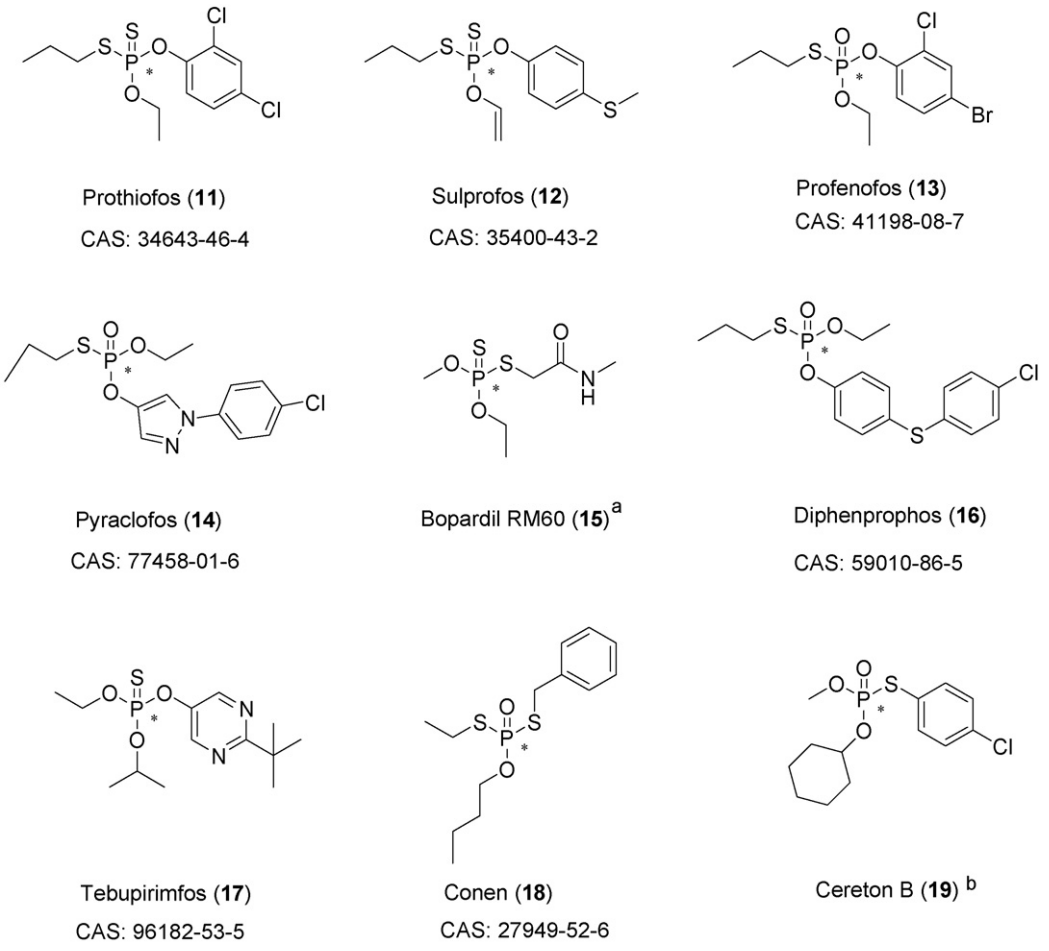
3.1.1. High-performance liquid chromatography (HPLC)

Due to improvements in HPLC instruments and the commercial availability of chiral stationary phases (CSPs), chiral separation techniques using HPLC have advanced considerably in the past three decades. Recently, HPLC has become useful not only for determining the optical purity of enantiomers but also for preparing enantiopure standards. So far, more than 100 CSPs have been sold commercially [14], and approximately 99% of enantiomeric separations were carried out by HPLC on various CSPs [15]. Based on their different chemical structures, CSPs can be divided into at least seven classes, including Pirkle's type, polysaccharides, cyclodextrins, macrocyclic glycopeptide antibiotics, proteins, crown ethers and ligand exchangers. In the chiral separation of OPs by HPLC, the most effective CSPs are the Pirkle's type and polysaccharides.

3.1.1.1. Enantiomeric separation of OPs by Pirkle type of CSPs. Since the 1980s, the Pirkle group has been devoted to the development of Pirkle CSPs, of which there are now nine types, including α-Burke 2, β-Gem 1, DACH-DNB, Leucine, Phenylglycine, Pirkle 1-J, ULMO, Whelk-O1, and Whelk-O2, both in analytical and preparative sizes [16]. A wide variety of chiral compounds have been resolved on Pirkle CSPs, such as aryl propionic acid, non-steroidal anti-inflammatory drugs, β-blockers, and many other pharmaceuticals and agrochemicals.

The Pirkle CSPs are derived from N-3,5-dinitrobenzoylamino acids and generally fall into three classes: π-electron acceptor/π-electron donors, π-electron acceptors and π-electron donors [17]. The design of the Pirkle CSPs was based on the three-point chiral recognition model developed by Dalgliesh [18]. That is, enantiomeric separation of a chiral compound requires a minimum of three simultaneous interactions between the CSP and the analyte, with at least one of these interactions being stereo-chemically dependent [12]. With the Pirkle CSPs, chiral recognition occurs at the binding sites. Major binding sites are classified as π-basic or π-acidic aromatic rings, which are potential sites for π–π interaction, acidic sites and basic sites, which are both involved in hydrogen bond formation, and steric interaction sites [19]. Because of the covalent nature, Pirkle type CSPs can be utilised in both normal-phase mode and reversed-phase mode.

As one of the most widely useful means for controlling pests, plant diseases and weeds, OPs have been investigated by researchers via chiral recognition on Pirkle type columns for



[a] Developed by Bombrini Parodi Delfino company in 1965. (no CAS registry number)

[b] Began to be used as a fungicide in Japan in 1966. (no CAS registry number)

Fig. 3. Chemical structures of chiral phosphoro(di)thiolates containing asymmetric phosphorus atoms.

some time. Resolution of organophosphorus enantiomers by HPLC using Pirkle CSPs was firstly reported by William H. Pirkle, who developed one of the most successful chiral selectors, (R)-N-(3,5-dinitrobenzyl) phenylglycine, in 1981 [20]. In 1997, a series of 14 *O*-ethyl *O*-phenyl *N*-isopropyl phosphoramidothioates containing stereogenic phosphorus atoms was successfully separated enantiomerically on a Sumichiral OA 4700 column by Gao's group [21]. This research group then resolved the enantiomers of 5 *O*-aryl *O*-alkyl *N*-alkyl phosphoramidothioates, which have high herbicidal activities, on the same Pirkle-type column, and the absolute configurations of the enantiomers were obtained through analysis of the circular dichroism (CD) spectra by virtue of the Cotton effect [22]. It was concluded that the enantiomeric separation of those 19 analytes was attributable to two mechanisms: (1) the formation of a π–π complex between the weak π-electron donor group of the Pirkle Model CSP and the weak π-electron acceptor of the organophosphorus compounds and (2) the hydrogen bonding between the donor NH group and acceptor O–R (i.e., O-ethyl, O-phenyl, etc.) group of the CSP and the phosphoramidothioates. In a study by Yen et al., LiChroCART 250-4 (S,S)-Whelk-O1 column and LiChroCART 250-4 (R,R)-Whelk-O1 columns were used for separating the enantiomers of two OPs, leptophos (**5**) and fenamiphos (**22**) [23,24], and the specific rotations of the enantiomers were measured using a polarimeter. It was interesting to note that the elution orders of the enantiomers for both compounds on the two Pirkle type CSPs were reversed. Inversion of the elution order by using Pirkle CSPs in the opposite absolute configuration allows researchers to make trace enantiomers elute before major ones.

