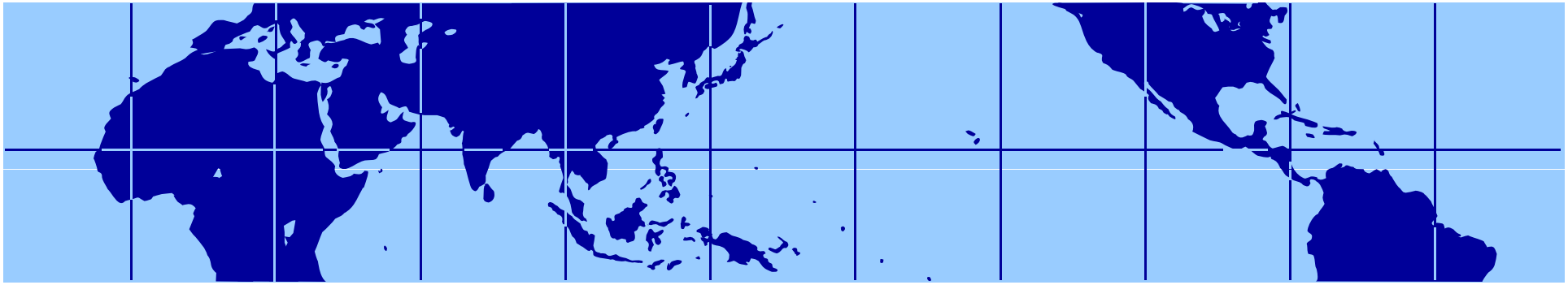
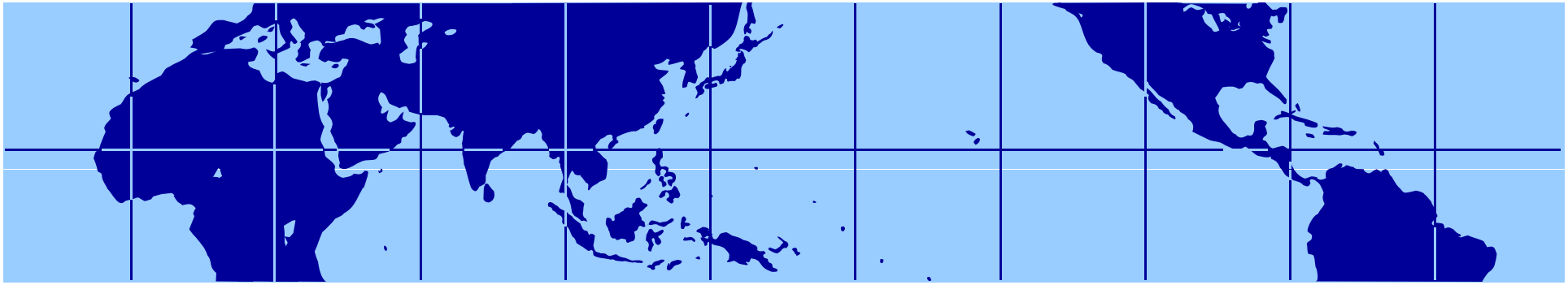


Refined Methodology for the Determination of Imidacloprid and its Metabolites in Honey Bees and Bee Products by Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS)

