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Detection and Quantification of Organophosphate Pesticides in Human Serum

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DETECTION AND QUANTIFICATION ORGANOPHOSPHATE PESTICIDES IN HUMAN SERUM

by

PETER KUKLENYIK

Under the Direction of Dr. Gabor Patonay

ABSTRACT

The United States Environmental Agency permits the use of 39 organophosphate pesticides. Many of these pesticides are acutely toxic and have lasting effect on human health. Organophosphates quickly metabolize in the body, therefore currently human exposure is studied by measuring the metabolic products in urine.

In this work a suite of analytical methods was developed to determine the presence of un-metabolized organophosphate pesticides in human serum. First mass spectroscopic detection methods were evaluated. Gas chromatograph coupled tandem mass spectrometer was used to compare the detection limits using chemical and electron impact ionization. Positive chemical ionization was selected, because it provided better detection limits for this set of analytes. Liquid chromatograph coupled tandem mass spectrometry was also evaluated and was found advantageous over the gas chromatographic method for approximately 50% of the compounds. Positive atmospheric pressure chemical ionization was chosen for this group of compounds. Once the analytes were separated by detection methods, analytical separation methods were

compared: column and eluent was selected for liquid chromatography, column alone was selected for gas chromatography.

Last step of the method development was to produce a suitable sample cleanup process. Solid phase extraction was not suitable because the very wide range of solubility characteristics and hydrolytic stability of the analytes. Lyophilization, liquid-liquid extraction methods were tested and compared. A multi step cleanup method was produced, which starts with liquid-liquid extraction using high pressure ethyl acetate in accelerated solvent extractor, solvent exchange and a lipid removal step. The concentrated extract then injected in a HPLC-MS-MS system then the same extract either directly injected in GC-MS-MS or further purified using headspace solid phase micro extraction before the GC-NS-MS step.

The method was used with good results for analyzing samples collected from farm workers using OP pesticides.

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No.	OP	Width: Length:	Betasil C18 2.1mm 100mm	Betasil C18 4.6mm 100mm	Betasil Phenyl 4.6mm MeOH	4.6mm ACN	
1	Acephate		8.1	7.94	6.15	5.62	
2	Azinphos-methyl		21.83	19.35	20.53	17.11	
3	Bensulide		24.2	21.4	21.72	19.08	
4	Cadusafos*		25.64	22.39	21.05	17.59	
5	Chlorethoxyfos		26.88	-	N/F	N/F	E/B
6	Chlorpyrifos		27.41	23.5	22.53	28.06	E/B
7	Chlorpyrifos methyl		25.86	22.57	21.67	27.61	E/B
8	Coumaphos		24.85	21.88	22.24	19.15	
9	Diazinon		24.8	21.83	21.18	17.92	
10	Dichlorvos (DDVP)		19.08	16.72	15.67	N/F	E/B
11	Dicrotophos		14.25	12.13	13.68	9.94	
12	Dimethoate		15.71	14.29	14.16	11.68	
13	Disulfoton		28.94	22.43	21.66	N/F	
14	Ethion		26.97	23.19	23.1	20.58	
15	Ethoprop		23.72	21.08	19.86	16.28	
16	Ethyl parathion		24.46	21.62	21.09	N/F	E/B
17	Fenamiphos		24.19	21.42	20.77	16.5	
18	Fenitrothion		23.36	20.76	20.64	N/F	E/B
19	Fenthion		24.83	21.9	21.82	N/F	E/B
20	Fonofos**		24.91	21.98	21.25	18.85	
21	Malathion		22.97	20.41	20.57	17.89	
22	Methamidophos		4.08	6.43	3.67	3.47	
23	Methidathion		21.66	19.18	19.75	16.86	
24	Methyl parathion		22.46	19.98	19.66	N/F	E/B
25	Mevinphos*		16.97	15.14	15.18	11.72	
26	Naled		21.52	18.96	18.47	N/F	
27	Oxydemeton methyl		12.79	10.67	12.34	10.22	split
28	Phorate		25.4	22.27	21.29	N/F	E/B
29	Phosalone*		25.26	22.17	22.12	N/F	E/B
30	Phosmet		29.03	N/F	N/F	N/F	E/B
31	Phostebupirim		26.79	23.13	22.04	19.27	
32	Pirimiphos methyl		24.72	21.71	20.69	17.56	
33	Profenofos		26.4	22.86	22	N/F	E/B
34	Propetamphos		23.21	20.64	19.87	17.68	
35	Sulfotepp		24.56	21.67	21.31	19.11	
36	Temephos		26.73	22.98	24.04	N/F	E/B
37	Terbufos		26.75	23.09	22.15	N/F	
38	Tetrachlorvinphos		24.28	21.51	21.03	17.53	
39	Tribufos (DEF)		28.58	24.31	23.17	20.08	
40	Trichlorfon		19.09	16.73	15.64	N/F	E/B

No.	OP	Width: / Length:	Aquasil C18 4.6mm 100 mm		Chromolith RP 18e 4.6mm 100 mm		Zorbax SBC3 4.6mm 100 mm	
1	Acephate		8.35		7.33		7.53	poor peakshape
2	Azinphos-methyl		20.12		18.42		19.43	
3	Bensulide		21.56		21.19		21.19	
4	Cadusafos*		22.76		22.51		22.19	
5	Chlorethoxyfos		20.1	Poor peak	N/F		N/F	
6	Chlorpyrifos		23.63		23.79		22.65	
7	Chlorpyrifos methyl		22.77	Poor peak	22.61		21.63	
8	Coumaphos		22.57		21.8		22.29	
9	Diazinon		22.32		21.67		21.75	
10	Dichlorvos (DDVP)		16.77		15.79		16.24	
11	Dicrotophos		13.8		11.73		14.13	
12	Dimethoate		14.12		12.42		14	
13	Disulfoton		22.47	Bad tailing	22.39	Smearing	21.4	
14	Ethion		23.55		23.59		22.58	
15	Ethoprop		21.45		20.39		20.63	
16	Ethyl parathion		21.86		21.25		20.97	
17	Fenamiphos		21.79		21.05		20.93	
18	Fenitrothion		21.04		19.91		20.15	
19	Fenthion		22.23		21.66		21.01	
20	Fonofos**		22.1		21.65		20.92	
21	Malathion		20.45		19.52		20.01	
22	Methamidophos		6.84		4.24	Excessive Peak broadening	4.03	
23	Methidathion		19.26		18.08		18.86	
24	Methyl parathion		20.06		18.88		19.21	
25	Mevinphos*		15.26		13.96		15.91	
26	Naled		*		18.06		N/F	
27	Oxydemeton methyl		12.79		10.39		12.38	
28	Phorate		22.35		22.14		21.13	
29	Phosalone*		22.41		22.17		21.64	
30	Phosmet		N/F		N/F		N/F	
31	Phostebupirim		23.26		23.38		22.48	
32	Pirimiphos methyl		21.87		20.85		20.77	
33	Profenofos		23.31		23.14		22.29	
34	Propetamphos		20.64		19.79		20.07	
35	Sulfotepp		21.71		21.43		20.86	
36	Temephos		23.47		23.55		22.78	
37	Terbufos		23.17		23.33		22.23	
38	Tetrachlorvinphos		21.88		21.15		20.9	
39	Tribufos (DEF)		24.31		24.51		23.6	
40	Trichlorfon		15.27		14.17		15.88	

Table 16 Retenetion times of OP pesticides on Aquasil C18 and Chromolit RP 18e, Zorbax

			Discovery HS F5 3µm		Hypercarb		Varian C18	
		Width:	4.6mm		4.6mm		4.6mm	
No.	OP	Length:	100 mm		100 mm		250mm	
1	Acephate		7.61		3.22		12.74	
2	Azinphos-methyl		20.61		N/F	E/B	23.99	
3	Bensulide		22.21		22.62	tailing	24.65	
4	Cadusafos*		21.7		18.73		26.17	
5	Chlorethoxyfos		N/F		N/F	E/B	N/F	
6	Chlorpyrifos		22.85		N/F	E/B	27.53	
7	Chlorpyrifos methyl		22.12		N/F	E/B	N/F	E/B
8	Coumaphos		23.83		N/F	E/B	25.62	
9	Diazinon		22.4		21.05		25.77	
10	Dichlorvos (DDVP)		16.8		N/F	E/B	21.64	
11	Dicrotophos		13.39		9.33		16.69	
12	Dimethoate		14.86		10.46		18.43	
13	Disulfoton		N/F		N/F		26.21	
14	Ethion		23.31		25.26	tailing	26.8	
15	Ethoprop		19.98		15.63		24.98	
16	Ethyl parathion		22.75		N/F	E/B	25.28	
17	Fenamiphos		20.97		N/F	E/B	24.88	
18	Fenitrothion		22.1		N/F	E/B	24.7	
19	Fenthion		21.99		N/F	E/B	25.77	
20	Fonofos**		22.02		8.23		25.92	
21	Malathion		21.27		18.7		24.23	
22	Methamidophos		4.28		1.43		12	
23	Methidathion		20.4		22.03		23.74	
24	Methyl parathion		21.57		N/F	E/B	24.13	
25	Mevinphos*		15.59		N/F	E/B	19.41	
26	Naled		18.55		N/F		N/F	
27	Oxydemeton methyl		11.57		8.23		15.35	
28	Phorate		22.02	tailing	N/F		26.9	
29	Phosalone*		22.57		N/F		25.66	
30	Phosmet		N/F		N/F	E/B	N/F	
31	Phostebupirim		22.73		20.45		27	
32	Pirimiphos methyl		26.26		N/F	E/B	25.89	
33	Profenofos		22.25		N/F	E/B	26.72	
34	Propetamphos		20.6		17.76		24.26	
35	Sulfotepp		21.92		17.32		25.21	
36	Temephos		23.59		N/F	E/B	26.44	
37	Terbufos		22.96		N/F		26.87	
38	Tetrachlorvinphos		21.19		N/F	E/B	25.09	
39	Tribufos (DEF)		23.01		N/F		28.5	
40	Trichlorfon		16.82		N/F	E/B	19.38	

Table 17 Retenetion times of OP pesticides on Discovery HS F5, Hyper carb and Varian C18 columns 96

LIST OF ACRONYMS

AChE	Acetyl Cholinesterase Enzyme
APCI	Atmospheric Pressure Chemical Ionization
CI	Chemical Ionization
DEDTP	O,O-Diethyl Dithiophosphate
DEP	Diethyl Phosphate
DETP	O,O-Diethyl Dithiophosphate
DMDTP	O,O-Dimethyl Dithiophosphate
DMP	Dimethyl Phosphate
DMTP	O,O-Dimethyl Thiophosphate
EI	Electron Impact Ionization
EPA	Environmental Protection Agency
GC	Gas Chromatography
HPLC	High Pressure Liquid Chromatography
LC	Liquid Chromatography
LOD	Limit of Detection
OP	Organophosphate Pesticide
OPICN	Organophosphorus Ester-Induced Chronic Neurotoxicity
OPIDP	Organophosphate Induced Delayed Polyneurophaty

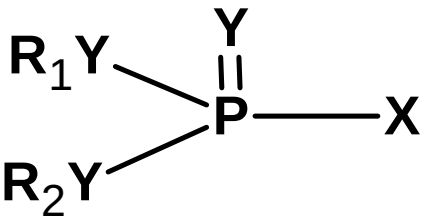
CHAPTER 1: INTRODUCTION AND GOALS

Organophosphates (OP) are a very effective and widely used class of pesticides.

Presently there are 39 organophosphate type pesticides registered with the Environmental Protection Agency (EPA) [1]

OP pesticides became popular after the persistent organochlorine pesticides were banned in the 1970s. Their wide availability relatively low price, short half life in the environment and low susceptibility to pest resistance made them an attractive alternative.

The total insecticide use in the United States has been declining: it has dropped approximately 45% from 1980 to 2001, according to the latest available statistics from the EPA. The same time the share of OP pesticides went up from 58 t 70% of the total use.



R: Methyl or Ethyl

Y: O or S

X: Specific Organic Group

Figure 1 General Structure of Organophosphate Pesticides

Using the above general structure, the organophosphate pesticides of interest can be grouped as seen in **Error! Reference source not found.**.

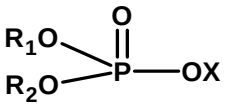
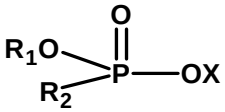
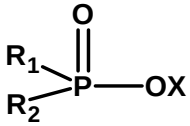
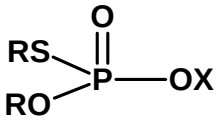
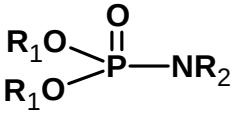
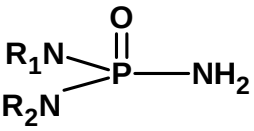
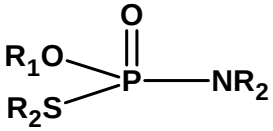
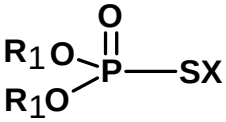
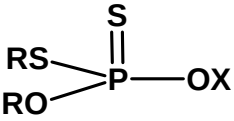
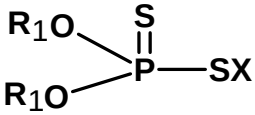
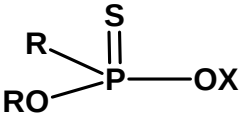
Type of Phosphorous Group	General Structure	Pesticide
Phosphate		Dichlorvos, Chlorfenvinphos, Heptenophos
Phosphonate		Trichlorfon
Phosphinate		Glufosinate (herbicide, not in the 40 compounds of interest)
S-alkyl phosphorothioate		
Phosphoramidate		
Phosphorotriamidate		
Phosphorothioamidate		
O-alkyl phoshorothioate		Omethoate
S-alkyl phosphorodithioate		
Phosphorodithioate		
Phosphonothioate		

Table 1 Groups of Organophosphates R, R1, R2, R3 : Ethyl, methyl. X: Specific Group

Toxicity

Inhibition of Acetyl Cholinesterase: Acute Cholinergic Syndrome

The primary reason for OP toxicity is its inhibition of the acetyl cholinesterase (AChE) enzyme:

Acetyl cholinesterase is found in many tissues but its role mostly is understood in the nervous system. It hydrolyzes the neurotransmitter acetylcholine, terminating its action. It is essentially the "off switch" of the neuroimpulse. If the AChE is inhibited it leaves the synapse in the "on" state hence the paralytic effect of the OP.

During inhibition the OP mimics the acetyl cholinesterase phosphorylation of the active site. Hydrolysis of the phosphoryl-AChE is very slow so the enzyme's catalytic potential is lost.