The high column efficiency and covalent phase bonding make Pirkle type columns more compatible for use as preparative columns, but it should be noted that Pirkle CSPs are only available for separating the enantiomers of compounds having aromaticity or molecules deriving from aromatic rings. Otherwise, aromatic groups such as aryl moieties must be added to chiral compounds so that good separation can be obtained on such Pirkle type chiral columns. As a result, when using Pirkle CSPs, the separation of enantiomers of chiral OPs with aryl groups may be feasible.

3.1.1.2. Enantiomeric separation by polysaccharide-based CSPs. Chiral polysaccharides have been considered highly efficient CSPs due to their unique advantages, such as high selectivity, sensitivity and reproducibility. According to Zhang et al., more than 95% of racemic compounds have been resolved successfully using polysaccharide CSPs [25]. Cellulose and amylose, the main polysaccharides used, were first recognised as potential chiral selectors by Kotake et al. [26]. However, native polysaccharides cannot be used as CSPs because of the poor resolution and broad analyte peaks obtained, due to the slow transfer and diffusion of compounds through native polymer networks. Accordingly, derivatives of these polymers were synthesised. When natural polysaccharides are modified by reac-

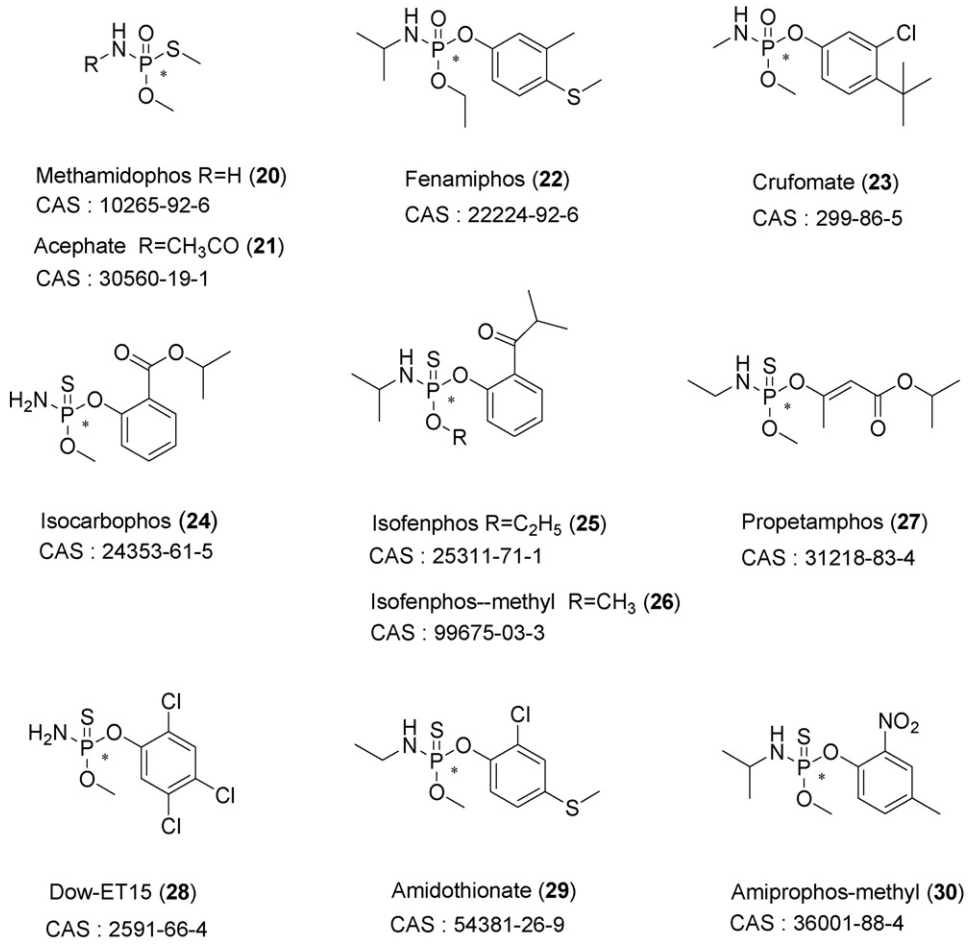


Fig. 4. Chemical structures of chiral phosphoramidothioates containing asymmetric phosphorus atoms.

tion of active hydroxyl groups with appropriate reagents, the chiral selectors produced have greater optical resolving power, indicating a high chiral discrimination capability. In particular, derivatives of cellulose and amylose such as tris-phenylcarbamate and tris-(3, 5-dimethylphenylcarbamate) are readily available optically active polymers, and they are the most widely applicable polysaccharide CSPs.

A series of cellulose-based and amylose-based CSPs including Chiralpak AD, AS, AS-H and Chiralcel OA, OB, OB-H, OC, OD, OF, OG, OJ, OK, etc., has been developed by Okamoto's group. Comparatively speaking, Chiralpak AD, Chiralcel OD and Chiralcel OJ are the most versatile commercial columns, since they are able to resolve more than 80% of chiral compounds [27]. In the late 1980s, researchers began to investigate the chiral recognition ability of polysaccharide CSPs for separation of the enantiomers of OPs. These types of CSPs have also been employed for the semi-preparative separation of pure optical isomers of OPs for toxicology research.