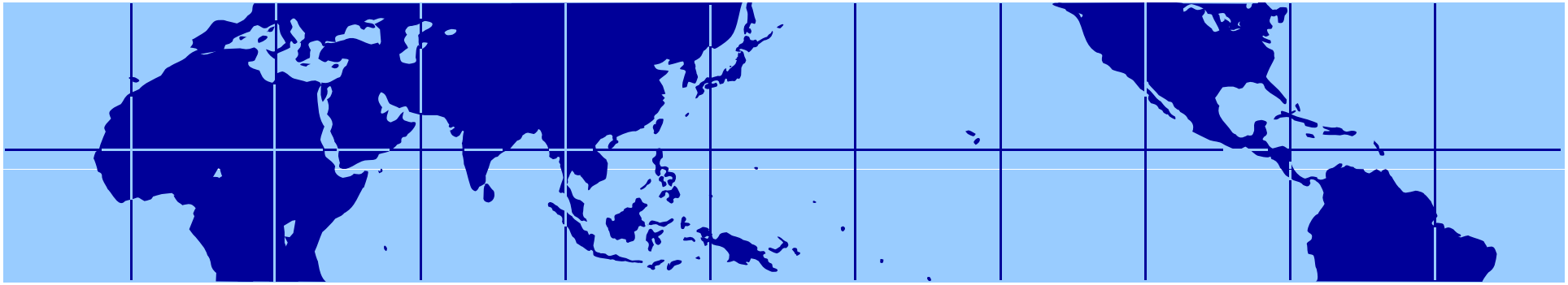
Alaa Kamel

Analytical Chemistry Branch,
Biological and Economic Analysis Division,
Office of Pesticide Programs,
Environmental Protection Agency



Colony Collapse Disorder (CCD)

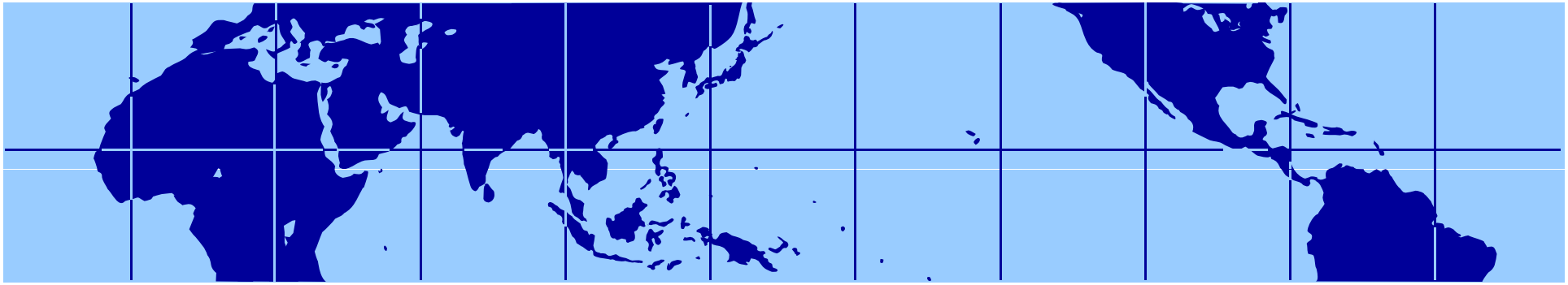
- **Is the sudden decline in adult honeybee populations without evidence of mortality.**
- **Pesticide use has been identified as a potential contributing factor to these declines among other factors such as new and re-emerging pathogens, habitat loss, pests, and nutritional stress.**
- **Researchers suspect that the declines likely result from a combination of factors representing environmental stressors that compromise the immune system of bees and make them more susceptible to disease.**



EPA-Office of Pesticide Programs (OPP) Pollinator Protection Strategic Plan

http://www.epa.gov/pesticides/ecosystem/pollinator-protection.html#plan

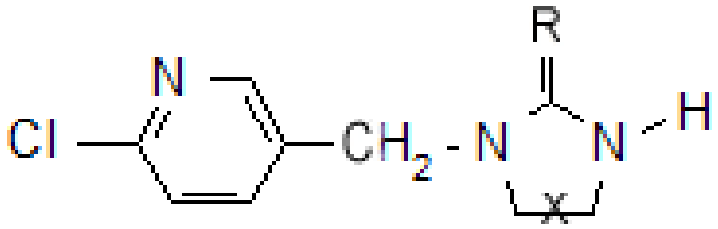
- **In 2008, OPP established a Pollinator Protection Team to facilitate a more coordinated approach for protecting pollinators.**
- **This strategic plan addresses the vision, goals and future proposed activities for the OPP Pollinator Protection Team.**
- **The three main goals for guiding the EPA's work and direction in protecting pollinators in the years ahead:**
  - **Advancing the Agency's scientific knowledge and assessment of potential pesticide risks to pollinator;**
  - **Improving risk management tools for mitigating potential risks to pollinators; and,**
  - **Increasing and broadening EPA's collaboration and communication with governmental and non-governmental organizations and the public in addressing pollinator issues.**



