Volume 3, Issue 2, July 2010
ISSN 1791-3691

Hellenic Plant Protection Journal

A semiannual scientific publication of the
BENAKI PHYTOPATHOLOGICAL INSTITUTE

The *Hellenic Plant Protection Journal* (ISSN 1791-3691) is the new scientific publication of the Benaki Phytopathological Institute (BPI) replacing the *Annals of the Benaki Phytopathological Institute* (ISSN 1790-1480) which had been published since 1935.

Starting from January 2008, the Benaki Phytopathological Institute is publishing the *Hellenic Plant Protection Journal* semiannually, in January and July each year, and accepts for publication any work related to plant protection in the Mediterranean region regardless of where it was conducted. All aspects of plant protection referring to plant pathogens, pests, weeds (identification, biology, control), pesticides and relevant environmental issues are topics covered by the journal.

Articles in the form of either a complete research paper or a short communication (including new records) may be submitted. Instructions on how to prepare the manuscript are available at the end of the current issue and in BPI's website (www.bpi.gr). Manuscripts should be submitted in electronic form either by e-mail at editors@bpi.gr or by post on a disk addressed to the Editorial Board, Benaki Phytopathological Institute, 8 St. Delta str., GR-145 61 Kifissia (Athens), Greece. Only original articles are considered and published after successful completion of a review procedure by two competent referees.

For subscriptions, exchange agreements, back issues and other publications of the Institute contact the Library, Benaki Phytopathological Institute, 8 St. Delta str., GR-145 61 Kifissia (Athens), Greece, e-mail: m.kitsiou@bpi.gr.

This Journal is indexed by: *CAB Abstracts*-Plant Protection Database, *INIST* (Institute for Scientific and Technical Information) and *SCOPUS*.

The olive tree of Plato in Athens is the emblem of the Benaki Phytopathological Institute

Hellenic Plant Protection Journal **3:** 33-56, 2010

REVIEW ARTICLE

Oviposition aggregation pheromone for *Culex* mosquitoes: bioactivity and synthetic approaches

A.P. Mihou[1] and A.N. Michaelakis[2]

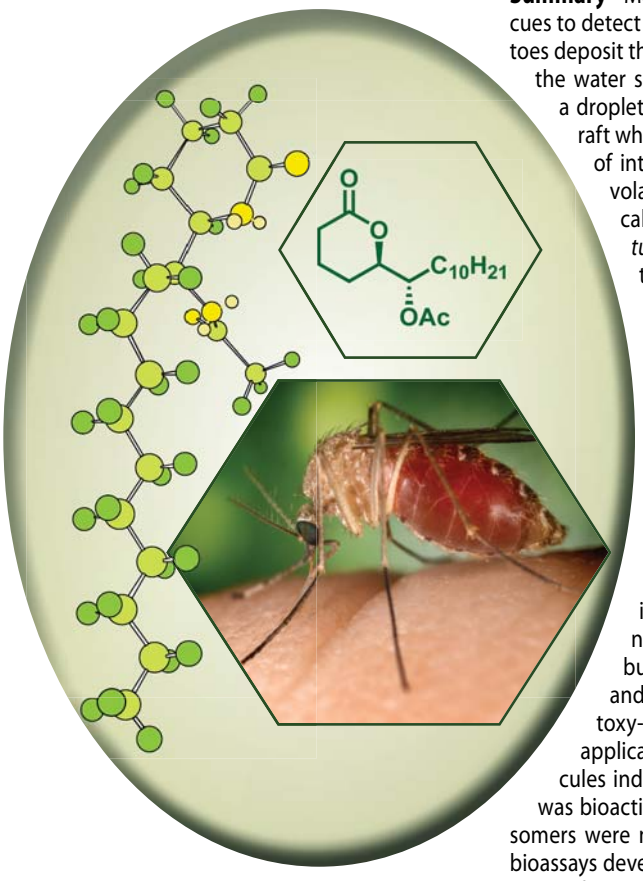


Figure 1. The oviposition aggregation pheromone for *Culex* mosquitoes and the vector of the West Nile virus. *Culex quinquefasciatus* mosquito has landed on a human finger.
Source of figure: http://phil.cdc.gov/phil/home.asp

Summary Mosquitoes use chemical and physical cues to detect water bodies. Female *Culex* mosquitoes deposit their eggs, in the form of egg rafts, on the water surface. Some of these species form a droplet at the apex of each egg in the egg raft which affects the oviposition behaviour of intraspecific gravid females. The main volatile compound, present in the apical droplets of the *Cx. quinquefasciatus* egg rafts, is the (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide, which acts as an attractant to other gravid females (of the same species complex) in order to oviposit nearby the pheromone source. There has been a plethora of biological approaches under laboratory, semi-field and field conditions in order to study the responses of gravid females. Additionally, since the general structure and stereochemistry were confirmed, several synthetic approaches have been developed, not only for the natural pheromone but also for the *erythro* enantiomers and all four diastereoisomers of 6-acetoxy-5-hexadecanolide. The interest and applications of natural and synthetic molecules indicated that only the (–)-(5*R*,6*S*) form was bioactive, while the other three diastereoisomers were not. This article reviews the various bioassays developed and also focuses on the most successful synthetic approaches published in literature.

Additional keywords: Bioassays, *Culex pipiens,* mosquito, oviposition, pheromone, synthesis, 6-acetoxy-5-hexadecanolide

[1] Chemistry Laboratory, Department of Science, Agricultural University of Athens, 75 Iera Odos Str., GR-118 55 Athens, Greece
[2] Laboratory of Agricultural Entomology, Department of Entomology and Agricultural Zoology, Benaki Phytopathological Institute, 8 St. Delta Str., GR-145 61 Kifissia (Athens), Greece
Corresponding author: a.michaelakis@bpi.gr

Introduction

In species with aquatic larvae, maternal reproductive success depends on the selection of a suitable site for depositing their eggs. This selection determines the continuation of such a species since, once in a hostile environment, immature stages are unable to move to another more suitable habitat and will perish (Mokany and Shine, 2003). The selection of the appropriate oviposition site by a gravid female mosquito is influenced by an oviposition aggregation pheromone. Each egg releases this pheromone, which acts as a signal to other *Culex* female adults to oviposit in the same water body.

Species of *Culex*, *Culiseta*, and *Uranotaenia* lay their eggs in raft (Figure 2, egg rafts) (Clements, 1999). These egg rafts, depending on the species, consist of 100 to about 400 eggs and are boat-shaped; the lower surface of each raft is convex and larger while the upper surface is concave and smaller (Clements, 1999; Christophers, 1944). Each egg forms a small droplet at the posterior. The first report on the existence of this droplet was in 1944 by Christophers (1944) who noticed that all egg droplets are of similar size and appearance, they appear shortly after oviposition and, if removed, they are formed again. Further studies reported that the apical droplet has also a righting function, turning capsized egg rafts to their normal position on the water surface (Iltis and Zweig, 1962) and that eggs are joined together by inter-digitation of the regularly spaced tubercles (Beament and Corbet, 1981).

The chemical composition of the droplet did not become known, until almost 40 years after it was first described. A series of chemical syntheses and bioassays confirmed that the droplet contained a pheromone, whose chemical structure was found to be the (–)-(5*R*,6*S*) isomer of 6-acetoxy-5-hexadecanolide, 1a, (Laurence and Pickett, 1982 and 1985) (see in Figure 3 all 4 diastereoisomers). Alongside the discovery of this pheromone, equally important and interesting have also been the various synthetic approaches that have been developed, not only for its natu-

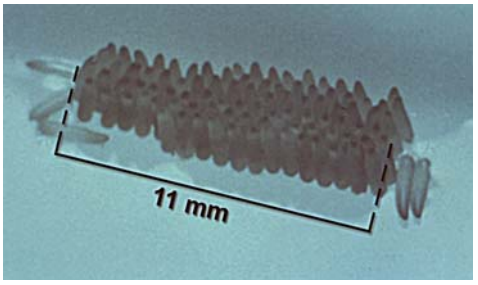


Figure 2. A typical mosquito egg raft measuring 11 mm in length. *Culex* mosquitoes lay their eggs one at a time, sticking them together in the form of a "raft".
Source of figure: http://phil.cdc.gov/phil/home.asp

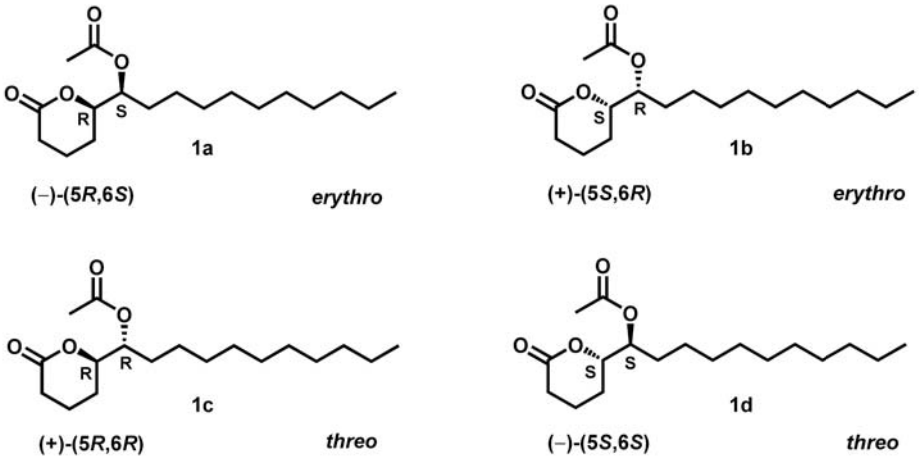


Figure 3. The four diastereoisomers of 6-acetoxy-5-hexadecanolide.

ral form, but also for the *erythro* enantiomers and for all four diastereoisomers of the 6-acetoxy-5-hexadecanolide.

1. Oviposition Bioassays

1.1. Egg-associated pheromone bioassays and observations

The first oviposition-inducing activity, caused by an egg-associated pheromone, was reported in the early '70s by Osgood (1971) (Table 1). This was demonstrated with *Culex tarsalis* employing 3 densities of egg rafts and also by split-airstream olfactometer. Preliminary analysis of the droplet indicated that this new chemical was a lipid, with low vapor pressure and consisted of a mixture of estrolide 1,3-diglycerides, that were composed of mono- and diacetoxy fatty acids (Osgood, 1971; Starratt and Osgood, 1972; Starratt and Osgood, 1973). Further studies concluded that the conclusion that the apical droplet of the mosquitoes' eggs contains straight-chain aliphatic fatty acids of C-12, C-14, C-16 and C-18. In addition, C-12β-OH and C-14β-OH fatty acids were found, with the latter to be the major component (Aharoni and Zweig, 1973).

The use of non-specific natural attractants (e.g. polyethylene or simulated egg rafts) shows no effect in oviposition behavior. Also, neither the presence of larvae, pupae and trapped adults, nor the 5% sucrose solution induced any response (Bruno and Laurence, 1979) (Table 2). In the same report, studies on the stability of the apical droplet indicated that, when the material was kept at 21°C in the light, it remained bioactive for 21 days, whereas when kept at 4°C in the dark, the material appeared to be mainly inactive for 28 days.

Table 1. Number of egg rafts laid from gravid females of *Culex* mosquitoes when the test bowl was primed with egg rafts of the same or another species. Reference: Bruno and Laurence (1979) modified by Clements (1999).

Test species	Priming species	Replicate tests	No. of priming egg rafts	Total no. of egg rafts laid in test bowls	Total no. of egg rafts laid in control bowls
Cx. quinquefasciatus	*Cx. quinquefasciatus*	10	1	374	46
	Cx. pipiens	10	1	333	163
	Cx. tarsalis	17	1	312	155
Cx. tarsalis	*Cx. tarsalis*	19	1	225	160
	Cx. tarsalis	10	10	273	113
	Cx. quinquefasciatus	14	1	145	124
	Cx. quinquefasciatus	10	10	295	115

Table 2. The response of *Culex* mosquitoes to factors other than egg rafts.

Test material	Replicate tests	Total no. of egg rafts laid in test bowl	Total no. of egg rafts laid in control bowl	Reference
polyethylene egg rafts	10	58	180	Bruno and Laurence (1979)
card egg rafts	12	322	160	Bruno and Laurence (1979)
1st-instar larvae	10	105	111	Bruno and Laurence (1979)
4th-instar larvae	10	408	337	Bruno and Laurence (1979)
pupae	10	376	138	Bruno and Laurence (1979)
5% sucrose	10	84	148	Bruno and Laurence (1979)
artificial white egg rafts	10	161	152	Osgood (1971)
artificial black egg rafts	10	153	152	Osgood (1971)

1.2. Bioassays with the pheromone under laboratory conditions

Responses of gravid females of *Cx. quinquefasciatus* to *erythro* racemate and to each enantiomer separately show that the pheromone is only in the (–)-(5*R*,6*S*) form. The natural product has relatively low vapour pressure, and several attempts have been made to alter its volatility. The methods employed, aimed at shortening the length of the carbon chain or opening the lactone ring of the molecule. However, both methodologies resulted in materials with no biological activity (Laurence and Pickett, 1985; Dawson *et al*., 1989). Instead, when hydrogen atoms were replaced by fluorine, the new molecules had biological activity as well.

More specifically, when the acetoxy group was replaced by a trifluoroacetoxy group, the new compound showed a higher activity than the natural product, probably due to its higher vapour pressure (Pickett and Woodcock, 1996). In addition, the heptadecafluoro analog, in which the *n*-octyl group is replaced by perfluoroctyl, retained high biological activity (Dawson *et al*., 1990).

The first bioassay employing all four stereoisomers of 6-acetoxy-5-hexadecanolide was conducted by Hwang *et al*. (1987). Predictably only the (–)-(5*R*,6*S*) enantiomer was active. The activity of the current isomer increased 50-fold when it was applied directly to the water surface and 100-fold when *Cx. quinquefasciatus* was employed instead of *Cx. tarsalis*. *Aedes aegypti* and *Anopheles quadrimaculatus* were not attracted to the pheromone (Figure 4). These experiments suggested once more that the current pheromone is genus-specific (Bruno and Laurence, 1979; Hwang *et al*., 1987). In contrast to the previous investigations, Sakakibara *et al*. (1984) reported that all four stereoisomers showed significant activity against *Cx. pipiens* biotype *molestus*. According to Clements (1999), these results cannot be taken as proof because there are no clear specifications of their synthetic procedures.

Antennae of *Cx. quinquefasciatus* gravid female mosquitoes were tested for sensitivity to test materials by recording electroantennograms (EAGs) during oviposition behaviour studies (Mordue *et al*., 1992). The tested materials were the oviposition pheromone (*erythro* racemate) and polluted water (made by fermenting rabbit droppings in distilled water). Both EAG responses and behaviour studies to the oviposition pheromone increased in a dose-dependent manner and the combination of the two materials increased oviposition with the total effect being additive. In 1985, Laurence and Pickett showed that the dose-responses over the concentrations were a flat curve (Laurence and Pickett, 1985). However, in 1993, Pickett with other co-workers, using a similar method of presentation, obtained a dose-dependent response for a certain range of pheromone. After a critical dose, the pheromone caused a significant reduction in oviposition behaviour (Blackwell *et al*., 1993).

High pheromone concentration could either result in confusion of the gravid females with respect to the oviposition sites or repel gravid females of laying their eggs. Although Millar *et al*. (1994) reported that attraction was constant even to very high pheromone concentration (1000 μg per cage), other researchers, who synthesized the oviposition pheromone as a mixture of all diastereoisomers and tested it against *Cx. p.* biotype *molestus*, obtained similar results to Blackwell *et al.* (1993) (Michaelakis *et al*., 2005).

Olagbemiro *et al*. (1999 and 2004) employed for the first time a plant-derived

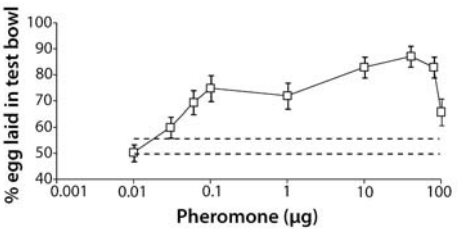


Figure 4. Oviposition behaviour of *Cx. quinquefasciatus* gravid females to different amounts of pheromone. Oviposition dishes contain the synthetic oviposition pheromone, *erythro*-6-acetoxy-5-hexadecanolide. The broken lines represent the upper and lower values of the control mean±SEM (52.6±3.7%, *n*=10) [for more details see Blackwell *et al*. (1993)].

pheromone material produced from the seed oil of the summer cypress, *Kochia scoparia* (Chenopodiaceae). Laboratory bioassays with 3 and 5 μg of each pheromone (plant-derived and synthetic) revealed that these two compounds were equally attractive. The bioactivity of the plant-derived pheromone was comparable to that of the synthetic oviposition pheromone (1:1:1:1 mixture of the four stereoisomers).

Apart from the reaction to the pheromone, *Culex* mosquitoes may also have an intrinsic neurophysiological response to specific indoles, which could explain why it oviposits in polluted water such as skatole water (3-methyl indole) (Pickett and Woodcock, 1996). According to Millar *et al.* (1994), responses to the pheromone combined with several doses of 3-methyl indole were shown to be additive rather than synergistic. The same additive pattern was also observed in the presence of skatole water at two concentrations with plant-derived pheromone (Olagbemiro *et al.*, 2004).

Three larvicides, an insect growth regulator (pyriproxyfen), an organophosphate (temephos) and a microbial (*Bacillus thuringiensis* subsp. *israelensis,* Bti) were tested separately or combined with synthetic pheromone, as agents that can keep water free of mosquito larvae (Kioulos *et al.*, 2007-2008). Preliminary results of oviposition bioassays revealed that, with the exception of temephos, all the other tested larvicidals, when used separately, repel gravid females of laying eggs for the first two days. However, when synthetic pheromone was combined with the three larvicidals, only temephos and the microbial agent (Bti) followed the same attractant pattern as the synthetic pheromone alone.

For the first time a slow release system has been developed that incorporates 6-acetoxy-5-hexadecanolide (mixture of the four stereoisomers). Laboratory bioassays studying attractancy over time, and showed a dose-dependent response. The microencapsulated pheromone was found to be sufficiently attractive to gravid female mosquitoes for almost 40 days (Figure 5) (Michaelakis *et al.*, 2007). The addition of temephos did not affect the activity of the pheromone while the bioassays showed 100% mortality. When an aged infusion was combined with aged pheromone (microencapsulated) there was a synergistic effect only for the first day, while all the other days the oviposition pattern was similar to that of the microencapsulated pheromone, with a minor exception on the 15th day (Michaelakis et al., 2009).

Recently, Leal *et al.* (2008) employed immunohistochemistry to prove the stereospecific interaction between the receptor of the *Culex* mosquitoes and the pheromone and that the receptor accepts only the (–)-(5*R*,6*S*)-enantiomer. The studies revealed that an odorant-binding protein from *Cx. p. quinquefasciatus*, CquiOBP1, is expressed in trichoid sensilla on the antennae. CquiOBP1 exists in two forms, monomeric and dimeric; the mosquito oviposition pheromone is bound in the monomeric form in a pH-dependent manner. While all previous reports measured the effect of pheromone (and also of other attractants) on egg laying, in this report authors evaluated the pheromone for attraction of gravid females (i.e., numbers captured). The results suggested that pheromone might be an arrestant rather than an attractant because it could increase egg laying but not necessarily gravid female capture.

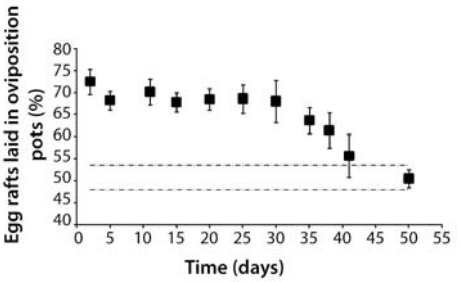


Figure 5. Oviposition behaviour of gravid females *Cx. p.* biotype *molestus* to aged microencapsulated pheromone (slow release system for the synthetic mixture of the four diastereomers of 6-acetoxy-5-hexadecanolide). The broken lines represent the upper and lower values of the control mean±SEM (50.45 ± 2.6%, $n = 10$) [for more details see Michaelakis *et al.* (2007)].

1.3. Bioassays with pheromone under semi-field and field conditions

In western Kenya oviposition pheromone was tested for the first time in the field. Five milligrams of the active (–)-(5*R*,6*S*) enantiomer was exploited in a tablet formulation and used in established breeding sites. Compared to the control, 82% more females oviposited around the pheromone source. The activity of the pheromone continued for four days after application (Otieno *et al*., 1988). The combination of the pheromone with an insect growth regulator showed both an acceptable oviposition activity and sufficient larvicidal effect. During that field test, there was an attempt to attract *Culex* mosquitoes in a non-breeding site. Despite using a very high dose of pheromone, only one egg raft was found and further analysis showed a higher pH than that in the breeding sides (established). This fact suggests that the lack of oviposition in non-established breeding sites involves factors in the attraction mechanisms other than the pheromone.

The same phenomenon was observed in Tanzania when synthetic oviposition pheromone was used to non-breeding sites (Mboera *et al*., 1999). When water from established breeding sites was treated with the pheromone, it received more egg rafts than the untreated water with nine days residual activity.

The mixture of the pheromone with soakage pit water or grass infusions indicated a synergistic effect. As mentioned before, *Culex* mosquitoes prefer breeding in water with a high organic content (polluted water) rather than clean water. This explains why females prefer water that consists of complex mixtures of compounds that vary over time, including products of bacterial degradation such as soakage pit water or grass infusions.

Apart from laboratory studies, plant-derived pheromone was also employed in field studies (Olagbemiro *et al*., 2004). Both laboratory and field bioassays revealed equal attractiveness level for plant-derived and synthetic pheromones. Of the two pheromones, only the plant-derived one was used in field tests in a mixture with skatole.

Under semi-field conditions, oviposition was induced by an amount of pheromone equal to a single egg raft (0.3 μg). This was the first report that compared a synthetic pheromone with the natural product (Braks *et al*., 2007). The oviposition jars were arranged in two squares (small and large, "near" and "distant" respectively). In the "near" bioassays, a single egg raft with hay infusion had the highest oviposition activity, while by increasing the egg rafts from 1 to 10 (or by adding 3.0 μg synthetic pheromone) synergistic effects were observed between the oviposition pheromone and the hay infusion at both distances. Authors suggested three possible explanations for the number of females ovipositing and the relation to the distance: a) females may fly along the edge of the cage searching for a suitable oviposition site by making a distinction between hay infusion based on the oviposition pheromone, b) females fly along the edges of the cage and after encountering the pheromone at close range land and/or oviposit and c) females visit and land at several oviposition sites before the final decision.

2. Synthetic approaches

According to Pickett and Woodcock (1996) "The characterization of the oviposition pheromone for mosquitoes in the genus *Culex* as a novel chiral lactone ester provided the impetus for a number of sophisticated asymmetric syntheses and economical large-scale routes to racemic products".