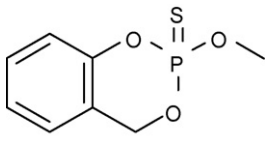
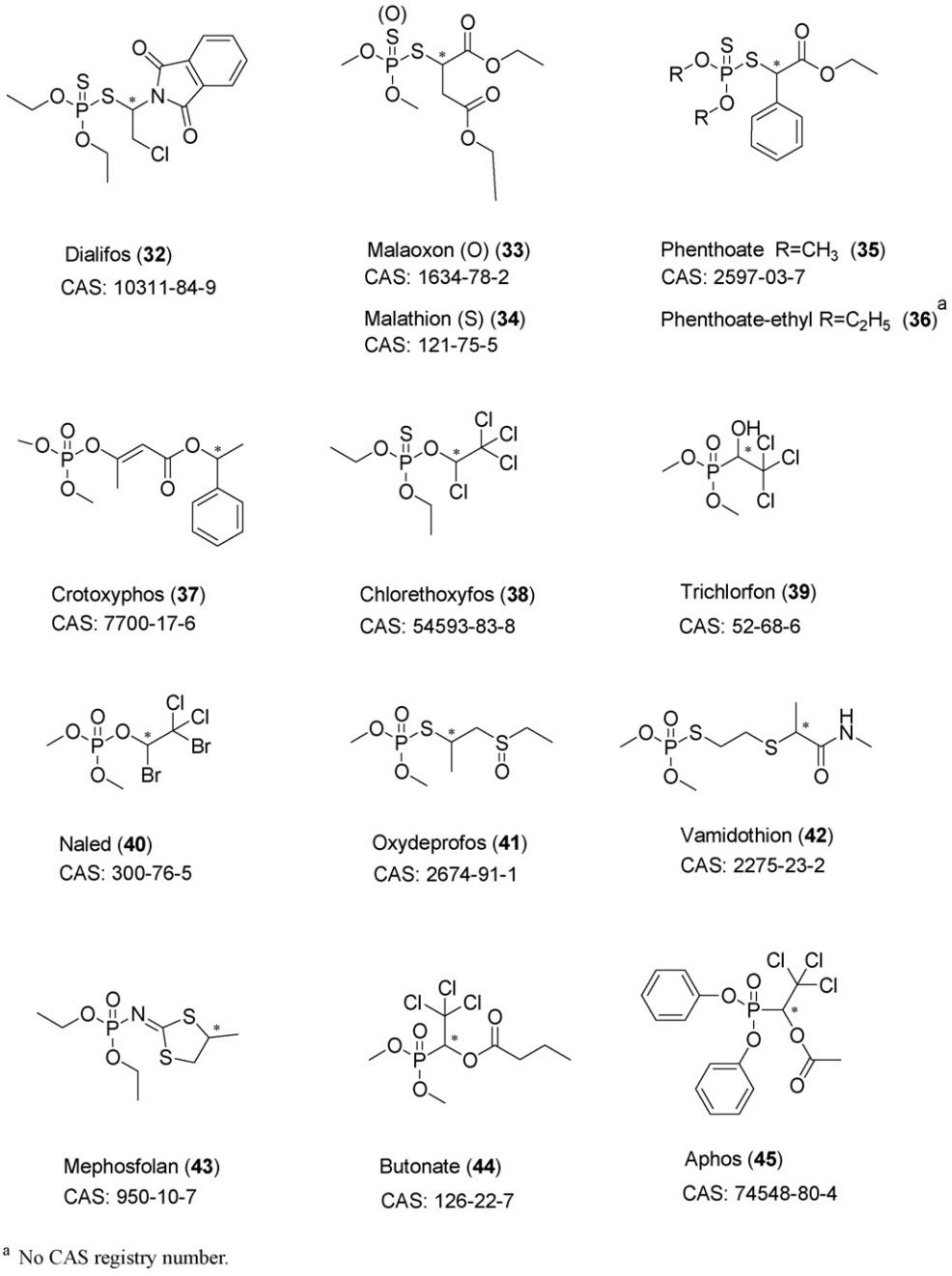


Fig. 5. Chemical structure of chiral cyclic phosphates containing an asymmetric phosphorus atom.

Some workers have attempted to optimise the chiral separation of OPs by varying the chiral column type, polar modifier, flow rate of the mobile phase, and column temperature [28]. Ellington's group reported the chiral separation of 12 OPs (8 compounds with asymmetric phosphorus atoms: trichloronate (**6**), fonofos (**7**), prothiophos (**11**), profenofos (**13**), methamidophos (**21**), fenamiphos (**22**), crufomate (**23**), isofenphos (**25**); 3 compounds with asymmetric carbon atoms: dialifor (**32**), malathion (**34**), crotoxyphos (**37**); and 1 compound with an asymmetric sulphur atom: fensulfothion) on five polysaccharide CSPs, including Chiralpak AD, Chiralpak AS, Chiralcel OD, Chiralcel OJ and Chiralcel OG [28]. It was found that the Chiralcel OJ column showed the best separation ability, permitting 7 of the OPs to be baseline resolved, while the other 5 were partially separated. The Chiralpak AD and Chiralcel OD columns could only resolve the enantiomers of 4 (profenofos (**13**), fenamiphos (**22**), crufomate (**23**) and fensulfothion) and 3 OPs (trichloronate (**6**), crufomate (**23**) and methamidophos (**21**)), respectively. Furthermore, enantiomers of fenamiphos (**22**) were separated on the Chiralpak AS column, and those of isofenphos (**25**) were separated on the Chiralcel OG column.

Besides the commercial CSPs, synthesised CSPs have also been used for enantiomeric separation of chiral OPs. Wang et al. described the chiral separation of isocarbophos (**24**) on a synthesised cellulose-tri(3,5-dimethylphenylcarbamate) CSP (CDMPC) under normal phase [14]. Effects of changes in polar modifier and column temperature (from 0 to 50 °C) on the enantioselectivity of CSPs were also investigated. It was noted that: (1) the resolution (R_S) values of enantiomers increased with decreasing amounts of polar modifiers, indicating that the polar modifiers competed with

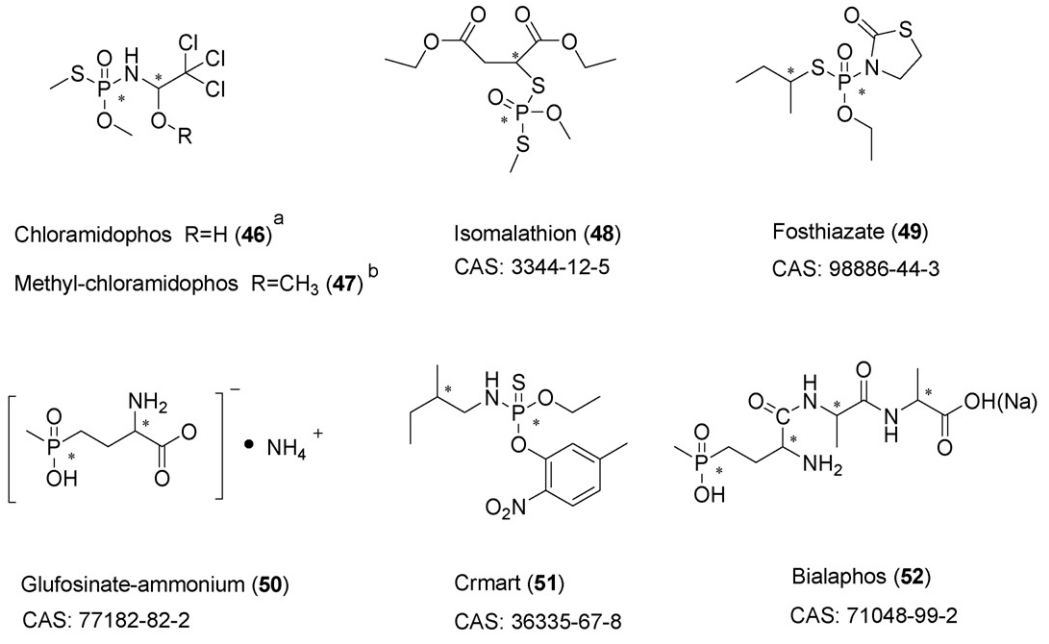


[a] No CAS registry number.