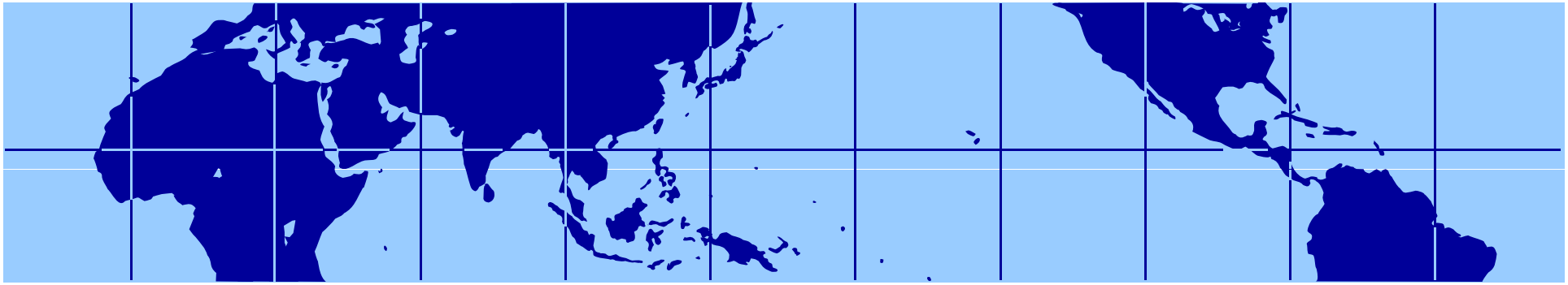
Neonicotinoid insecticides

- Neonicotinoids containing the nitroguanidine moiety such as imidacloprid, dinotefuran, thiamethoxam and clothianidin have very selective toxicity to insects.
- Imidacloprid is widely used on cucurbits such as cantaloupe, cucumber, and watermelon crops and other crops pollinated by bees.
- Imidacloprid is a systemic insecticide which metabolizes, more or less completely according to plant species and time, into several different metabolites, all of which contained elements of the 6-chloropyridine group

Imidacloprid & its metabolites



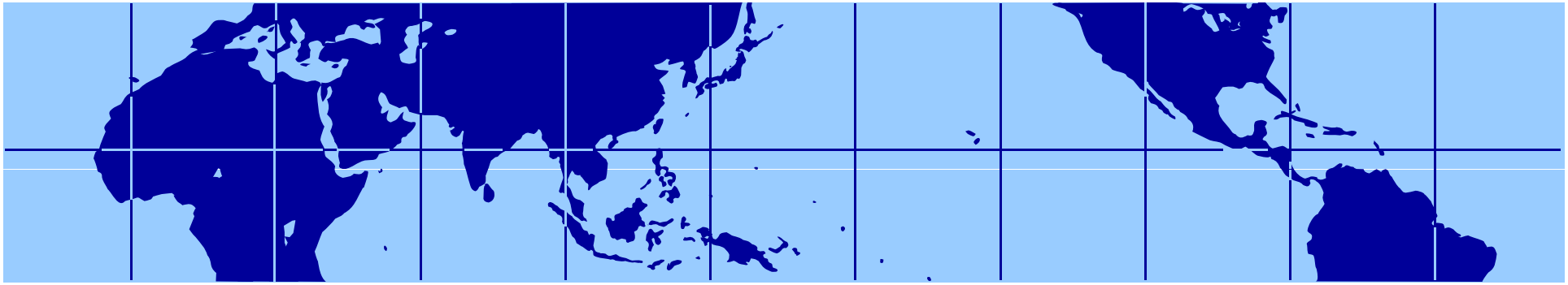
Imidacloprid:	**R = N-NO2 , C-X-C = -CH2-CH2-**
Imidacloprid olefin:	**R = N-NO2 , C-X-C = -CH=CH-**
Imidacloprid, 5-hydroxy:	**R = N-NO2 , C-X-C = -CH2-CHOH-**
Imidacloprid urea:	**R = O , C-X-C = -CH2-CH2-**
Imidacloprid, desnitro olefin:	**R = NH , C-X-C = -CH=CH-**
Imidacloprid, desnitro HCl:	**R = NH , C-X-C = -CH2-CH2- , HCl**



Acute toxicities of imidacloprid & its metabolites

- Acute LD_{50} of imidacloprid to bees range between 5-200 ng/bee from colony to colony.
- This difference can be due to a variation in detoxification capacity in honeybee colonies.
- Acute LD_{50} of the 5-hydroxy is similar to imidacloprid, while the acute toxicity of the olefin is higher than imidacloprid.
- Acute LD_{50} of the urea is almost half of imidacloprid, while the desnitro metabolites & 6-chloronicotinic acid acute LD_{50}s are >1000 ng/bee
- Metabolites of significant acute toxicity are: olefin, 5-hydroxy & urea (to some extent).