This section focuses on the most successful asymmetric synthetic approaches published in literature. The synthetic strategies are grouped depending on the method used to induce chirality to the molecule. Carbohydrates and aminoacids were used as starting materials. Kinetic resolution (Sharpless asymmetric epoxidation or oxidation protocol) of racemic starting materials and Sharpless asymmetric epoxidation or dihydroxylation of olefins have also been utilized for the enantioselective synthesis of

mosquito pheromone.

Finally, it is noteworthy that in several cases, apart from the Wittig reaction and the use of Grignard reagents, radical chain reactions or organolithium substrates have also been successfully employed for the construction of the carbon skeleton.

2.1. Asymmetric syntheses from carbohydrates

Several approaches to (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide have exploited carbohydrates as chiral starting materials. In this regard, Kang and Cho (1989) published an enantiospecific synthesis of (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide, **1a**, from readily available (–)-2-deoxy-*D*-ribose (Figure 6).

Acetonide **2**, prepared as an anomeric mixture in ~60% yield from commercial (–)-2-deoxy-*D*-ribose was condensed with *n*-octyltriphenylphosphorane to give the unsaturated alcohol **3** in a ratio Z/E ~ 9/1. Catalytic hydrogenation on Pd/C provided the saturated alcohol which was converted to the iodide **4**. Elongation of the carbon skeleton was achieved by a radical chain reaction of the iodide **4** with ethyl acrylate leading to the condensed product **5**. Hydrolysis of ester with potassium hydroxide, deprotection of the acetonide, lactonization under acidic conditions and acetylation of the semifinal hydroxylactone afforded the target compound **1a** *(7 steps, 10% overall yield)*.

Ichimoto reported the synthesis of (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide in an enantiomerically pure form via the key intermediate (+)-(*S*)-1-[(*R*)-oxiran-2-yl]undecan-1-ol, **6**, derived from 2-deoxy-D-ribose (Figure 7) (Ichimoto *et al.*, 1988). Coupling reaction of the hydroxyl protected product of **6** with 3-butenylmagnesium bromide and oxidative cleavage of the terminal olefinic group afforded pyran derivative **8**, which was oxidized with pyridinium dichromate to give the desired lactone. Hydrolytic cleavage of the THP group and subsequent acetylation furnished **1a** *(12 steps, from 2-deoxy-D-ribose, 12.7% overall yield)*.

A formal synthesis of **1a** from *D*-ribose was reported by Gallos *et al.* (2000) based on

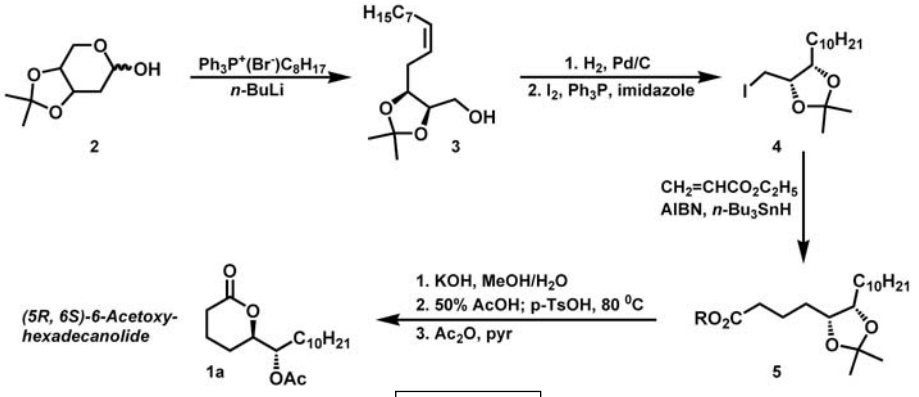


Figure 6

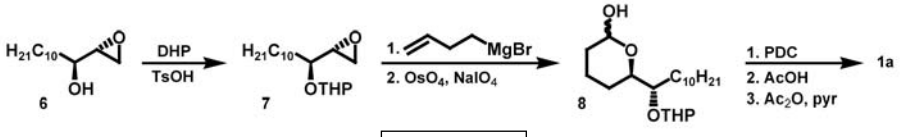


Figure 7

three simple transformations: i) elongation at C-5 position by oxidation and Wittig olefination, ii) reductive cleavage of the C-4 carbon with magnesium in methanol, iii) introduction of the long chain at C-1 of *D*-ribose by Wittig reaction. The olefin produced was hydrogenated to give the desired methyl ester. Further basic hydrolysis of the ester afforded the known acid **13**, which could be easily transformed to the desired pheromone by simple manipulations according to literature (Kang and Cho, 1989) (Figure 8) *(7steps, 11.8% overall yield)*.

Wu (1991) published the synthesis of **1a** from *D*-glucose. 2,3-*O*-Ethylidene-*D*-erythrose, easily prepared as an anomeric mixture in three steps from *D*-glucose, was treated with triphenylnonylphosphorane to afford a mixture of alkenes, **15**. Subsequent oxidation, Wittig reaction of the produced aldehyde, hydrogenation of the two double bonds, deprotection, acid lactonization and finally acetylation furnished the natural pheromone (Figure 9).

The stereoselective synthesis of (–)-(5*R*, 6*S*)-6-acetoxy-5-hexadecanolide from the readily available chiral pool compound, *L*-(+)-tartaric acid was accomplished by Prasad (Prasad and Anbarasan, 2007b) (Figure 10). The synthetic sequence includes the elaboration of an *α*-benzyloxy aldehyde, **18**, derived from tartaric acid with ring closing metath-

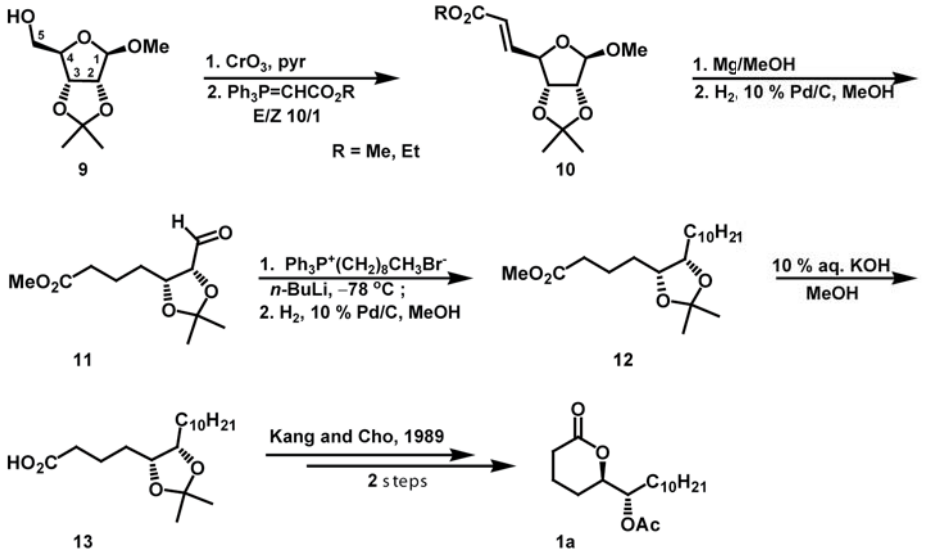


Figure 8

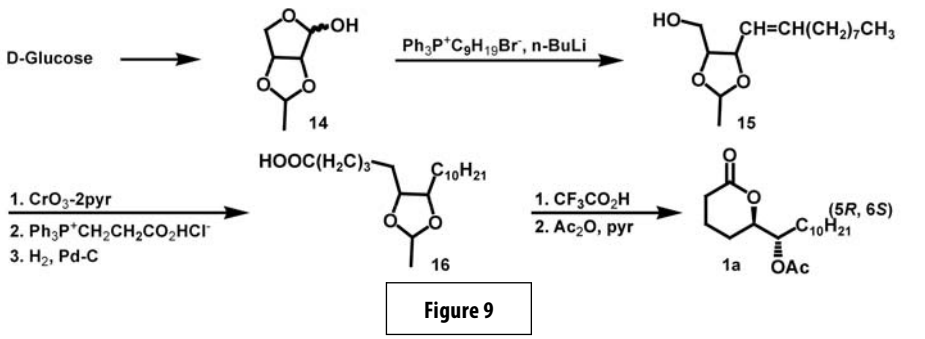


Figure 9

esis as the key step. The secondary hydroxyl group was epimerized under Mitsunobu conditions furnishing alcohol **22**. Acid hydrolysis led to the corresponding lactol, which was subjected to oxidation to afford the final product *(7 steps from* **19***, 30.7% yield)*.

Kotsuki reported an enantiospecific synthesis of mosquito pheromone originating from *L*-tartrate as chiral source (Kotsuki *et al*., 1990). The synthesis employed an efficient carbon-carbon bond forming reaction of triflate with copper(I)-catalyzed Grignard reagent (Figure 11).

The one-pot procedure from tosyl-triflate **24** through initial reaction first with 3-butenylmagnesium bromide/CuBr and further alkylation with lithium di-*n*-nonylcuprate provided intermediate **25** in 58% overall yield from **24**. Conversion of **25** to hydroxy *δ*-lactone **26** was accomplished by one-pot ozonolysis, dimethyl sulfide reduction, Ag_2O oxidation, acidification to make free hydroxy acid and finally lactonization. Acetylation of hydroxy lactone with simultaneous inversion of configuration was accomplished by applying Ikegami's procedure (Torisawa *et al*., 1984) *(10 steps, 28.4% yield)*.

From *D*-isoascorbic acid, a general approach to (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide, via a four carbon atoms bis-epoxide equivalent was reported by Gravier-Pelletier (Gravier-Pelletier *et al*., 1994) (Figure 12). Alkylation resulted in the opening of the free epoxide allowing the introduction of the carbon chain. Nucleophilic opening of the second epoxide, being masked into the protected glycol, by ethylpropiolate, led after hydrogenation of the triple bond and lactonization to the optically pure pheromone **1a** *(8 steps, 22% yield)*.

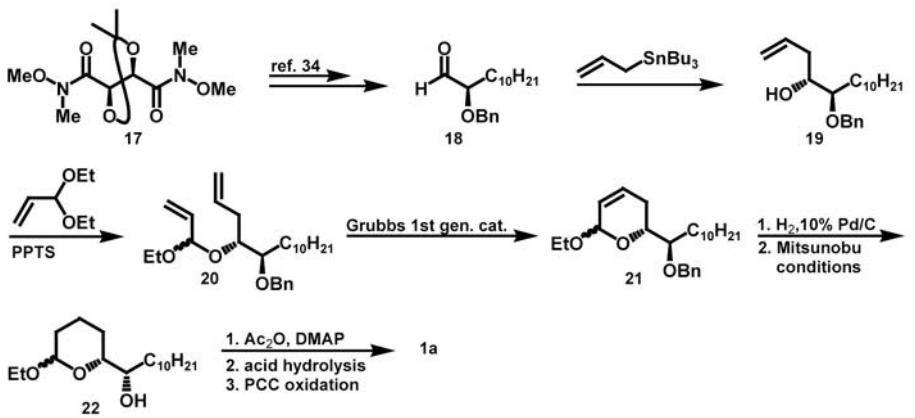


Figure 10

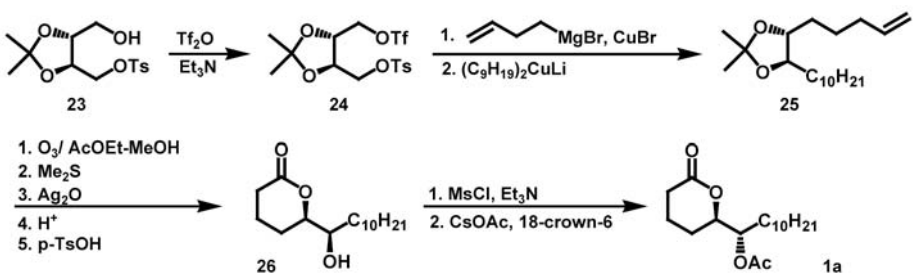


Figure 11

2.2. Asymmetric syntheses via kinetic resolution of racemic starting materials

2.2.1. Kinetic resolution of racemic 2-furylundecanol

Kinetic resolution of racemic secondary 2-furylundecanol using catalytic amounts of the titanium(IV)–tartrate complex was achieved successfully, providing optically active 2-furylundecanol (80–>98% *ee*) and the corresponding optically active pyranone (Figure 13). Thus, utilizing (*S*)-1-(2-furyl)undecanol as a chiral building block and following functional and protective group manipulations Honda furnished the desired pheromone (Figure 14) (Kametani *et al*., 1990) *(10 steps, 25% yield).*

2.2.2. Kinetic resolution of allylic alcohols

Several approaches to (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide, **1a**, have exploited the kinetic resolution of secondary or cyclic allylic alcohols. In this regard, Mori and Otsu-

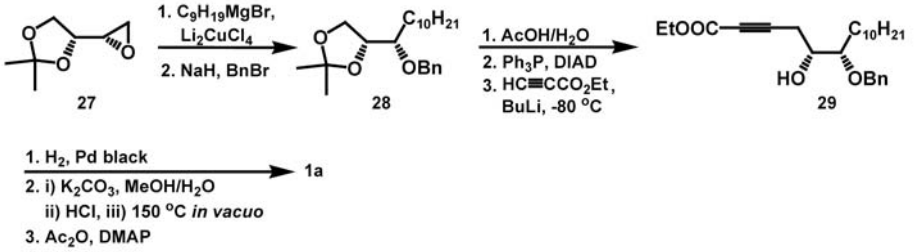


Figure 12

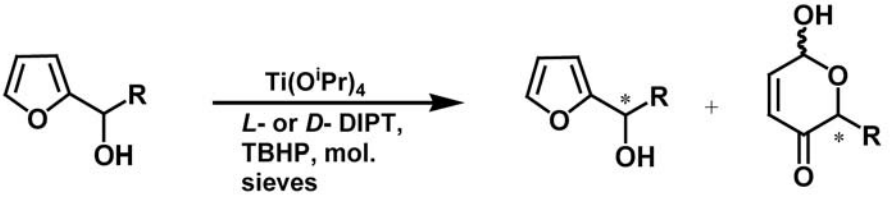


Figure 13

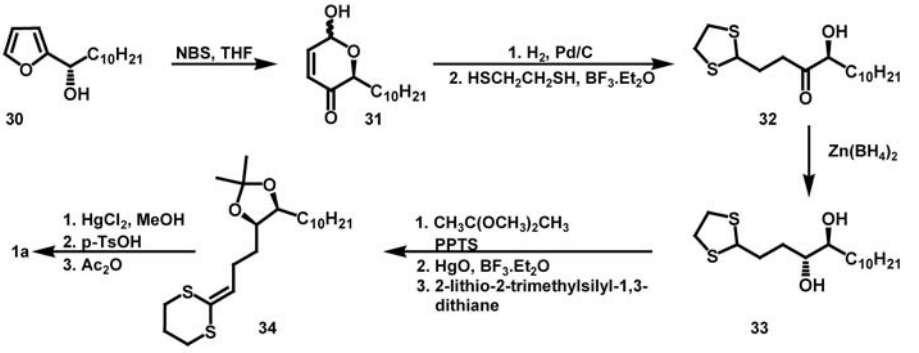


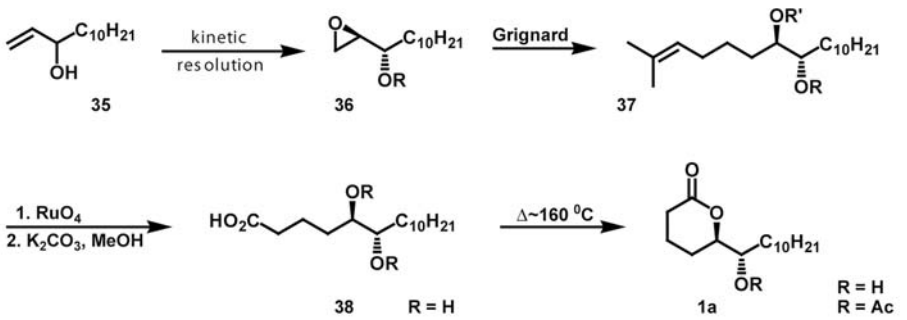
Figure 14

ka (1983) employed the Sharpless asymmetric epoxidation as the starting step. Kinetic resolution of 1-tridecen-3-ol by enantioselective epoxidation using diisopropyl tartrate gave an optically active epoxy alcohol in good enantiomeric excess (91-94.5% *ee*). A Grignard reagent prepared from 4-methyl-3-pentenyl bromide was added to the epoxide to effect the C-chain elongation. The terminal double bond was oxidized to an acid by the Sharpless modification of the catalytic RuO_4 oxidation. Upon heating the acid lactonized to give a *δ*-lactone. Acetylation of the lactone gave the final product (Figure 15) *(9 steps, 33% yield).*

Some years later, the same enantiomerically pure epoxy alcohol **36** was employed by Barua and Schmidt (1986) for their synthesis. Reaction of *β*-lithiated-*β*-ethylthioacrylate with epoxide **36** and subsequent Raney nickel treatment gave the oviposition pheromone **1a** (Figure 16) *(4 steps, 35% yield).*

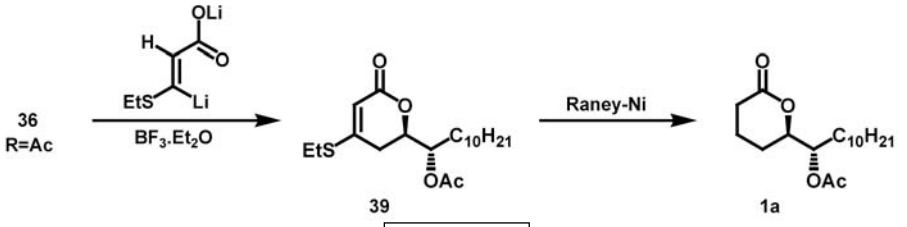
The synthesis of **1a** from readily available 1,2-cyclohexanediol using kinetic resolution of cyclic allylic alcohol by modified Sharpless asymmetric epoxidation reagent as the key step was reported by Wang *et al.* (1990) (Figure 17). The asymmetric allylic alcohol was initially converted to the silyl ether. Hydroboration of the silyl ether with 9-BBN followed by alkaline hydrogen peroxide workup, yielded a mixture of the alcohol **42** and its diastereoisomer in a ratio 2:1. After chromatographic separation, compound **42** was oxidized with PDC to the corresponding ketone. Beayer-Villiger oxidation of ketone, followed by acetylation afforded the target lactone **1a** *(8 steps, 23% yield).*

Bonini described the enantio- and stereoselective synthesis of (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide via chemo-, regio- and stereoselective opening with LiI of a chiral epoxy alcohol precursor which was obtained by kinetic resolution of the racemic allylic alcohol **44** (Figure 18) (Bonini *et al.*, 1995). The resulting halohydrin was easily reduced to the corresponding *erythro* vicinal diol by *n*-Bu_3SnH, which was then protected as acetonide. Oxidation with $RuCl_3$, lactonization with catalytic amount of *p*-



35 36 37 38 R = H 1a

Figure 15



39 1a

Figure 16

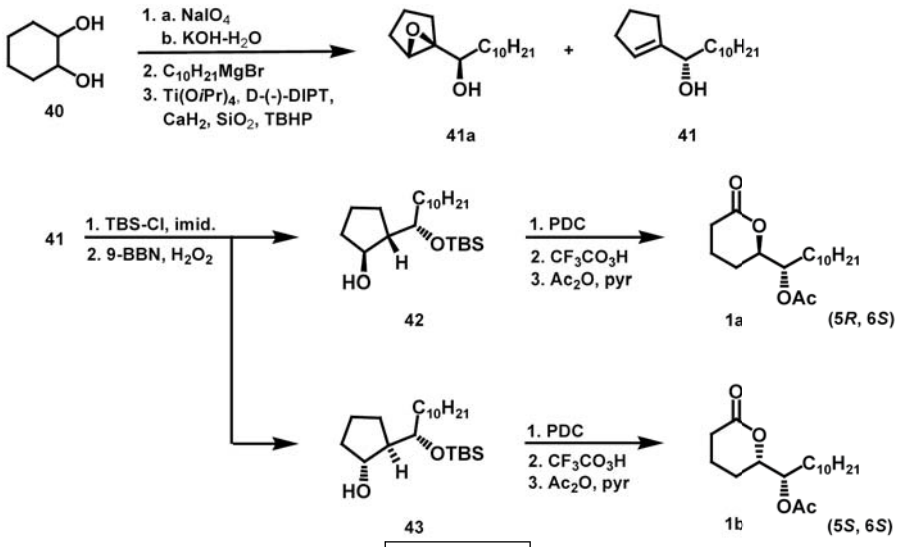


Figure 17

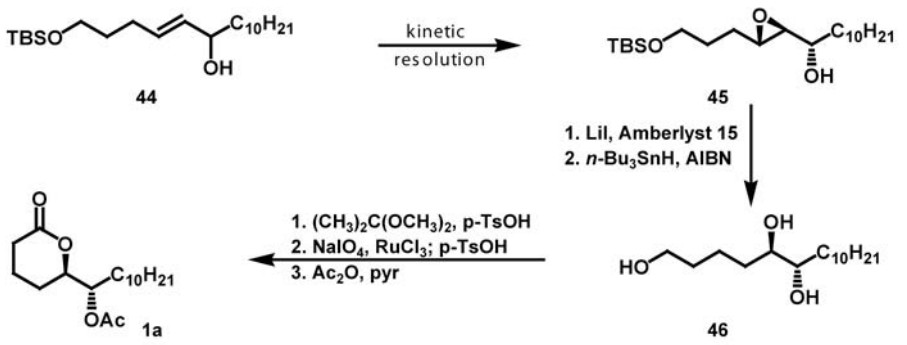


Figure 18

TsOH and finally acetylation afforded lactone **1a** *(6 steps, 23% yield).*

2.3. Syntheses via asymmetric epoxidation or dihydroxylation reactions

All the four optical isomers of *Cx. quinquefasciatus* oviposition pheromone were synthesized by Lin *et al.* (1985) via Sharpless asymmetric epoxidation protocol (Figure 19). The allylic alcohol, prepared from propargylic alcohol by partial hydrogenation, was exposed over Sharpless conditions to give all the optically active epoxy alcohols. The (2*S*,3*S*)- isomer was treated with Collins reagent to give the epoxy aldehyde which after Wittig reaction led to the complete construction of the carbon skeleton. Oxidation of the free hydroxyl group to an acid proceeded without affecting the chiral epoxy moiety. The acid was then lactonized in acidic conditions to form (5*R*,6*S*)-6-hydroxy-5-hexadecanolide with inversion of the configuration at C5. Subsequent acetylation completed the synthesis of **1a** *(9 steps,* 15-20% *yield).*

Singh and Guiry (2009) also used Sharpless asymmetric epoxidation reaction as one of the key steps of their synthesis (Figure 20).

They also developed $ZrCl_4$-catalyzed deprotection of 1,3-dioxane and ring closure to prepare a benzyloxy-substituted tetrahydropyrane as the key intermediate (*7 steps, 28% yield*).