Fig. 6. Chemical structures of chiral phosphates containing asymmetric carbon atoms.

the solutes for interaction with the CSP; (2) the distinct effect of the five alcohols on the enantiomeric separation of isocarbophos (isopropanol > isobutanol > *n*-propanol > *n*-butanol > ethanol) indicated that the structure of alcohol affects stereoselectivity, probably by changing the environment of the chiral cavity; (3) as the analyte–CSP interactions between the two enantiomers were enhanced at lower temperatures, lower column temperatures resulted in better chiral separation of isocarbophos. Optimal separation was finally achieved with a selectivity factor of 1.89 when using hexane/isopropanol = 95/5 (v/v) as the mobile phase at room temperature. According to Wang et al. [29,30], the synthesised CDMPC column was also employed to resolve the enantiomers of profenofos (**13**), methamidophos (**20**), acephate (**21**), malathion (**34**) and phenthoate (**35**). In all these tests, isopropanol always appeared to be the best modifier. Except for the polarity and viscosity of the modifiers, the competition between the alcohol molecules and solutes with the CSP can also influence the chiral separation capability [31]. In another study by Wang et al., an amylose tris-(S)-1-phenylethylcarbamate CSP (similar to a commercial Chiralpak AS column) was synthesised and applied to separate the enantiomers of thirty-two chiral pesticides, 7 of which were OPs [32]. The results demonstrated that only enantiomers of malathion (**34**) could be partially separated on this amylose tris-(S)-1-phenylethylcarbamate CSP, while the other 6 OPs (fonofos (**7**), methamidophos (**20**), acephate (**21**), isocarbophos (**24**), isofenphos-methyl (**26**) and trichlorfon (**39**)) did not achieve any effective chiral resolution [32]. The elution orders of the enantiomers separated above in Wang's work were all determined by a CD detector.

Liu et al. have also studied chiral separation of OPs, especially on polysaccharide-based columns: (1) Trichloronate (**6**) belonged to the phosphonothioates and was investigated on three kinds of



[a] Registered provisionally in China in 2005. (no CAS registry number)

[b] Isolated from the stored commercial formulation of chloramidophos in 2009. (no CAS registry number)

Fig. 7. Chemical structures of chiral organophosphorus containing two or more chiral centres.

columns with different CSPs by HPLC [33]. Baseline resolution of the enantiomers of trichloronate was achieved on a Chiralcel OJ column, while no separation was obtained on either a Pirkle-type Sumichiral column (OA-2500-I) or a reverse-phase column (Chiralcel OD-R). The enantiomers were characterised by the optical rotation on the polarimeter and by GC–MS. (2) Enantiomers of methamidophos (**20**) and isocarbophos (**24**), which are both phosphoramidothioates, were successfully separated on the Chiralcel OD column [34,35]. The enantiomers were distinguished by the presence of positive or negative peaks in the CD chromatograms (Fig. 8, taking methamidophos (**20**) as an example). It was important that the absolute stereochemistry of (+)-methamidophos was determined to be of the $(R)_p$ configuration according to Miyazaki et al. [36]. (4) A suite of commercial chiral polysaccharide columns, i.e., Chiralcel OD column, Chiralcel OJ column and Chiralpak AD column, was employed for the enantiomeric separation of salithion (**31**), a cyclic phosphate [37]. It was found that satisfactory separation of salithion (**31**) enantiomers could be achieved on all the tested columns, with the Chiralcel AD column offering the best chiral discrimination. The chiral recognition ability of Chiralpak AD for salithion was also better than that of Chiralcel OB and Chiralpak OT (+), according to the studies of Wu et al. [38,39], while the absolute configuration of the individual enantiomers of salithion (**31**) was deduced from their elution order on the Chiralpak OT (+) column. (5) The enantioselective separation of a series of phosphonates that showed notable herbicidal activity was compared under various chromatographic conditions using a Chiralcel OD column, Chiralcel OJ column, Chiralpak AD column and Chiralpak AS column [40]. It was found that the Chiralpak AD column showed the best chiral separation capacity. It should be noted that the Chiralcel OD column, which had the same D-glucose constituents as the chiral adsorption sites on the Chiralpak AD column, did not show a high chiral separation capacity for phosphonates, implying that the higher order structures arising from the different arrangements of the glucose units might also play a role in the chiral recognition. In addition, the effects of both mobile phase composition and column temperature were discussed, with isopropanol proving the most suitable mobile phase additive in most separation cases.

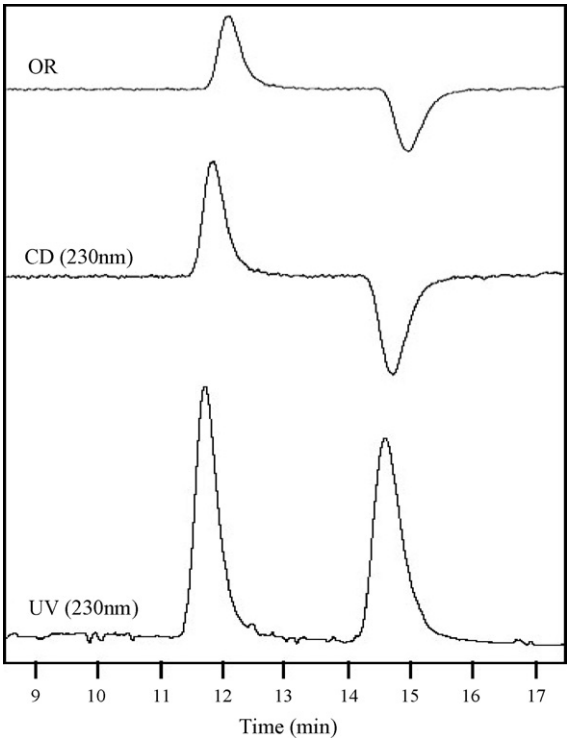


Fig. 8. Representative optical rotation (OR), CD and UV chromatograms for enantiomeric separation of methamidophos (**20**) on a Chiralcel OD column. Reprinted from [34] with permission.

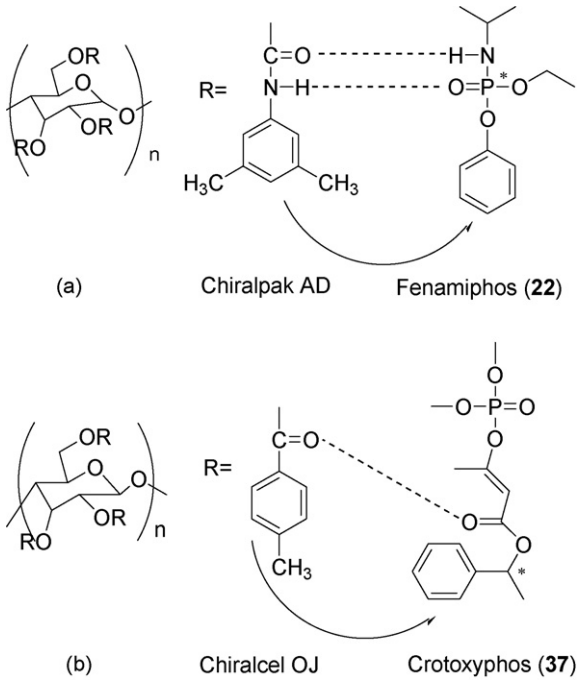


Fig. 9. Proposed mechanism for chiral recognition: (a) interactions between Chiralpak AD and fenamiphos (**22**); (b) interactions between Chiralcel OJ and crotoxyphos (**37**).