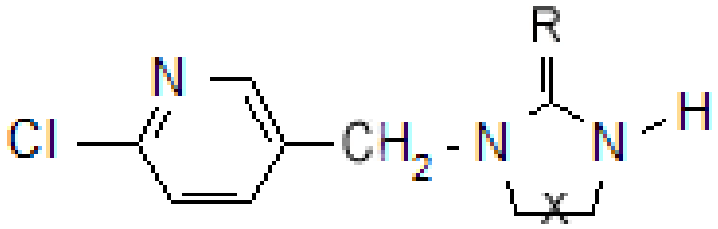
Suchail et al *Environ. Toxicol. Chem.* 20, 2001



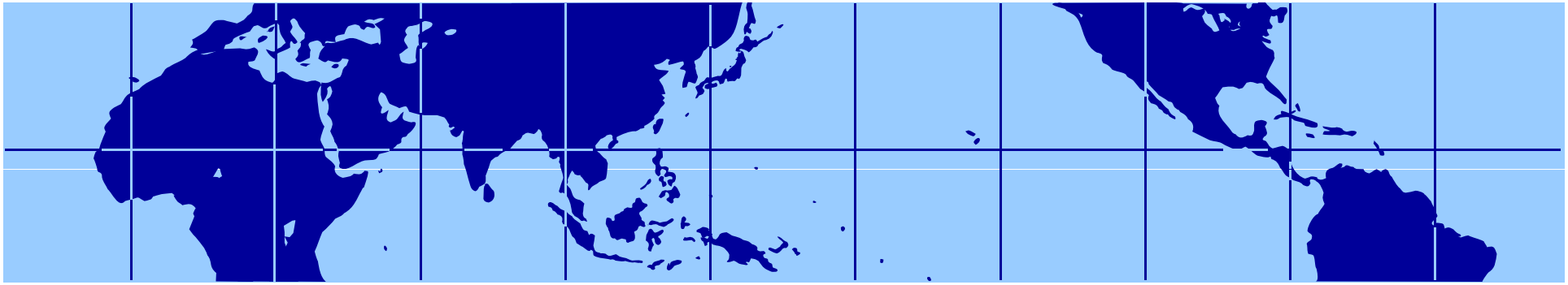
Chronic toxicities of imidacloprid & its metabolites

- In chronic studies, 50% of mortality is obtained after 8 d of exposure to imidacloprid or any of its metabolites during which each bee ingested as low as 0.1 ng/bee/day of the toxic substance.
- The high toxicity of imidacloprid and its metabolites, at very low doses, to honeybees could reflect the existence of binding sites with different affinities and would be more specific to the 2-chloropyridinyl moeity.
- The discrepancies between acute & chronic toxicities suggest that at high doses imidacloprid and the metabolites that resemble it (olefin, 5-hydroxy & urea) fit into specific receptors different than the high affinity receptors which all compounds may act on at very low doses