Inhibition of the AChE enzyme becomes irreversible when the methoxy or ethoxy groups hydrolytically removed from the absorbed portion of the organophosphate pesticide. This process is called aging Phosphorylated AChE undergoes two reactions at a very slow rate: recovery and aging. Recovery is the hydrolytic removal of the phosphate moiety from the AChE enzyme. The half life of the OP-AChE adduct depends on R1 and R2. In case of pesticides it takes place in order of hours.

The other important process is aging. Aging is the dealkylation of the OP moiety in the OP-AChE complex. Once aging takes place, the inhibited AChE can not be reactivated.

- Symptoms of acute OP poisoning are:
- Nausea, vomiting, abdominal cramps, diarrhea;
- Excessive salivation, rhinorrhea;
- Headache, vertigo;

- Fixed pinpoint pupils, blurred vision ocular pain;
- Muscle twitches, especially of face, tongue and neck;
- Difficulty in breathing, primarily due to excessive secretion and bronchoconstriction
- Random jerky movements, convulsions;
- Respiratory paralysis, death.

Depending on the dose, type/toxicity of the OP pesticide and quality of health facilities, the reported mortality rates are between 4 % and 29 %.

Recovery of inhibited AChE in the nervous system is about 1 week in experimental animals and it is believed to be the same in people. In untreated patients it takes 5-7 weeks for the AChE activity to return to normal.

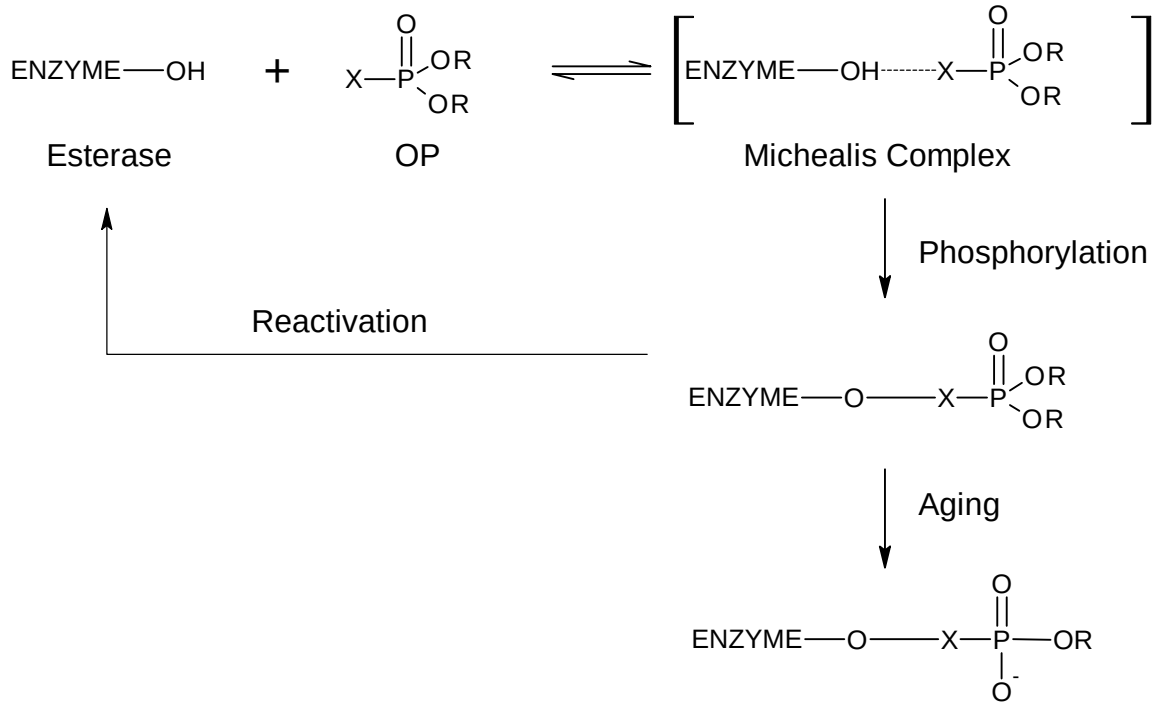


Figure 2 Esterase Inhibition by Organophosphate R: Ethyl or methyl, X: Specific group

Intermediate Syndrome

The intermediate syndrome is a set of symptoms that may develop in patients after recovery from acute OP pesticide poisoning. It too is caused by esterase inhibition and manifests itself through muscle weakness tremors and respiratory distress. Since it develops after the acute cholinergic syndrome but before the "OP induced delayed neuropathy, it was coined as the "Intermediate syndrome"

Organophosphate Induced Delayed Polyneurophaty (OPIDP)

OPIDP is a set of symptoms caused by OP poisoning but with an onset of 1-3 weeks after the initial acute poisoning. Its manifestations are: muscle weakness, cramps in the arms and legs. Recovery is slow and usually incomplete.

The cause of OPIDP is the OP induced inhibition of the neuropathy target esterase (NTE)

Organophosphorus Ester-Induced Chronic Neurotoxicity (OPICN) [2]

OPICN is produced by acute or multiple subclinical doses of organophosphorus compounds. Clinical signs, ranging from weeks to years after exposure, consist of neurological and neurobehavioral abnormalities. These abnormalities continue for a prolonged time, and are caused by damage to the central and peripheral nervous system.

Most examples in the literature are observed after acute OP poisoning. The OP exposure causes lesions on the brain which results in neurological symptoms: increased incidence of depression, irritability, confusion, and social isolation. Also there is observable decreased verbal attention, visual memory, motoricity, and affectivity.

There is more information on acute exposure induced OPICN, probably because the cause is well defined and the dose is quantifiable.

For our studies however, effects of repeated small doses – which do not cause acute poisoning – is more relevant.

Professional pesticide applicators and farmers exposed to organophosphate pesticides show the following symptoms: anxiety, diminished vigilance and reduced concentration. Kaplan et al.[3] observed long-term cognitive problems in concentration, word finding, and short-term memory in individuals exposed to low subclinical levels of chlorpyrifos. Sheep dippers exposed to OP pesticides experienced significant neuropsychological problems.[4, 5] Male fruit farmers who were chronically exposed to OP insecticides showed significant increase of their reaction time.[6] The same symptoms were observed in female pesticide applicators, with the addition of reduced motor steadiness, and increased muscle tension, depression, and fatigue . Workers exposed to the OP insecticide quinalphos during manufacturing, exhibited changes in central nervous system function: memory loss, impaired vigilance, slow learning and motor deficits. The AChE activity of these workers was normal..[7]

Many rescue workers and some people who did not develop any acute symptoms during the Tokyo nerve gas attack later developed chronic, persistent memory loss three years and nine months after the attack.[8, 9] Pilkington et al.[10] reported strong correlation between chronic low-level exposure to OP pesticide concentrates in sheep dips and neurological symptoms in workers

Exposure [11]

The main routes of exposure are oral, dermal or inhalation.

Oral Exposure

The main route of the exposure of the general population to OP pesticides is ingestion via food or drinking water. Regulatory controls of pesticide use is designed to keep the resulting pesticide levels insufficient to cause adverse health effects.

Ingestion of substantial amounts can occur either deliberately (suicide attempts) or accidentally as a result of mistaken identity or poor handling techniques.

Dermal Exposure

The main cause of dermal exposure is handling of OP pesticides, during manufacturing or application. Dermal penetration is varying, depending the carrier solvent: usage of solvents which can penetrate the skin – such as acetone – cause higher uptake than water based suspensions. Absorption of OP pesticidesis also dependent on the hydratation and temperature of skin.

Inhalation

Inhalation of OP pesticides is usually the result of use of sprays, mists and powders. Absorption is fast and almost complete. The pesticide enters in the circulatory system without passing through the liver. Exposure through inhalation is usually combined with exposure of eyes and mucous membranes.

Distribution

In rhesus monkeys, 40% of intravenously administered radiolabelled diazinon, was excreted in 24 hrs. (urinary excretion) This was followed by a slow and almost constant 5% / day excretion for 3 – 7 days. Dermal exposure of humans followed a similar pattern.

OP Metabolism [11]

Many tissues like skin, gut lung and kidneys contain enzymes that can metabolize Ops, the main sites are the liver and blood. Mammalian OP metabolism involves many enzymes and possible pathways. The dominant metabolic process will depend on the type of OP, the exposure route, and the dose. These are some of the possible routes, grouped by the participating enzyme:

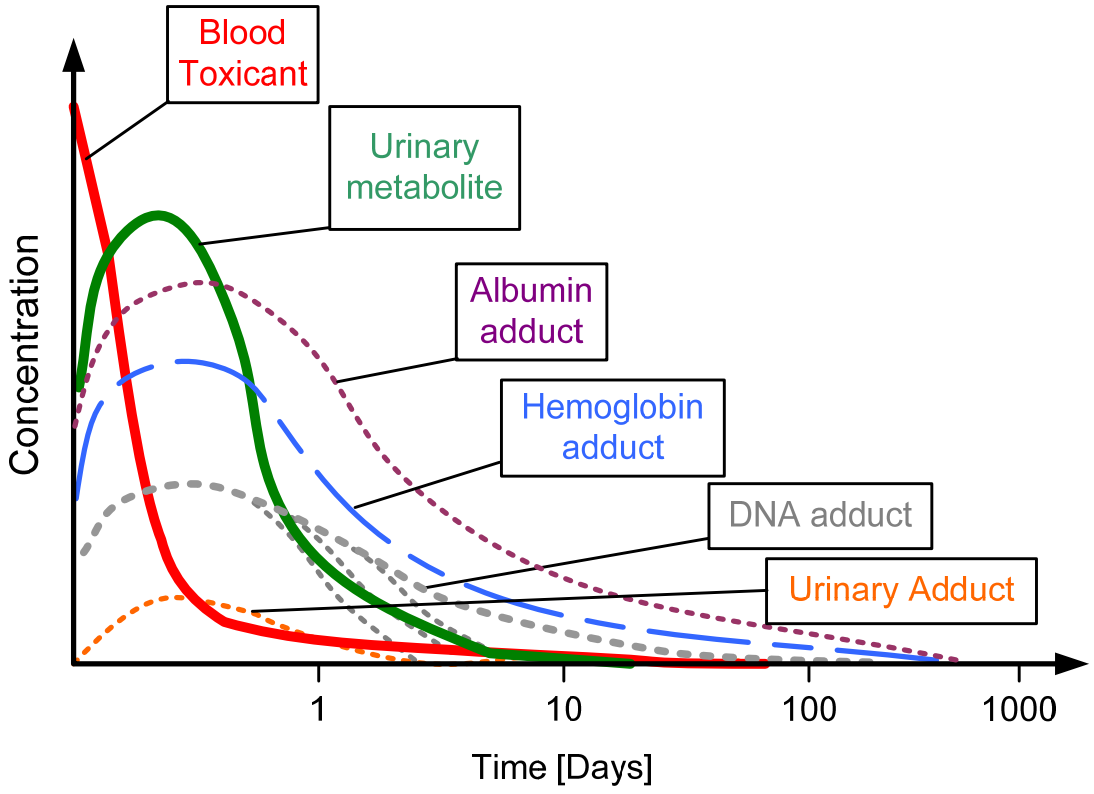
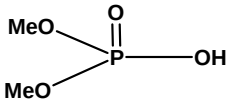
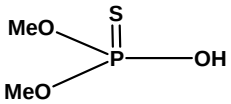
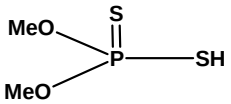
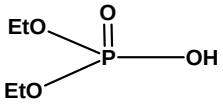
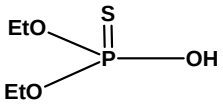
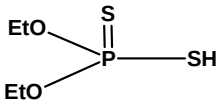


Figure 3 Non Persistent Pesticide: Concentration Change in Blood and in Urine [12]

Table 2 Non-Specific Metabolites of OP Pesticides and their Structures

Chemical Name	Abbreviation	Structure
Dimethyl phosphate	DMP	
O,O-Dimethyl Thiophosphate	DMTP	
O,O-Dimethyl Dithiophosphate	DMDTP	
Diethyl phosphate	DEP	
O,O-Dimethyl Thiophosphate	DETP	
O,O-Dimethyl Dithiophosphate	DEDTP	

A-esterases

The A- esterases are a group of enzymes which are able to hydrolyze a broad range of substrates, including OP pesticides. They are present in a number of tissues: in the liver, the

intestine and in serum. Most studied example is the hydrolysis of paraoxon to diethyl phosphate. (Figure 4) Many similar OP hydrolyze to the respective dialkyl phosphate, following a similar pattern.

The products of the hydrolysis are excreted in the urine. This hydrolytic process results in the removal of the "leaving group", which subsequently can be analyzed and used as a biomarker for that specific OP. These removed leaving groups are also known as "specific metabolites"

Parathion and other thion compounds are not substrates for A-esterases.