Thus, four commercial polysaccharide CSPs, i.e., Chiralpak AD, Chiralcel AS, Chiralcel OD and Chiralcel OJ, as well as the synthesised CDMPC seem to have high degrees of stereogenic recognition in the separation of chiral OPs. In particular, using Chiralpak AD and Chiralcel OJ columns with isopropanol as the organic modifier at lower temperatures was found to be the preferred set of conditions for separating the enantiomers of chiral OPs. The diversity of chiral recognition mechanisms under these conditions resulted in enantioselectivity. Detailed HPLC separation conditions for the OP enantiomers are shown in Table 1. These interactions are so complex that few articles have elucidated the roles of the different intermolecular forces and modes in chiral separation, but the main mechanisms may include: (1) dipole–dipole interactions, i.e., a carbonyl group (C=O) with a C=O group; (2) hydrogen bonding associations, i.e., a C=O group with a NH group; (3) π–π interactions, i.e., between a π-electron acceptor group and a π-electron donor group; (4) steric interactions, i.e., the degree of steric fit into the chiral cavities of CSPs. The proposed interactions for chiral recognition are illustrated in Fig. 9 (using the interactions of Chiralpak AD with fenamiphos (**22**) and Chiralcel OJ with crotoxyphos (**37**) as examples). As a result of the various interactions mentioned above, transient diastereomeric complexes of different stabilities may be formed between enantiomers and CSPs. Furthermore, changes in the type of CSP, the structure of the analyte, or the chromatographic conditions such as mobile phase composition and column temperature can influence the chiral recognition. For example, (1) the structures of CSPs and analytes can affect chiral recognition directly due to the different interactions of the groups between CSPs and analytes; (2) the organic alcohol modifier can alter the steric environment of the chiral cavities on CSPs by bonding to achiral sites at or near the chiral cavity. As a result, changes in the polarity of the mobile phase due to varying the percentage or the type of polar modifier can greatly influence the elution time and resolution; (3) column temperature is a potential factor not only affecting the viscosity and diffusion coefficient of solutes but also impacting the thermodynamics of interactions. Thus, by calculating the standard enthalpy and entropy values of sorbed vs. dissolved constituents, the driving force for the transfer of enantiomers from the mobile phase to the stationary phase can be deduced.

3.1.2. Gas chromatography

GC is among the earliest chromatographic techniques used for enantiomeric separation. The first successful chiral separation of enantiomers by GC on a CSP was reported by Gil-Av et al. at the Weizmann Institute of Science in Israel during the late 1960s. GC has a number of advantages: (1) contaminants and impurities can usually be separated from the analytes, and the enantiomeric compositions in mixtures can also be precisely determined; (2) the delicate choices of solvents, modifiers and gradient elution systems that characterise HPLC separations are not necessary for GC methods. As of 2000, the GC separation of 22,000 enantiomers involving 5500 basic chiral compounds had already been reported [41]. Many reversible diastereomeric associations between enantiomers and chiral selectors such as inclusion, hydrogen-bonding, dispersion forces, dipole-dipole interactions, electrostatic interactions and hydrophobic interactions have been reported to play a role in the chiral recognition of compounds by GC [42]. In 1979, direct GC separation of enantiomers of several O-alkyl O-aryl N-isopropylphosphoroamidothioates was described by Ôi et al. [43]. However, no clear picture of the mechanisms of chiral discrimination has emerged from the data reported thus far. Due to the difficulties in finding appropriate enantioselective chromatography columns, especially commercial ones, there are relatively few publications concerning the chiral separation of OPs using GC in comparison with HPLC chromatographic methods. Therefore, based on an empirical selection of CSPs, most chiral OPs cannot be separated by GC, in spite of the high efficiency and sensitivity of this method. However, there are still some notable exceptions, which are described below.

Fidalgo-Used et al. compared two different commercially available chiral columns for chiral separation of 13 OPs by GC with flame ionisation detection (FID) [44]. According to the authors, a Chirasil-Val column with a stationary phase consisting of L-valine-*tert*-butylamide directly bonded to dimethylpolysiloxane did not possess an enantiomeric discrimination for the OPs. By contrast, a CP-Chirasil-Dex CB column, the stationary phase of which consisted of heptakis (2,3,6-tri-O-metil)-β-CD molecules directly bonded to dimethylpolysiloxane, was demonstrated to resolve the enantiomers of ruelene (**23**) and trichlorfon (**39**). In addition, the enantiomers of methamidophos (**20**), isophenphos (**25**), chlorethoxyfos (**38**) and naled (**40**) were also partially separated. It was concluded that not only the formation of an inclusion complex with cyclodextrins but also the presence of halogen atoms in the phenyl ring resulted in the diverse resolution results. Furthermore, two different detection systems, a classical electron capture detector (ECD) and an inductively coupled plasma mass spectrometry (ICP-MS), were employed for monitoring the enantiomers of ruelene (**23**) in environmental samples by Fidalgo-Used et al. [45]. It was found that the ICP-MS detector was superior to the ECD in terms of both selectivity and sensitivity. In addition, the detection limit (three times the standard deviation of the background divided by the slope of the calibration curve) using ICP-MS was found to be as low as 27 ng L^{-1} for each enantiomer of ruelene. The excellent resolution of ruelene (**23**) suggested that a phenyl ring with a chlorine atom in its structure may play an important role in forming inclusion complexes with cyclodextrin. In addition, CSPs based on modified cyclodextrins were utilised for separating the enantiomers of five other OPs [46,47]. The detailed GC separation conditions of these OP enantiomers are illustrated in Table 2 .

3.1.3. Capillary electrophoresis

As a complementary analytical technique for HPLC and GC, CE has also been employed widely in the field of enantiomeric

Table 1
Enantiomeric separation of OPs by HPLC.