Imidacloprid & its metabolites

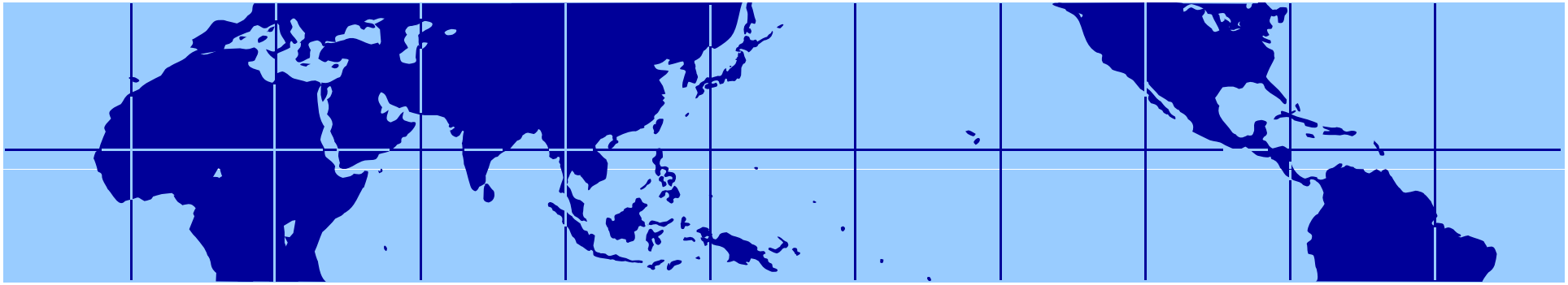


Imidacloprid	**R = N-NO2 , C-X-C = -CH2-CH2-**
Imidacloprid olefin	**R = N-NO2 , C-X-C = -CH=CH-**
Imidacloprid, 5-hydroxy	**R = N-NO2 , C-X-C = -CH2-CHOH-**
Imidacloprid urea	**R = O , C-X-C = -CH2-CH2-**
Imidacloprid, desnitro olefin	**R = NH , C-X-C = -CH=CH-**
Imidacloprid, desnitro HCl	**R = NH , C-X-C = -CH2-CH2- , HCl**



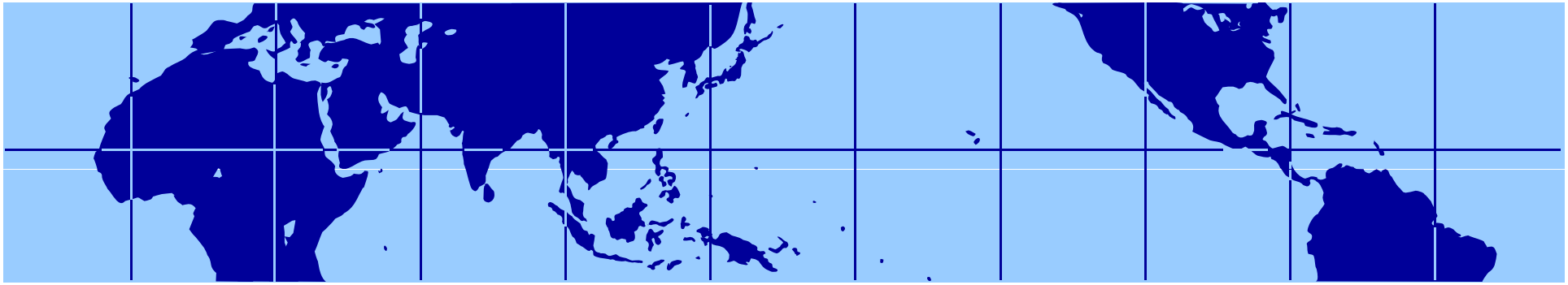
International actions involving pesticides and pollinators

- **France - Sunflower and corn seed treatments of the active ingredient imidacloprid are suspended in France; other imidacloprid seed treatments, such as for sugar beets and cereals, are allowed as foliar uses.**
- **Germany - The use of a number of seed treatment pesticides was temporarily suspended. Suspension was lifted with the exception of clothianidin, which remains suspended as a seed treatment for corn.**
- **Italy - Imidacloprid and other neonicotinoid seed treatment uses were suspended temporarily, but foliar uses are allowed.**
- **Slovenia - Neonicotinoid seed treatments for maize and oil seed rape (canola) were temporarily suspended. Suspension was lifted in August 2008.**