Methyl *trans*-hexa-5-decenoate was dihydroxylated by Lohray and Venkateswarlu, (1997) using Sharpless asymmetric dihydroxylation reaction followed by treatment with thionyl chloride to furnish cyclic sulfite as the major product (Figure 21). The cyclic sulfite was oxidized to the cyclic sulfate. The methyl ester of cyclic sulfate **36** was hydrolysed to affect an intramolecular cyclization by the *in situ* generated carboxylate ion with concomitant S_N2 intramolecular ring opening of cyclic sulfate with inversion of configuration at the reacting center. Acetylation

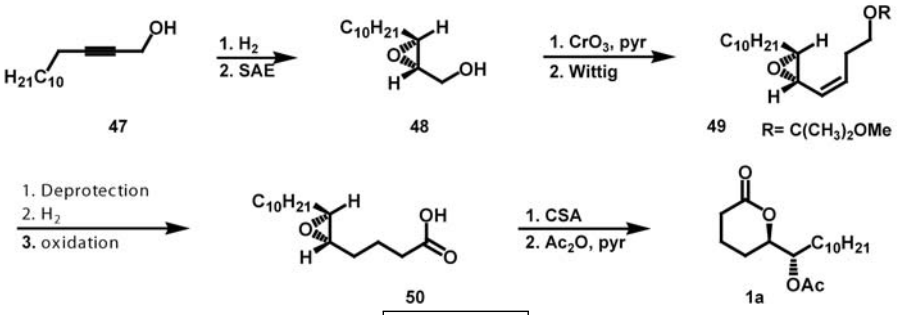


Figure 19

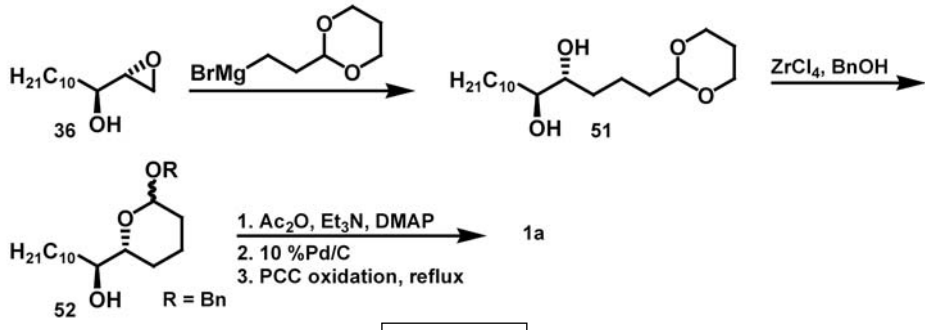


Figure 20

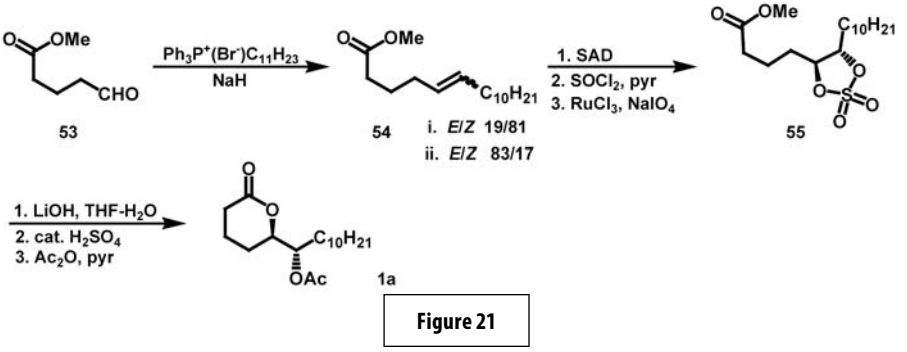


Figure 21

of hydroxylactone afforded natural oviposition attractant pheromone **1a** (*7 steps, 11% yield*).

Couladouros and Mihou (1999) published a methodology of synthezing six membered asymmetric lactones from *δ*-valerolactone using the following reaction sequence: Reduction, Wittig-Schlosser coupling, Sharpless asymmetric dihydroxylation, oxidation and lactonization. The offset of the synthesis involved the *trans* olefinic substrate **57** as derived from the Wittig- Schlösser reaction between the phosphonium salt of bromoundecane with 5-[(*t*-butyldiphenylsilyl) oxy]pentanal (E/Z ratio 9/1). Application of the Sharpless asymmetric dihydroxylation protocol to substrate **57** furnished the optically pure diol. (5*R*,6*S*)-6-acetoxy-5-hexadecanolide was prepared via a carbonate ester, utilizing a novel lactonization with inversion of stereochemistry (Figure 22) (*8 steps, 38% yield*).

In 1990, Wu Y.-L. and Wu W.-L. achieved the synthesis of **1a** in 10% overall yield starting from (*S*)-*O*,*O*-isopropopylideneglyceraldehyde and in 35% overall yield starting from (*R*)-*O*,*O*-isopropopylideneglyceraldehyde, using diastereoselective dihydroxylation (with catalytic amounts of OsO_4) as the key step (the ratio of the two isomers being about 9:1). When (*S*)-*O*,*O*-isopropopylideneglyceraldehyde was used as the starting material, the lipophilic side chain was introduced after "late" lactone ring formation, whereas in the second case the lipophilic side was "early" introduced (Figure 23).

2.4. Chiral pool syntheses

The benzyl derivative of *S*-2-hydroxy-dodecanal, prepared by a previously described asymmetric synthesis based on a chiral

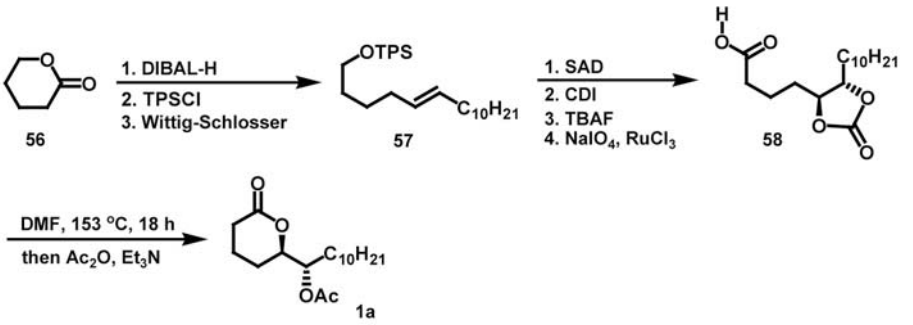


Figure 22

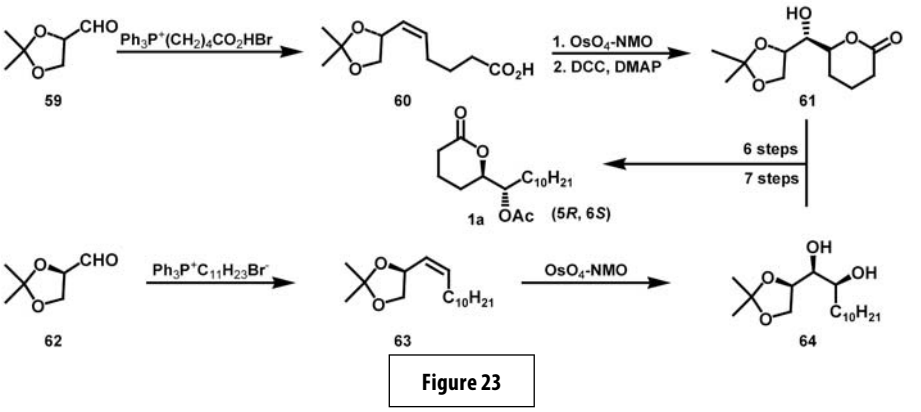


Figure 23

1,3-oxathiane, was converted by Ko and Eliel (1986) into (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide in a highly diastereoselective fashion (Figure 24).

The construction of the carbon skeleton was accomplished by Grignard addition of 5-pentenylmagnesium bromide to *α*-benzyloxy aldehyde **65**. Mitsunobu inversion at C6, ozonization, oxidation and finally lactonization smoothly led to (5*R*,6*S*)-6-benzyloxyhexadecanolide. Deprotection of the benzyl group and acetylation of hydroxy lactone afforded the target compound **1a** (*8 steps, 36% yield*).

(–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide has also been targeted by Machiya *et al.* (1985). Machiya's approach commenced from isopropylidene-*L*-glyceraldehyde (Figure 25) in which the nucleophilic addition of Grignard reagent involved the diastereoface-differentiating process as the key step affording an alcohol, which was smoothly transformed in five steps to aldehydes **69** and **70**. Those aldehydes were a chromato-

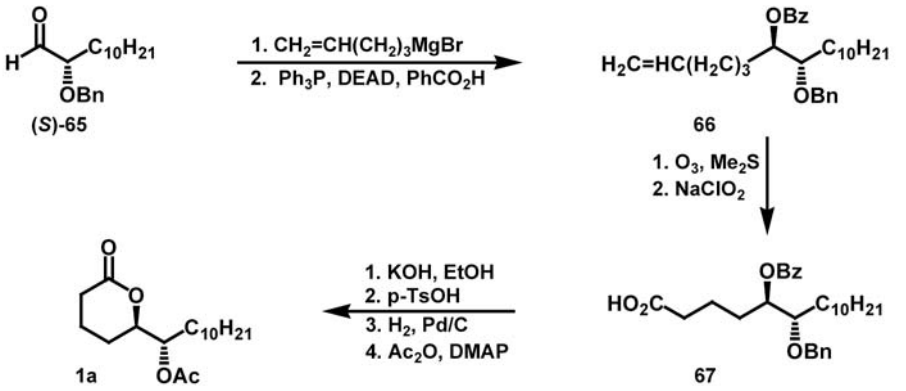


Figure 24

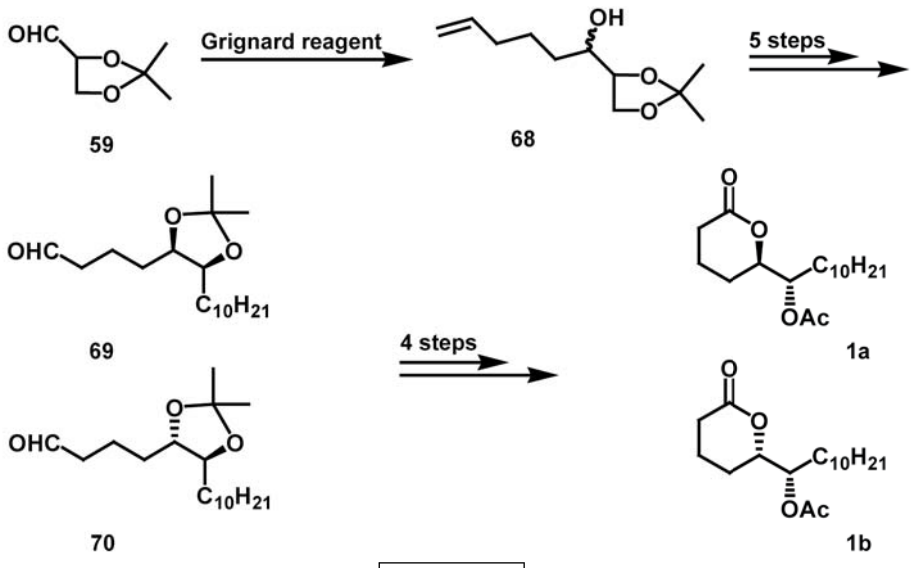


Figure 25

graphically separable mixture of (5*R*,6*S*)- and (5*S*,6*S*)-isomer. Oxidation of **69** followed by deprotection, lactonization and acetylation afforded the optically pure pheromone **1a** (*10 steps, 24.8% yield*).

Years later Sabitha *et al.* (2006) reported the asymmetric total synthesis of (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide via the key intermediate **75** which was prepared by a Grignard reaction from alcohol **71** as a major product with anti-selectivity (80% *ee*). The alcohol was easily accessed via two different routes from THP protected hex-5-en-1-ol **72** and epoxy chloride **73** (Figure 26) (*9 steps from 71, 14.5% yield*).

R-2,3-Cyclohexylideneglyceraldehyde has been used by Chattopadhyay to prepare functionalized *δ*-lactones (Dhotare *et al.*, 2005). Stereo-differentiating organolithium addition to aldehyde **77** in hexane and ether was found to be highly *anti* selective leading to the required (*R*,*S*)-1,2-diol unit. Subsequent functional and protective group manipulations furnished (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide (Figure 27) (*9 steps, 15% yield*).

A synthetic scheme starting from the reaction between the lithiated *N*-allyl-*N*-methyl-(bisdimethylamino)phosphoramide anion and the triflate derivative of (*R*)-(–)-2,3-*O*-isopropylideneglycerol was described by Coutrot to prepare the key chiral synthon (*R*)-5-formyl-*δ*-valerolactone **83** (Figure 28) (Coutrot *et al.*, 1994). Chemo- and enantioselective addition of *n*-decylmagnesium bromide to aldehyde **83** afforded (5*R*,6*S*)-6-hydroxy-5-hexadecanolide which after acetylation yielded the desired pheromone *(7 steps, 13% yield)*.

2.5 Proline – catalyzed asymmetric aldol reactions

Years after his first publication on the asymmetric synthesis of mosquito pheromone, Kotsuki reverted to the synthesis of the target molecule with a convenient method for proline-catalyzed asymmetric aldol reactions using synthons of straight-chain aliphatic aldehydes and aldehydes bearing a 1,3-dithiane moiety at the *β*-position (Ikishima *et al.*, 2006). The required aldehyde **86** was readily prepared from ethyl acetoacetate after chain elongation. The aldol reac-

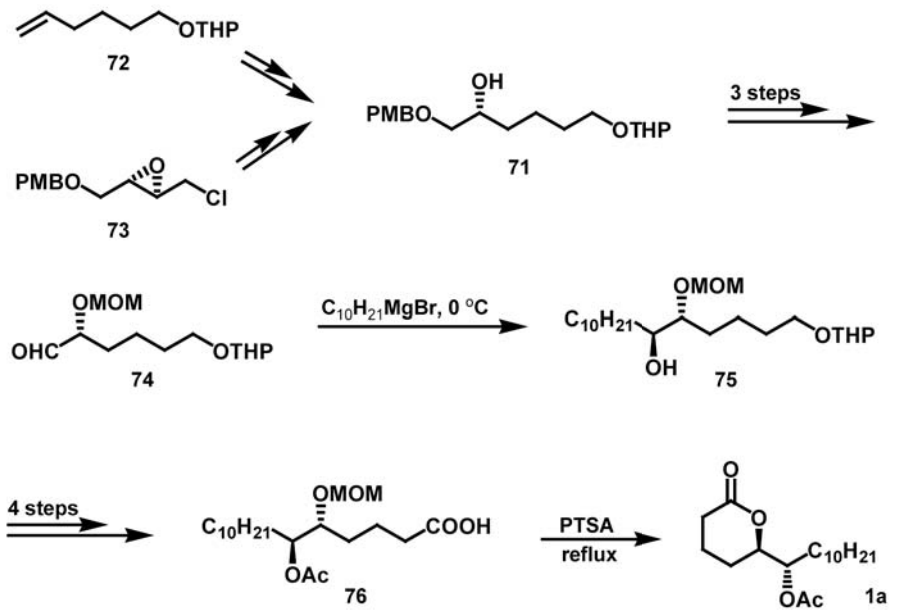


Figure 26

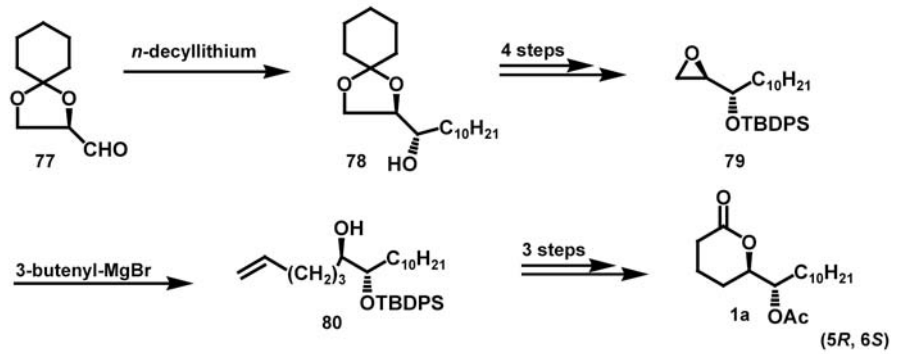


Figure 27

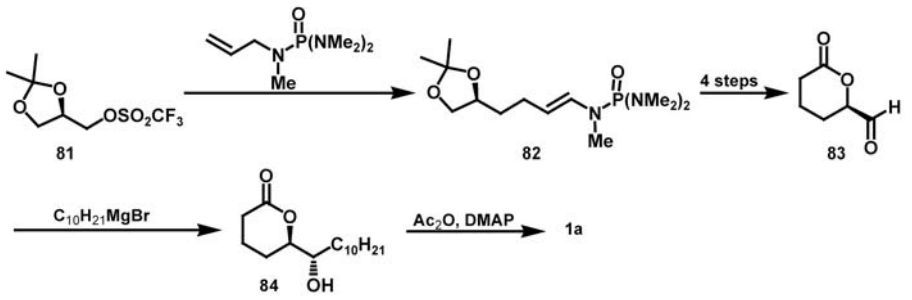


Figure 28

tion of **86** with cyclopentanone in the presence of 30 mol% of *L*-proline gave *syn*- and *anti*-adducts (75:25 ratio). The *syn*-adduct **87** was then treated with Raney-Ni to give the desired *β*-hydroxy ketone which upon oxidation and acetylation led to molecule **1a** (Figure 29) *(4 steps, 35.5% yield)*.

2.6. Chemo – enzymatic synthesis

A chemo-enzymatic synthesis of (–)-(5*R*, 6*S*)-6-acetoxy-5-hexadecanolide was published by Schick (Henkel *et al.,* 1995). The methyl ester of *racemic* 5,6-dihydroxyhexadecanoic acid was prepared by a Wittig reaction of (4-carboxybutyl)-triphenylphosphonium bromide with undecanal followed by esterification with methanol and *cis* dihydroxylation with osmium tetroxide. After conversion of the dihydroxy ester **90** and **91** into 6-hydroxy-hexadecanolide by lactonization, (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide was obtained by an enantioselective lipase-catalyzed acetylation with vinyl acetate (Figure 30) *(6 steps, 11% yield)*.

2.7. Chemo – microbial synthesis

Oehlschlager explored the role of baker's yeast as a chiral reagent presenting a multi-step formal synthesis of (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide (Ramaswamy and Oehlschlager, 1991). His approach to the synthesis involved the elaboration of the chiral diol **94** (Figure 31). Diol **94** was prepared by baker's yeast reduction of hydroxyketone **93** in 47% yield and an optical purity of 98.5%. The primary alcohol was converted enzymatically to butyrate whereas the secondary alcohol was THP protected. Regeneration of the primary alcohol, oxidation and addition of *n*-decylmagnesium bromide afforded an *erythro*:*threo* mixture (45:55). Conversion of this mixture into *erythro* diol was achieved through Swern oxidation, removal of the THP group and

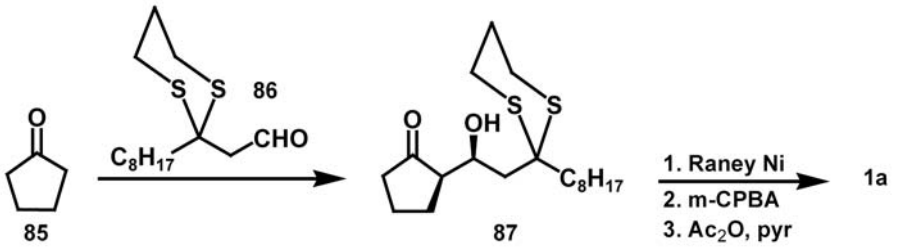


Figure 29

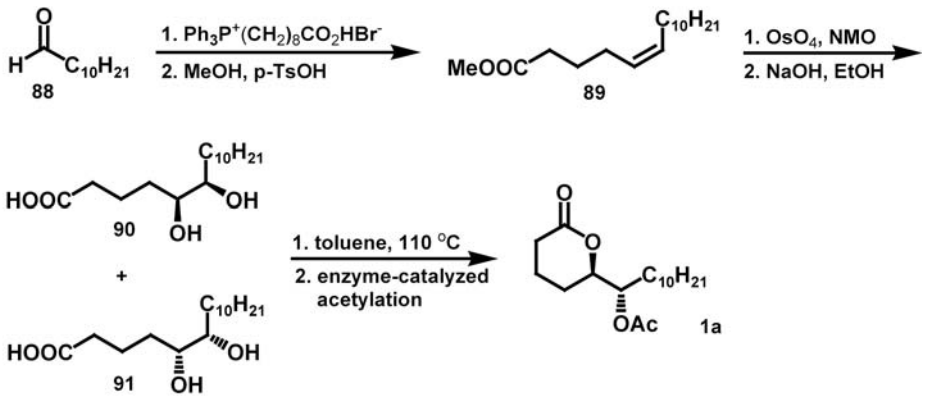


Figure 30

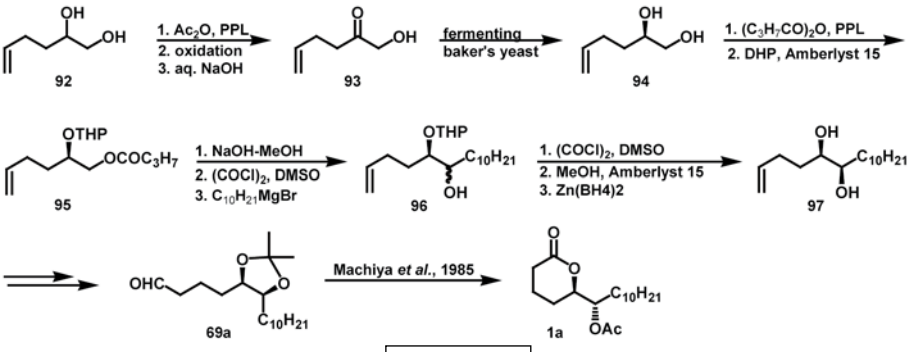


Figure 31

$Zn(BH_4)_2$ reduction of the produced ketone to the diol **97**. More functional and protective group manipulations led to the intermediate **69a** which could be transformed to the desired pheromone according to literature (Machiya *et al.*, 1985).

2.8. Oxidative cyclization with Ru(+2) complexes

The synthetic utility of the oxidative cyclization of bis-homopropargylic alcohols with Ru(+2) complexes as catalysts and *N*-hydroxysuccinimide to form *δ*-lactones was il-

lustrated by the synthesis of the oviposition pheromone of the mosquito *Cx. quinquefasciatus* (Trost and Rhee, 2002). A catalytic system composed of $CpRu[(p\text{-}CH_3O_6H_4)_3P]_2Cl$ and excess $(p\text{-}CH_3O\text{-}C_6H_4)_3P$ directs the reaction toward the oxidative cyclization to form *δ*-lactones in good yields (Figure 32).