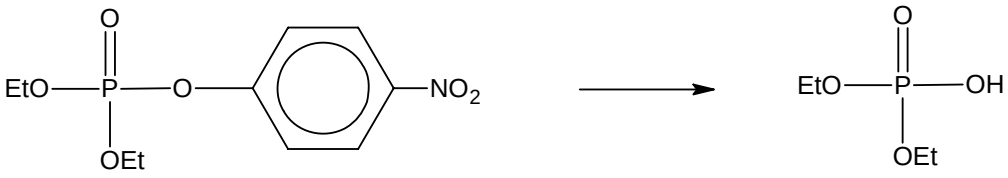


Figure 4 Paraoxon Hydrolysis to diethyl phosphate

Carboxylesterases

Carboxylesterases (CaEs) are present in mammalian plasma and in many tissues including liver, kidney, brain, intestines, muscles, etc. Their normal physiological role is the metabolism of lipids. They also contribute to the detoxification of OP pesticidesthrough two mechanisms:

1. Hydrolysis of carboxyl ester side chains.(Figure 5)
2. Irreversible binding of the OP without hydrolysis

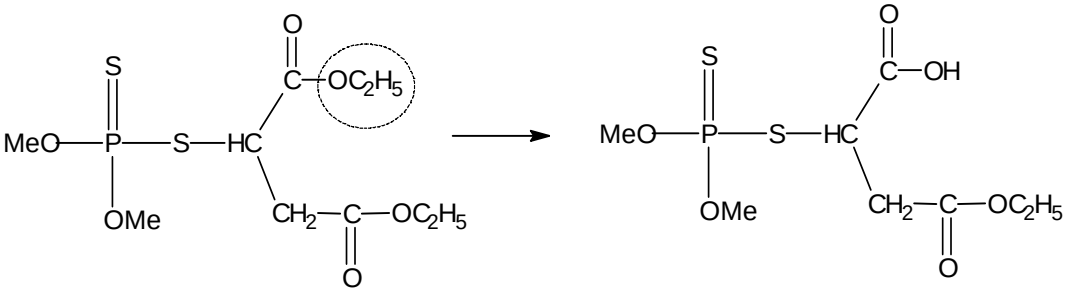


Figure 5 Hydrolysis of the Carboxyl Ester Side Chain of Malathion

Amidases

Amidases catalyse the hydrolytic cleavage of carboxylamide. (Figure 6)

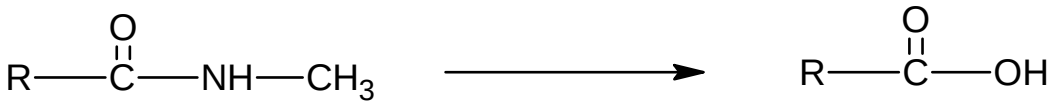


Figure 6 Amidase Cleavage of Carboxylamide R: Methyl, Ethyl

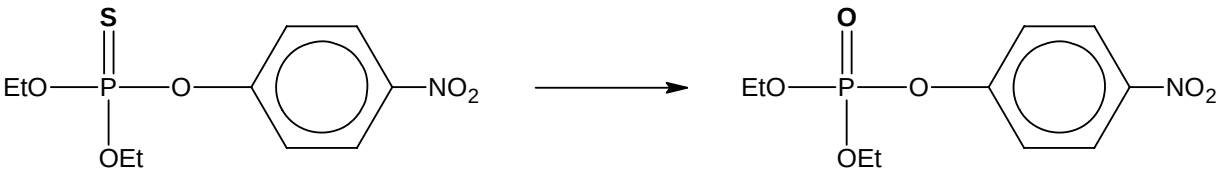


Figure 7 Oxidative Desulphuration of Parathion

Microsomal Oxigenases

Microsomal oxigenases are a family of enzymes that contain cytochrome P450 and/or flavin. These enzymes are found in most tissues but their predominant location is the liver. Microsomal oxigenases catalyze several reactions:

- Oxidative desulfuration of OP pesticidessuch as conversion of parathion to paraoxon (Figure 7). The resulting oxon is much more toxic.
- Oxidative O and N-dealkylation and dearylation
- Thioether oxidation
- Side chain oxidation

An example of OP metabolism is shown on Figure 8: Metabolism of Chlorpyrifos

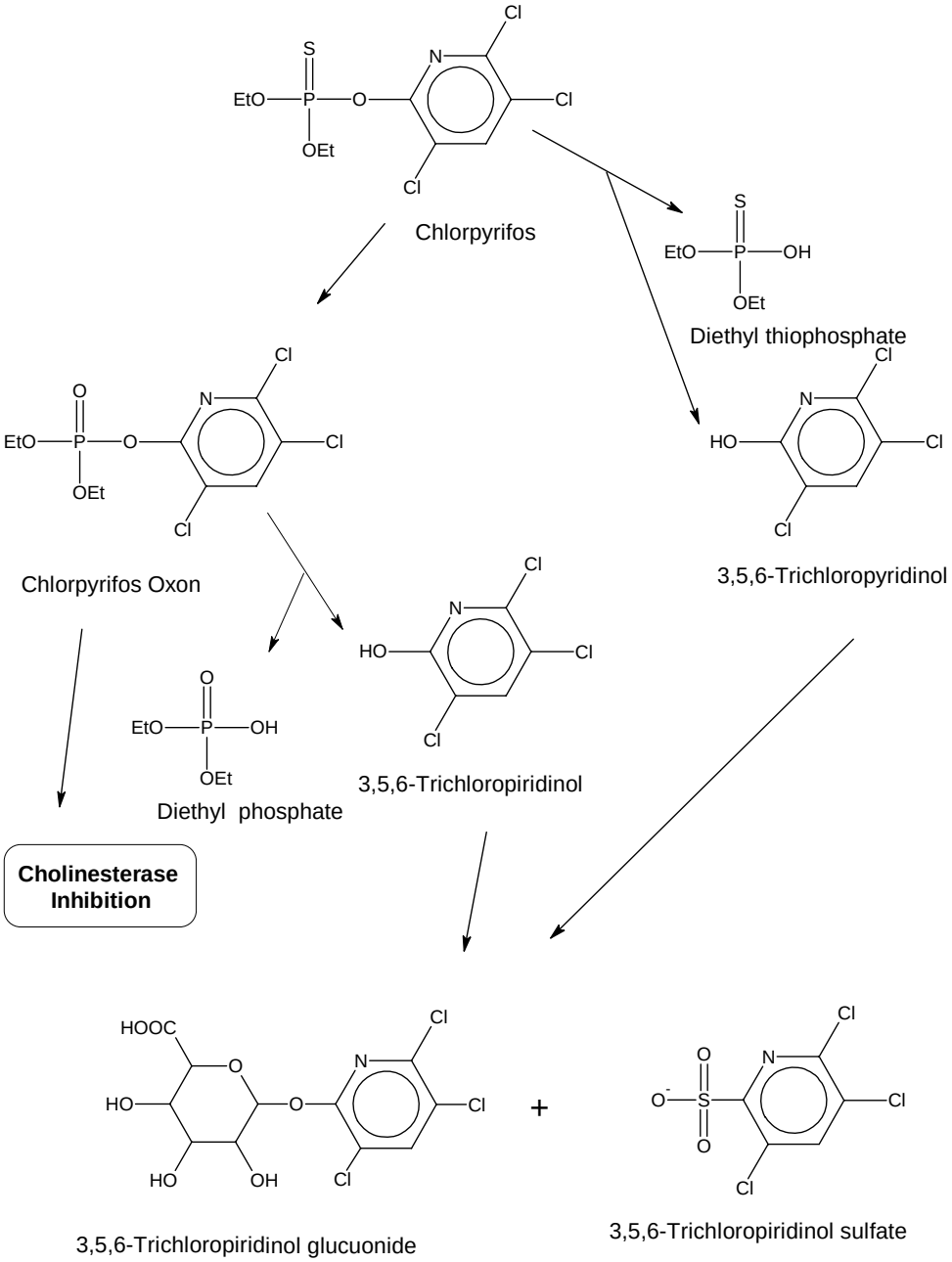


Figure 8 Metabolism of Chlorpyrifos

Determining OP pesticide exposure

Non – assay methods.

Exposure to pesticides may be estimated by using empirical mathematical formulas based on usage, time of exposure, exposed skin area and other factors. [4, 5]. Of course these methods are only estimates, not actual measurements.

Urinary Assay of OP Metabolites

Most modern pesticides and all of the organophosphates of interest are “non persistent”, which means that they quickly metabolized, the intact pesticide is only present in the bloodstream – in any measurable amount - for a few hours to a few days

Traditionally most of the studies are carried out using urine samples analyzing for OP metabolites. The two main reasons are that urine samples are much easier to obtain than blood and that the metabolite concentration – time function gives a wider window of delectability.

Examples of urinary assays of organophosphate metabolites:

Hardt Et. Al. [13]used liquid-liquid extraction followed by centrifugation and drying under vacuum. After derivatization overnight with 2,3,4,5,6 pentafluorobenzyl bromide. After a second liquid-liquid extraction the reconstituted sample was separated with gas-chromatography (GC). The detector was mass spectrometer.

Bravo et al. [14] greatly simplified the above method by first lyophilizing the urine samples then using liquid extraction with a blend of acetonitrile and ethyl ether. Just like in the previous example, the OP metabolites being highly polar compounds, had to be derivativized to make them suitable for GC separation. In this case the derivatizing agent was 1-Chloro-3-iodopropane.

Using metabolites in urine as biomarker has drawbacks however; metabolites are much less

specific. Most OP pesticidesmetabolize to one of the seven dialkyl phosphates:

Second source of error is that the OP pesticidespath of breakdown is often similar to the metabolic pathway. As a result a person may ingest a breakdown product, which is also the target compound of the assay. Nonetheless today the preferred method of determining OP pesticide exposure is the urinary assay of metabolites.

Detection of OP Pesticides in Blood/Serum/Plasma

Being able to detect the unchanged pesticide solves the above mentioned, but because the concentrations in blood drop rapidly, (Figure 3) it requires a more sensitive detector. On the other hand the unmetabolized form of these compounds is more lipophilic than the metabolite, which should make sample cleanup and separation somewhat easier.

Measuring the parent compounds in blood or blood products has other advantage; it is a regulated fluid. It means that blood volume is constant, unlike the urine volume in the human body. Therefore no dilution-correction needed when using serum, plasma or whole blood.[15]

Two review articles [16, 17] summarize the most frequently used methods in OP analysis in whole blood, plasma or serum matrix. This review also uses independent searches of "ISI web of science", focusing on assays OP compounds, blood / serum / plasma as matrix and –for the most part – mass spectrometer as a detector. The results are tabulated in Table 6 - Table 8, grouped by methods of separation

The first group of methods, listed in Table 6 list recent works based on GC.

The most recent work by Mushoff et. Al. [18] used solid phase microextraction, (SPME), GC and mass spectrometric detection with electron impact ionization and single ion monitoring to determine 22 OP pesticides. Detection limits were reported between 10 and 300 ng/mL. SPME is

a clean and simple technique the method is hampered though with low recoveries.

The lowest limits of detection was achieved by Barr and her coworkers [19] They analyzed 29 contemporary pesticides in blood, 9 of them OP-s. using GC coupled with high resolution mass spectrometer they achieved 0.5 to 2 ppt detection limits This is probably the most important and applicable method four our purposes; the LOD-s are likely low enough to study background levels of exposure.

Goals and Objectives

To develop an analytical method to measure the non metabolized form of all 39 EPA registered organophosphate pesticides in human serum as matrix.

Test various cleanup methods such as solid phase extraction, liquid-liquid extraction, lyophilization, and their automated variants for feasibility. Determine recoveries for each analyte. While high recovery is important, focus of the cleanup has to be removal of interfering contaminants.

Test HPLC and GC for analytical separation methods, optimize separation parameters.

Determine Limits of Detection, Limits of Quantitation.

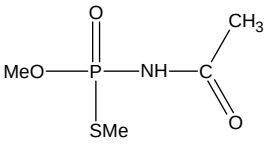
Study the robustness of the method. (Sensitivity to parameter variations.)

Study the stability of OP pesticides at different temperatures.

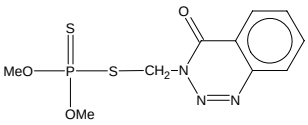
Study the stability of organophosphorus pesticides id blood and means to stabilize it.

In order to achieve these goals, the project has to be broken down to the following blocks:

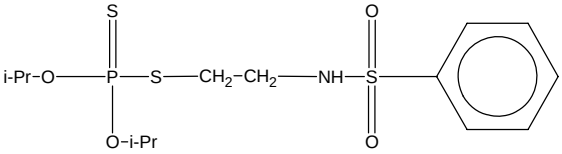
Development Step	Experimental Choices	Considerations
Cleanup	First Choice: SPE LLE	For all sample cleanup / pre-concentration steps the main considerations are: 1. Recovery 2. Purity (not to extract compounds interfering with further steps of the analysis) 3. Feasibility for automation / Sample throughput
Analytical Separation	GC	Will work for the intact OP pesticides
	HPLC	Probably preferred for the metabolites or heat labile OP-s. Interfacing with HRMS may be a problem
Detection	HRMS MS-MS	- First Choice, - Second choice, but should give good results with the newer instruments. (Quantum, Sciex 4000)
Stability Study	This is like a project in a project: it may run parallel with the above outlined parts on a limited number of OP pesticides or: subsequently on all or most of the OP pesticides.	



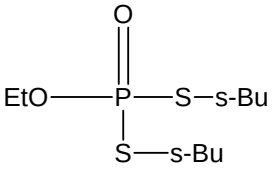
Acephate



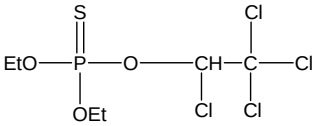
Azinphos-methyl



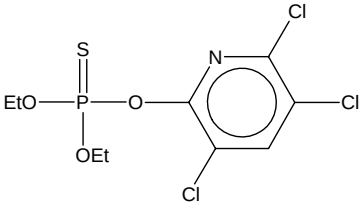
Bensulide



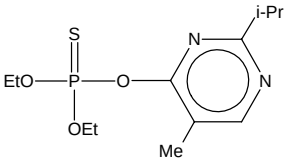
Cadusafos



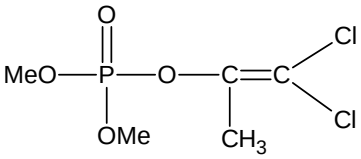
Chlorethoxyphos



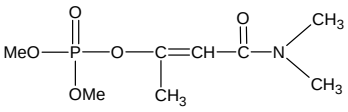
Chlorpyrifos



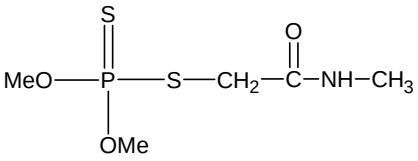
Diazinon



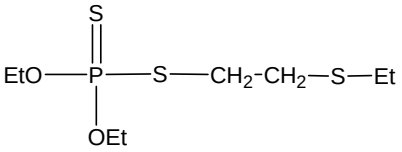
Dichlorvos (DDVP)