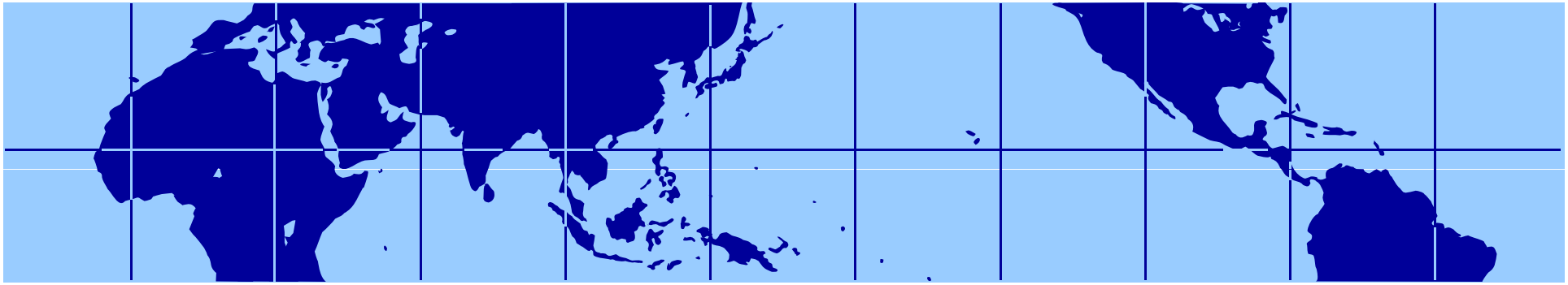
Objectives

- Help achieve OPP's goals of the strategic plan by developing more comprehensive testing protocols for evaluating potential sub-lethal and chronic effects of pesticides to brood and adult honey bees under both laboratory and field conditions.
- Develop an analytical method capable of recovering imidacloprid and all of its metabolites.
- Recovery percentages between 70-120%.
- Reach very low limits of detection <1 ppb
- Validate method on bees, pollen and honey (or nectar).



Analytical Methodologies used to detect Imidacloprid & metabolites in Honey Bees

- Methods include:
  - Liquid-liquid partitioning
  - Solid phase extraction
  - Dispersive solid phase extraction (QuEChERS)
- Current methodologies yield poor recoveries for the imidacloprid desnitro metabolites and 6-chloronicotinic acid.



Refined Methodology

3g bees, pollen or honey

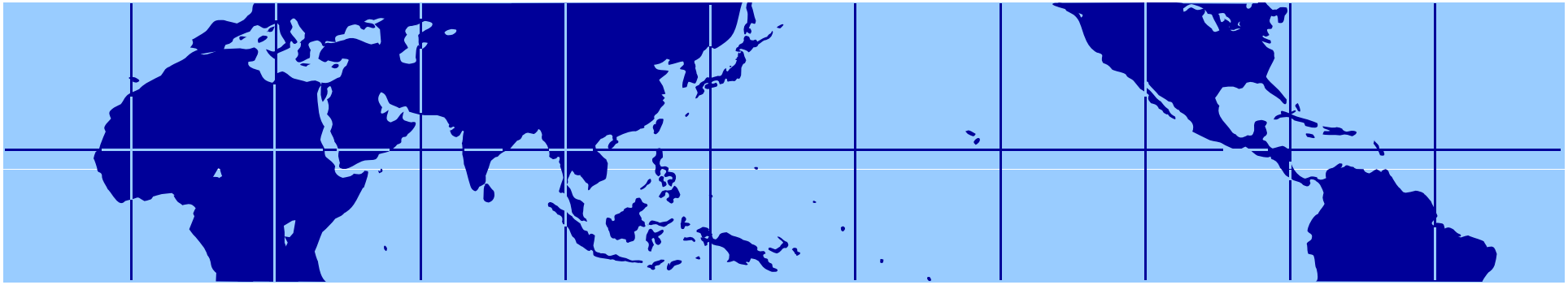
↓ **extract with 2% TEA/ACN/H_2O**

Shaking, salting out (NaCl, NaOAc, $MgSO_4$), centrifugation

↓ **pass through C-18**

Concentrate

Waters Xevo LC-MS/MS



Comparison between the recovery percent of imidacloprid & metabolites using different methods