Classes	Commercial names	Separation conditions	Rotation	Ref.
A	Leptophos (**5**)	LiChroCART250-4(S,S)-Whelk-O1[a]	−/+	[23]
		LiChroCART250-4(R,R)-Whelk-O1[a], *n*-hexane/dichloromethane = 97/3, rt[b], UV = 210 nm	+/−	
	Trichloronate (**6**)	Chiralcel OD (R_s = 1.3), *n*-heptane/ethanol = 100/0, 15 °C, 1.0 mL/min	ud[c]	[28]
		Chiralcel OJ[a], *n*-hexane/*n*-heptane/ethanol = 90/5/5, rt, 1.0 mL/min, UV = 300 nm	+/−	[33]
	Fonofos (**7**)	Chiralcel OJ[a], *n*-heptane/ethanol = 90/10, 25 °C, 1.0 mL/min, UV = 202 nm	+/−	[28]
	Prothiophos (**11**)	Chiralcel OJ[a], *n*-heptane/ethanol = 98/2, 15 °C, 1.0 mL/min, UV = 202 nm	+/−	[28]
	Profenofos (**13**)	Chiralpak AD[a], *n*-heptane/ethanol = 98/2, 15 °C, 1.0 mL/min	+/−	[28]
		Chiralcel OJ[a], *n*-hexane/ethanol = 99.5/0.5, 5 °C, 0.8 mL/min, UV = 202 nm	+/−	[28]
	Methamidophos (**20**)	Chiralcel OD[a], *n*-heptane/ethanol = 90/10, rt, 1.0 mL/min	+/−	[28]
		Chiralcel OJ[a], *n*-hexane/ethanol = 93.5/6.5, 5 °C, 0.8 mL/min, UV = 200 nm	+/−	[28]
		Synthesised CDMPC[a], *n*-hexane/isopropanol = 90/10, 20 °C, 1.0 mL/min, UV = 230 nm	+/−	[30]
		Chiralcel OD[a], *n*-hexane/isopropanol = 80/20, 25 °C, 0.5 mL/min, UV = 230 nm	+/−	[34]
	Acephate (**21**)	Chiralcel OC[a], *n*-hexane/isopropanol=80/20, 1.0 mL/min, UV = 235 nm	+/−	[36]
	Fenamiphos (**22**)	LiChroCART250-4(S,S)-Whelk-O1[a]	−/+	[24]
		LiChroCART250-4(R,R)-Whelk-O1[a], *n*-hexane/isopropanol = 95/5, rt, UV = 210 nm	+/−	
		Chiralpak AS[a], *n*-heptane/ethanol = 90/10, rt, 1.0 mL/min	−/+	[28]
		Chiralpak AD[a], *n*-heptane/ethanol = 90/10, 25 °C, 1.0 mL/min	+/−	[28]
		Chiralcel OJ (R_s = 1.08), *n*-hexane/ethanol = 99.1/0.9, 40 °C, 0.5 mL/min, UV = 203 nm	+/−	[28]
	Isocarbophos (**24**)	Synthesised CDMPC[a], *n*-hexane/isopropanol = 90/10, 25 °C, 1.0 mL/min, UV = 225 nm	ud	[14]
		Chiralcel OD[a], *n*-hexane/isopropanol = 90/10, 25 °C, 0.8 mL/min, UV = 230 nm	+/−	[35]
	Isofenphos (**25**)	Chiralcel OG (R_s = 1.1), *n*-heptane/isopropanol = 98/2, 25 °C, 1.0 mL/min	+/−	[28]
		Chiralcel OJ (R_s = 1.11), *n*-hexane/ethanol = 99.4/0.6, 10 °C, 0.3 mL/min, UV = 201 nm	+/−	[28]
	Salithion (**31**)	Chiralcel OJ[a], *n*-hexane/isopropanol = 95/5, 25 °C, 1.0 mL/min, UV = 220 nm	+/−; *R/S*	[37]
		Chiralcel OD (R_s = 1.42), *n*-hexane/isopropanol = 99.5/0.5, 25 °C, 1.0 mL/min, UV = 220 nm	+/−; *R/S*	[37]
		Chiralpak AD[a], *n*-hexane/isopropanol = 99.5/0.5, 25 °C, 1.0 mL/min, UV = 220 nm	+/−; *R/S*	[37]
		Chiralpak OT(+)[a], methanol, rt, 1.0 mL/min, UV = 254 nm	−/+; *S/R*	[38]
		Chiralcel OB[a], *n*-hexane/isopropanol = 10/1, rt, 0.3 mL/min, UV = 254 nm	+/−; *R/S*	[39]
B	Dialifor (**32**)	Chiralcel OJ[a], *n*-hexane/ethanol = 90/10, 20 °C, 0.9 mL/min, UV = 220 nm	+/−	[28]
	Malathion (**34**)	Chiralcel OJ[a], *n*-hexane/ethanol = 90/10, 20 °C, 0.9 mL/min, UV = 210 nm	+/−	[28]
		Synthesised CDMPC[a], *n*-hexane/isopropanol = 99/1, 5 °C, 1.0 mL/min, UV = 210 nm	+/−	[30]
	Phenthoate (**35**)	Synthesised CDMPC[a], *n*-hexane/isopropanol = 99.5/0.5, 20 °C, 0.5 mL/min	ud	[29]
		Chiralcel OD[a], *n*-hexane/isopropanol = 100/0.8, 1 mL/min, UV = 230 nm	+/−	[67]
	Crotoxyphos (**37**)	Chiralcel OJ[a], *n*-hexane/ethanol = 90/10, 20 °C, 0.9 mL/min, UV = 211 nm	−/+	[28]
C	Chloramidophos (**46**)	Chiralpak AD[a], *n*-hexane/ethanol = 90/10, 25 °C, 1.0 mL/min, UV = 230 nm	−/+/+/−	[55]
	MCP (**47**)	Chiralpak AD[a], *n*-hexane/ethanol = 85/15, 25 °C, 1.0 mL/min, UV = 220 nm	+/+/−/−	[57]
	Isomalathion (**48**)	Chiralpak AD[a], *n*-hexane/isopropanol = 90/10, 0.4 mL/min, UV = 215nm	(1R,3R)/(1S,3R)/(1S,3S)/(1R,3S)	[56]
	Glufosinate-ammonium (**50**)	Inertsil ODS-2 column, 10 mM ammonium acetate (pH 5.0)/acetonitrile = 77/23, 40 °C, 0.8 mL/min, fluorescence detection: 260 nm (excitation) and 305 nm (emission)		[59]
D	Fensulfothion	Chiralpak AD[a], *n*-heptane/ethanol = 90/10, 25 °C, 1 mL/min	−/+	[28]
		Chiralcel OJ (R_s = 1.21), *n*-hexane/ethanol = 96/4, 40 °C, 0.8 mL/min, UV = 201 nm	−/+	[28]

A: OPs with only one chiral centre at a phosphorus atom; B: OPs with only one chiral centre at a carbon atom. C: OPs with two chiral centres at both a phosphorus atom and a carbon atom. D: OPs with only one chiral centre at a sulphur atom.

[a] R_s > 1.5.
[b] Room temperature.
[c] Undetermined.

Table 2
Enantiomeric separation of OPs by GC.

Classes	Commercial names	Separation condition	Ref.
A	Methamidophos (**20**)	GC-FID (R_s = 0.67) CP-Chirasil-Dex CB 50 °C (1 min) 20 °C min^{-1} to 120 °C	[44]
	Ruelene (**23**)	GC-FID[a] CP-Chirasil-Dex CB 50 °C (1 min) 20 °C min^{-1} to 190 °C (50 min)	[44]
		GC-ECD (or) GC-ICP-MS[a] CP-Chirasil-Dex CB 50 °C (1 min) 40 °C min^{-1} to 190 °C (40 min)	[45]
	Isofenphos (**25**)	GC-FID (R_s = 1.03) CP-Chirasil-Dex CB 50 °C (1 min) 10 °C min^{-1} to 125 °C 0.2 °C min^{-1} to 190 °C	[44]
B	Chlorethoxyfos (**38**)	GC-FID (R_s = 0.69) CP-Chirasil-Dex CB 50 °C (1 min) 10 °C min^{-1} to 110 °C (40 min) 0.2 °C min^{-1} to 190 °C (10 min)	[44]
	Trichlorfon (**39**)	GC-FID[a] CP-Chirasil-Dex CB 160 °C (30 min)	[44]
	Naled (**40**)	GC-FID (R_s = 0.89) CP-Chirasil-Dex CB 50 °C (1 min) 10 °C min^{-1} to 110 °C (40 min) 0.2 °C min^{-1} to 190 °C (10 min)	[44]

A: OPs with only one chiral centre at a phosphorus atom; B: OPs with only one chiral centre at a carbon atom.
[a] R_s > 1.5.

separation. It has many interesting characteristics such as high separation efficiency, ease of operation, short analysis time and low sample and electrolyte consumption. CE is also a versatile separation method because it can be applied to a wide variety of analytes, thanks to the various modes that can be used [48]. To date, six separation modes of CE have been successfully used in chiral separation: capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MECC or MEKC), capillary isoelectric focusing (CIEF), capillary isotachophoresis (CITP) and capillary electrochromatography (CEC). In the chiral separation of OPs by CE, cyclodextrin and its derivatives are often used as the chiral selector, and MEKC is the most commonly used mode.