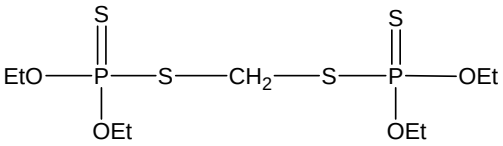
Dicrotophos



Dimethoate

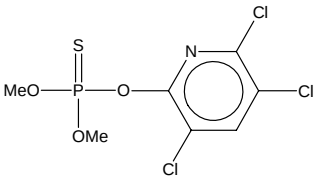


Disulfoton

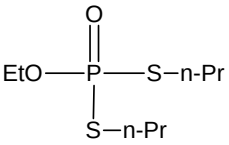


Ethion

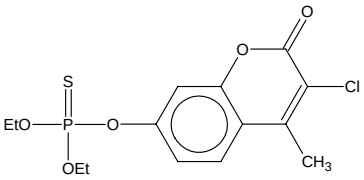
Table 3 OP pesticides of interest



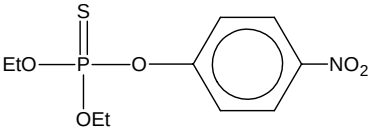
Chlorpyrifos methyl



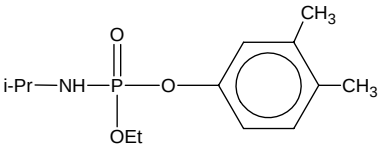
Ethoprop



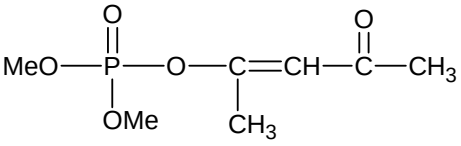
Coumaphos



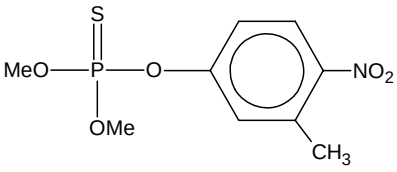
Ethyl Parathion



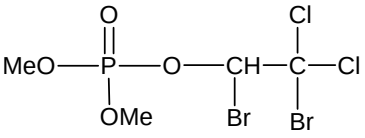
Fenamiphos



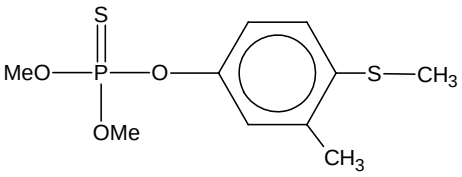
Mevinphos



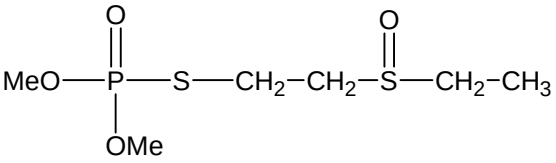
Fenitrothion



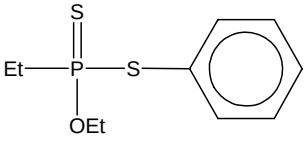
Naled



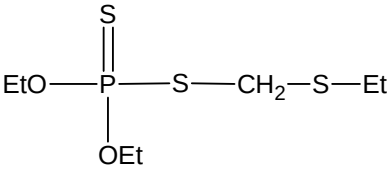
Fenthion



Oxydemeton methyl

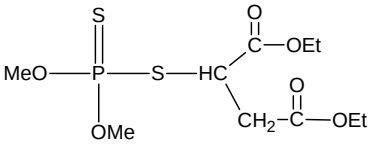


Fonofos

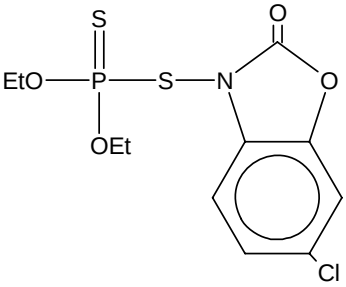


Phorate

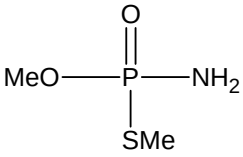
Table 3 (cont.) OP pesticides of interest



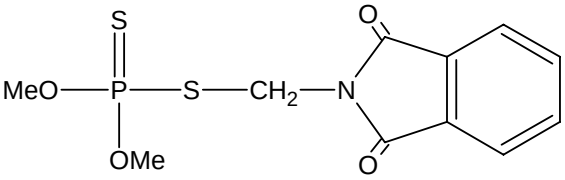
Malathion



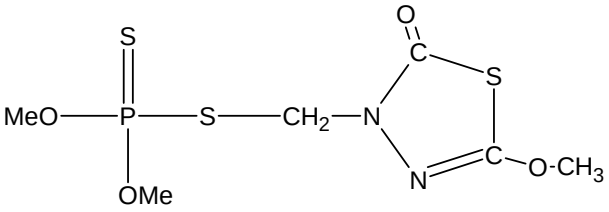
Phosalone



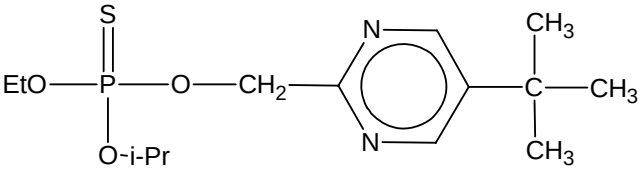
Methamidophos



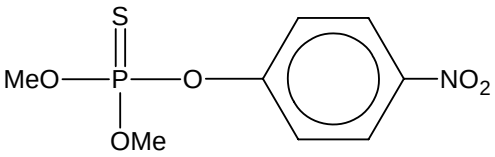
Phosmet



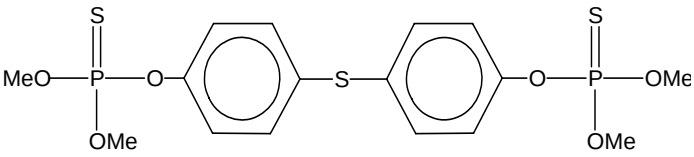
Methidathion



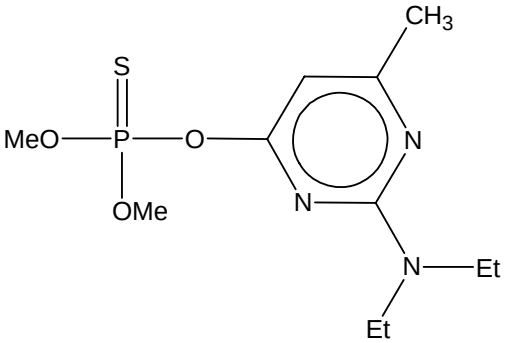
Phostebupirim (tebupirimfos)



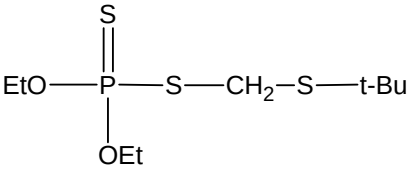
Methyl parathion



Temephos

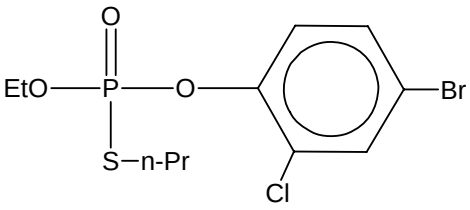


Pirimiphos methyl

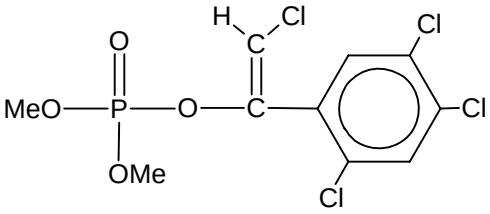


Terbufos

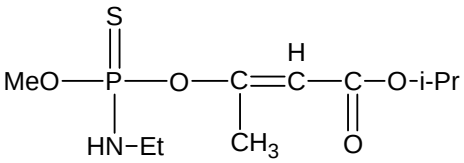
Table 3 (cont.) OP pesticides of interest



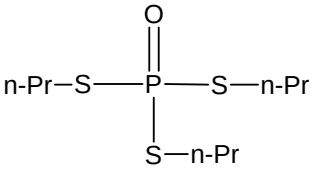
Profenofos



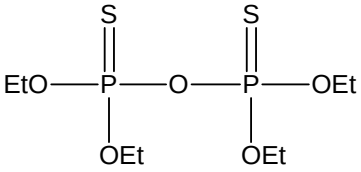
Tetrachlorvinphos



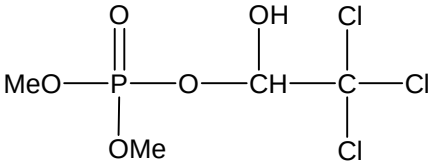
Propetamphos



Tribufos (DEF)



Sulfotepp



Trichlorfon

Table 3 (cont.) OP pesticides of interest

Abbreviation	Chemical Name
BTA	1,2,3-benzotriazin-4(3H)-one
CIT	5-chloro-1-isopropyl-(1H)-1,2,4-triazol-3-ol
CMHC	3-chloro-7-hydroxy-4-methyl-2H-chromen-2-ol
DEAMPY	2-(diethylamino)-6-methylpyrimidin-4-ol
DEDTP	O,O-Diethyl Dithiophosphate
DMDTP	O,O-Dimethyl Dithiophosphate
IMPY	2-isopropyl-4-methyl-6-hydroxypyrimidine
MSMB	Methysulfonyl methylbenzazimide

Table 4 Specific OP Pesticide Metabolites

Pesticides	Non Specific metabolites						Specific
	DMP	DMTP	DMDTP	DEP	DETP	DEDTP	metabolites
Acephate	—	—	—	—	—	—	Acephate, methamidophos
Azinphos-methyl	X	X	X	—	—	—	BTA, MSMB
Bensulide	—	—	—	—	—	—	—
Cadusafos	—	—	—	—	—	—	—
Chlorethoxyphos	—	—	—	X	X	—	—
Chlorpyrifos	—	—	—	X	X	—	3,5,6-TCPY
Chlorpyrifos-methyl	X	X	—	—	—	—	3,5,6-TCPY
Coumaphos	—	—	—	X	X	—	CMHC
Diazinon	—	—	—	X	X	—	IMPY
Dichlorvos (DDVP)	X	—	—	—	—	—	—
Dicrotophos	X	—	—	—	—	—	—
Dimethoate	X	X	X	—	—	—	—
Disulfoton	—	—	—	X	X	X	—
Ethion	—	—	—	X	X	X	—
Ethoprop	—	—	—	—	—	—	—
Ethyl parathion	—	—	—	X	X	—	p-Nitrophenol
Fenamiphos	—	—	—	—	—	—	—
Fenitrothion	X	X	—	—	—	—	—
Fenthion	X	X	—	—	—	—	—
Malathion	X	X	X	—	—	—	Malathion dicarboxylic acid, mono-carboxylic acid
Methamidophos	—	—	—	—	—	—	Methamidophos
Methidathion	X	X	X	—	—	—	—
Methyl parathion	X	X	—	—	—	—	p-Nitrophenol
Mevinphos	X	—	—	—	—	—	—
Naled	X	—	—	—	—	—	—
Oxydemeton-methyl	X	X	—	—	—	—	—
Phorate	—	—	—	X	X	X	—
Phosalone	—	—	—	X	X	X	—
Phosmet	X	X	X	—	—	—	—
Phostebupirim (tebupirimphos)	—	—	—	—	—	—	—
Pirimiphos-methyl	X	X	—	—	—	—	DEAMPY
Profenofos	—	—	—	—	—	—	—
Propetamphos	—	—	—	—	—	—	—
Sulfotepp	—	—	—	X	X	—	—
Temephos	X	X	—	—	—	—	—
Terbufos	X	X	X	X			
Tetrachlorvinphos	X	—	—	—	—	—	—
Tribufos	—	—	—	X	X	—	—
Trichlorfon	X	—	—	—	—	—	—

Table 5 Specific and non specific metabolites of OP pesticides

Abbreviations: —: not applicable, X: exposure to the pesticide listed, DMP, DMTP,DMDTP, DEP, DETP, DEDTP see; BTA, CIT, CMHC, DEAMPY; DEDTP, DMDTP, IMPY MSMB: see Table 4

Ref	First	Year	Matrix Type	Sample Cleanup		Analytical Separation			MS		Results
No	Author	Publ.	Amount	Type	Recovery [%]	Type	Column	Ioniz.	MS Type	#of OP-s (Ops of Interest)	LOD
[18]	Mushoff	2002	Whole Blood 0.5 g	Headspace SPME	0.1-19.6	GC	HP5-MS 30mx.25mmx.25µm	EI	HP5972	22 (13)	10-30 ng/mL
[19]	Barr	2002	Serum, Plasma, 4g	SPE Oasis HLB	14-27	GC	DB1701 (J&W) 30mX.25mmX.25µm	EI	High Res. Finnigan MAT-900	9 (9)	0.5 –2 ppt
[20]	Hernandez	2001	Whole Blood 0.5	HS-SPME		GC	HP Ultra 2 30mX.32mmX.50µm	EI	Ms-MS Ion trap	3 (3)	0.02-0.7 ng/mL
[21]	Tarbah	2001	Blood, Plasma 0.7g	LLE	50-133	GC	HP5-MS 30mX.25mmX.25µm	EI	SIM HP MSD 5970	23 (10)	- stability studies
[22]	Lacassie	2001	Whole Blood	SPE (HLB, C18)	41.2-107.9	GC	Supelco PTE5 30mX.32mmX.25µm	EI	SIM Shm QP-5000	29	5-25 ng/mL
[23]	Lacassie	2001	Serum 1 mL	SPE (HLB)	40-99% ? not detailed	GC	Supelco PTE5 30mX.25mmX.25µm	EI	SIM Shm QP-5000	29 (21)	5 ng/mL
[24]	Liu	2001	Serum6 mL	LLE, hexane 2X	100%	GC	RTX CLPesticides 30mX.25mmX.25µm	EI	SIM 5971A	4	2-5 ng/mL
[25]	López	2000	Serum (.5 mL, dil.)	SPME of 3 mL of dil. sample	60-100%. relative to water	GC	Brand? 30mX .32mmX .5µm	FPD det.		7 (7)	1-15 ng/mL

Table 6 Assay of OP Pesticides In Serum, Plasma or Whole Blood - Gas Chromatography

Ref	First	Year	Matrix Type	Sample Cleanup		Analytical Separation		MS			Results
No	Author	Publ.	Amount	Type	Recovery [%]	Type	Column	Ioniz.	MS Type	# of OP-s (Ops of Interest)	LOD
[26]	Sancho	2000	Serum 0.5 mL	0.5mL ser.+ .75mL ACN 10µL inj 2	87-113	col switching LC	Columns: #1: Discovery C18 #2: Supelco ABZ	ESI	Micromass Quattro Zspray Ms-ms	Chlor-pyriphos & metab	1.5 ng/mL
[27]	Abu-Qare	2001	(Rat) Plasma /0.5ml	SPE C18 (Sep-Pack)	80.1-83.9	LC	C18	UV detection		2 + metab	20-50 ng/mL

Table 7 Assay of OP Pesticides In Serum, Plasma or Whole Blood - Liquid Chromatography

Ref	First	Year	Matrix		Sample Cleanup	Analytical Separation		MS			Results
No	Author	Publ.	Type Amount	Type	Recovery [%]	Type	Column	Ioniz.	MS Type	No of OP-s (Ops of Interest)	LOD
[28]	Amini	2003	Plasma /.5ml	RAM (restricted access materials)	60-92	LC	C18	ESI & APCI	ms-ms MM, Quattro	9 OP esters	0.2 –1.8 ng/mL
[29]	Jonsson	2003	Plasma 5g	Micro-porous membrane	Stir bar assisted 32-92 % Counter Current 23-78 %	GC	DB5 MS 30m X .25mm X .1µm	NPD		8 OP esters	0.2 – 0.9 ng/g
[30]	Jonsson	2001	Plasma 5g	Membrane Extraction Device	72-83 %	GC	DB5 MS 30m X .32mm X .1µm	NPD		9 OP esters	0.06-4 ng/g
[31]	Jonsson	2003	Plasma 50 µL !	Hollow Fiber Extraction Device	40-80 %	GC	DB17 MS 30mX.25mmX .15µm	EI	TSQ 700	8 OP esters	0.06-4 ng/g