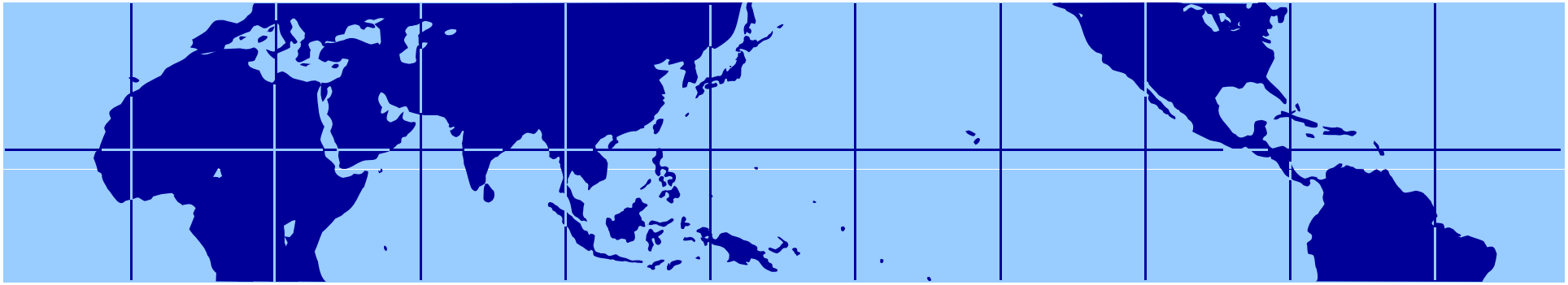
	Extraction with acetone	Liquid/liquid ext.	Dispersive SPE	Refined
	no cleanup [1]	SPE cleanup [2]	QuEChERS [3]	Method
Analyte	Recovery %			
imidacloprid	77-87	98.6 ± 4.1	100 ± 4.6	102.2 ± 5.5
imidacloprid olefin	80-90	78.9 ± 11.9	92.7 ± 11.7	76.6 ± 36.9
5-hydroxy imidacloprid	65-71	85.2 ± 6.3	93 ± 8.2	96.0 ± 25.7
imidacloprid urea	-	-	88.3 ± 8.6	107.6 ± 6.0
imidacloprid desnitro olefin	-	-	24.7 ± 6.1	97.7 ± 13.8
imidacloprid desnitro HCl	-	-	13.7 ± 1.5	58.7 ± 4.9
6-chloronicotinic acid	-	63 ± 11 [4]	ND	73.7 ± 2.3

1 Ranccan et al Analytica Chimica Acta 555 (2006) 20–24

2 Suchail et al Pest Manag Sci 60:291–296, 2003

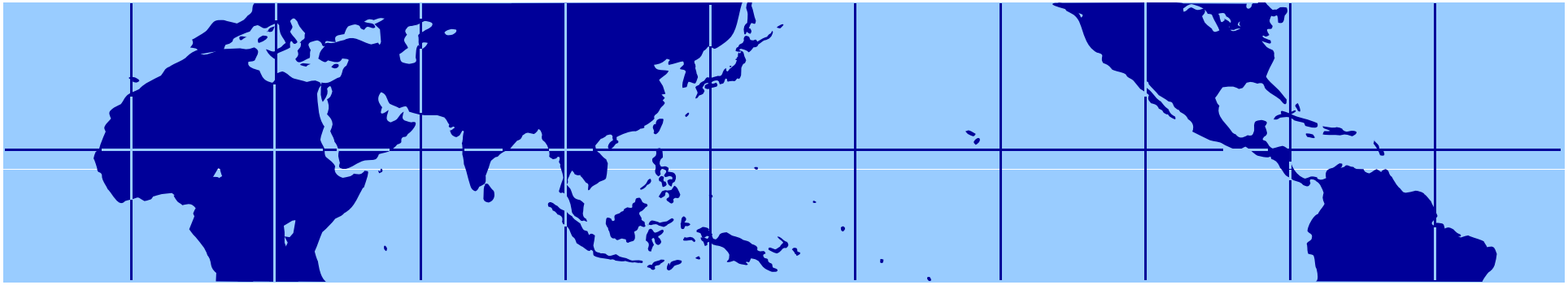
3 AOAC method 2007.1

4 S. Totti et al. / Talanta 69 (2006) 724–729



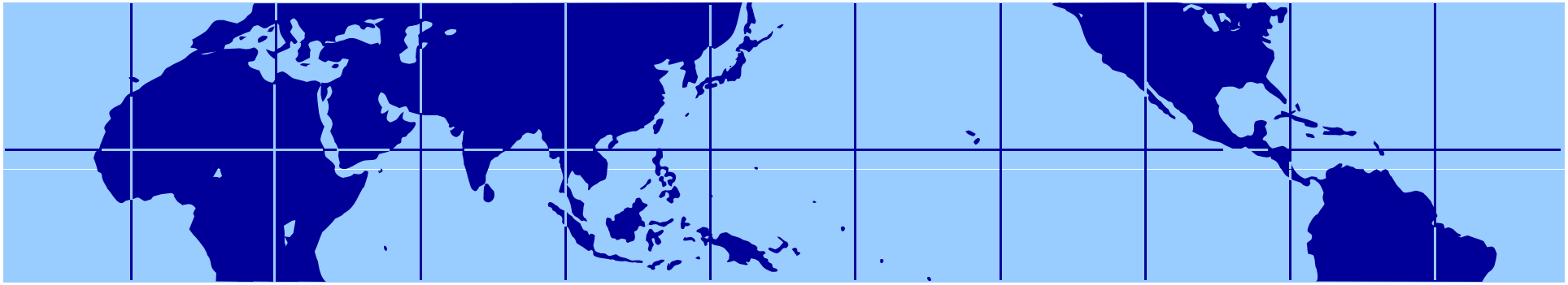
Average recovery percent and standard deviation of imidacloprid & metabolites in bee products fortified in triplicate at LOQ, 5x LOQ & 10x LOQ