Schmitt et al. chose five chiral selectors, including α-CD, β-CD, γ-CD, hydroxypropyl-β-cyclodextrin (HP-β-CD), dimethyl-β-cyclodextrin (DM-β-CD) and trimethyl-β-cyclodextrin (TM-β-CD), which differed in both their cavity diameters and lipophilic properties, to investigate their effects on the MEKC-separation of the enantiomers of 5 OPs [49]. It was observed that the enantiomers of ruelene (crufomate **23**), dialifos (**32**) and malathion (**34**) can be separated using HP-β-CD, β-CD and/or γ-CD in 10 min, whereas the enantiomers of isofenphos (**25**) and fenamiphos (**22**) could not be separated under the same experimental conditions. It was presumed that too many bulky groups bonded to the chiral atom could limit the formation of inclusion complexes with cyclodextrin molecules with too small a diameter, such as α-CD. In another study, Lewis et al. also demonstrated the effective chiral separation of ruelene (**23**) by CE in the MEKC mode [50]. Huang's studies focused on separating the enantiomers of poorly water-soluble phosphoro(di)thiolates, including prothiophos (**11**), sulprofos (**12**), profenofos (**13**) and pyraclofos (**14**) [51]. Both non-aqueous and aqueous–organic CE media were employed. Generally, a surfactant could not be used to improve the resolution in non-aqueous solutions because micelle formation was limited, due to the weakness of hydrophobic interactions, preventing surfactants from aggregating [52]. However, good enantiomeric resolution was achieved for pyraclofos (**14**) when large amounts of sodium cholate (SC) and sodium dodecyl sulphate (SDS) were added as chiral selectors, with an analysis time of only 3.5 min. It was concluded that the high polarity of SC was favourable to the formation of micelles and enhanced the formation of inclusion complexes of these micelles with the hydrophobic pyraclofos (**14**). On the other hand, the presence of H_2O also appeared to be particularly useful for the chiral separation of poorly water-soluble OPs. García-Ruiz et al. investigated the efficiencies of three chiral selectors, carboxymethylated γ-cyclodextrin (CM-γ-CD), carboxymethylated β-cyclodextrin (CM-β-CD) and β-cyclodextrin sulphated (β-CD sulphated), in the separation of 8 chiral OPs under different experimental condition [53]. This method was confirmed to be suitable for the determination of malathion (**34**) enantiomers in water samples spiked at μg/mL levels, and it played an important role in validating analyses of trace chiral OPs. Anigbogu et al. reported a chiral separation process for OPs using mixed-mode electrokinetic capillary chromatography (mixed-mode ECC), i.e., a combination of MECC and dual-cyclodextrin electrokinetic capillary chromatography (dual-CECC) [54]. It was demostrated that the versatility of the mixed-mode ECC in handing separation of OP enantiomers was achieved by combining three or more pseudostationary phases in the background electrolyte (BGE). The authors suggested that approaches to optimise the enantioselectivity in mixed-mode ECC were generally more straightforward than changes in the CSPs used in HPLC and GC. Detailed CE separation conditions for OPs are illustrated in Table 3.

Mechanisms responsible for the enantiomeric separation are similar in both chromatographic and electrophoretic techniques. Briefly, all chiral resolution is attributable to the enantioselective interactions between the analyte enantiomers and chiral selectors. However, a lack of injection precision and detection sensitivity limits the application of CE for enantiomeric separation when compared to HPLC and GC. In addition, only small amounts of the sample can be separated by this technique. To date, no reports have been published describing separation of OP enantiomers by CE on a semi-preparative scale.

3.2. Two asymmetric centres

Considerable attention has been paid to the enantiomeric resolution of OPs with only one asymmetric centre; however, research

Table 3
Enantiomeric separation of OPs by CE.

Classes	Commercial names	Separation conditions	Ref.
A	Prothiophos (**11**)	75 mM SC and 20 mM γ-CD in methanol/H_2O/ACN (5/4/1) (R_s = 0.99)	[51]
	Sulprofos (**12**)	50 mM SC and 10 mM γ-CD in methanol/H_2O/ACN (5/4/1) (R_s = 1.12)	[51]
	Profenofos (**13**)	50 mM SC and 20 mM γ-CD in methanol/H_2O/ACN (5/4/1) (R_s = 1.36)	[51]
	Pyraclofos (**14**)	100 mM SDS and 50 mM SC in methanol/ACN (4/1)[a]	[51]
	Fenamiphos (**22**)	20 mM CM-β-CD in 25 mM Tris buffer (pH 7.0), 25 °C, 24 kV (R_s = 0.6)	[53]
	Ruelene (**23**)	100 mM SDS and 40 mM DM-β-CD and 15% methanol in 40 mM borate buffer (pH 9), 30 °C, 30 kV (α[b] = 1.024)	[49]
		70 mM SDS and 15 mM CM-β-CD and 45 mM HP-β-CD and 20% ACN in 20 mM borate buffer (pH 8.6), 25 °C, 25 kV[c]	[54]
		100 mM SDS and 40 mM 2- HP-β-CD and 20% CAN in 20 mM tetraborate buffer (pH 8.5), 20 kV	[50]
B	Dialifor (**32**)	100 mM SDS and 40 mM γ-CD and 10% methanol in 40 mM borate buffer (pH 9), 30 °C, 30 kV (α = 1.057)	[49]
	Malathion (**34**)	100 mM SDS and 65 mM HP-β-CD and none methanol in 40 mM borate buffer (pH 9), 30 °C, 30 kV (α = 1.014)	[49]
		10 mM SDS and 50 mM CM-β-CD and 40 mM HP-β-CD and 20% methanol in 20 mM borate buffer (pH 8.6), 25 °C, 25 kV[c]	[54]
		20 mM CM-β-CD in 25 mM Tris buffer (pH 7.0), 25 °C, 24 kV (R_s = 1.4)	[53]
	Phenthoate (**35**)	20 mM CM-β-CD in 25 mM Tris buffer (pH 7.0), 25 °C, 24 kV[a]	[53]
C	Isomalathion (**48**)	20 mM CM-β-CD in 25 mM Tris buffer (pH 7.0), 25 °C, 24 kV (only three peaks) (R_s = 2.5, 1.1)	[53]
D	Fensulfothion	75 mM SDS and 12.5 mM CM-β-CD and 45 mM HP-β-CD and 20% methanol in 20 mM borate buffer (pH 8.6), 25 °C, 25 kV[c]	[54]

A: OPs with only one chiral centre at a phosphorus atom; B: OPs with only one chiral centre at a carbon atom; C: OPs with two chiral centres at both a phosphorus atom and a carbon atom; D: OPs with only one chiral centre at a sulphur atom.