Table 8 Assay of OP esters In Serum, Plasma or Whole Blood

No.	OP	Native	Labeled
1	Acephate	X	X, D6
2	Azinphos-methyl	X	—
3	Bensulide	X	X, D14
4	Cadusafos*	X	—
5	Chlorethoxyfos	X	—
6	Chlorpyrifos	X	X, D10
7	Chlorpyrifos methyl	X	X, D6
8	Coumaphos	X	X, D10
9	Diazinon	X	—
10	Dichlorvos (DDVP)	X	—
11	Dicrotophos	X	—
12	Dimethoate	X	X, D6
13	Disulfoton	X	X, D10
14	Ethion	X	—
15	Ethoprop	X	—
16	Ethyl parathion	X	X, D10
17	Fenamiphos	X	—
18	Fenitrothion	X	—
19	Fenthion	X	X, D6
20	Fonofos**	X	X, 13C6
21	Malathion	X	X, D10
22	Methamidophos	X	X, D6
23	Methidathion	X	—
24	Methyl parathion	X	—
25	Mevinphos*	X	—
26	Naled	X	—
27	Oxydemeton methyl	X	X, D6
28	Phorate	X	X, 13C4
29	Phosalone*	X	—
30	Phosmet	X	X, D6
31	Phostebupirim	X	—
32	Pirimiphos methyl	X	—
33	Profenofos	X	—
34	Propetamphos	X	—
35	Sulfotepp	X	X, D20
36	Temephos	X	—
37	Terbufos	X	X, 13C4
38	Tetrachlorvinphos	X	—
39	Tribufos (DEF)	X	—
40	Trichlorfon	X	—

Table 9 Available Native OP pesticides and their Labeled Reference Compounds [32]
Abbreviations: X: compound is available; —: compound is not available, D*n*: n number of Hydrogen Atoms Replaced with Deuterium (Labeled Compound); 13C*n* n number of carbons replaced with ^{13}C isotope.(Labeled Compound)

METHOD DEVELOPMENT

General development strategy

Our goal was as stated in the introduction, to develop a method to detect and measure trace concentration of the EPA approved OP pesticides. The difficulty of the project lies in the fact that these compounds encompass a very wide range of concentrations many of them are labile and all of them are subject of enzymatic degradation while in the matrix.

Possible matrices – other than whole blood – are serum and plasma.

We choose serum as matrix of preference, mainly because of easier cleanup and availability.

The development process started with determining the correct mass spectrometric detection method. Detection has to be developed in conjunction with the separation techniques applied. Analytical separation techniques such as choice of solvents, effect decisions made at the detection part of the project, at the same time one needs a detection method to develop any analytical separation.

- First, GC-MS was evaluated electron impact ionization was compared with chemical ionization.

  Chemical ionization positive polarization was chosen.
- Tandem mass spectrometry: MS-MS fragmentation was optimized and ions chosen

- LC/MS method was chosen (APCI positive mode) then MS-MS transitions were selected , parameters optimized.
- HPLC method was finalized

Sample Cleanup section

Cleanup methods evaluated:

- SPE was not considered because previous experience and the range of compounds involved with varying physical and chemical properties.
- Freeze drying followed with solvent extraction was tried – not chosen
- Liquid-liquid extraction on solid carrier (ChemElut) was tested with better results
- Based on the promising results of the ChemElut method, accelerated solvent extraction (ASE)was tried and proved very successful.
- ASE was optimized proved to be successful
- A “normal phase chromatography” like additional cleanup step was developed using the “Rapid Trace” automated SPE instrument. It is to further purify the serum extract and make it usable. In a GC application
- As an alternative to the second step cleanup headspace solid phase microextraction
- Checked for accuracy and precision
- Applied the method to a study of farm workers, potentially exposed to pesticides

CHAPTER 2: GAS CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

First step in the development process was to establish a method of detection: without detection one can not study separations. In our laboratory the most sensitive detectors are the mass spectrometers. The available instruments were GC coupled TSQ-7000 triple quadropole mass spectrometers and the newer LC coupled TSQ-Quantum Ultras, also of the triple quadropole type. We also have a MAT-95 high resolution sector instrument, but decided against using it mainly because of the limited availability and reliability issues at the time.

The initial task was to determine the preferred ionization form for both: liquid chromatography (LC) and gas chromatography (GC) coupled systems. The selection process started with the GC-MS-MS system. After initial experiments it became apparent that chemical ionization – being a soft ionization mode – yield a higher proportion of molecular ions than electron impact ionization. For that reason chemical ionization (CI) was chosen. Later in this chapter actual limits of detections will be compared and the choice of CI quantitatively justified.

Process to optimize mass spectrometric parameters for GC-MS-MS systems.

When deciding on a detection method for a group of compounds, first the followings have to be decided:

1. Type of ionization: The main modes are electron impact ionization(EI) and chemical ionization.(CI) The polarity of ionization is always positive in EI, in CI however it can be either positive or negative.
2. Ion selection: Two transitions have to be selected: one for quantitation, one for confirmation.
3. Once the transitions are selected, the collision energy needs to be optimized.

Some of the parameters were not optimized but used at or close to their default value.

	CI	EI
Filament Current	300 mA	1300 mA
Source temperature	150°C	180°C
Reagent Gas	Methane @ 1500 mT	

Table 10 Non optimized MS parameters

Increasing filament current may increase signal strength but greatly decreases service life. Elevating source temperature will increase reaction rate, but also cause decomposition of thermally labile molecules. Lower source temperatures also cause increased fouling of the CI source and accelerate signal loss.

Determining Ionization type and ion selection

First individual solutions of the analytes were prepared at 2 µg/mL concentration in toluene. Based on prior literature, [18, 21-24] and availability at the time, W&J-s DB-5 MS column was selected for initial testing. The column is 30m in length, has 0.25mm internal diameter and 25µm coating thickness. 1 µL of the pure analyte solution was injected and the developing chromatogram observed. Once the analyte peak was detected,

retention time recorded and one of more dominant ion selected. Preferably one of the ions of interest a molecular ion, but OP pesticides being often labile, it is not always possible. After one or more potential parent ion was selected, a second injection was made. This time the MS was set to product ion scan: quadropole one selects the parent ion, quadropole two fragments the parent ion and quadropole three was scanning the fragmentation products. Observing the product ions of each parent ion, two or three promising transition was selected. For each of these transitions (parent ion -> product ion) a selected reaction monitoring (SRM) table was set up with different collision energies. Collision energy is the voltage difference between the last lens before quadropole 2 (Lens 2-3) and the first lens after quadropole two (Lens 3-1). This is the potential difference, that accelerate the parent ion (select in quadropole one) down on quadropole two. Quadropole two is filled wit low pressure (~2 mTorr) argon. The parent ions hitting the argon atoms break up, producing the product ions. At a set argon (collision gas) pressure, the pattern of fragmentation and the amount of product ions depend on the collision energy. For each transition of interest, the collision energy had to be optimized. The before mentioned SRM table is the instrument of collision energy optimization. A third injection was made in SRM mode, covering all transitions and collision energies of interest. After the GC-MS-MS run, for each transition and collision energy setting the chromatographic curve was plotted and the respective peaks integrated. For each transition the area count – collision energy curve was plotted and the optimum collision energy determined. This process is shown in Figure 9-Figure 12.

This process was repeated for each analyte in positive chemical ionization. For electron impact and negative chemical ionization, only the full scan (parent ion selection) and the product ion scan were performed.

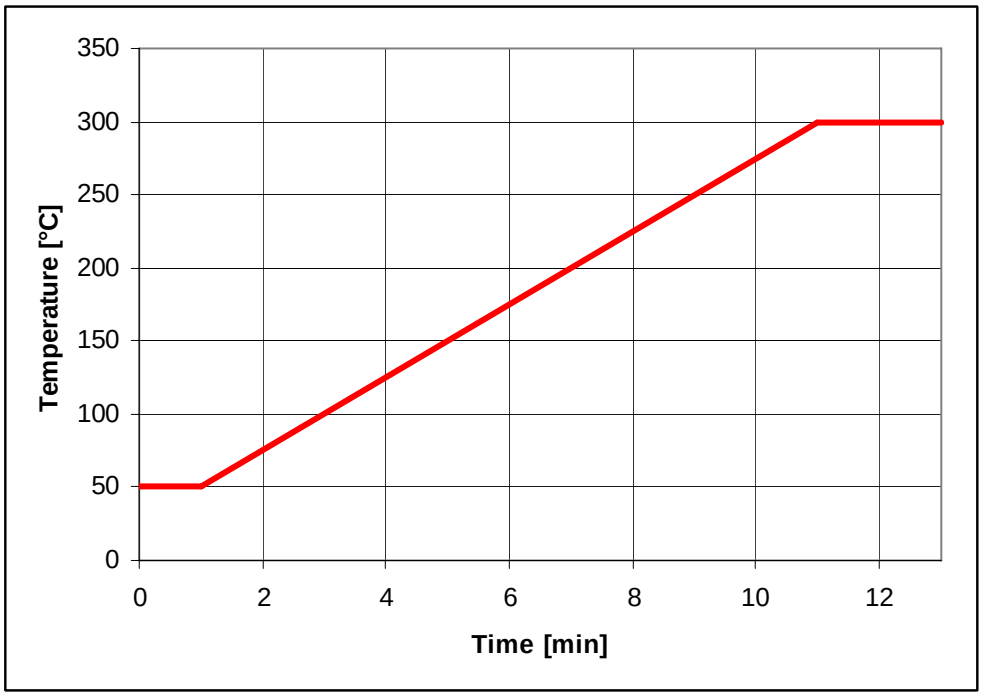


Figure 9 Simple temperature gradient to determine GC properties of OP compounds

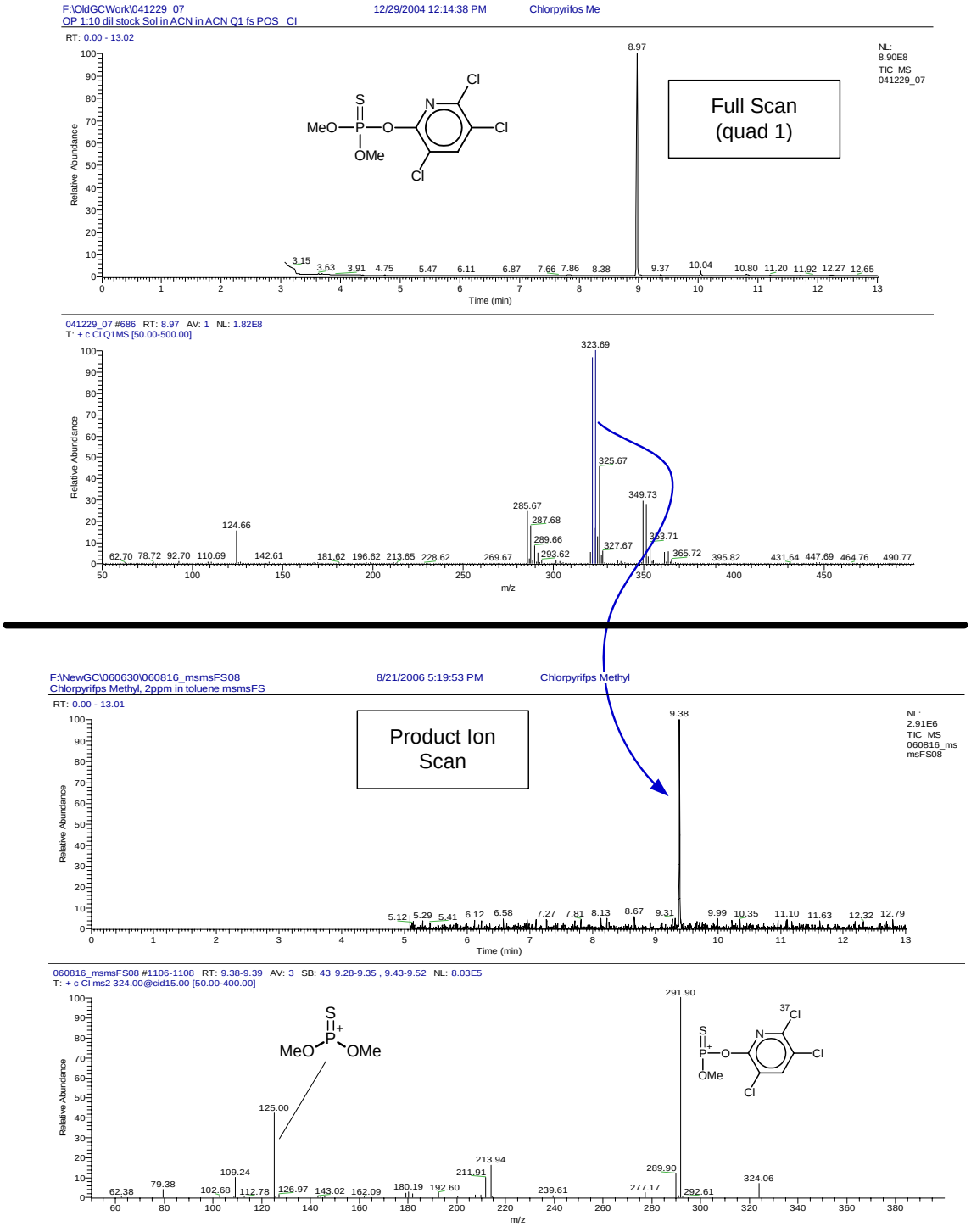


Figure 10 Transition selection for chlorpyrifos methyl

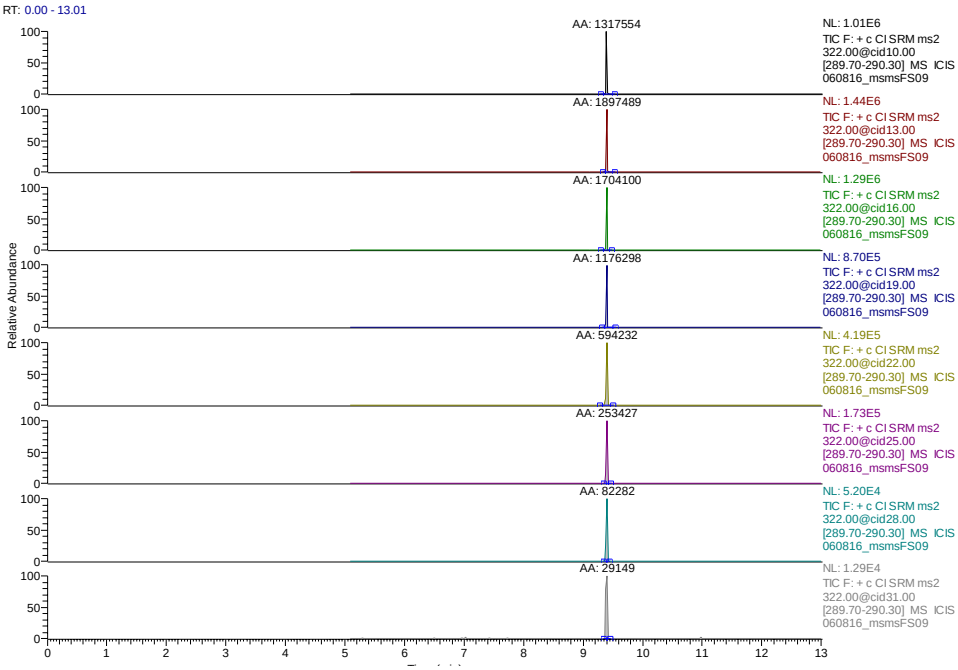


Figure 11 Area counts for clorpyrofos methyl 322 -> 290 transition at different collision energies