Analyte/matrix	bees	pollen	honey
imidacloprid	102.2 ± 5.5	71.7 ± 7.6	74.5 ± 15.6
imidacloprid olefin	76.6 ± 36.9	96.3 ± 22.9	86.5 ± 9.4
5-hydroxy imidacloprid	96.0 ± 25.7	93.1 ± 4.1	99.4 ± 24.0
imidacloprid urea	107.6 ± 6.0	88.4 ± 3.3	90.4 ± 6.1
imidacloprid desnitro olefin	97.7 ± 13.8	70.0 ± 2.8	102.9 ± 9.2
imidacloprid desnitro HCl	58.7 ± 4.9	32.9 ± 7.5	40.8 ± 6.5
6-chloronicotinic acid	73.7 ± 2.3	72.4 ± 6.7	68.3 ± 7.2



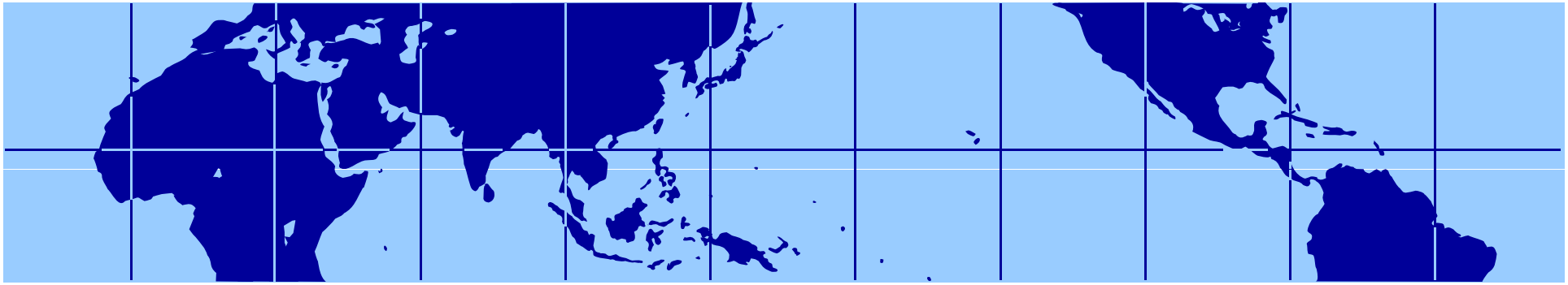
Comparison between limits of detection using the Waters Xevo MS/MS and Waters Quattro Premier MS/MS

Analyte	Quattro Premier LOD (ng/g)	Xevo LOD (ng/g)
imidacloprid	1	0.2
imidacloprid olefin	10	0.8
5-hydroxy imidacloprid	10	1
imidacloprid urea	0.5	0.2
imidacloprid desnitro olefin	0.75	0.2
imidacloprid desnitro HCl	0.75	0.2
6-chloronicotinic acid	25	8



Application of developed method in chronic toxicity studies

- The method will be used for the determination of imidacloprid & its metabolites in samples from a study of the behavior of honey bees treated with low imidacloprid concentrations in their diet.
- The study which is funded by a USDA grant to the Department of Entomology, University of Maryland, will shed more light on the effect of low doses of imidacloprid on honey bees.
- The method could be expanded to include more neonicotinoids and other pesticide classes.




The Pollination Equation
plants & pollinators = pollinators & plants
female flower
male flower
+
=
Some other pollinator friends:
hummingbirds
beetles
flies
butterflies and moths