2.9. Syntheses of racemic or diastereoisomeric mixtures

Ochiai *et al.* (1985) synthesized the *erythro*-6-acetoxy-5-hexadecanolide starting from (*E*)-hexadec-5-enal **99** (Figure 33). Oxidation of aldehyde **99** with sodium chlorite, epoxidation by treatment with *m*-chloroperbenzoic acid, lactonization of the resulting oxirane by heating in toluene-cyclohexane at reflux for 1 day and finally acetylation afforded a mixture of (5*R*,6*S*)- and (5*S*,6*R*)-6-acetoxy-5-hexadecanolide *(4 steps, 40% yield)*.

From the seeds of the summer cypress plant, *Kochia scoparia*, (5*R*,6*S*)-isomer was efficiently synthesized, in admixture with the inactive (5*S*,6*R*)-isomer (Olagbemiro *et al*., 1999). The intermediate (5*R*,6*S*)-6-hydroxy-5-hexadecanolide, **106**, was prepared with three different approaches from glycerides of (*Z*)-5-hexadecenoic acid, **100** (Figure 34). In the final step an acetylation gave the enantiomerically enriched 6-acetoxyhexadecanolide, **1a** *(Routes A, B and C 8, 12 and 16.5% yield respectively)*.

A diastereoisomeric mixture of the oviposition pheromone was synthesized via a simple (five steps), efficient and high yielding procedure (Figure 35) (Michaelakis *et al*., 2005).

Oxidation, *cis*-dihydroxylation of the 9:1 mixture of *cis* and *trans* double bonds and consequent lactonization led to the formation of the *erythro* and *threo* isomers respectively. Thus, a mixture of the four stereoisomers of 6-acetoxy-5-hexadecanolide (**1a-d**) containing 45% of the natural oviposition aggregation pheromone [(–)-(5*R*,6*S*)], 45% of its enantiomer [(+)-(5*S*,6*R*)] and 10% of the respective *threo* enantiomeric pair [(–)-(5*S*,6*S*) and (+)-(5*R*,6*R*)] was prepared *(5 steps, 45% yield)*.

3. Conclusion

Pheromone synthesis is important to obtain sufficient quantities in order to be used for the determination of their absolute configuration as well as for the biological studies, both in laboratory and field conditions (Mori and Tashiro, 2004).

The simple chemical structure of the current oviposition aggregation pheromone (only two asymmetric centres) and the fact that the management of mosquitoes is important for reducing their impacts on public health provoked many research groups to synthesize or/and bioassay it.

Figure 32

Figure 33

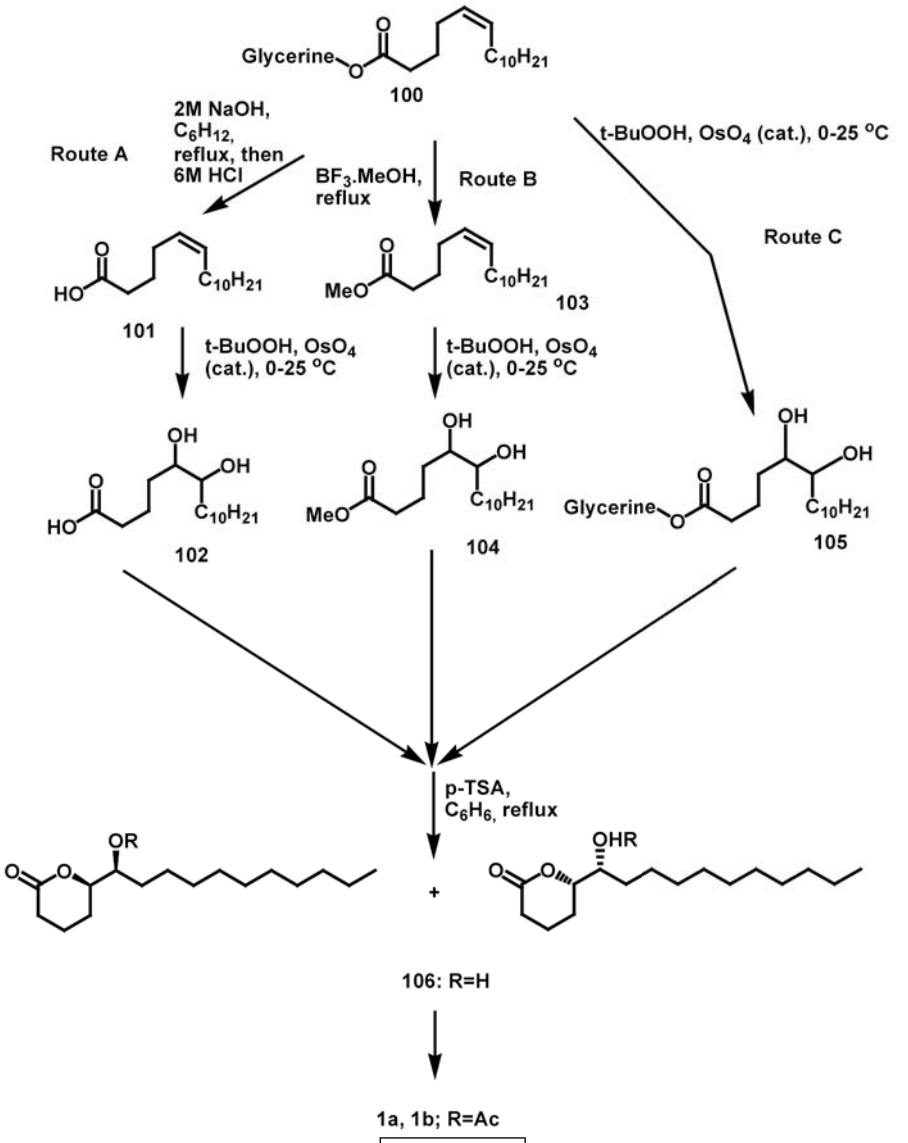


Figure 34

The use of pheromone in mosquito control would be of great value since *Culex* mosquitoes are known to be vectors of human tropical diseases such as the West Nile virus disease. During the last decade, the spread of West Nile virus in the Western Hemisphere resulted in the death of hundreds and the infection of thousands of people (Centers for Disease Control and Prevention, http://www.cdc.gov). Furthermore, pheromone could be used in gravid traps for West Nile virus surveillance. Moreover, accurate and reliable information regarding the breeding habits and distribution of *Culex* mosquitoes is an essential requisite for improved management strategies for these important disease vectors.

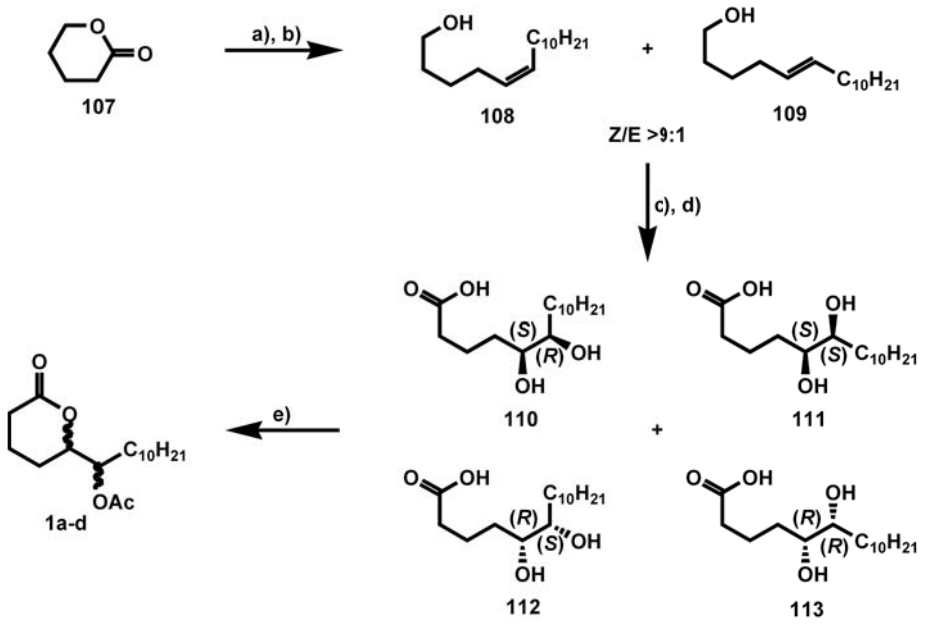


Figure 35

Literature cited

Aharoni, A. and Zweig, G. 1973. On the chemical structure of the apical droplet from eggs of *Culex pipiens*. *Journal of Insect Physiology*, 19: 861-875.

Barua, N.C. and Schmidt, R.R. 1986. Stereoselective synthesis of the major component of a mosquito oviposition attractant pheromone from a *β*-lithiopropionate equivalent. *Tetrahedron*, 42: 4471-4474.

Beament, J. and Corbet, A.S. 1981. Surface properties of *Culex pipiens* eggs and the behaviour of the female during egg-raft assembly. *Physiological Entomology*, 6: 135-148

Bonini, C., Checconi, M., Righi, G. and Rossi, L. 1995. Enantio and stereoselective synthesis of (5*R*,6*S*)-6-acetoxy-5-hexadecanolide, a mosquito oviposition attractant pheromone. *Tetrahedron*, 51: 4111-4116.

Blackwell, A., Mordue (Luntz), A.J., Hansson, B.S., Wadhams, L.J. and Pickett, J.A. 1993. A behavioral and electrophysiological study of oviposition cues for *Culex quinquefasciatus*. *Physiological Entomology*, 18: 343-348.

Braks, A.H.M., Leal, W.S. and Cardé, T.R. 2007. Oviposition responses of gravid female *Culex quinquefasciatus* to egg rafts and low doses of oviposition pheromone under semifield conditions. *Journal of Chemical Ecology*, 33: 567-578.

Bruno, D.W. and Laurence, B.R. 1979. The influence of the apical droplet of *Culex* egg rafts on oviposition of *Culex pipiens fatigans* (Diptera: Culicidae). *Journal of Medical Entomology*, 4: 300-305.

Centers for Disease Control and Prevention (CDC), http://www.cdc.gov/ncidod/dvbid/westnile/

Christophers, S.R. 1944. Structure of the *Culex* eggs and eggs-raft in relation to function (diptera). *Transactions of the Royal Entomological Society of London*, 95: 25-35

Clements, A.N. 1999. *The biology of Mosquitoes,* CABI Publishing: New York, USA.

Couladouros, E.A. and Mihou, A.P. 1999. A general synthetic route towards *γ*- and *δ*-Lactones. Total asymmetric synthesis of (–)-muricatacin and the mosquito oviposition pheromone (5*R*,6*S*)-6-Acetoxy-hexadecanolide. *Tetrahedron Letters*, 40: 4861-4862.

Coutrot, Ph., Grison, C. and Bômont, C. 1994. 5-Formyl-*δ*-valerolactone: A useful synthon for the chiral synthesis of the *Vespa Orientalis* pheromone and the mosquito oviposition attractant pheromone. *Tetrahedron Letters*, 45: 8381-8384.

Dawson, G.W., Laurence, B.R., Pickett, J.A., Pile, M.M. and Wadhams, L.J. 1989. A note on the mosquito oviposition pheromone. *Pesticide Science*, 27: 277-280.

Dawson, G.W., Mudd, A., Pickett, J.A., Pile, M.M. and Wadhams, L.J. 1990. Convenient synthesis of

mosquito oviposition pheromone and highly fluorinated analog retaining biological activity. *Journal of Chemical Ecology*, 16: 1779-1789.

Dhotare, B., Goswami, D. and Chattopadhyay, A. 2005. (*R*)-2,3-Cyclohexylideneglyceraldehyde, a novel template for stereoselective preparation of functionalized *δ*-lactones: Synthesis of mosquito oviposition pheromone. *Tetrahedron Letters*, 46: 6219-6221.

Gallos, J.K., Mihelakis, D.S., Dellios, C.C. and Pozarentzi, M.E. 2000. A new synthesis of (–)-(5*R*,6*S*) -6-acetoxy-5-hexadecanolide. The mosquito *Culex pipiens* fatigans oviposition attractant pheromone. *Heterocycles*, 53: 703-707.

Gravier-Pelletier, C., Sanière, M., Charvet, I., Le Merrer, Y. and Depezay, J.C. 1994. Synthesis of (–)-muricatacin and (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide, the mosquito oviposition attractant pheromone, from *D*-isoascorbic acid. *Tetrahedron Letters*, 35(1): 115-118.

Henkel, B., Kunath, A. and Schick, H. 1995. Chemoenzymatic synthesis of (5*R*,6*S*)-6-acetoxyhexadecan-5-olide – The major component of the mosquito oviposition attractant pheromone. *Liebigs Annalen*, 921-923.

Hwang, Y.S., Mulla, M.S., Chaney, J.D., Lin, G.G. and Xu, H.J. 1987. Attractancy and species specificity of 6-Acetoxy-5-hexadecanolide, a mosquito oviposition attractant pheromone. *Journal of Chemical Ecology*, 13: 245-252.

Ichimoto, I., Yoshizawa, T., Machiya, K., Kirihata, M., and Ueda, H. 1988. An efficient synthesis of mosquito oviposition attractant pheromone, (5*R*,6*S*)-(–)-6-acetoxy-5-hexadecanolide. *Chemistry Express*, 11: 687-690.

Iltis, W.G. and Zweig, G. 1962. Surfactant in apical drop of eggs of some *culicine* mosquitoes. Annals of the *Entomological Society of America*, 55: 409-415.

Ikishima, H., Sekiguchi, Y., Ichikawa, Y. and Kotsuki, H. 2006. Synthesis of (–)-(5*R*,6*S*)-6-acetoxyhexadecanolide based on *L*-proline-catalyzed asymmetric aldol reactions. *Tetrahedron*, 62: 311-316.

Kametani, T., Tsubuki, M., Tatsuzaki, Y. and Honda, T. 1990. Synthesis of optically active 2-furylmethanols as useful chiral building blocks and its application to the synthesis of (5*R*,6*S*)-6-acetoxyhexadecan-5-olide and (+)-disparlure. *Journal of The Chemical Society-Perkin Transactions 1*: 639-646.

Kang, S.K., and Cho, I.H. 1989. An enantiospecific synthesis of (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide, the mosquito oviposition attractant pheromone. *Tetrahedron Letters*, 30: 743-746.

Ko, K.Y., and Eliel, E.L.. 1986. Asymmetric synthesis of (5*R*,6*S*)-6-acetoxy-hexadecanolide, the major component of the oviposition attractant pheromone of the mosquito *Culex pipiens fatigans*, and two of its stereoisomers. *Journal of Organic Chemistry*, 51: 5353-5362.

Kotsuki, H., Kadota, I., and Ochi, M. 1990. A novel carbon-carbon bond-forming reaction of triflates with copper(I)-catalysed Grignard reagents. A new concise and enantiospecific synthesis of (+)-exo-Brevicomin, (5*R*,6*S*)-(–)-6-acetoxy-5-hexadecanolide and *L*-Factor. *Journal of Organic Chemistry*, 55: 4417-4422.

Kioulos, E., Koliopoulos, G., Couladouros, E. A., and Michaelakis, A. 2007-2008. Laboratory evaluation of three larvicides and preliminary studies of the attract–and–kill strategy on hatched larvae of *Culex pipiens* (Diptera: Culicidae). *Entomologia Hellenica*, 17: 3-11.

Laurence, B.R., and Pickett, J.A. 1982. Erythro-6-acetoxy-5-hexadecanolide, the major component of an oviposition attractant pheromone. *Journal of the Chemical Society, Chemical Communications*, 1: 59-60.

Laurence, B.R., and Pickett, J.A. 1985. An oviposition attractant pheromone in *Culex quinquefasciatus* Say (Diptera: Culicidae). *Bulletin of Entomological Research*, 75: 283-290.

Leal, W.S., Barbosa, M.R., Xu, W., Ishida, Y., Syed, Z., Latte, N., Chen, M.A., Morgan, T.I., Cornel, J.A., and Furtado, A. 2008. Reverse and conventional chemical ecology approaches for the development of ovipositor attractants for *Culex* mosquitoes. *PLoS ONE*, 3:1-11

Lin G.Q., Xu H.J., Wu B.C., Guo G.Z., and Zhou, W.S. 1985. Studies on the identification and syntheses of insect pheromones. Stereoselective synthesis of all the possible optical isomers of the mosquito oviposition attractant pheromone. *Tetrahedron Letters*, 26: 1233-1236.

Lohray, B.B., and Venkateswarlu, S. 1997. Intramolecular S_N2 ring opening of a cyclic sulfate: synthesis of erythro-(–)-6-acetoxy-5-hexadecanolide a major component of mosquito oviposition attractant pheromone. *Tetrahedron Asymmetry*, 8: 633-638.

Machiya, K., Ichimoto, I., Kirihata, M., and Ueda, H. 1985. A convenient synthesis of four stereoisomers of 6-acetoxy-5-hexadecanolide, the major component of the mosquito oviposition attractant pheromone. *Agricultural and Biological Chemistry,* 49: 643-649.

Mboera, L.E.G., Mdira, K.Y., Salum, F.M., Takken, W., and Pickett, J.A. 1999. Influence of synthetic oviposition pheromone and volatiles from soakage pits and grass infusions upon oviposition site selection of *Culex* mosquitoes in Tanzania. *Journal of Chemical Ecology*, 25: 1855-1865.

Michaelakis, A., Mihou, A.P., Koliopoulos, G. and Couladouros, E.A. 2009. Influence of the microencapsulated pheromone from aged infusion as an oviposition medium of the West Nile virus vector *Culex pipiens*. *Parasitology Research*, 104: 1005-1009.

Michaelakis, A., Mihou, P.A., Koliopoulos, G. and Cou-

ladouros, E.A. 2007. Attract and kill strategy. Laboratory studies on hatched of *Culex pipiens*. *Pest Management Science*, 63: 954-959.

Michaelakis, A., Mihou, A.P., Couladouros, E.A., Zounos, A.K. and Koliopoulos, G. 2005. Oviposition responses of *Culex pipiens* to a synthetic racemic *Culex quinquefasciatus* oviposition aggregation pheromone. *Journal of Agricultural and Food Chemistry*, 53: 5225-5229.

Millar, J.G., Chaney, J.D., Beehler, J.W. and Mulla, M.S. 1994. Interaction of the *Culex quinquefasciatus* egg raft pheromone with a natural chemical associated with oviposition sites. *Journal of the American Mosquito Control Association*, 10(3): 374-379.

Mokany, A. and Shine, R. 2003. Oviposition site selection by mosquitoes is affected by cues from conspecific larvae and anural tabpoles. *Austral Ecology*, 28: 33-37

Mordue, A.J., Blackwell, A., Hansson, B.S., Wadhams, L.J. and Pickett, J.A., 1992. Behavioural and electrophysiological evaluation of oviposition attractants for *Culex quinquefasciatus* Say (Diptera: Culicidae). *Experientia*, 48: 1109-1111.

Mori, K. and Tashiro, T. 2004. Useful reactions in modern pheromone synthesis. *Current Organic Synthesis*, 1: 11-29.

Mori, K. and Otsuka, T., 1983 Synthesis of both the enantiomers of erythro-6-acetoxy-5-hexadecanolide. *Tetrahedron*, 39: 3267-3269.

Ochiai, M., Ukita, T., Nagao, Y. and Fujita, E. 1985. Stereochemistry of an oxidative 1,4-fragmentation of γ-stannyl alcohols with a hypervalent organoiodine compound and the synthesis of *erythro*-6-acetoxyhexadecan-5-olide. *Journal of the Chemical Society, Chemical Communications*, 637-638.

Olagbemiro, T.O., Birkett, M.A. Mordue (Luntz), A.J. and Pickett, J.A. 1999. Production of (5*R*,6*S*)-6-acetoxy-5-hexadecanolide, the mosquito oviposition pheromone, from the seed oil of the summer cypress plant, *Kochia scoparia* (Chenopodiaceae). *Journal of Agricultural and Food Chemistry*, 47: 3411-3415.

Olagbemiro, T.O., Birkett, M.A., Mordue (Luntz), A.J. and Pickett, J.A. 2004. Laboratory and field responses of the mosquito, *Culex quinquefasciatus*, to plant-derived *Cullex spp.* Oviposition pheromone and the oviposition cue skatole. *Journal of Chemical Ecology*, 30: 965-976

Osgood, E.C. 1971. An oviposition pheromone associated with the egg raft of *Cules tarsalis*. *Journal of Economic Entomology*, 64: 1038-1041.

Otieno, W.A., Onyango, T.O., Pile, M.M., Lawrence, B.R., Dawson, G.W., Wadhams, L.J. and Pickett, J.A. 1988. A field trial of the synthetic oviposition pheromone with *Culex quinquefasciatus Say* (Diptera: Culicidae) in Kenya. *Bulletin of Entomological Research*, 78: 463-478.

Pickett, A.J. and Woodcock, M.C. 1996. The role of mosquito olfaction in oviposition site location and in the avoidance of unsuitable hosts. *Ciba Foundation Symposium*, 200: 109-123.

Prasad, K.R. and Anbarasan, P. 2007a. Enantiodivergent synthesis of both enantiomers of gypsy moth pheromone disparlure. *Journal of Organic Chemistry*, 72: 3155-3157.

Prasad, K.R. and Anbarasan, P. 2007b. Stereoselective synthesis of (–)-6-acetoxyhexadecanolide: a mosquito oviposition attractant pheromone. *Tetrahedron Asymmetry*, 18: 2479-2483.

Ramaswamy, S. and Oehlschlager, A.C. 1991. Chemico-microbial syntheses of Japanese beetle and mosquito oviposition pheromones. *Tetrahedron*, 47: 1145-1156.

Sabitha, G., Swapna, R., Venkata, R.E. and Yadav, J.S. 2006. Synthesis of (–)-(5*R*,6*S*)-6-acetoxyhexadecanolide, a mosquito oviposition attractant pheromone of *Culex pipiens* fatigans. *Synthesis*, 24: 4242-4246.

Sakakibara, M., Ikeshoji, T., Machiya, K. and Ichimoto, I. 1984. Activity of four stereoisomers of 6-acetoxy-5-hexadecanolide, the oviposition pheromone of culicine mosquitoes. *Japanese Journal of Sanitary Zoology,* 35: 401-403.

Singh, S. and Guiry, P.J. 2009. A facile synthesis of both enantiomers of 6-acetoxy-5-hexadecanolide, a major component of mosquito oviposition attractant pheromones. *European Journal of Organic Chemistry*, 12: 1896-1901.

Starratt, A.N. and Osgood, C.E. 1972. An oviposition pheromone of the mosquito *Culex tarsalis*: diglyceride composition of the active fraction. *Biochimica et Biophysica Acta,* 280: 187-193.

Starratt, A.N. and Osgood, C.E. 1973. 1,3-diglycerides from eggs of *Culex pipiens quinquefasciatus* and *Culex pipiens pipiens*. *Comparative Biochemistry Physiology*, 46B: 857-859.