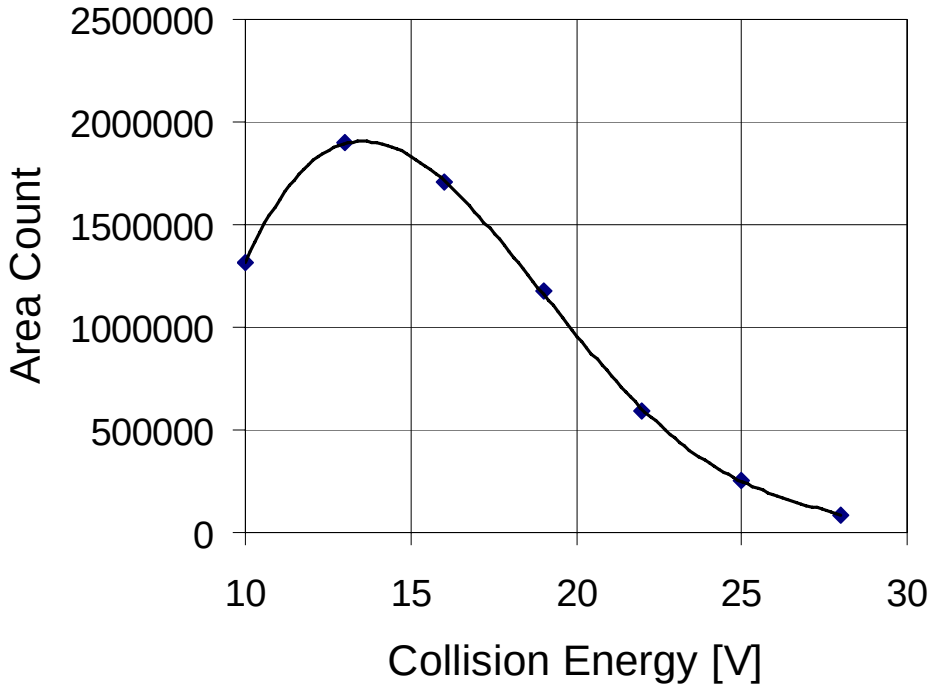


Figure 12 Otimization of collision energies for clorpyrofos methyl 322 -> 290 transition

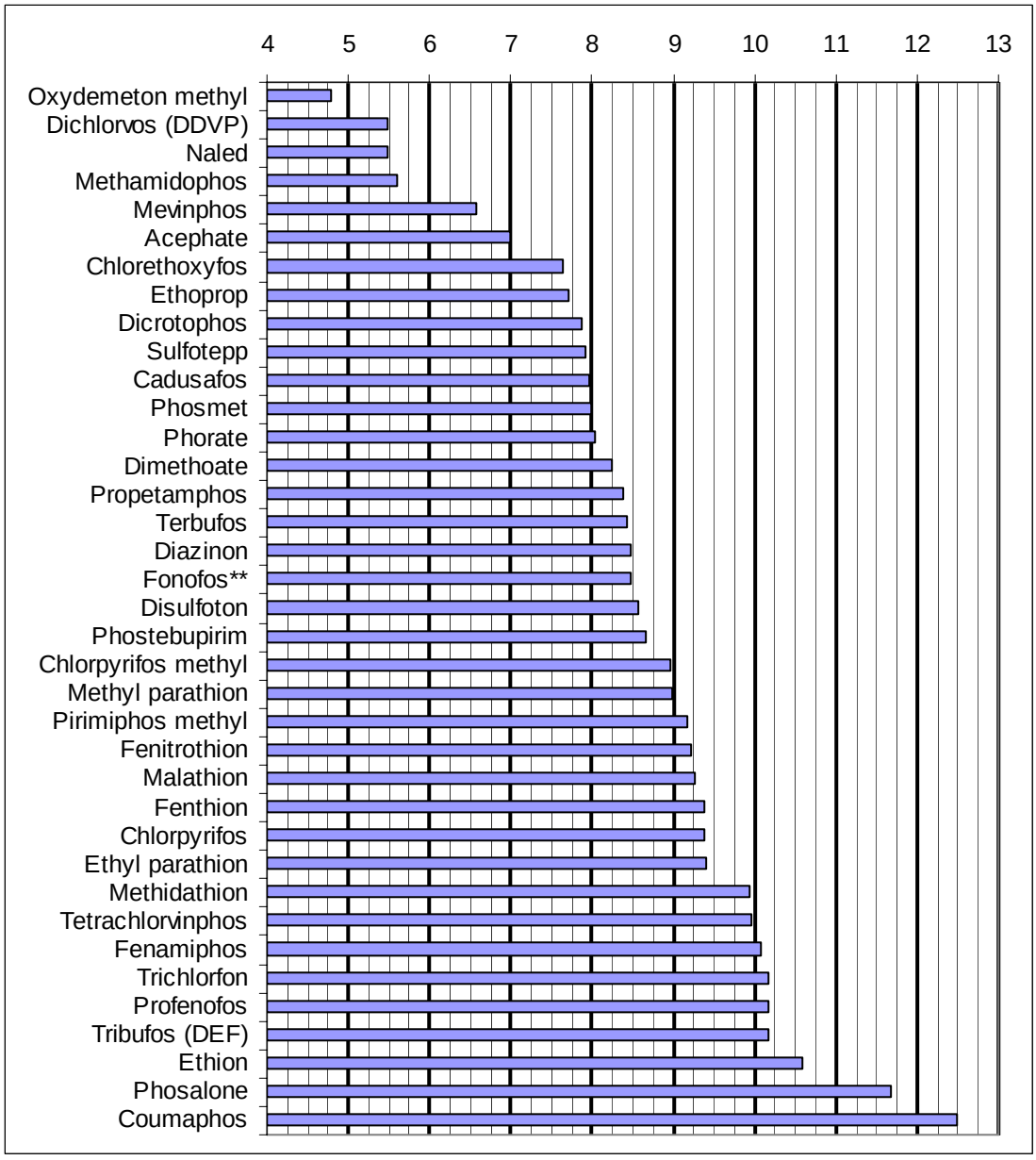


Figure 13 Retention times for OP compounds on 30m .25mm X 25µm DB-5 MS column, linear temperature gradient.

Chapter 2 Dicussion

When developing the GC-MS-MS part, there were two questions to consider.

The first one is the selection of ionization mode. Using MS and MS-MS, the available ionization modes are EI, CI positive and negative polarity. Most of the available literature uses EI. [8]-[14]. The reason for this is probably twofold: EI is more economical in the sense that it produces multiple fragments in the ion source. As a result a compound can be identified from the main ions or fragments and their ratios. One does not need tandem mass spectrometer to produce fragments. The second reason is probably more practical: EI ions form in a partially open ion source. It remains clean longer than CI sources. CI sources or "ion volumes" are closed to the outside, save the few holes needed for material transport. Inside is the end of the GC column, and the reagent gas – most often methane – bombarded by the electron beam. As a result, contaminants from the sample leaving the GC column and their decomposition products tend to coat the inside of the ion volume. Once the inside of the source becomes less conductive it also less effective, resulting in loss of sensitivity.

Chemical ionization on the other hand has the advantage of the more controlled ion formation. To maximize sensitivity, our focus was to generate as much as possible molecular ion or a close fragment where unavoidable. Tandem mass spectrometers were available, these ions could be fragmented at a later stage.

Using CI the choice is between positive and negative ionization. All things being equal, analyzing biological samples, negative ionization sometimes has an advantage. It is easier to ionize a compound in positive mode because it is easier to add a charge – usually a proton – to a molecule. In negative ionization, the removable proton (removable proton)

has to be there the first place. So if the molecule of interest ionizes in negative mode, the background is likely to be lower.

OP pesticides however pose a particular problem: they tend to break apart in negative ionization. When this happens, often the non-specific part (phosphate-methyl, ethyl ester, thio-ester) is dominant.

These issues can be demonstrated on the example of malathion, a very common pesticide. Figure 15 shows the many fragments produced by EI. CI, positive ionization (Figure 16) produces mainly the molecular ion (331), an adduct ($C_2H_5^+$) and one more ion which created with a loss of an ethoxy. Negative chemical ionization chiefly produces the ion with the m/z of 157. As it shown on Figure 17, it is a very non specific fragment. In a crowded separation it may come from many different compounds, such as Fonofos and Dimethoate. For this reason, CI positive was chosen as preferred ionization mode.

For gas chromatography portion of the analytical separations part, most authors used either DB-5 / DB-5MS (5% phenyl, methyl polysiloxane) type columns or HP1701 ((14%-Cyanopropyl-phenyl)-methylpolysiloxane). (Figure 14)

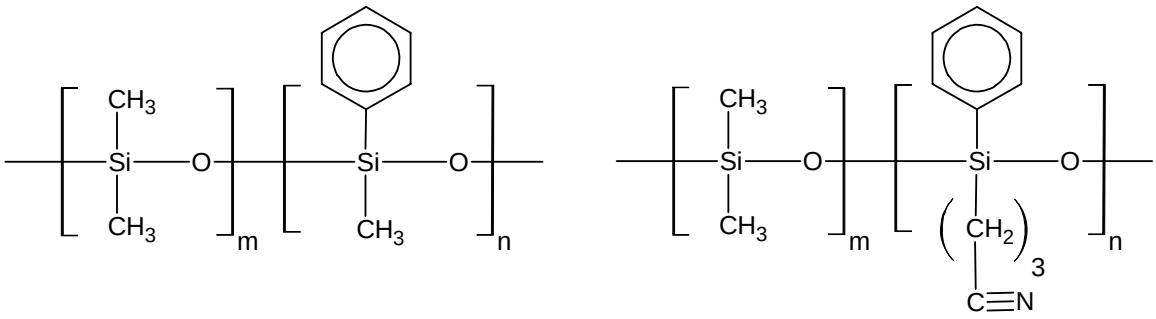


Figure 14 Polymer structure of phenyl-methyl (DB-5 MS) and cyanopropyl phenyl (HP1701) stationary phases