[a] R_s > 1.5.

[b] Separation factor α.

[c] Either R_s or α is unmentioned in article.

related to the enantiomeric separation of OPs with two asymmetric centres is still limited to 5 compounds, i.e., four insecticides, chloramidophos (**46**), MCP (**47**) (O,S-dimethyl-N-(2,2,2-trichloro-1-methoxyethyl)phosphoramidothioate), isomalathion (**48**), fosthiazate (**49**), and one herbicide, glufosinate-ammonium (**50**). It was interesting to find that successful HPLC separations of the optical isomers of the four insecticides were all obtained using Chiralpak AD columns [55–58]. Hori et al. developed an indirect chromatographic method to quantify glufosinate-ammonium (**50**) enantiomers in biological specimens using reverse-phase HPLC with an Inertsil ODS-2 column [59]. Furthermore, García-Ruiz et al. investigated the enantioselectivity of isomalathion using CE [53]. Detailed HPLC separation conditions for OP enantiomers are illustrated in Table 1. However, there are no published reports concerning the enantiomeric separation of OPs with two chiral centres by GC.

4. Applications of enantioselectivity to environmental fate and toxicity

Enantioselective toxicity and environmental behaviour of chiral OPs are two topics of great interest in environmental chemistry. Traditionally, the individual enantiomeric standards necessary for environmental chemistry research on these topics were obtained via asymmetric syntheses [36,56,60]. With the development of chromatographic and electrophoretic methods for the separation of OP enantiomers, an opportunity was created not only for preparing enantiopure standards but also for analysing the enantiomers in environmental samples. This advance in chromatographic and electrophoretic techniques has further developed our understanding of the environmental safety of various chiral compounds.

HPLC has been the primary technique used for preparing individual enantiomers, even though only small amounts (<10 mg, generally) can be obtained by manual collection at the outlet of HPLC detectors. As a result, enantioselective toxicity research is presently limited to experiments using in vitro enzyme kinetics assays, either in cell models or with sensitive aquatic organisms. However, the biggest advantage of HPLC in enantiomeric separation is that the manually collected enantiomers often have high enantiopurity (>98%).

Enantiomeric separation of chiral OPs by chromatographic and electrophoretic separation methods has been well studied, as described in the previous sections. Based on these separation techniques, many acute toxicity studies have been carried out for OPs in enantiomeric form. In the study of Lin et al., a 50-fold difference in toxicity to *Daphnia magna* (*D. magna*) was observed between the two enantiomers of isocarbophos (**24**), and the joint toxicity of the enantiomers was additive [35]. Furthermore, regardless of the magnitude of enantioselectivity, either diverse species or stereoselectivity in metabolic processes could cause apparent diversity or even reversal of the relative potency of enantiomers for in vivo and in vitro assays. The (+)-form of fenamiphos (**22**) was found to be roughly 20 times more toxic to *D. magna* (in vivo), but only about four times more inhibitory to the activity of butyrylcholinesterase (in vitro) than the (−)-form [24]. The (+)-enantiomer of profenofos (**13**) as well as that of fonofos (**7**) was less toxic in vivo to *D. magna* and *Ceriodaphnia dubia* (*C. dubia*) [61]; however, it had higher activity against both electric eel acetylcholinesterase (EE-AChE) and human recombinant acetylcholinesterase (HR-AChE) (in vitro) than its antipode [62]. The (−)-isomer of methamidophos (**20**) was more potent as measured by in vitro enzyme assays, but it was less toxic to *D. magna* in vivo than its (+)-isomer [34]. Con-

verse results were observed on salithion (**31**) enantiomers [37]. The above OPs all contain an asymmetric phosphorus atom. In addition, crotoxyphos (**37**), which contains a carbon chiral centre, also has different stereoselective toxicity in diverse species [62]. The EE-AChE inhibitor potencies of four isomers of fosthiazate (**49**), which has two chiral centres, were slightly stereoselective, whereas a 3.1-fold difference was observed in the acute toxicity of these isomers to *D. magna* [58].

Additionally, in addition to acute toxicity, long-term exposure to OPs can also be dangerous. Many techniques such as solid-phase microextraction (SPME)–GC and liquid–liquid microextraction–GC have been developed for determination of OPs in their racemic forms in biological fluids such as urine and blood [63,64]. However, no reports concerning the determination of OP enantiomers in biological fluids have been published, though it is known that the limits of detection of several OP enantiomers by GC and CE can reach ng/mL and μg/mL levels, respectively [45,53]. Therefore, after proper cleanup steps, the determination of certain OP enantiomers in biological fluids by chromatographic and electrophoretic techniques should be feasible in theory.

Besides potential differences in their toxic effects on target and non-target species, enantiomers can also differ in their degradations and transformations in the environment. Therefore, determination of the relative persistence of OP enantiomers is important for assessing their environmental risk. Lewis et al. and Jarman et al. studied the environmental behaviour of ruelene (**23**) using MEKC methods [50,65]. It was found that the transformation rates of racemic ruelene (**23**) only depended on the soil texture, but differences in air temperature and vegetation cover can change the preferentially degraded enantiomer. Li et al. reported that the more active (+)-enantiomer of phenthoate (**35**) degraded faster than its antipode in both Tianjin and Hubei soils [66]. Furthermore, due to chemical processes, significant enantiomerisation of (+)-phenthoate to (−)-phenthoate occurred in both non-sterilised and sterilised soils. In addition, this chiral inversion also depended on pH. However, in Jarman's study, no selectivity was observed in the transformation of fonofos, since the enantiomer fraction (EF) after 8 weeks incubation was the same as that of the racemic standard [65].

5. Conclusions

Over the last two decades, the development of techniques for the separation and determination of OP enantiomers has made great contributions to the study of their relative toxicity and environmental fate. Chromatographic and electrophoretic approaches continue to take a leading role in the chiral separation of OPs. In this review, we conclude that, of the 52 chiral OPs listed in the Encyclopedia of Agricultural Chemicals, enantiomers of 26 have been reportedly separated by HPLC, GC and CE over the last two decades. Among the three methods, HPLC was the most widely used, with 20 of these 26 chiral OPs successfully separated using HPLC with various CSPs. In addition, pure enantiomeric standards prepared by HPLC have been widely used in environmental research, while no reports of the isolation of OP enantiomers on a semi-preparative scale by GC or CE have been published. However, HPLC methods for separating enantiomers only produce quantities sufficient for bioassays calling for relatively low amounts of these compounds (generally less than 10 mg). In view of the high demand for optically pure enantiomers and in order to evaluate the enantiomer-specific environmental fate and toxicity of these compounds, the development and improvement of these separation techniques, especially on the preparative scale, are highly desirable. Furthermore, lower limits of detection for OP enantiomers by chromatographic and electrophoretic techniques are needed in order to analyse OP enantiomer residues in biological fluids for evaluating the effects of enantioselective metabolic processes on OPs.

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