Trost, B. and Rhee, Y.H. 2002. A Ru catalyzed divergence: Oxidative cyclization vs cycloisomerization of bis-homopropargylic alcohols. *Journal of the American Chemical Society*, 124(11): 2528-2533.

Wang, Z.M., Qian, X.H. and Zhou, W.S. 1990. Stereoselective synthesis of (–)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide, the mosquito oviposition attractant pheromone. *Tetrahedron*, 46: 1191-1198.

Wu, W.L. and Wu, Y.L. 1990. Diastereoselective synthesis of (–)-(5*R*,6*S*)-6-acetoxy-hexadecan-5-olide. *Journal of Chemical Research*, Synopses 4: 112-113.

Wu, W.L. and Wu, Y.L. 1991. A concise synthesis of the natural mosquito oviposition attractant pheromone from *D*-glucose. *Journal Carbohydrate Chemistry*, 10(2): 279-281.

Re ceived: 9 May 2010; Accepted: 12 July 2010

ΑΡΘΡΟ ΑΝΑΣΚΟΠΗΣΗΣ

Φερομόνη συνάθροισης των κουνουπιών του γένους *Culex* για εναπόθεση ωών: μελέτες βιοδραστικότητας και συνθετικές προσεγγίσεις

Α.Π. Μίχου και Α.Ν. Μιχαηλάκης

Περίληψη Τα γονιμοποιημένα θηλυκά κουνούπια του γένους *Culex* γεννούν σε υδατικό περιβάλλον όπου προϋπάρχουν σχεδίες ωών. Πειραματικές μελέτες έδειξαν ότι αυτό οφείλεται στο γεγονός ότι τα γονιμοποιημένα θηλυκά ανταποκρίνονται θετικά σε μια φερομόνη. Η φερομόνη αυτή ονομάζεται φερομόνη ωοθεσίας. Εκλύεται από σταγονίδια που σχηματίζονται στο ακραίο τμήμα του κάθε ωού, λίγο μετά την εναπόθεσή τους στο νερό και δρα ως υποκινητής για να εναποθέσουν και άλλα άτομα στον ίδιο χώρο. Η χημική ανάλυση των ακραίων σταγονιδίων αποκάλυψε ότι η φερομόνη ωοθεσίας του κουνουπιού *Cx. quinquefasciatus* είναι το (–)-(5*R*,6*S*)-6-ακετοξυ-5-δεκαεξανολίδιο. Έκτοτε, η φερομόνη χρησιμοποιήθηκε σε μεγάλο αριθμό πειραμάτων προσελκυστικότητας γονιμοποιημένων θηλυκών προκειμένου να διερευνηθούν τα χαρακτηριστικά της. Ποικίλες συνθετικές προσεγγίσεις έχουν αναφερθεί στη βιβλιογραφία και αφορούν όχι μόνο το φυσικό ισομερές αλλά τόσο τα *ερυθρο*- εναντιομερή όσο και τα τέσσερα διαστερεομερή του 6-ακετοξυ-5-δεκαεξανολιδίου. Μελέτες βιολογικής δράσης αποκάλυψαν ότι μόνο το (–)-(5*R*,6*S*)-ισομερές ήταν βιοδραστικό ενώ τα υπόλοιπα τρία ισομερή δεν προκαλούσαν καμία επίδραση. Στο άρθρο ανασκόπησης γίνεται αναφορά στις ποικίλες βιοδοκιμές που αναπτύχθηκαν ενώ αναφέρονται και οι πιο επιτυχημένες συνθετικές προσεγγίσεις της φερομόνης στη διεθνή βιβλιογραφία.

Hellenic Plant Protection Journal **3:** 33-56, 2010

Hellenic Plant Protection Journal **3:** 57-69, 2010

Validation of a multiresidue method for the determination of multiclass pesticides by using representative analytes by gas chromatography

N. Vlastaras[1], E. Dasenakis[1] K.S. Liapis[2], G.E. Miliadis[2] and C.J. Anagnostopoulos[2]

Summary The protection of crops against pests and diseases by various mostly synthetic pesticides is a common approach in conventional farming. Even when pesticides are applied in accordance with Good Agricultural Practices (GAP), they can leave residues on plants. Therefore, the need of finding determination methods for pesticide residues with speed, facility and reliability is rendered imperative. The estimation of reliability of a multiresidue method is achieved by the process of validation. Validating a multiresidue method is a time- and effort-consuming task, which usually requires 5-6 replicates of recovery in at least two different levels for each compound and matrix combination of interest. In this study, a different approach using a more effortless and fast method of validation is followed. A selection of representative analytes was made; the number of the tested analytes was at least 20% of the total number of each chemical pesticide class, covering selected physicochemical properties (polarity, solubility in water, vapor pressure). In addition, the representative analytes included those for which the worst performance was expected. Furthermore, validation in only one matrix per group commodity is proposed. With the above process and by testing 44 representative analytes, the multiresidue method by gas chromatography with electron capture (ECD) and nitrogen-phosphorous (NPD) detectors used in this study for the determination of over 180 pesticides in crops, including organophosphorus, organochlorine, triazole, triazine, strobilurin, pyrethroid, dinitroaniline and nitrogen-containing pesticides, was validated. The matrixes were tomatoes and grapes, products of high consumption in Greece.

Additional keywords: analytes, GC-NPD, GC-ECD, multiresidue methods, representative, validation

Introduction

Monitoring of pesticides in food usually involves the rapid, accurate and cost-effective detection of a wide range of compounds that belong to different chemical classes. Thus, it is imperative to develop multiresidue analytical methods in order to screen the maximum possible number of compounds. Laboratories perform method validation to provide evidence that a method is fit for the purpose for which it is to be used. The method usually has to be fully validated for all analytes included in the scope of the method and probably for all matrixes. With respect to matrixes, representative ones may be used (4). Regarding analytes, we believe that, by choosing representative ones from each chemical class, multiclass multiresidue methods with less time and effort, appropriate for laboratories performing pesticide residue analysis and acceptable for accreditation purposes can be validated.

In the present study, representative analytes for various chemical groups including organophosphorus, organochlorine, triazole, triazine, strobilurin, pyrethroid, dinitroaniline and nitrogen-containing pesticides, were selected according to their physicochemical properties. The physicochemical properties used to select the representative analytes were:

[1] Laboratory of Environmental Chemistry, Division III, Department of Chemistry, University of Athens, Panepistimioupolis, GR-105 71 Athens, Greece
[2] Laboratory of Pesticide Residues, Department of Pesticides Control and Phytopharmacy, Benaki Phytopathological Institute, 8, St. Delta Str., GR-145 61 Kifissia (Athens), Greece.
Corresponding author: c.anagnostopoulos@bpi.gr

(a) Polarity

It is usually reported as a logarithm, most commonly as log Kow or log Pow. It is defined as the ratio of the equilibrium concentrations of the two-phase system consisting of water and n-octanol. Polarity refers to the extent to which charge is unevenly distributed within the molecule and to the occurrence of polar functional groups in it. This parameter is characteristic of the liphophility of the molecule and gives an indication of the compound's tendency to accumulate in biological membranes and living organisms. It's determination gives data required for the registration of new organic chemicals. It is generally considered that substances with a log Kow value higher than 3 can show accumulation. This risk is measured experimentally by the bioconcentration factors in aquatic organisms, and some correlations have been found between these two parameters for very hydrophobic pesticides, such as the organochlorines. Some persistent organochlorines withdrawn from the market were all characterised by log Kow > 4 (1).

As a rough rule, non-polar analytes are characterised by log Kow values above 4-5, whereas polar analytes have log Kow values below 1 or 1.5. Between these two values, compounds are classified as moderately polar (Figure 1).

(b) Water solubility

It is a fundamental, chemical-specific property defined as the concentration of a chemical dissolved in water when that water is both in contact and at equilibrium with the pure chemical. As a general rule, a very soluble ingredient (water solubility above several g/l) cannot be extracted from water with the available extraction procedures. Very insoluble ones (water solubility <0.5-1 mg/1) are difficult to analyse at trace levels because they have a tendency to adsorb everywhere, especially on glassware; this leads to low extraction recoveries, unless some organic solvent is added to the samples prior to extraction (1). Water solubility indicates the tendency of a pesticide to be removed from soil by run-off or irrigation water and to reach the surface water. It also indicates the tendency to precipitate at the surface soil. However, this parameter alone cannot be used for predicting leaching through soil, although the distribution of pesticides in the environment is conditioned by a variety of partition coefficients into water, and several authors have shown correlations between these partition coefficients and the water solubility (Figure 2) (1).

(c) Vapour pressure

It is another chemical-specific property, defined as the partial pressure of a chemical, in the gas phase, in equilibrium with the pure solid or liquid chemical. Vapour pressures are very temperature-dependent. This parameter governs the distribution between liquid and gas phase or between solid and gas phase. Experimental measurements are

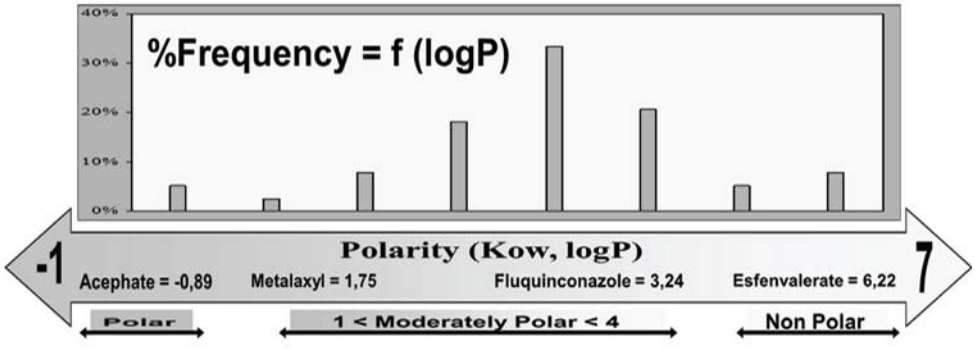


Figure 1. Distribution of the analytes studied according to their log Pow.

not easily made, especially because many plant protection products have low vapour pressure values. They are not always measured at ambient temperature, but can be extrapolated from measurements at higher temperatures using the Clapeyron equation. Reported vapour pressure values often exhibit wide discrepancies, up to tenfold between different authors. In general, only compounds with vapor pressures exceeding about 10^{-10} torr can be analyzed by gas chromatograph (Figure 3) (1).

The objective of this study is to present and validate a simple and rapid method for the determination of the multiclass pesticides (organophosphorus, organochlorine, triazole, triazine, strobilurin, pyrethroid, dinitroaniline and nitrogen-containing pesticides) with different physicochemical properties in fruits and vegetables of high water content. One crop per group commodity was chosen (i.e. tomatoes and grapes). The sample preparation included liquid extraction based on acetone and dichloromethane/petroleum ether (50:50) (5) and the determination step was performed by gas chromatography with electron capture (ECD) and nitrogen phosphorus (NPD) detectors.

Materials and methods

1. Chemicals and Reagents

(a) Solvents

Acetone, 2,2,4-trimethylpentane and toluene were used for the preparation of stock and working standard solutions. Acetone,

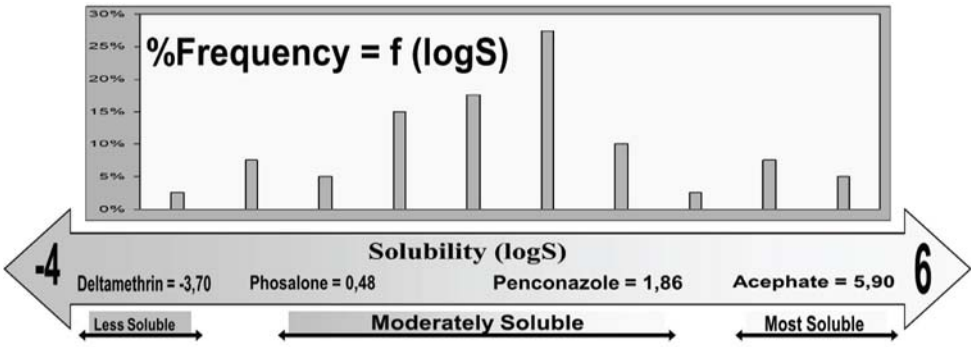


Figure 2. Distribution of the analytes studied according to their water solubility.

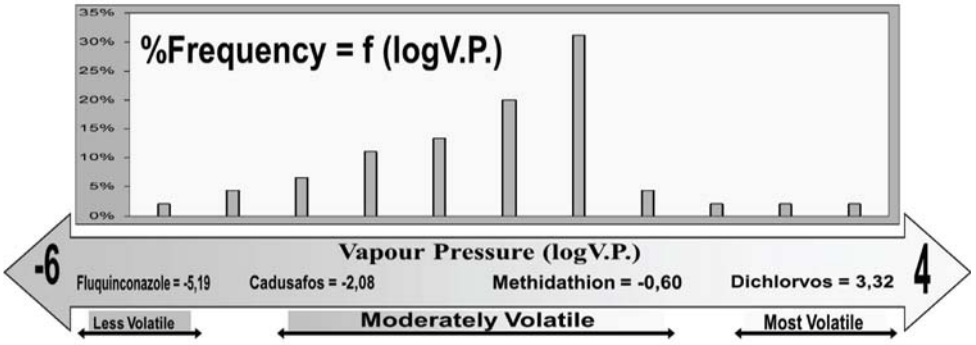


Figure 3. Distribution of the analytes studied according to their vapour pressure.

dichloromethane and petroleum ether were used in the extraction procedure. All solvents were of pesticide residues grade and were obtained from Lab Scan (Ireland).

(b) Pesticide standards

In this study, 44 analytes were selected with distinct physicochemical characteristics as shown in Table 1 (2, 7). They included polar and non-polar compounds, as well as compounds of various molecular masses.

Representative analytes for various chemical groups, including organophosphorus, organochlorine, triazole, triazine, strobilurin, pyrethroid, dinitroaniline and nitrogen-containing pesticides, were selected according to their physicochemical properties, such as polarity, water solubility and vapour pressure (see Introduction). It is imperative to add that these are not the only physicochemical parameters that may used to select the representative analytes. The

Table 1. Analytes studied, their chemical class and basic physicochemical characteristics.

Analyte	K_{ow}, logP	Vapor Pressure (mPa)	Solubility (mg/l)
Organophosphorous			
acephate	-0.89	0.226 (24°C)	790 g/l (20°C)
azinphos-methyl	2.96	c. 4-5 (20°C)	0.32 (20°C)
cadusafos	3.9	$1.2x10^2$ (25°C)	248
chlorpyrifos-methyl	4.24	3 (25°C)	2.6 (20°C)
diazinon	3.30	12 (25°C)	60 (20°C)
dichlorvos	1.9 (OECD 117); 1.42 (separate study)	$2.1x10^3$ (25°C) (OECD 104)	c. 18 g/l (25°C)
demeton s-methyl	1.32 (20°C)	40 (20°C)	22 g/l (20°C)
dimethoate	0.704	0.25 (25°C)	23.8 g/l (20°C)
ethion	4.28	0.20 (25°C)	2 ppm (25°C)
fenthion	4.84	0.74 (20°C); 1.4 (25°C)	4.2 (20°C)
methidathion	2.2 (OECD 107)	$2.5x10^{-1}$ (20°C)	200 (25°C)
omethoate	-0.74 (20°C)	3.3 (20°C)	Readily soluble in water
parathion–ethyl	3.83	0.89 (20°C)	11.0
parathion-methyl	3.0	0.2 (20°C); 0.41 (25°C)	55 (20°C)
phosalone	4.01 (20°C)	<0.06 (25°C)	3.05 (25°C)
pyrazophos	3.77 (20°C)	$1.6x10^{-3}$ (20°C)	33 (20°C)
triazophos	3.34	0.39 (30°C); 13 (55°C)	39 (pH 7, 20°C)
Amides			
dichlofluanid	3.7 (21°C)	0.014 (20°C)	1.3 (20°C)
metalaxyl	1.75 (25°C)	0.75 (25°C)	8.4 g/l (22°C)
benalaxyl	3.54 (20°C)	0.66 (25°C, gas saturation method)	28.6 (20°C)
Triazoles			
fluquinconazole	3.24 (pH 5.6)	$6.4x10^{-6}$ (20°C)	1 (pH 6.6, 20°C)
myclobutanil	2.94 (pH 7-8, 25°C)	0.213 (25°C)	142 (25°C)
penconazole	3.72 (pH 5-7, 25°C)	0.17 (20°C) 0.37 (25°C)	73 (25°C)
propiconazole	3.72 (pH 6.6, 25°C)	$2.7x10^{-2}$ (20°C); $5.6x10^{-2}$ (25°C)	100 (20°C)
tetraconazole	3.56 (20°C)	0.18 (gas saturation method)	156 (pH 7, 20°C)
triadimenol	3.11	0.02 (20°C); 0.06 (25°C)	64 (20°C)
Organochlorines			
aldrin	6.50	8.6 (20°C)	0.027 (27°C)
dieldrin	5.40	0.4 (20°C)	0.186 (20°C).
endosulfan-a	4.74 (pH 5)	0.83 (20°C)	0.32 (22°C)
Pyrethroids			
acrinathrin	5.6 (a.i. 20°C)	$4.4x10^{-5}$ (25°C)	< or = 0.02 (25°C)
bifethrin	>6	0.024 (25°C)	<1 µg/l

Table 1 (continued)

Analyte	K_{ow}, logP	Vapor Pressure (mPa)	Solubility (mg/l)
cyfluthrin	6.0 (20°C) 5.9 (20°C) 6.0 (20°C) 5.9 (20°C)	$9.6x10^{-4}$ (20°C) $1.4x10^{-5}$ (20°C) $2.1x10^{-5}$ (20°C) $8.5x10^{-5}$ (20°C)	(I) $9.6x10^{-4}$; (II) $1.4x10^{-5}$; (III) $2.1x10^{-5}$; (IV) $8.5x10^{-5}$ (all in, 20°C)
deltamethrin	(I,II) 4.6 (25°C)	$1.2x10^{-5}$ (25°C)	< 0.2 µg/l
esfenvalerate	6.22 (25°C)	$2x10^{-4}$ (25°C)	0.002 (25°C)
fenpropathrin	2.9 (pH 7, 25°C)	17 (25°C)	530 g/m^3 (pH 7, 25°C)
flucythrinate	4.7 (25°C)	0.0012 (25°C)	0.5 (21°C)
permethrin	6.1 (20°C)	cis- 0.0025 ; trans- 0.0015 (both 20°C) (12)	$6x10^{-3}$ (pH 7, 20°C)
Triazines			
atrazine	2.5 (25°C)	$3.85x10^{-2}$ (25°C) (OECD 104)	33 (pH 7, 22°C)
prometryn	3.1 (25 °C, unionised)	0.165 (25°C) (OECD 104)	33 (25°C)
simazine	2.1 (25°C, unionised)	$2.94x10^{-3}$ (25°C) (OECD 104)	6.2 (pH 7, 20°C)
terbuthylazine	3.21 (unionised)	0.15 (25°C)	8.5 (pH 7, 20°C).
Strobilurins			
kresoxim-methyl	3.4	$2.3x10^{-3}$	2
Dinitroanilines			
pendimethalin	5.18	4.0 (25°C)	0.3 mg/l (20°C)
trifluralin	4.83 (20°C) (EECAS)	6.1 (25°C) (EECA4)	0.184 (pH 5), 0.221 (pH 7), 0.189 (pH 9)

numbers of the tested analytes were at least 20% percent of the total number of each chemical pesticide class, covering the whole range of selected physicochemical properties (polarity, solubility in water, vapor pressure). In addition, in the representative analytes those for which the worst performance was expected were also included (4, 6).

The selected analytes belong to different chemical classes: 17 organophosphorous, 3 organochlorines, 3 amides, 6 triazoles, 4 triazines, 1 strobilurins, 2 dinitroanilines and 8 pyrethroids.

Pesticides may be base- or acid-sensitive (correspondingly captan and pymetrozine), high volatile (dichlorvos), very polar (acephate, omethoate) or unstable in the chromatographic system (metribuzin, carbaryl). For example, dichlorvos high volatility resulted in increased uncertainty during the evaporation phase and all the polar organophosphorus pesticides demonstrated low recoveries (10). So it is expected that, in a multiresidue method, pesticides with similar physicochemical properties yield similar results. This similar behavior is used during validation of a method by choosing representative analytes covering physicochemical properties of choice.

The pesticide active ingredients used in this study were obtained from Dr Ehrenstorfer Laboratories (GmbH Germany).

(c) GC–ECD/NPD system

The pesticides were separated and determined in a Agilent 6890 gas chromatograph, with two splitless injectors, a DB-5-MS column (30 m, 0.32 mm i.d. and 0.25 µm film thickness) connected to the ECD and a DB-17 MS column (30 m, 0.3 mm i.d. and 0.25 µm film thickness) connected to the NPD. The oven temperature programme started from 60°C for 1.5 min, increased to 220°C at a rate 14°C/min, held for 4 min, then increased to 280°C at 20°C /min and held for 20 min. The helium carrier gas flow rate was 1.5 ml/min for both columns. Injectors' temperature was set at 230°C and splitless in-

jection was carried out with the purge valve closed for 1 min. Hydrogen (3 ml/min) and air (60 ml/min) were used as fuel gases for the NPD, while nitrogen (60 ml/min) and helium (6 ml/min) were used as auxiliary gases for the ECD. The temperature of both detectors was set at 310°C. The data acquisition and analysis was conducted with Chemstation chromatography data manager software.

(d) Chopper

For the extraction of samples, a Janke & Kunkel IKA Labortechnik Ultra-Turrax T25 was used.

(e) Centrifuge

A centrifuge Hellenic Labware ALC 4236 was used.

(f) Waterbath

A waterbath SWB was used.

2. Preparation of samples

(a) Stock and Working solutions

Stock standard solutions for each pesticide were prepared in acetone at 1,000 mg/l and stored at -20°C. Standard mixture solutions of the compounds were prepared in 2,2,4-trimethylpentane/toluene (90/10) at intermediate concentrations (1-10mg/l) and stored at -20°C. Three standard mixture solutions of the compounds were prepared, as shown in Table 2. The compounds selected for each solution were chosen to achieve better chromatographic separation and according to the sensitivity of the compounds to each detector. Matrix matched standard

Table 2. Retention times (R.T.) of the 44 analytes studied according to the column type, the detector and their distribution in three mixtures.