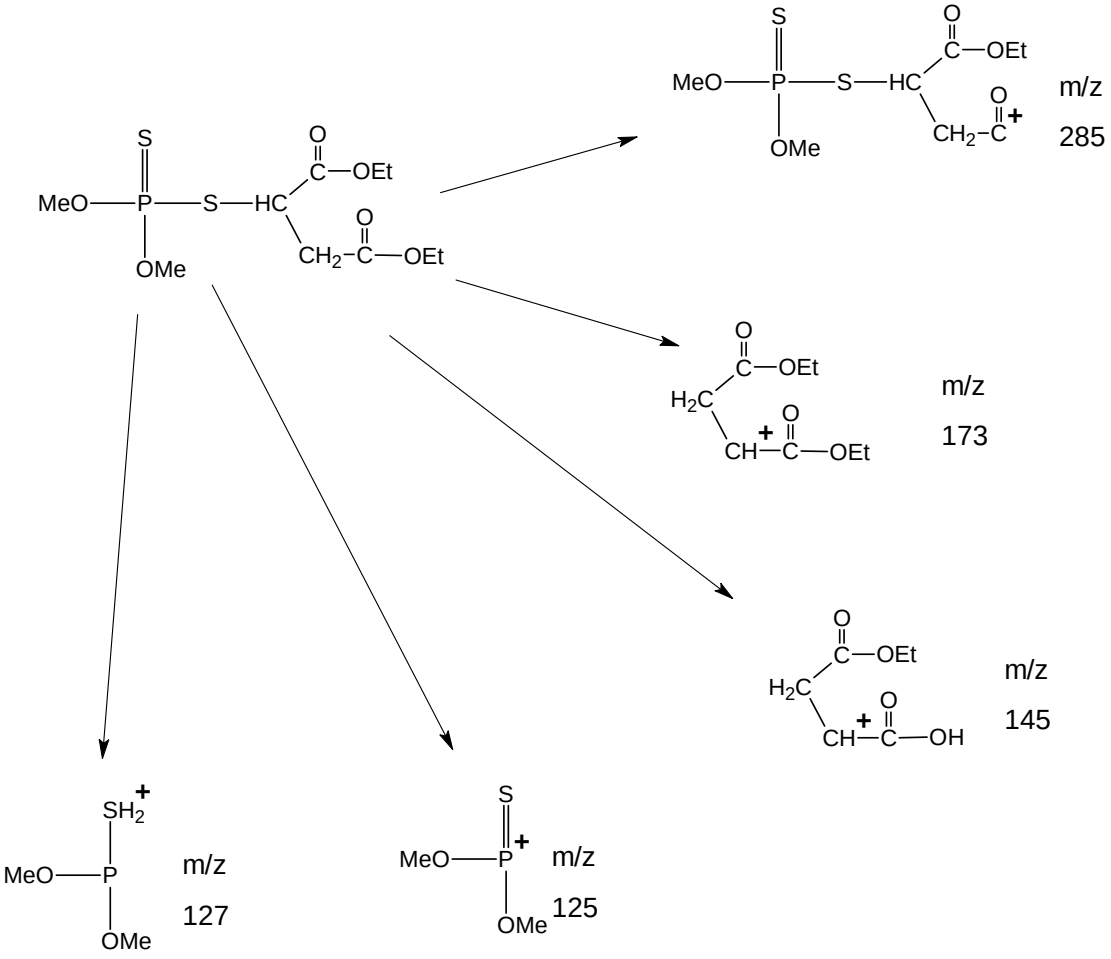


Figure 15 Major fragments of malathion in EI mode

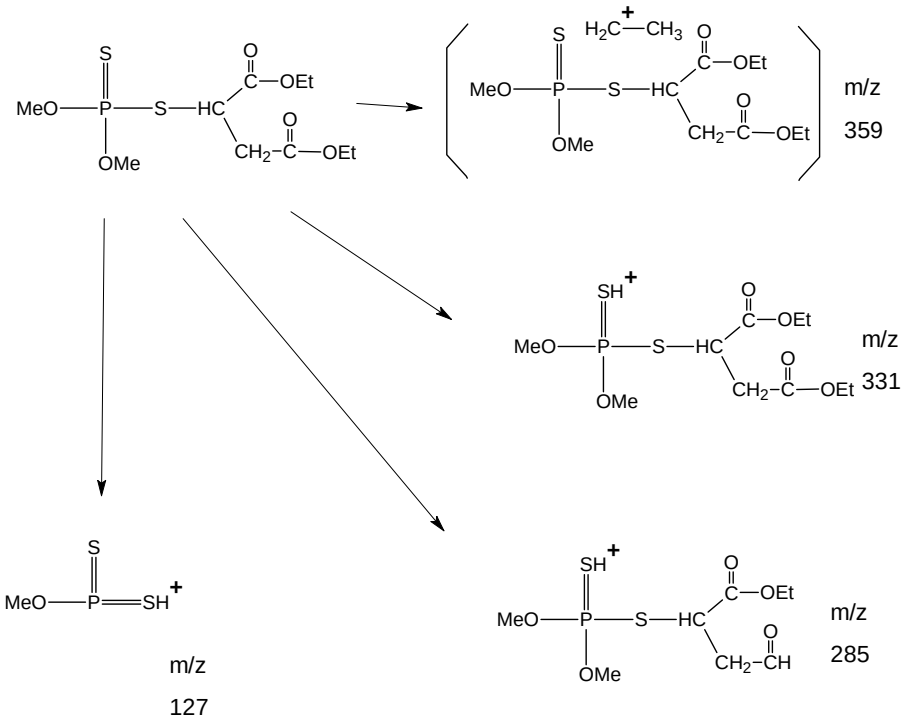


Figure 16 Major fragments of malathion in CI positive mode

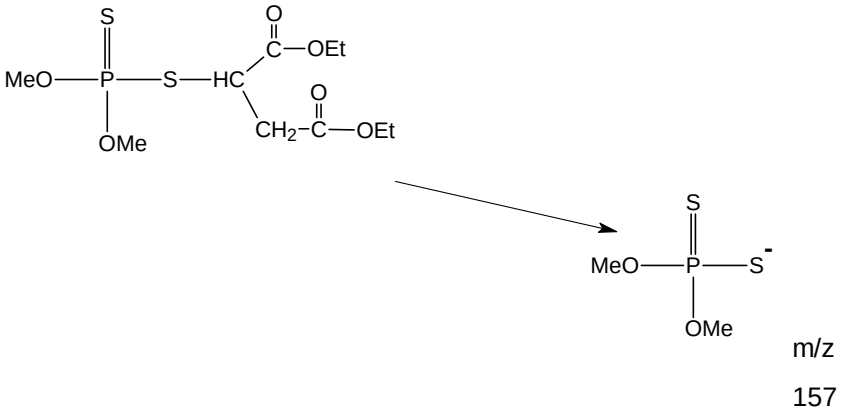


Figure 17 Major fragments of malathion in CI negative mode

When looking for correlations in the GC section, the first approach is to plot retention time as a function of published[33] vapor pressure data. Since vapor pressure covers a wide range of values, logarithmic scale is useful. Figure 18 shows these vapor pressure values. It is tempting to find two groups, represented by lines A and B, but examining line B and the compounds it corresponds to, there is no structural commonality. (Table 11)

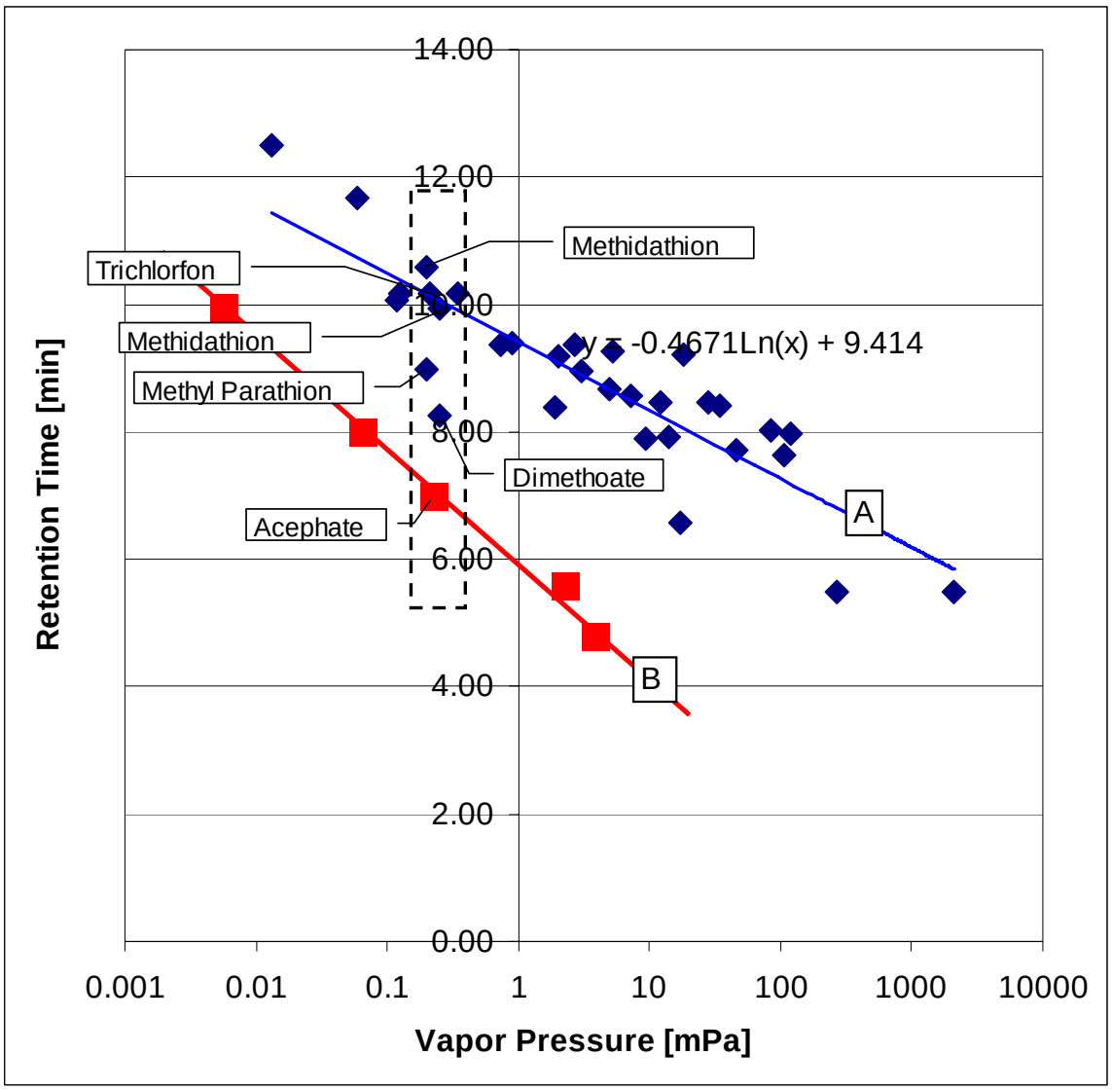
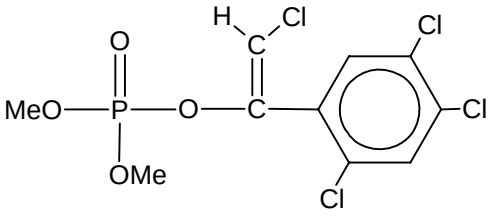
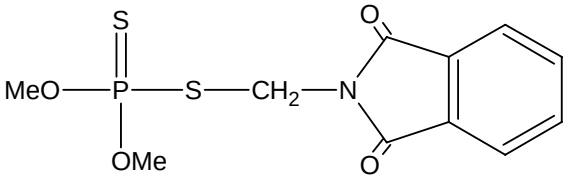


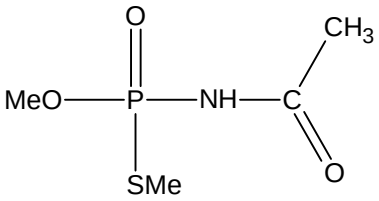
Figure 18 GC retention times as the function of vapor pressure data (logarithmic scale)



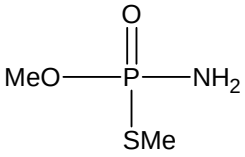
1 Tetrachlorvinphos



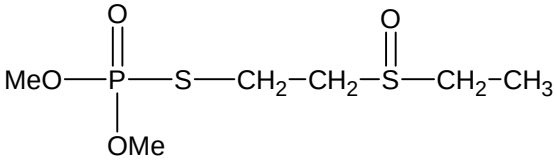
2 Phosmet



3 Acephate



4 Methamidophos



5 Oxydemeton methyl

Table 11 Compounds of line B (GC)

Next step is to look at the outliers at an area where the vapor pressure is the same but the retention time is markedly different. Such is the boxed area on Figure 18. The two most extreme outliers are acephate and methamidophos. Both are very water soluble, so it is useful to factor in hydrophilic – hydrophobic properties in the investigation. Kow values, describe a compound hydrophobicity.

Next, a retention factor "R" was proposed to predict an OP retention time (DB-5 column, linear temperature gradient) along with the equation $R = \log(K_{ow}) - a * \log(P)$. The correlation factor for Retention time –R factor arrays was calculated for several "a" parameters and charted. A=1.5 was approximated to be the optimum. (Figure 19)

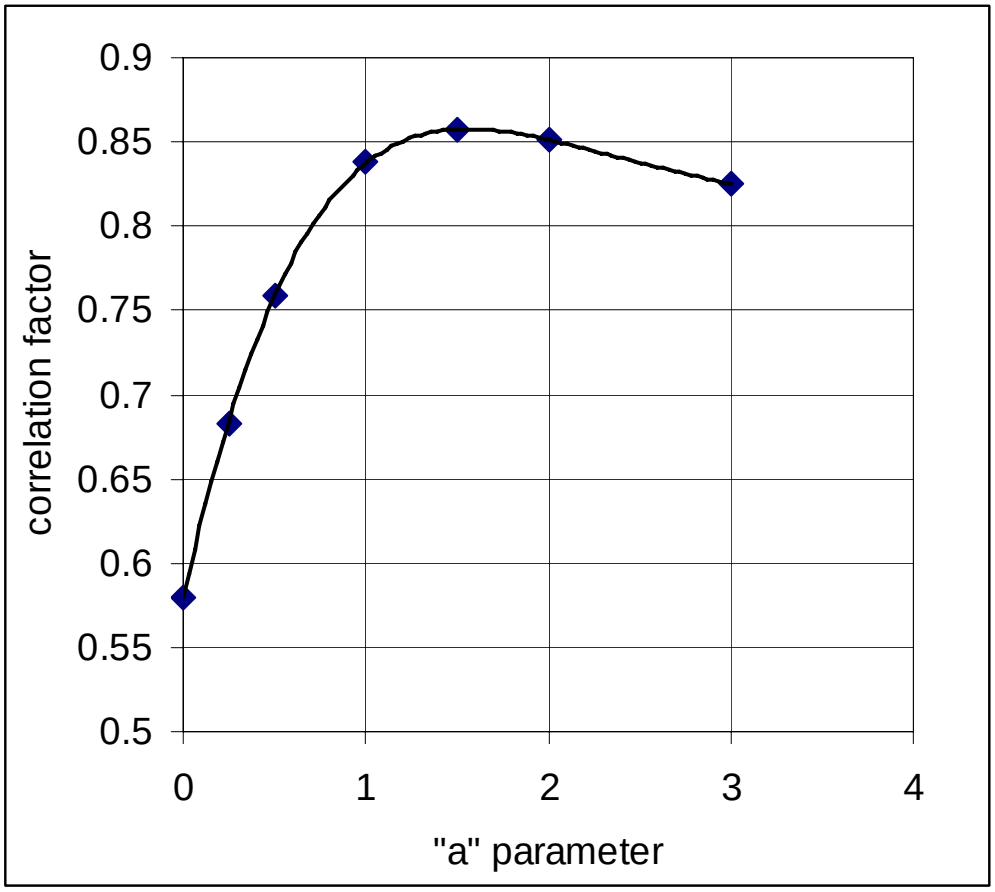


Figure 19 Correlation factor analysis

Calculating the R value for an OP using the $R = \log(K_{ow}) - a * \log(P)$ equation, the retention time of an OP may be easily predicted.