Analyte	R.T.	Column	Detector	Mixture
trifluralin	7.4	DB-5	ECD	1
terbuthylazine	8.7	DB-5	ECD	1
metribuzin	10.2	DB-5	ECD	1
aldrin	12	DB-5	ECD	1
pendimethalin	13.8	DB-5	ECD	1
endosulfan a	15.5	DB-5	ECD	1
dieldrin	16.9	DB-5	ECD	1
kresoxim-methyl	18.2	DB-5	ECD	1
propiconazole 1/2	22	DB-5	ECD	1
propiconazole 2/2	22.4	DB-5	ECD	1
bifenthrin	26.7	DB-5	ECD	1
fenpropathrin	27.1	DB-5	ECD	1
acrinathrin	32.8	DB-5	ECD	1
permethrin 1/2	33.5	DB-5	ECD	1
permethrin 2/2	33.8	DB-5	ECD	1
cyfluthrin 1/4	34.5	DB-5	ECD	1
cyfluthrin 2/4	34.6	DB-5	ECD	1
cyfluthrin 3/4	34.7	DB-5	ECD	1
cyfluthrin 4/4	34.8	DB-5	ECD	1
flucythrinate 1/2	35.2	DB-5	ECD	1
flucythrinate 2/2	35.5	DB-5	ECD	1
esfenvalerate	36.6	DB-5	ECD	1
deltamethrin 1/2	37.1	DB-5	ECD	1
deltamethrin 2/2	37.5	DB-5	ECD	1
parathion-methyl	10.5	DB-5	ECD	2
dichlofluanid	11.8	DB-5	ECD	2
fenthion	12.3	DB-5	ECD	2
penconazole	13.9	DB-5	ECD	2
triadimenol 1/2	14.4	DB-5	ECD	2
triadimenol 2/2	14.7	DB-5	ECD	2
acetamiprid	25.2	DB-5	ECD	2
dichlorvos	5.1	DB-17	NPD	3
acephate	7.2	DB-17	NPD	3

Table 2 (continued)

Analyte	R.T.	Column	Detector	Mixture
demeton -S- methyl	8	DB-17	NPD	3
cadusafos	8.6	DB-17	NPD	3
omethoate	9.7	DB-17	NPD	3
diazinon	10.5	DB-17	NPD	3
atrazine	10.8	DB-17	NPD	3
simazine	11.1	DB-17	NPD	3
dimethoate	12.2	DB-17	NPD	3
chlorpyrifos-methyl	13.9	DB-17	NPD	3
prometryn	14.3	DB-17	NPD	3
metalaxyl	15.1	DB-17	NPD	3
tetraconazole	15.9	DB-17	NPD	3
parathion ethyl	16.4	DB-17	NPD	3
fenthion	17.8	DB-17	NPD	3
methidathion	23.3	DB-17	NPD	3
myclobutanil	24.8	DB-17	NPD	3
ethion	26.9	DB-17	NPD	3
benalaxyl	29.9	DB-17	NPD	3
triazophos	32.4	DB-17	NPD	3
phosalone	35	DB-17	NPD	3
pyrazophos	35.6	DB-17	NPD	3
azinphos-methyl	36.5	DB-17	NPD	3
fluquinconazole	37.5	DB-17	NPD	3

mixture solutions for measurements were prepared in matrix extract, previously analysed for the absence of compounds interfering with the analytes. The blank extract, in which the solutions were prepared, was produced as described in section "*Procedure*". 0.5 ml of the blank extract was evaporated to dryness by a stream of N_2 and 0.5 ml of a standard solution of the desired concentration, prepared in 2,2,4-trimethylpentane/toluene (90/10), was added. The final solution was placed in an ultrasonic bath for 30 sec.

(b) Procedure

The following extraction method was used (5): An aliquot of 15 ± 0.15 g of sample was weighted into a 250 ml PTFE centrifuge bottle (Nalgene, Rochester, NY); 30 ml of acetone were added and stirred for 1 min in an ultra-turrax homogenizer at 15,000 rpm; 30 ml of dichloromethane and 30 ml of petroleum ether were added and the mixture was stirred for 1 min and then centrifuged at 4,000 rpm for 2 min. An aliquot of 25 ml of the supernatant liquid were evaporated to dryness on a water stream bath at 65–70°C, and 1 ml of 2,2,4-trimethyl pentane/toluene (90/10) was added. An additional quantity of 15 ml of the supernatant liquid was evaporated to dryness on a water steam bath at 65–70°C, and 3 ml of 2,2,4-trimethyl pentane/toluene (90/10) were added. The two extracts were placed in ultrasonic bath for 30 sec and then they were transferred into separate vials with a Teflon stopper, in order to be used for the NPD and ECD chromatographic analysis, respectively. Simultaneous injections were performed in the injectors with the aid of two separate autosamplers.

(c) Preparation of fortified samples

Control samples were prepared from organically produced tomatoes and grapes. Aliquots of 15 g of the sample were fortified at two levels, the lowest fortification level (LFL) and the tenfold fortification level (10*LFL), as shown in Table 2. For validating the method (3), five replicates were used for each level.

Results and Discussion

The accuracy of the method was estimated by calculating the attained recovery. For validating a method, mean recoveries of 70–120% are considered acceptable, while in

the case of routine analysis, the acceptable recoveries are between 60 and 140% (3). The precision of the method was estimated by assessing the relative standard deviation (RSD) values under repeatability conditions (same analyst, same instrument, same day). Repeatability with RSD≤ 20% is considered acceptable (3).

As shown in Table 3, mean recoveries of the tomato samples at the LCL were between 73.2 – 117.9% with a RSD between 1 – 16.72% and at the 10*LFL between 86.5 – 108.5% with a RSD between 0.37 – 7.3% for most of the analytes studied. These results generally indicate good method accuracy.

As shown in Table 4, mean recoveries of the grape samples at the LCL were between 71.5 – 120.6% with a RSD between 0.87 –

Table 3. Average recovery values and relative standard deviation (RSD) for the 44 analytes in tomatoes, as derived from the fortification experiments in both detection systems.

Analyte	LFL[1] (mg/Kg)	Recovery % ± RSD	10*LFL[1] (mg/Kg)	Recovery % ± RSD
acephate	0.02	35.7±9.23	0.2	20.3±4.81
acrinathrin	0.05	87.9±6.74	0.5	100.7±1.67
aldrin	0.01 (MRL)	87.0±8.23	0.1	100.2±4.61
atrazine	0.10 (MRL)	93.5±2.18	1.0	89.9±1.23
azinphos-methyl	0.10	103.9±3.81	1.0	90.4±7.30
benalaxyl	0.10	103.3±6.78	1.0	98.8±1.35
bifenthrin	0.1	90.0±5.97	1	99.9±2.87
cadusafos	0.05	91.4±4.74	0.5	96.6±1.07
chlorpyrifos-methyl	0.10	97.5±3.06	1.0	98.2±1.81
cyfluthrin	0.05 (MRL)	90.2±8.42	0.5	101.2±1.77
deltamethrin	0.05	91.2±12.18	0.5	98.3±2.27
demeton -S- methyl	0.10	73.2±7.18	1.0	86.5±2.15
diazinon	0.02	96.0±2.85	0.2	98.1±1.37
dichlofluanid	0.1	100.1±9.55	1	86.8±2.00
dichlorvos	0.10 (MRL)	39.4±53.55	1.0	66.1±12.3
dieldrin	0.01 (MRL)	91.0±9.32	0.1	103.9±2.63
dimethoate	0.02	101.2±2.00	0.2	90.2±2.64
endosulfan a	0.05	90.3±8.03	0.5 (MRL)	100.1±1.24
esfenvalerate	0.05 (MRL)	90.8±9.65	0.5	99.7±1.40
ethion	0.10 (MRL)	101.3±2.26	1.0	97.6±1.6
fenpropathrin	0.05	89.5±9.03	0.5	101.1±1.60
fenthion	0.05	96.0±2.30	0.5	95.8±1.0
flucythrinate	0.05 (MRL)	94.9±16.72	0.5	98.4±2.32
fluquinconazole	0.10	104.4±5.75	1.0	93.8±3.68
kresoxim-methyl	0.1	90.8±8.66	1	101.7±1.26
metalaxyl	0.05 (MRL)	109.8±4.84	0.5	93.0±1.7
methidathion	0.02 (MRL)	85.1±5.42	0.2	94.9±2.8
myclobutanil	0.10	117.9±3.79	1.0	96.9±1.0
omethoate	0.10	46.2±6.41	1.0	37.4±5.4
parathion ethyl	0.05 (MRL)	100.3±2.35	0.5	96.4±1.90
parathion-methyl	0.05	96.6±1.05	0.5	92.3±1.59
penconazole	0.05 (MRL)	114.3±4.43	0.5	95.3±1.23
pendimethalin	0.05 (MRL)	87.3±9.90	0.5	99.4±1.5
permethrin	0.05 (MRL)	84.7±14.57	0.5	108.5±4.66
phosalone	0.05	107.7±3.44	0.5	105.1±2.59
prometryn	0.10	100.2±2.19	1.0	97.2±1.55
propiconazole	0.05 (MRL)	120.1±10.79	0.5	103.4±1.80
pyrazophos	0.05 (MRL)	106.6±3.50	0.5	99.4±4.58
simazine	0.10	96.4±2.48	1.0	89.7±1.03
terbuthylazine	0.1	111.7±14.73	1	97.7±3.22
tetraconazole	0.10	115.3±2.81	1.0	95.5±1.28
triadimenol	0.1	114.3±4.23	1	97.7±0.37
triazophos	0.02 (MRL)	102.2±4.33	0.2	97.8±2.5
trifluralin	0.05	87.6±11.39	0.5	98.3±2.89

[1]LFL: lowest fortification level; 10*LFL: tenfold fortification level

20.43% and at the 10*LFL from 88 – 99.7% with a RSD between 0.55 – 19.14% for most of the analytes studied. These results also indicate good method accuracy.

Some compounds (aldrin-dichlofluanid and pendimethalin-penconazole) co-eluted in the DB-5-MS column (Table 2). In case a peak was found at the certain retention times, an acceptable method of confirmation was applied. It is of paramount importance first to confirm the presence of the compound and then to quantify it. One of the acceptable confirmation methods is the use of different polarity columns to confirm the presence of a compound. According to Table 5, the proper column for the separation of the above pairs is the DB-17. Additionally, a GC-MS system can be used. The m/z

Table 4. Average recovery values and relative standard deviation (RSD) for the 44 analytes in grapes, as derived from the fortification experiments in both detection systems.

Analyte	LFL[1] (mg/Kg)	Recovery % ± RSD	10*LFL[1] (mg/Kg)	Recovery % ± RSD
acephate	0.02 (MRL)	50.8±4.05	0.2	28.1±7.34
acrinathrin	0.05	109.6±3.66	0.5	97.9±4.16
aldrin	0.01 (MRL)	103.8±1.22	0.1	96.8±5.32
atrazine	0.10 (MRL)	95.2±.1.88	1.0	94.3±1.03
azinphos-methyl	0.10	118.3±2.96	1.0 (MRL)	90.2±0.55
benalaxyl	0.10	105.5±4.79	1.0	96.6±0.81
bifenthrin	0.1	116.8±5.21	1	100.1±3.46
cadusafos	0.05	98.0±2.82	0.5	93.9±1.51
chlorpyrifos-methyl	0.10	100.4±1.77	1.0	97.5±0.87
cyfluthrin	0.05	102.9±2.53	0.5	94.0±3.36
deltamethrin	0.05	96.6±3.57	0.5	88.0±7.35
demeton -S- methyl	0.10	73.4±20.43	1.0	44.4±32.34
diazinon	0.02 (MRL)	100.5±1.83	0.2	97.4±0.76
dichlofluanid	0.1	112.2±1.56	1	90.0±0.94
dichlorvos	0.10 (MRL)	59.5±27.05	1.0	57.5±19.14
dieldrin	0.01 (MRL)	104.2±1.86	0.1	97.1±2.29
dimethoate	0.02 (MRL)	102.2±1.82	0.2	91.4±0.87
endosulfan a	0.05	103.6±0.87	0.5 (MRL)	95.7±2.51
esfenvalerate	0.05	97.3±1.57	0.5	93.1±4.85
ethion	0.10	103.0±2.21	1.0	98.5±1.34
fenpropathrin	0.05	103.6±1.61	0.5	96.1±2.33
fenthion	0.05	95.1±2.39	0.5	89.9±6.93
flucythrinate	0.05 (MRL)	101.9±1.57	0.5	93.1±4.85
fluquinconazole	0.10	120.6±3.97	1.0	76.9±3.84
kresoxim-methyl	0.1	106.2±1.39	1 (MRL)	96.5±2.20
metalaxyl	0.05	117.3±3.93	0.5	97.7±0.27
methidathion	0.02	107.4±7.55	0.2	98.7±2.39
myclobutanil	0.10	120.2±2.00	1.0 (MRL)	92.9±1.02
omethoate	0.10 (MRL)	52.0±6.46	1.0	30.1±5.03
parathion ethyl	0.05 (MRL)	104.3±1.80	0.5	97.2±0.70
parathion-methyl	0.05	98.3±4.02	0.5	89.1±0.87
penconazole	0.05	136.1±1.38	0.5	91.4±2.52
pendimethalin	0.05 (MRL)	102.9±1.41	0.5	95.5±1.86
permethrin	0.05 (MRL)	76.8±4.64	0.5	96.5±2.43
phosalone	0.05 (MRL)	107.2±4.79	0.5	92.4±1.14
prometryn	0.10	101.1±1.60	1.0	96.3±1.40
propiconazole	0.05	171.5±3.66	0.5 (MRL)	93.5±4.38
pyrazophos	0.05	124.6±1.16	0.5	92.9±1.38
simazine	0.10	99.3±1.77	1.0	91.9±1.41
terbuthylazine	0.1	71.5±6.27	1	85.9±9.72
tetraconazole	0.10	116.3±1.41	1.0	95.8±1.07
triadimenol	0.1	117.7±0.89	1	99.7±2.45
triazophos	0.02 (MRL)	106.9±2.13	0.2	96.9±0.86
trifluralin	0.05	95.4±1.60	0.5	93.8±8.34

[1]LFL: lowest fortification level; 10*LFL: tenfold fortification level

Table 5. Relative retention times (R.R.T.) of the compounds aldrin, dichlofluanid, pendimethalin and penconazole (relative to parathion ethyl) in four different columns.

Analyte	R.R.T. (DB-1)	R.R.T. (DB-5)	R.R.T. (DB-17)	R.R.T. (DB-WAX)
aldrin	1.01	0.99	0.89	0.61
dichlofluanid	0.98	0.96	0.99	
pendimethalin		1.05	0.99	
penconazole	1.05	1.03	1.07	

fragments for the same compounds are the following: 260, 270, 290 and 300 for aldrin, 123, 167, 224 and 332 for dichlofluanid, 162, 191, 208 and 252 for pendimethalin and 159, 163, 213 215, 248 and 250 for (162, 191, 208, 252), penconazole. If we compare the ions of the compounds aldrin and dichlofluanid, the m/z fragments of 123, 167 and 332 of dichlofluanid are not produced by aldrin. Similarly if we compare the compounds penconazole and pendimethalin, the m/z fragments of 213, 215, 248 and 250 of the former are not produced by the latter. Therefore, and according to the principles of mass spectrometry, even if these compounds are co-eluted we can still identify and confirm which compound is present.

There has been a high uncertainty in the results of dichlorvos (RSD = 53.55% in tomatoes and 27.05% in grapes) mostly at the LFL, which most probably was due to some losses of the analyte at the stage of evaporation, as dichlorvos has a very high vapour pressure. In order to estimate the reasons of this uncertainty, we conducted the following test: first, a standard solution of dichlorvos at 0.84 mg/ml in 2,2,4-trimethylpentane/toluene (90/10) was prepared. Then, a standard solution (A) of 60 ml acetone, 60 ml of dichloromethane and 60 ml of petroleum ether spiked with 1.6 ml of the previous solution was prepared. An aliquot of 25 ml of the solution (A) was transferred into five 50 ml volumetric flasks and the solution was evaporated to dryness on a water stream bath at 65–70°C. Finally, 1 ml of 2,2,4-trimethyl pentane/toluene (90/10) was added. The final solutions were transferred into five vials with Teflon stoppers. In order to evaluate the level of uncertainty during the evaporation and injection stages, we injected the five solutions five times in the chromatographic system. Based on the results of this test, the uncertainty at the stage of injection was significant (RSD = 12,9%) but the uncertainty at the stage of evaporation, as a result of dichlorvos high vapor pressure, was about 5 times higher (RSD = 58%). Lehotay *et al.* (10) reported that dichlorvos may be lost during either the evaporation step or the sample processing step. As our results showed, the loss of dichlorvos was attributed to a large extent to the evaporation step.

The most polar analytes, acephate and omethoate, gave low recovery values out of the acceptable range (35.5% in tomatoes and 50.8% in grapes for acephate and 46.2% in tomatoes and 52% in grapes for omethoate) but consistent (RSD <10%). Therefore, these values are considered acceptable and adjustment for recovery in the case of quantification of these compounds is suggested (3). Nevertheless, the method is still able to serve as a semiquantitative method for the detection and confirmation of the presence of acephate and omethoate in samples.

In the case of metribuzin, the recovery results at the ECD detector gave low recoveries (25-30%). In addition, the presence of an unknown peak (R.T. = 11.1 min) for both samples was observed. The area of the unknown peak is significant smaller than that of the peak that belongs to metribuzin (R.T. = 10.2 min). After injection of a standard solution of metribuzin in the mass spectrometer, the ions of the unknown peak were the same as that of metribuzin. Therefore, the unknown peak may have been the result of decomposition of metribuzin in its metabolites, mainly desamino-metribuzin. Figure 4(a) shows the chromatograph of metribuz-

in in solvent isooctane – toluole (90:10) and in tomato matrix. In tomato matrix, the presence of two peaks (R.T.=13.7 and 14.9) is observed; the peak with R.T.=13.7 is that of metribuzin and the peak at R.T.=14.9 is estimated to be a metribuzin metabolite. As shown in Figure 4(b), both peaks have the same spectra. Therefore, all the steps of the analytical procedure and the final determination should be conducted on the same day to avoid conversion of metribuzin into its metabolites in the extracts (8, 9). Sometimes, due to the unavailability of the instrument or the large number of required injections, the recovery experiments may take 24-48 h to be completed and therefore, especially for metribuzin, we might have conversion to its metabolites.

The molecular formula of metribuzin is $C_8H_{14}N_4OS$ (M.W.=214 for MS purposes) with an estimated Kovats RI of 1867 i.u., while the molecular formula of desamino-metribuzin is $C_8H_{13}N_3OS$ (M.W.=199) with an estimated Kovats RI of 1779 i.u. (11) Therefore, in the case of these metabolites, the expected retention time (R.T.) should be earlier than the

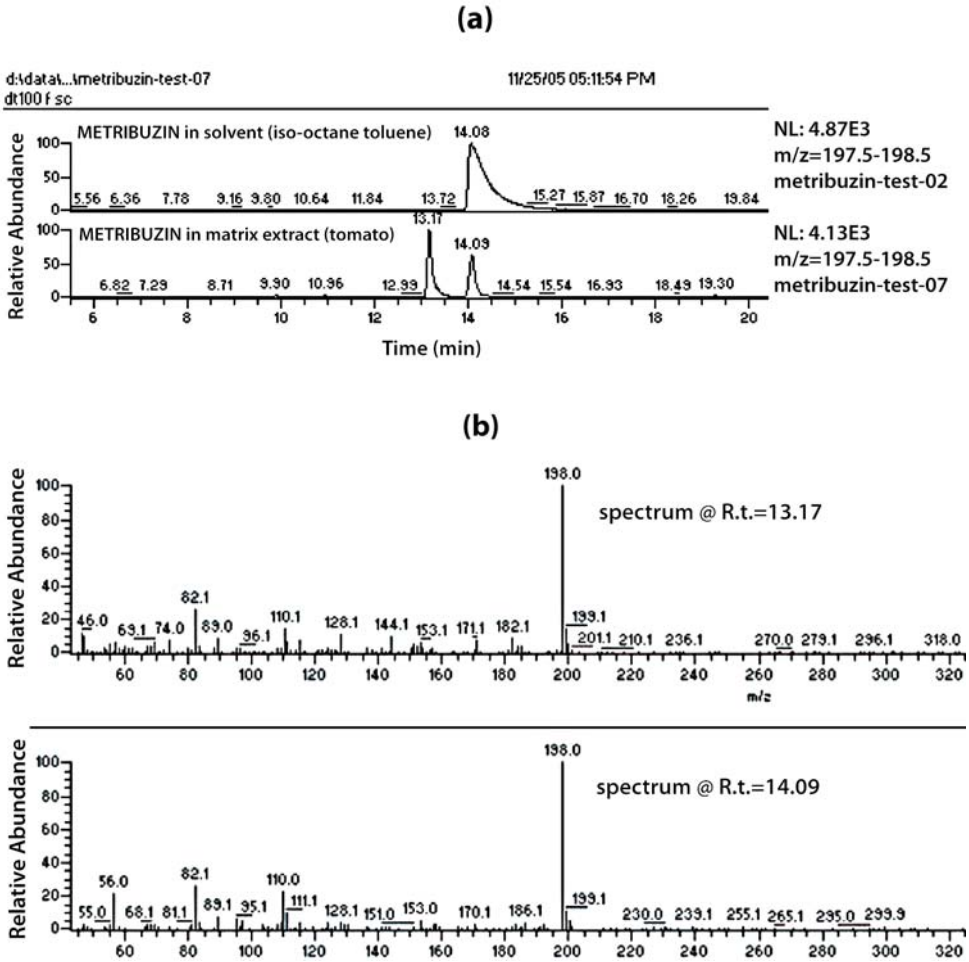


Figure 4. (a) Chromatograph of metribuzin at 0.1 mg/ml in solvent isoctane-toluole (90:10) and in tomato matrix. **(b)** Spectra of metribuzin at 0.1 mg/ml in tomato matrix. The upper spectrum is that of an unknown compound and the lower that of metribuzin.

R.T. of metribuzin, as in our case. The Kovats RI of the other metabolite is much smaller than the Kovats RI of metribuzin, which makes metribuzin-diketo (M.W.=184) and metribuzin-desamino-diketo (M.W.=169) as smaller compounds to have earlier R.T. At the time of the study, no reference material was available in our laboratory to confirm the presence of any metabolite. Therefore, the identification of any degradation product or metabolite was not possible and this investigation was used to partly explain the low recovery of the certain compound. The spectra presented in Figure 4(b) are in full scan mode and in accordance with the commercial available library of Dr Ehrenstofer, which is a special collection of pesticides and pollutants.

In conclusion, the peak with R.T.=13.17 min in the GC-MS system, shown in Figure 4(b), may have been a metabolite, a degradation product or a pollution of the system or matrix. However, in the framework of the present study, it was difficult to determine its identity. Further studies dedicated to the identification of this compound are required.

Conclusions

The method of analysis proposed by the present study is fast, easy and effective. By using 44 representative analytes, the method applied for the determination of more that 180 pesticides by gas chromatography with electron capture (ECD) and nitrogen phosphorus (NPD) detectors was validated. Nevertheless, several difficulties occurred with certain problematic molecules (dichlorvos, metibuzin) or specific chemical subgroups (polar organophosphorus pesticides). In addition, the matrix effect played an important role in the sensitivity of the analytes. For example, in the case of demeton-s-methyl, propiconazole and penconazole, the signal to noise ratio was higher in tomatoes than in grapes. Although the validation of the method by choosing representative analytes is of low cost, effective and easy technique for testing if a method is suitable for the determination of a wider range of pesticides, it is not adequate. According to the European Commission (3), for checking the methods performance for all the analytes in its scope, each laboratory should have a rolling programme to include all other analytes at least every 12 months, but preferably every 6 months.

Literature cited

1. Barceló, D., Hennion, M.C. 1997. *Trace determination of pesticides and their degradation products in water*, Elsevier, Amsterdam, 542 p.
2. Worthing, C.R. 1987. *The Pesticide Manual.* 12th edition, British Crop Protection Council, 1344 p.
3. European Commission 2007. *Method validation and Quality control procedures for pesticide residues analysis in food and feed,* Document No. SANCO/2007/3131. Available from: www.ec.europa.eu/food/plant/protection/resources/ qualcontrol_en.pdf.
4. Council Directive 91/414/EEC. Concerning the placing of plant protection products on the market. *Official Journal of European Community* L 230, 19.8.1991, p. 6
5. Working group "Development and improvement of Residue-analytical methods". 1996. *Analytical Methods for Pesticide Residues in Foodstuffs. Part I: Multiresidue methods,* 6th Edition, Ministry of Public Health, Welfare and Sport, The Netherlands, p. 1-21.
6. Hellenic Accreditation System S.A. 2007. *Κατευθυντήρια Οδηγία για την Επικύρωση Μεθόδων Προσδιορισμού Υπολειμμάτων Φυτοπροστατευτικών Προϊόντων* http://www.esyd.gr/gr_pages/b_kanonismoi_eggrafa/f_mnimonia/mnimonio_fytoprostateytikon.doc (accessed on 29/12/2008).
7. Interactive PhysProp Database, url, available at: http://www.syrres.com/esc/physdemo.htm" (accessed on 29/12/2008).
8. Jarczyk, H.J. 1983a. *Pflanzenschutz-nachrichten Bayer*, 36/1983,1: 63-72.
9. Jarczyk, H.J. 1983b. *Pflanzenschutz-nachrichten Bayer*, 36/1983,1: 84-94.
10. Lehotay, S. 2007. Pesticide Residues in Foods by Acetonitrile Extraction and Partitioning with Magnesium Sulfate. *Journal of AOAC International* 90: 485-520.
11. NIST/EPA/NIH Mass Spectral Library, Data Version: NIST 06 (Software Version 2.0 d).
12. Wells, D., Terence, G.B., Langner, E. 1986. Vapour pressure of permethrin. *Pesticide Science,* Vol. 17 (5): 473-476.

Received: 4 January 2010; Accepted: 17 June 2010

Επικύρωση μεθόδου προσδιορισμού υπολειμμάτων φυτοπροστατευτικών προϊόντων διαφόρων χημικών κατηγοριών με επιλογή αντιπροσωπευτικών ουσιών με τη χρήση αέριας χρωματογραφίας

Ν. Βλαστάρας, Ε. Δασενάκης, Κ.Σ. Λιαπής, Γ.Ε. Μηλιάδης και Χ.Ι. Αναγνωστόπουλος

Περίληψη Η εντατική εφαρμογή χημικών φυτοπροστατευτικών προϊόντων είναι μια συνήθης πρακτική στη συμβατική γεωργία, με στόχο την αποτελεσματικότερη αντιμετώπιση ασθενειών και εχθρών των φυτών. Ακόμα και όταν η εφαρμογή των χημικών φυτοπροστατευτικών προϊόντων γίνεται σύμφωνα με τους κανόνες της Ορθής Γεωργικής Πρακτικής, οι ουσίες αυτές είναι πιθανό να αφήσουν υπολείμματα στο τελικό προϊόν. Ως εκ τούτου, η ανάγκη ανάπτυξης γρήγορων, εύκολων και αξιόπιστων μεθόδων προσδιορισμού για την ανίχνευση υπολειμμάτων φυτοπροστατευτικών προϊόντων έχει καταστεί επιτακτική. Η εκτίμηση της αξιοπιστίας μιας μεθόδου πραγματοποιείται μέσω της διαδικασίας της επικύρωσης. Η επικύρωση μιας πολυ-υπολειμματικής μεθόδου είναι μια διαδικασία η οποία απαιτεί αρκετό χρόνο και χρήμα, καθόσον απαιτούνται 5-6 επαναλήψεις σε δύο τουλάχιστον επίπεδα για κάθε ουσία και για κάθε υπόστρωμα. Στην παρούσα μελέτη ακολουθείται μια διαφορετική προσέγγιση, χρησιμοποιώντας μια πιο εύκολη και μικρότερου κόστους διαδικασία. Αρχικά γίνεται επιλογή αντιπροσωπευτικών αναλυτών της κάθε χημικής κατηγορίας, οι οποίοι καλύπτουν όλο των εύρος των φυσικοχημικών ιδιοτήτων (πολικότητα, διαλυτότητα στο νερό, τάση ατμών) του συνόλου των αναλυτών και πρέπει σε αριθμό να καλύπτουν τουλάχιστον το 20% των αναλυτών της κάθε κατηγορίας. Σε αυτούς συμπεριλαμβάνονται και οι αναλύτες αυτοί για τους οποίους εκτιμάται ότι θα έχουν τη χειρότερη απόδοση. Επιπλέον, η επικύρωση πραγματοποιείται σε ένα μόνο υπόστρωμα από κάθε κατηγορία τροφίμων. Ακολουθώντας αυτή την προσέγγιση επικυρώθηκαν πάνω από 180 αναλύτες διαφόρων χημικών κατηγοριών (οργανοφωσφορικά, οργανοχλωριωμένα, τριαζόλες, τριαζίνες, στρομπιλουρίνες, πυρεθρίνες, δινιτροανιλίνες και διάφοροι αναλύτες που περιέχουν άζωτο στο μόριο τους) επιλέγοντας μόλις 44 αντιπροσωπευτικούς αναλύτες, με τη χρήση αέριας χρωματογραφίας σε συνδυασμό με ανιχνευτή σύλληψης ηλεκτρονίων και αζώτου φωσφόρου. Τα υποστρώματα που χρησιμοποιήθηκαν ήταν τομάτες και σταφύλια, δύο κατηγορίες τροφίμων η οποίες παρουσιάζουν υψηλή κατανάλωση στην Ελλάδα.

Hellenic Plant Protection Journal **2:** 57-69, 2010

Hellenic Plant Protection Journal **3:** 71-80, 2010

Validation of the QuEChERS method for the determination of 25 priority pesticide residues in cereal-based baby foods by gas chromatography with electron capture and nitrogen phosphorous detection

C.J. Anagnostopoulos[1], P. Aplada Sarli[1], G.E. Miliadis[1] and C.A. Haroutounian[2]

Summary A multiresidue method for the simultaneous analysis of 25 pesticides and metabolites included in Commission Directives 2006/141/EC and 2006/125/EC for baby food was validated in cereal-based baby foods. The extraction procedure was based on the QuEChERS method and the determination was performed by gas chromatography combined with electron capture detector (ECD) and nitrogen phosphorus detector (NPD). The analytical performance was demonstrated by the analysis of extracts of spiked samples at two concentration levels, 3 and 30 μg/kg, for each analyte. Good sensitivity and selectivity of the method were obtained with limits of quantification at 3 μg/kg in all cases. All pesticides and metabolites, with the exception of omethoate, gave recovery values ranging from 79.2 to 124.6% with relative standard deviations less than 28% at both spiking levels for most analytes.

*Additional keywords***:** GC-NPD, GC-ECD, linearity, precision, trueness, validation

Introduction

In recent years, the safety of children's and infants diet has received special attention. Because they are still growing and developing, their metabolism, as well as other physiological and biochemical processes, differ from that of adults (11). However, as with adults, diet can be a significant source of exposure to pesticides. Trace quantities of pesticides and their breakdown products present on or in foodstuffs are termed as "residues". Residue levels reflect the amount of pesticide applied to a crop, the time that has elapsed since application, and the rate of pesticide dissipation and evaporation (11). To ensure the safety of foods specifically produced for infants (children under the age of 12 months) and young children (between 1 and 3 years), the European Commission (EC) has set rules for these categories of food (6). Regarding pesticides, the EC specifies a maximum residue level (MRL) of 10 μg/ kg of pesticide residue in baby food and has banned highly toxic pesticides (ADI ≤0.5 mg/kg body weight) in agricultural produce intended for processed baby foods. The MRLs for highly toxic pesticides set by the EC are even more strict, ranging from 3 to 8 μg/kg (European Union Directive 91/321/EC and subsequent revisions, 1999/50/EC, European Union Directive 96/5/EC and subsequent revisions, 1999/39/EC, European Union Directives 2003/13/EC, 2003/14/EC, 2006/125/EC and 2006/141/EC).

Considering the low concentration levels that must be detected for successful monitoring of pesticide residues in infant foods, sensitive and reliable confirmation methods are required.

Many different extraction methods, such as supercritical fluid extraction (SFE) (8) and acetone-based extraction (1, 17), have been used for the extraction of pesticides in cereals and flour. Acetonitrile was also adopted as extraction solvent with various applications in low water and fat content commod-

[1] Laboratory of Pesticide Residues, Department of Pesticides Control and Phytopharmacy, Benaki Phytopathological Institute, 8 St. Delta Str., GR-145 61 Kifissia (Athens), Greece.
[2] Chemistry Laboratory, Department of Science, Agricultural University of Athens, 75 Iera Odos Str., GR-118 55 Athens, Greece
Corresponding author: c.anagnostopoulos@bpi.gr

ities (wheat, rice, oats, cereals, etc.). Schenck *et al*. (14) used 65% acetonitrile in water as extraction solvent. The extracts were salted out with NaCl and $MgSO_4$ and cleaned-up on a series of graphitized carbon black and amino SPE cartridges before analysis of 33 pesticides in grain. Wang *et al.* (16) used acetonitrile and hexane as extraction solvents and clean-up with SPE cartridges for the determination of 13 pesticides in soy-based infant formula using LC-MS/MS. Lehotay *et al*. (2, 9, 10) conducted a study to establish a miniaturized acetonitrile-based extraction, followed by a dispersive solid phase extraction clean-up step (QuEChERS). Walorczyk (15) used the QuEChERS method for the determination of 122 GC amenable pesticides in cereals and animal feed. Payá *et al*. (12) validated the QuEChERS method for 38 GC amendable pesticides in wheat flour using GC-MS/MS and 42 LC amendable pesticides using LC-MS/MS.

In the last few years, modern analytical techniques, such as GC-MS/MS, LC-MS/MS, LC-TOF/MS, are used for the detection of pesticides in baby foods. These techniques require expensive and sophisticated equipment, increasing the initial purchase cost to the laboratory as well as that of the analytical processes themselves. In addition, the number of accredited laboratories in some EU-bordering countries is still low and the legislation and organization of agricultural plant production in these countries have not reached yet the required level (13). Therefore, the methods used for the analysis of baby food samples or the relevant agricultural products need to be simple, fast, low-cost, and sensitive. They also need to be suitable for routine analysis in a variety of laboratories and harmonized with the EU legislation requirements concerning the validation criteria, so as to ensure the quality and comparability of analytical results (5).

The aim of this paper was to validate a multiresidue analytical method suitable for the simultaneous extraction and determination of 25 priority pesticides in cereal-based baby food samples by using gas chromatography with two different detectors, an electron capture and a nitrogen phosphorus detector.

Materials and Methods

Reagents and chemicals

In this work, the following 25 priority pesticides and metabolites, obtained from Dr Ehrenstorfer Laboratories (GmbH Germany) and included in the European Union Directives 2006/125/EC and 2006/141/ EC were studied (4): aldrin, cadusafos, demeton-s-methyl, demeton-s-methyl sulfone, demeton-s-methyl sulfoxide, dieldrin, dimethoate, disulfoton, disulfoton-sulfone, disulfoton-sulfoxide, ethoprophos, fensulfothion, fensulfothion sulfone, fensulfothion-oxon, fensulfothion-oxon-sulfone, terbufos, terbufos-sulfone, terbufos-sulfoxide, fipronil, fipronil-desulfinyl, heptachlor, heptachlorepoxid (trans), hexachlorobenzene, nitrofen, omethoate. LC-MS grade acetonitrile, 2,2,4-trimethyl pentane and toluene pesticide residue analysis grade were used. Magnesium sulphate anhydrous, disodium hydrogencitrate sequihydrate were obtained from Fluka (Buchs, Switzerland). Sodium chloride (ACS reagent grade ≥99.0%) and trisodium citrate dihydrate were obtained from Sigma-Aldrich (Madrid, Spain). PSA (Primary Secondary Amine) sorbent Bondesil-PSA 40μm was obtained from Varian.

Stock and working standard solutions

Stock standard solutions at 1000 mg/l were prepared in acetone for each pesticide and stored at -20°C. Standard mixture solutions of the compounds were prepared in 2,2,4-trimethylpentane/toluene (90/10) at intermediate concentrations (1-10 mg/l) and stored at -20°C. In order to acquire the retention time of each analyte, working solutions containing only one analyte at 0.5 mg/l were prepared and injected to the chromatographic system.

Working standard mixture solutions for calibration were prepared in wheat flour extract, previously analysed for the absence of compounds interfering with the analytes.

According to Document SANCO/3131/2007 of the European Commission, the potential for matrix effects should be assessed during the validation of the method (5). The effect can be due to different reasons e.g. the presence of a blank, due to solvent and/or reagents or the presence of a compound in the sample that contributes to the analytical signal (7). Matrix-induced enhancement is a phenomenon commonly found in gas chromatographic analysis of pesticides in food (5) and has been noticed in the analysis of these compounds by either GC-ECD (11) or GC-NPD (5). For this purpose, matrix-matched standard solutions (including matrix blanks) were used. An aliquot of 2ml of control sample extracts were evaporated to dryness by a stream of N_2. After evaporation 1 ml of a standard solution at the desired concentration prepared in 2,2,4-trimethylpentane/toluene (90/10) was added. The final solution was reconstructed in an ultrasonic bath for 30 sec.

Preparation of fortified samples

Control samples were prepared from organically produced cereal-based infant food. Aliquots of 5 g portion of cereal-based infant food sample were fortified at two levels. The lowest fortification level was 3 μg/kg and the second fortification level was 10 times higher (30 μg/kg). For validating the method according to SANCO 2007/3131, a minimum of five replicates is required. Therefore, five replicates were used for each level.

Sample preparation

For the extraction of the pesticides, the protocol of QuEChERS method concerning commodities with high fat content was followed (2, 3). According to this method, a 5 g portion of the cereal-based infant food was weighted in a 50 ml PTFE centrifuge tube, and 10 ml of water were added. The water should be at low temperature (< 4°C) to compensate for the heat development caused by the addition of the salts that follows. A short vibration using a Vortex mixer (K-550-GE, Scientific industries inc. Bohemia, USA) helped to disperse solvent and pesticides well through the sample. For the extraction of the pesticides, 10 ml of acetonitrile were added and the tube was vigorously shaken for 1min. A mixture of 1 g of NaCl, 4 g of $MgSO_4$, 1 g of trisodium citrate dehydrate and 1 g of disodium hydrogencitrate sesquihydrate were added and the tube was vigorously shaken for 1 or more minutes to prevent coagulation of $MgSO_4$. The sample was then centrifuged (4000 rpm) for 5 min. An aliquot of 8 ml of the supernatant (acetonitrile phase) was then taken and transferred into a 15 ml centrifuge tube and stored for at least 2 h in the freezer. Freezing-out helps to partly remove some additional co-extractives with limited solubility in acetonitrile, while the major part of fat and waxes solidify and precipitate. An aliquot of 6 ml of the still cold acetonitrile phase was transferred into a 15 ml centrifuge tube containing 250 mg of PSA and 750 mg of $MgSO_4$ and the tube was shaken vigorously for 30 sec and centrifuged for 5 min at 4000 rpm. An aliquot of 5 ml of the cleaned-up extract was transferred into a screw cup storage vial, taking care to avoid sorbent particles of being carried over. The extract was slightly acidified by adding 50 μl of a 5% formic acid solution in acetonitrile. An aliquot of 2 ml of the extract was obtained, evaporated near dryness and reconstituted in 1 ml of 2,2,4-trimethylpentane/toluene (90/10). The final extract was sonicated in an ultrasonic bath for 30 sec and transferred into autosampler vials with a Teflon stopper.

Gas-chromatographic analysis

Determination was performed by the use of an Agilent 6890 gas chromatograph, with two splitless injectors, a DB-5-MS column (30 m, 0.32 mm i.d. and 0.25 μm film thickness) connected to the ECD and a DB-17 MS column (30 m, 0.3 mm i.d. and 0.25 μm film thickness) connected to the NPD and equipped with a Chemstation chromatography manager data acquisition and processing software. For confirmation purposes the two columns were swapped between the two detectors.

The oven temperature programme started from 60°C for 1.5 min, increased to 220°C at a rate of 14°C/min, held for 4 min then increased to 280°C at 20°C/min and held for 20 min. The helium carrier gas flow rate was 1.5 ml/min for both columns. Injectors' temperature was set at 230°C and splitless injection was carried out with the purge valve closed for 1 min. Hydrogen (3 ml/min) and air (60 ml/min) were used as fuel gases for the NPD. Temperature was set at 310°C for both detectors.

Results and discussion

The method was found to be effective for extraction of the tested compounds. The method was validated by assessing the basic parameters, sensitivity, mean recovery (as a measure of trueness) and repeatability (as a measure of precision). These parameters were evaluated for both detectors.

The confirmation of the analytes was conducted, as mentioned earlier, only by the retention time of the analyte using two columns of different polarity and two different detectors. The retention times acquired for each analyte using a combination of two different columns and two different detectors are shown in Table 1. Because the detectors used (ECD and NPD) offer a degree of selectivity, the use of two columns in combination with these detectors was necessary to achieve sufficient confirmation. As shown in Table 1, confirmation was achieved successfully for all analytes. For organochloride pesticides, confirmation was achieved using

Table 1. Pesticides studied, retention times of the analytes with DB-5-MS and DB-17-MS columns and sensitivity to ECD and NPD detectors.

Analytes	DB-5- MS	DB-17-MS	ECD sensitivity	NPD sensitivity
aldrin	12.0	14.0	+	-
cadusafos	7.6	8.6	+	+
demeton-s-methyl	7.1	8.9	-	+
demeton-s-methyl sulfone	11.1	19.2	-	+
demeton-s-methyl sulfoxide	7.0	4.5	-	+
dieldrin	16.9	22.0	+	-
dimethoate	8.2	12.3	+	+
disulfoton	9.2	11.3	+	+
disulfoton-sulfone	15.7	24.1	+	+
disulfoton-sulfoxide	5.1	5.5	+	+
ethoprophos	6.9	8.3	+	+
fensulfothion	19.3	29.4	+	+
fensulfothion sulfone	20.5	30.7	+	+
fensulfothion-oxon	17.4	27.9	+	+
fensulfothion-oxon-sulfone	18.4	29.2	+	+
fipronil	14.6	16.2	+	+
fipronil-desulfinyl	10.9	11.8	+	+
heptachlor	10.8	12.5	+	-
heptachlor epoxid (trans)	13.9	17.4	+	-
hexachlorobenzene	8.1	9.2	+	-
nitrofen	18.3	25.6	+	+
omethoate	6.7	9.7	-	+
terbufos	8.7	10.1	+	+
terbufos-sulfone	14.0	20.3	+	+
terbufos-sulfoxide	8.6	10.0	-	+

two different separation systems (two different columns) because they are not sensitive to the NPD. For pesticides like cadusafos, disulfoton, terbufos and others that are sensitive to both detectors, confirmation was achieved by two different columns. Figure 1 shows a spiked cereal-based baby food sample with fensulfothion and fensulfothion sulfone at 3 µg/l in various columns and detectors.

Linearity

Calibration curves were constructed from data acquired after injecting of matrix-matched calibration standards in control matrix of cereal-based baby foods in 2,2,4-trimethylpentane/toluene (90/10) of the 25 pesticides at eight concentration levels, i.e. 3, 6, 8, 10, 15, 20, 30 and 40 µg/l. In Table 2, the basic calibration line parameters for the analytes are presented. Linearity parameter values were found acceptable for most pesticides, with coefficients of determination (r^2) higher than 0.98 in most cases. However, some pesticides, such as omethoate and terbufos sulfoxide, did not exhibit good linear behaviour, due to the fact that they are polar compounds so they achieved low sensitivity and poor chromatography at the under study concentration range. Due to the variability in the peak area resulting from the use of the calibration curve, the appropriate value C (mg/kg) was estimated by the following equation:

$$S_u = \frac{S_{Area/C}}{b}$$

where, $$S_{Area/C} = \sqrt{\frac{\sum_i (y_i - \bar{y})^2}{n-2}}$$

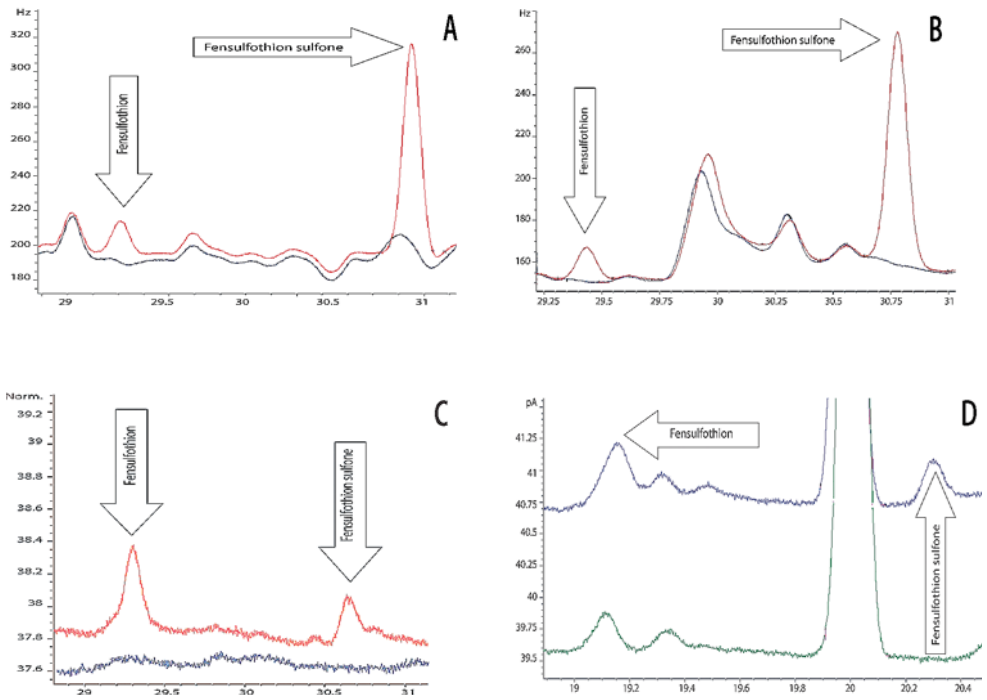


Figure 1. Chromatogram of spiked cereal-based baby food sample with fensulfothion and fensulfothion sulfone at 3 µg/kg analyzed by (A) column DB-5MS, detector ECD, (B) column DB-17MS, detector ECD, (C) column DB-5MS, detector NPD, (D) column DB-17MS, detector NPCD.

n is the number of the data points in the calibration, (y_i - $\bar{y}$) is the residual for the ith point and *b* is the slope of the regression line. The contribution of the uncertainty of the calibration curve to the total uncertainty of the method was calculated at the middle (C=15 μg/l) of the calibration curve for each compound and was estimated to be 3.8 – 23.5%.

Trueness, precision

The trueness was estimated by calculating the attained recovery from fortification experiments. For validating a method, mean recoveries of 70–120% with a repeatability RSD ≤ 20% are considered acceptable, while in routine analysis, the acceptable recoveries are in the range of the mean recovery ±2 relative standard deviations (SDr) %, which usually fall within the range 60-140% (4). The mean recoveries were determined from spiked flour samples, at two concentration levels with five replicates for each level.

The recoveries were calculated using matrix-matched calibration standards. As shown in Table 3, mean recoveries of the samples fortified at the lowest fortification level (3 μg/kg) were between 79.2 – 124.6% with SDr less than 17.2% with some exceptions, as shown in Table 3 and at the second fortification level (30 μg/kg) between 68.2 – 118.1% with SDr less than 23.9%. These results indicate generally good method accuracy and precision.

Dimethoate, fensulfothion sulfone, fensulfothion-oxon-sulfone and terbufos-sulfone are sensitive to both detectors and the qualification can be achieved by both ECD and NPD at the lowest fortification level. However, the mean recoveries are acceptable only by one of the detectors. Therefore, we can use both detectors for confirmation, but for quantification of the analyte we can use only the detector that provides good accuracy. For example, fensulfothion sulfone can be confirmed with a DB-5-MS column combined with an ECD and a NPD detector, or a DB-17 MS column combined with an ECD and a NPD detector, or a DB-5-MS column and a DB-17 MS column each of them combined with an ECD, or a DB-5-MS column and a DB-17 MS column each of them combined with a NPD, or a DB-5-MS column combined with an ECD and a DB-17 MS column combined with a NPD, or a DB-5-MS column combined with a NPD and a DB-17 MS column combined with an ECD. However, the quantification of fensulfothion sulfone must be done with a NPD detector. The experiments for recovery of the analytes fensulfothion-oxon and terdufos sulfoxide failed at the lowest fortification level and for omethoate failed at both levels.

Limit of Quantification (LOQ)

The limit of quantification (LOQ) was established as the lowest concentration tested for which recovery and precision were satisfactory (70–110% and <20% RSD, respectively) in accordance with the criteria established for analysis of pesticide residues in foods (4). Therefore, in most cases the method's limit of quantification (LOQ) was the lowest fortification level, i.e. 3 μg/kg.

Conclusions

In the present study, 25 target pesticides and metabolites, selected according to the European Union Directives 2006/141 and 2006/125, were studied in cereal-based baby foods. The extraction procedure was based on the QuEChERS method and the determination was performed with GC–ECD and NPD. The validation results presented good accuracy with recoveries of 79.2 – 124.6%, precision with RSD of 3.4 - 28% and limits of quantification meeting the EU legislation requirements for the maximum residue limits. Concerning the reporting of the results, from the data of the EU proficiency tests, a default expanded uncertainty figure of 50% (corresponding to a 95% confidence level and a coverage factor of 2), in general covers the inter-laboratory variability between the laboratories for residue analysis (5).

The disadvantage of the method was that fensulfothion-oxon and terdufos sulfoxide failed to be determined at the 3 μg/

Table 2. Summary of Calibration line parameters for the compounds in 3 – 40 µg/l concentration range.

Analytes	Detector	r	r^2	b	S_b	a	S_a	S_u
aldrin	ECD	0.998	0.996	222.78	6.05	18.32	123.43	0.92
cadusafos	NPD	0.998	0.997	11.14	0.26	3.41	5.40	0.81
demeton-s-methyl	NPD	0.980	0.961	0.54	0.04	0.77	0.91	2.80
demeton-s-methyl sulfone	NPD	0.990	0.981	1165719	73085	-1571724	1493110	2.44
demeton-s-methyl sulfoxide	NPD	0.994	0.988	2475677	126093	-4452833	2576058	1.98
dieldrin	ECD	0.998	0.995	187.75	5.24	79.51	107.05	0.95
dimethoate	ECD	0.993	0.985	10.87	0.54	26.97	11.08	1.70
	NPD	0.980	0.960	0.16	0.01	0.01	0.27	2.84
disulfoton	ECD	0.996	0.993	6.28	0.22	-2.75	4.39	1.16
disulfoton-sulfone	ECD	0.996	0.991	7.71	0.30	3.02	6.05	1.31
disulfoton-sulfoxide	ECD	0.999	0.998	4.58	0.08	3.70	1.66	0.60
	NPD	0.998	0.995	0.25	0.01	-0.11	0.14	0.97
ethoprophos	ECD	0.992	0.984	10.06	0.53	28.32	10.85	1.80
	NPD	0.984	0.969	0.26	0.02	1.64	0.50	3.14
fensulfothion	ECD	0.990	0.980	8.92	0.52	-4.94	10.71	2.00
	NPD	0.989	0.978	0.28	0.02	-0.73	0.35	2.08
fensulfothion sulfone	ECD	0.998	0.995	66.44	1.90	-4.81	38.70	0.97
	NPD	0.969	0.939	0.19	0.02	0.12	0.41	3.53
fensulfothion-oxon	ECD	0.994	0.987	5.65	0.26	8.51	5.36	1.58
fensulfothion-oxon-sulfone	ECD	0.991	0.982	5.81	0.33	11.66	6.64	1.90
fipronil	ECD	0.999	0.998	155.34	2.59	-38.92	52.88	0.57
	NPD	0.999	0.998	0.54	0.01	0.16	0.22	0.66
fipronil-desulfinyl	ECD	0.995	0.989	297.02	12.77	-615.64	260.63	1.46
heptachlor	ECD	0.996	0.991	122.25	4.73	-56.98	96.46	1.31
heptachlor epoxid (trans)	ECD	0.996	0.993	168.47	5.82	19.21	118.71	1.17
hexachlorobenzene	ECD	0.997	0.994	137.51	4.33	63.42	88.32	1.07
nitrofen	ECD	0.995	0.990	132.44	5.33	7.73	108.76	1.37
omethoate	-	-	-	-	-	-	-	-
terbufos	ECD	0.997	0.994	7.37	0.24	4.32	4.80	1.08
terbufos-sulfone	ECD	0.990	0.981	25.02	1.43	57.43	29.13	1.94
	NPD	0.980	0.960	0.30	0.02	0.45	0.51	2.85
terbufos-sulfoxide	-	-	-	-	-	-	-	-

b : Slope of the regression line

S_b : Mean standard deviation of the slope of the regression line

a : Mean of the population that corresponds to x=0

S_a : Mean standard deviation of the mean of the population that corresponds to x=0

r : correlation coefficient

r^2 : determination coefficient

S_u : standard uncertainty of the concentration (µg/ml) due to the calibration line

Table 3. Average recovery values and relative standard deviation for the compounds as derived from the fortification experiments in both detection systems.

Analyte	Detector: ECD				Detector: NPD			
	Lower fortification level (3 µg/kg)		Higher fortification level (30 µg/kg)		Lower fortification level (3 µg/kg)		Higher fortification level (30 µg/kg)	
	Recovery (%)	SD_R (%)	Recovery (%)	SD_R (%)	Recovery (%)	SD_R (%)	Recovery (%)	SD_R (%)
aldrin	97.1	15.2	95.5	9.3	-	-	-	-
cadusafos	113.6	3.4	101.9	6.5	108.6	6.8	101.3	10.4
demeton-S-methyl	-	-	-	-	79.2	3.5	97.6	23.9
demeton-S-methyl sulfone	-	-	-	-	108.3	28.0	89.3	5.4
demeton-S-methyl sulfoxide	-	-	-	-	106.8	17.2	85.6	7.6
dieldrin	94.8	10.0	112.4	6.8	-	-	-	-
dimethoate	124.6	11.5	103.2	6.3	-	-	-	-
disulfoton	102.5	7.47	68.2	17.1	104.6	11.3	87.2	12.6
disulfoton-sulfone	110.4	7.2	89.6	3.7	95.2	16.0	80.4	11.4
disulfoton-sulfoxide	98.9	12.2	111.5	4.9	100.3	7.83	106.4	4.7
ethoprophos	97.6	6.45	80.7	13.9	91.1	8.8	93.3	12.2
fensulfothion	95.6	7.55	97.6	3.7	118.2	13.6	86.8	13.0
fensulfothion sulfone	-	-	-	-	95.8	10.1	78.3	7.6
fensulfothion-oxon	-	-	112.8	8.7	-	-	76.2	9.7
fensulfothion-oxon-sulfone	96.4	11.7	118.1	4.6	-	-	-	-
fipronil	93.4	7.59	113.5	14	-	-	-	-
fipronil-desulfinyl	118.8	10.7	87.0	3.3	-	-	-	-
heptachlor	103.5	14.3	100.4	9.6	-	-	-	-
heptachlor epoxid (trans)	106.8	10.9	108.3	7.2	-	-	-	-
hexachlorobenzene	104.2	16.1	87.0	10.7	-	-	-	-
nitrofen	111.0	6.9	99.6	15.4	-	-	-	-
omethoate	-	-	-	-	-	-	-	-
terbufos	97.4	7.52	99.9	10.4	88.2	5.7	107.6	13.72
terbufos-sulfone	110.9	13.7	113.7	9.3	-	-	-	-
terbufos-sulfoxide	-	-	-	-	-	-	90.0	11.3

kg level and omethoate failed at both tested levels. Therefore, a different technique should be used for the determination of these analytes. However, by using only a GC with an ECD and a NPD detector and two different columns for the confirmation and quantification of the analytes, instead of more expensive instruments (LC-MS-MS), reduces the cost of the analysis and makes it suitable to be used by a variety of laboratories. Therefore, the method is suitable for small routine laboratories of the private or public sector where the cost of analysis is very important. Although the limits are set for the final product, monitoring of raw materials used in the production chain of cereal-based baby foods is also crucial, not only for the public authorities responsible for the monitoring of pesticide residues in foodstuffs, but also for the food industry which checks pesticide residues in their supplies.

Literature cited

1. Anagnostopoulos, C.J. and Miliadis, G.E. 2009. Method validation for the determination of pesticide residues in wheat flour by gas chromatography. *Hellenic Plant Protection Journal,* 2: 15-22
2. Anastassiades, M.. Lehotay S.J., Stajnbaher, D. and Schenck, F.J. 2003. Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and "Dispersive Solid-Phase Extraction" for the Determination of Pesticide Residues in Produce. *Journal of the Association of Official Analytical Chemists International,* 86: 412-431.
3. Anastassiades, M., Tasdelen, B., Scherbaum, E., Stajnbaher, D. 2007. Recent Developments in QuEChERS Methodology for Pesticide Multiresidue Analysis. In: Ohkawa, H. Miyagawa, H. Lee, P.W. 2007. Pesticide chemistry: Crop protection, public health, environmental safety. Wiley-VCH. Weinheim. 538p.
4. European Commission. 2006. Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae. *Official Journal of European Communities* L401: 1–33.
5. European Commission. 2007. Method validation and Quality control procedures for pesticide residues analysis in food and feed. Document No. SANCO/2007/3131.
6. European Communities. 2009. Foods for Infants & Young Children available in: http://ec.europa.eu/food/food/labellingnutrition/children/index_en.htm (accessed on 14/12/2009).
7. Gil Garcia, M.D., Martinez Vidal, J.L., Martinez Galera, M., Lopez Lopez, T., Almansa Lopez, E. and Cuadros Rodriguez, L. 2003. Correction of the Matrix Effect in the Determination of Benzoylphenylurea Insecticides in Vegetables. *Journal of Liquid Chromatography & Related Technologies,* 26: 297-316.
8. Kim, Dal Ho. 1998. Determination of organophosphorus pesticides in wheat flour by supercritical fluid extraction and gas chromatography with nitrogen–phosphorus detection. *Journal of Chromatography A*, 824: 63–70.
9. Lehotay, S.J., Mastovska, K. and Schenck, F.J. 2003. Abstracts of Papers, 225th ACS National Meeting.
10. Lehotay, S.J. and Mastovska, K. 2004. Abstracts of Papers, 228th ACS National Meeting
11. National Research Council. 1993. Pesticides in the Diets of Infants and Children p. 13-17.
12. Payá, P., Anastassiades, M., Mack, D., Sigalova, I., Tasdelen, B., José Oliva, J. and Barba, A. 2007. Analysis of pesticide residues using the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) pesticide multiresidue method in combination with gas and liquid chromatography and tandem mass spectrometric detection. *Analytical and Bioanalytical Chemistry*, 389: 1697-1714.
13. Steimer, M., Gerbl-Rieger, S., Schaff. P. and Parlar, H. 2007. Current situation of the application and identification of pesticides in EU bordering countries with respect to the implementation of a monitoring system. *Fresenius Environmental Bulletin*, 16: 443-451.
14. Schenck, F.J. and King, V.H. 2002. Determination of organochlorine and organophosphorus pesticide residues in low moisture, non-fatty products using a solid phase extraction cleanup and gas chromatography. *Journal of Environmental Science and Health, Part B* 35:1–12.
15. Walorczyk, S. 2007. Development of a multi-residue screening method for the determination of pesticides in cereals and dry animal feed using gas chromatography–triple quadrupole tandem mass spectrometry. *Journal of Chromatography* A 1165: 200–212.
16. Wang, J. and Cheung W. 2006. Determination of Pesticides in Soy-Based Infant Formula Using Liquid Chromatography with Electrospray Ionization Tandem Mass Spectrometry. *Journal of AOAC International,* 89: 214-224.
17. Working Group "Development and Improvement of Residue-analytical methods". 1996. Analytical Methods for Pesticde Residues in Foodstuffs. 6th Edition Part I: Multiresidue methods. Minstry of Public Health, Welfare and Sport, The Netherlands.

Received: 4 January 2010; Accepted: 16 June 2010

Επικύρωση της μεθόδου QuEChERS για τον προσδιορισμό 25 φυτοπροστατευτικών ουσιών σε παιδικές τροφές με την τεχνική της αέριας χρωματογραφίας

Χ.Ι. Αναγνωστόπουλος, Π. Απλαδά-Σαρλή, Γ.Ε. Μηλιάδης και Σ.Α. Χαρουτουνιάν

Περίληψη Μια γρήγορη πολυϋπολειμματική μέθοδος για τον προσδιορισμό 25 φυτοπροστατευτικών ουσιών και μεταβολιτών τους σε παιδικές τροφές επικυρώθηκε. Η εκχύλιση των ουσιών πραγματοποιήθηκε με τη μέθοδο QuEChERS (εκχύλιση με ακετονιτρίλιο). Ο ποιοτικός και ποσοτικός προσδιορισμός των ουσιών πραγματοποιήθηκε με την τεχνική της αέριας χρωματογραφίας σε συνδυασμό με ανιχνευτές σύλληψης ηλεκτρονίων και αζώτου/φωσφόρου. Από τα στοιχεία επικύρωσης προκύπτει ότι η μέθοδος παρουσιάζει αποδεκτή ορθότητα με ποσοστά ανάκτησης 79.2 – 124.6% καθώς και πιστότητα με σχετικές τυπικές αποκλίσεις μικρότερες από 28% για όλες τις ουσίες εκτός από το omethoate. Το όριο ποσοτικοποίησης της μεθόδου είναι 3 μg/kg. Η μέθοδος χαρακτηρίζεται από αξιοπιστία και ευαισθησία και κρίνεται κατάλληλη για αναλύσεις ρουτίνας υπολειμμάτων φυτοπροστατευτικών προϊόντων σε παδικές τροφές βασισμένες στα δημητριακά.

Hellenic Plant Protection Journal **3:** 71-80, 2010

Appreciation to the external Associate Editors

The Editorial Board of the Hellenic Plant Protection Journal (HPPJ) expresses its deep appreciation and gratitude to the external Associate Editors listed below for having generously offered their time and expertise to review and help improve the manuscripts submitted for publication in Volume 3 of HPPJ.

Balayiannis P. George, Research Scientist, Laboratory of Chemical Control of Pesticides, Department of Pesticides Control and Phytopharmacy, Benaki Phytopathological Institute

Karassali Eleni, Associate Research Scientist, Laboratory of Chemical Control of Pesticides, Department of Pesticides Control and Phytopharmacy, Benaki Phytopathological Institute

Kasiotis M. Konstantinos, Research Scientist, Laboratory of Toxicological Control of Pesticides, Department of Pesticides Control and Phytopharmacy, Benaki Phytopathological Institute

Kontodimas C. Dimitrios, Senior Research Scientist, Laboratory of Agricultural Entomology, Department of Entomology and Agricultural Zoology, Benaki Phytopathological Institute

Kyriakopoulou Katerina, Senior Research Scientist, Laboratory of Toxicological Control of Pesticides, Department of Pesticides Control and Phytopharmacy, Benaki Phytopathological Institute

Paplomatas J. Epaminondas, Associate Professor, Laboratory of Phytopathology, Department of Crop Science, Agriculgural University of Athens

Instructions to Authors

General

The *Hellenic Plant Protection Journal* (HPPJ) is a scientific publication of the Benaki Phytopathological Institute (BPI), which publishes scientific work on any aspect of plant protection in the Mediterranean region. Only original articles which have not been published or submitted for publication elsewhere are considered for publication in the journal. Upon publication all articles are copyrighted by the BPI and the HPPJ.

Types of papers accepted

Papers submitted for publication can be either in the form of a complete research article or in the form of a sufficiently documented short communication. New records of diseases, pathogens, pests and weeds can also be submitted in either form. Review articles in related topics, either submitted or invited by the Editorial Board, are also published, normally one article per issue.

Manuscript submission and review

Submit your manuscript to the Editorial Board in an electronic form by e-mail at the address editors@bpi.gr or on a disk by post to the following address: Benaki Phytopathological Institute, 8 St. Delta str., GR-145 61, Kiphissia (Athens), Greece. Text including tables, footnotes, legends for figures and references must be in one MS Word file in the format of doc or RTF. Figures must be in separate files (not incorporated into the text) as explained under Figures.

The Editorial Board assigns the manuscript to an associate editor and two anonymous reviewers who evaluate its content and presentation. It is the associate editor's responsibility to forward the review process and report to the Editorial Board who decides on acceptance or rejection of the manuscript notifying the author(s). The review process is completed with the submission by the author(s) of the revised manuscript to the Editorial Board.

Preparation of manuscripts

Authors are advised to refer to a recent issue of the journal, as a guide to the required text layout, heading and table settings etc. Use single-line spacing on an A4 page (297x210 mm) size.

Page limits, page charges and reprints

Full-length research papers and review articles should not exceed 25 typewritten pages including tables, figures and references. Short communications should not exceed 5 pages in total. There is no page charge for authors.

Arrangement of the text

Major sections of each full paper should be arranged in the following order: title, author(s) name, summary, additional keywords, main text, acknowledgements, literature citations, a title and a summary in Greek.

Title: The title should be short and reflect at the best the content of the article.

Running head: A short title of less than 65 characters including spaces should be typed in a separate line after the title.

Author(s) names and addresses: Authors names should be listed under the title and the addresses of the place(s) where the work has been carried out, with the appropriate numbering, should be provided in a footnote.

Summary: The english summary should describe concisely (in no more than 200 words) the aim

and main conclusions of the paper. There is no summary in short communications.
Additional keywords: Only words not contained in the title, which are essential in indexing and retrieval systems, are listed in alphabetical order. They should be nouns and not more than six.
Main text: Major sections should be arranged in the following order: Introduction, Materials and methods, Results, Discussion and Conclusions (or Results and discussion). Short communications may include the same sections of the main text.
Acknowledgments: They follow the text without a heading.
Title and Summary in Greek: A title and a summary in Greek, not exceeding one page, should give a concise presentation of the main points of the paper. For non Greek-speaking authors the Editorial Board will provide the appropriate Greek title and summary.
Literature cited: References should be listed in alphabetical order of the name of the first author. The listed references should be numbered and cited in the text with the corresponding numbers in brackets. They should be set out (according to ISO 690, 1987) in the style shown below.

Units
ISO 31 units should be used e.g. mg, km, mm, cm, m, l (liter), s (second). Units should be preferably explicit, e.g. 1 g/l rather than 0.1% w/v.

Tables
Each table should be self explanatory and typed on a separate page following Literature cited.

Figures
Legends for figures should be self explanatory. They are grouped together and typed on a separate page following the table pages.
Photographs should be black and white, well-contrasted, high resolution images. Colour images may be accepted in certain cases providing they are of good quality and of best resolution for printing. Do not place photographs in the text, submit them as independent files in an appropriate format (usually in separate JPG or TIFF files).
Drawings and graphs must be in black and white and may be submitted either in the form of images of an appropriate size and resolution for printing (similar to photographs) or as independent XLS files prepared using MS Excel.

Reference styles for Literature cited

A. Monographs

1. **Katsoyannos, P. 1996.** *Integrated Insect Pest Management for Citrus in Northern Mediterranean Countries.* Kifissia - Athens: Benaki Phytopathological Institute, 110 p.
2. **Velissariou, D. 1993.** *Ozone Sensibility of Important Crop Plants around Athens, Greece.* Newcastle upon Tyne (UK) : University of Newcastle, 100 p. PhD Thesis.

B. Articles in serials or monographs

1. **Chitzanidis, A. 1988.** *Penicillium digitatum* Sacc. In SMITH, IM. *et al.* (eds). *European Handbook of Plant Diseases.* Oxford: Blackwell Scientific Publications, p. 251-252.
2. **Giannopolitis, C.N. and Vassiliou, G. 1989.** Propanil tolerance in *Echinochloa crus-galli* (L.) Beauv. *Tropical Pest Management,* **35:** 6-7.
3. **Georgiou, P.P., Liapis, K.S., Miliadis, G.E. and Siskos, P.A. 2004.** Solid phase extraction cleanup of tomato samples for the determination of pesticide residues by Gas Chromatography-Electron Capture Detection. In *Proceedings of the 3rd European Conference of Pesticides and Related Organic Micropollutants in the Environment*, Halkidiki, Greece, 7-10 Oct. 2004, p. 276-280.

Hellenic Plant Protection Journal
Volume 3, 2010

Contents

Hellenic Plant Protection Journal
Τόμος 3, 2010

Περιεχόμενα

Τόμος 3, Τεύχος 2, Ιούλιος 2010
ISSN 1791-3691

Περιεχόμενα

Volume 3, Issue 2, July 2010
ISSN 1791-3691

Contents

Hellenic Plant Protection Journal

www.bpi.gr