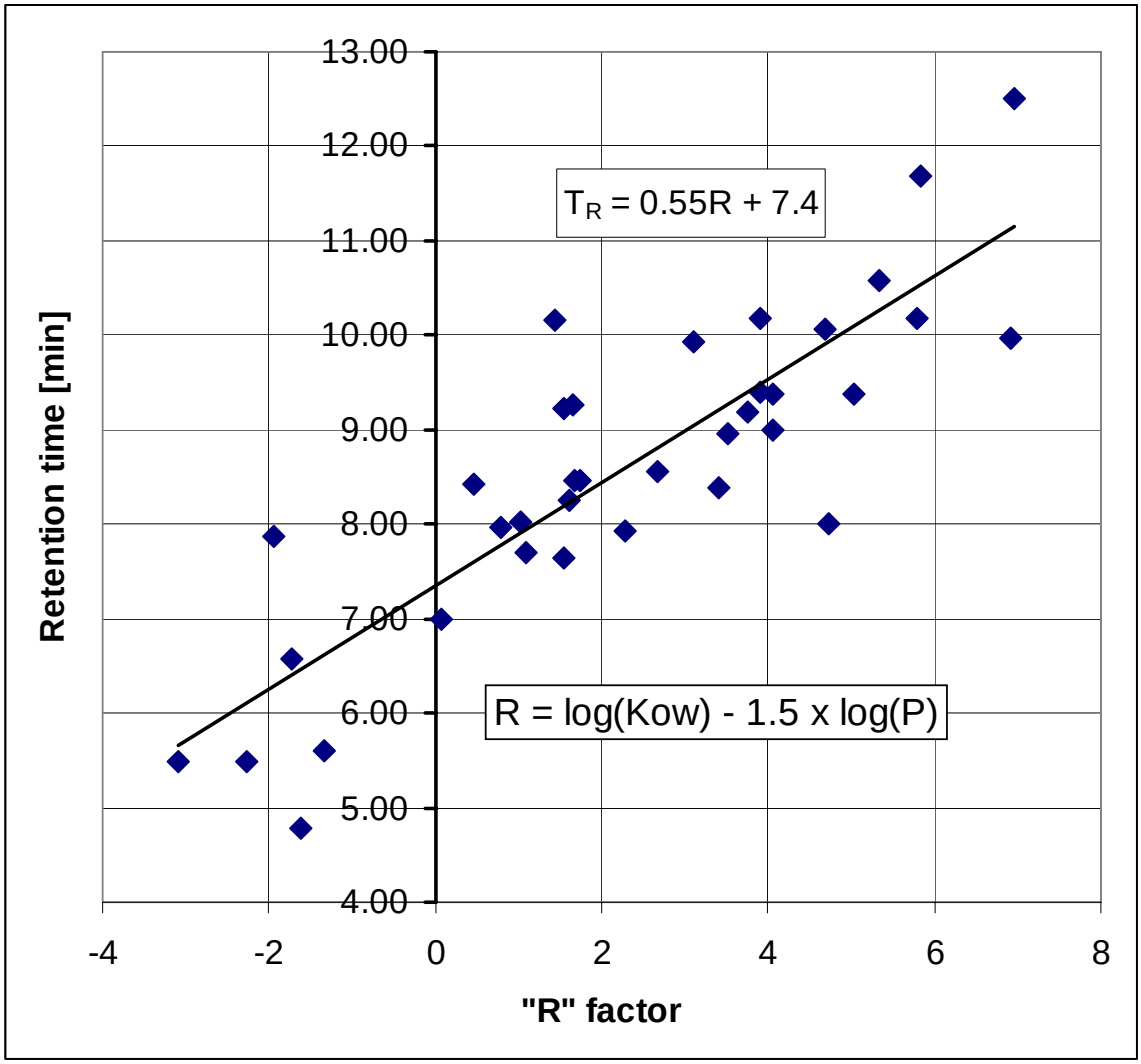


Figure 20 Predicting OP retention times in GC

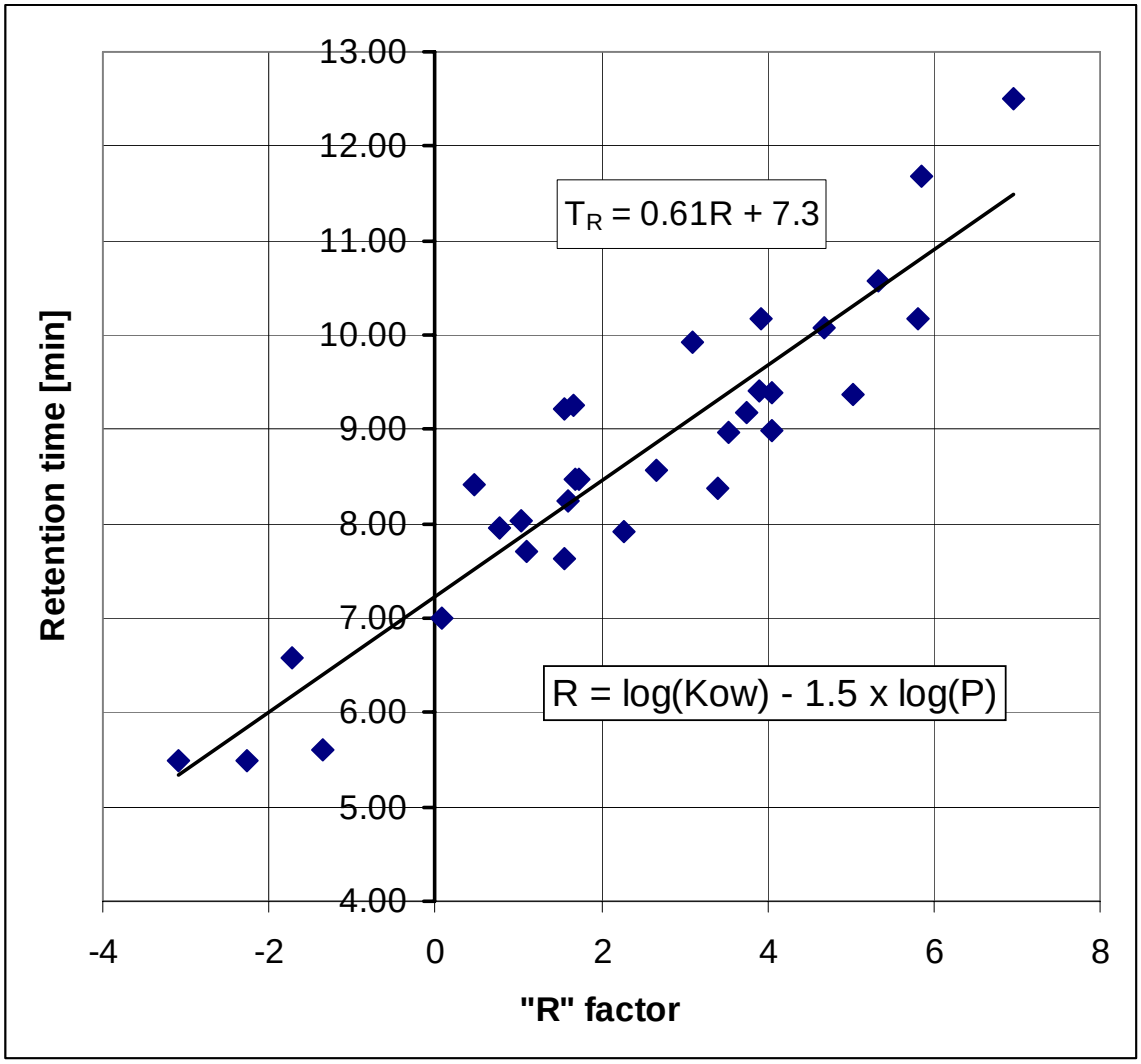


Figure 21 Predicting OP retention times in GC – outliers (Oxydemethon methyl, dicrotophos, tetrachlorvinphos, trichlorfon, all LC group members) taken out:

	Retention Time [min]	
	predicted	actual
Cadusafos	7.8	8.0
Chlorethoxyfos	8.2	7.6
Chlorpyrofos Me	9.8	9.4
Diazinon	8.3	8.5
Disulfoton	8.9	8.6
Et Parathion	9.7	9.4
Fenamiphos	10.2	10.1
Fenitrothion	8.2	9.2
Fenthion	10.4	9.4
Fonofos	8.4	8.5
Malathion	8.3	9.3
Me Parathion	9.8	9.0
Mevinphos	6.3	6.6
Naled	5.9	5.5
Phorate	7.9	8.0
Phosalone	10.9	11.7
Profenofos	10.8	10.2
Sulfotepp	8.7	7.9
Terbufos	7.6	8.4

Table 12 GC predicted and actual retention times

CHAPTER 2 APPENDIX

Appendix A CI Positive, negative and EI full scans

These MS full scan experiments were necessary for the development of the GC-MS-MS par t of the method. Unlike the LC –MS portion, few automated tools are available. Acetonitrile based stock solution was diluted with toluene to produce 1 µg/mL samples.2 µL of these were injected on DB-5 MS column, using a linear temperature gradient. Source conditions were the recommended starting values:

	Source Temperature [°C]	Reagent Gas Pressure [mTorr]	Filament Current [µA]	Electron Energy [eV]
CI positive	150	1500	300	-200
CI negative	150	1500	300	-200
EI	150	N/A	1300	-70

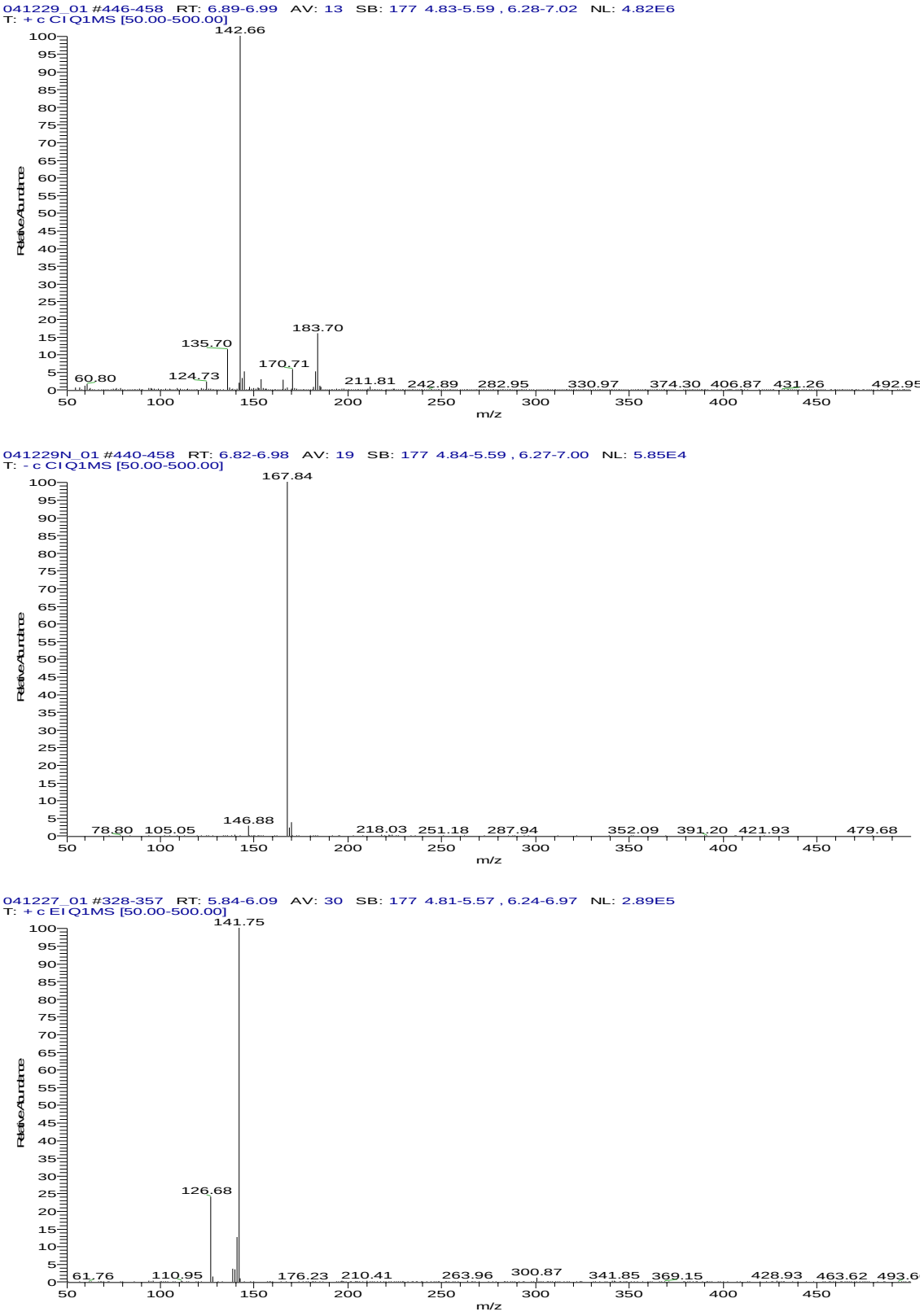
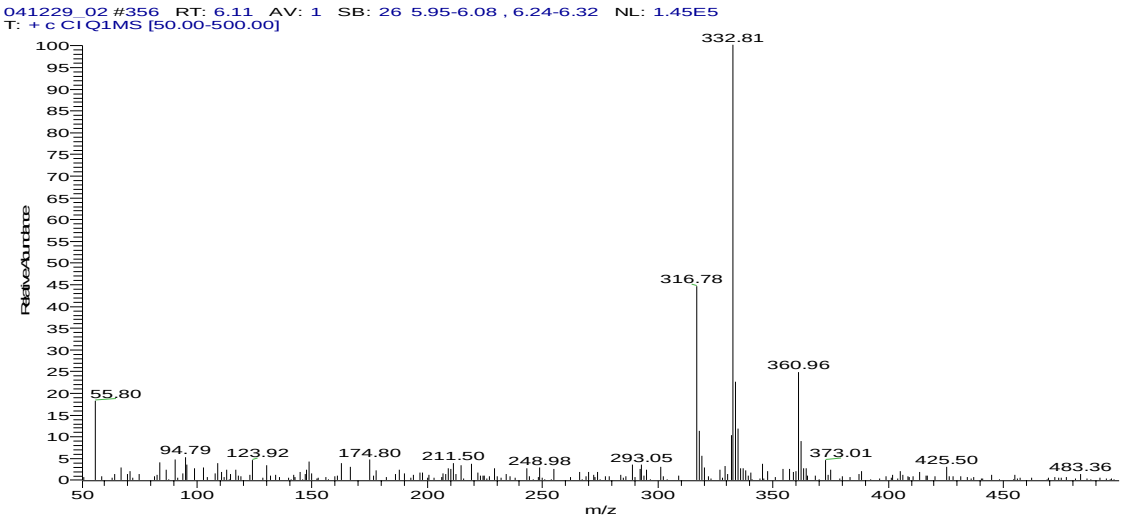


Figure 22 Acephate - Positive CI, Negative CI, EI spectra



N/F

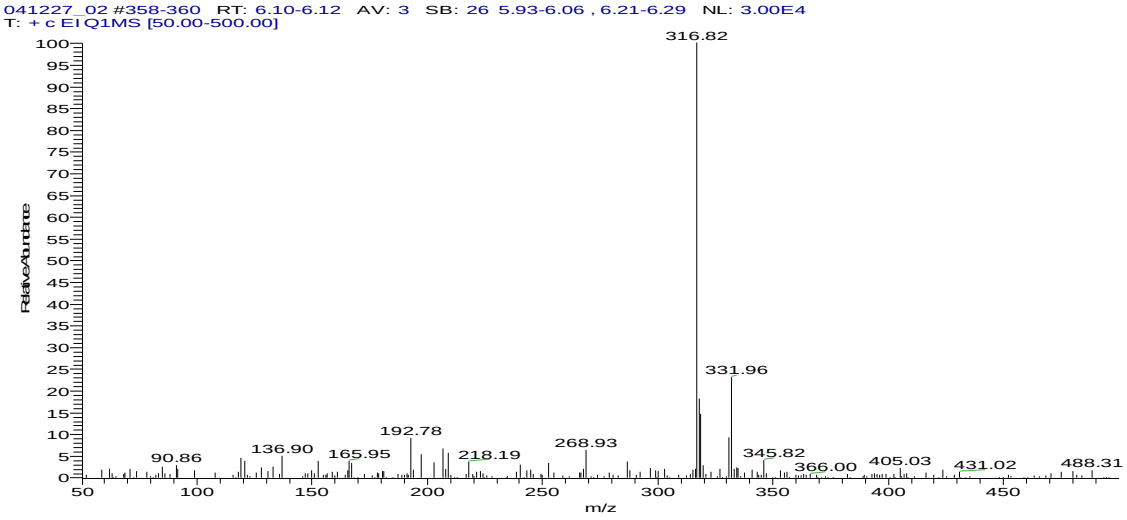


Figure 23 Azinphos Methyl - Positive CI, Negative CI, EI spectra

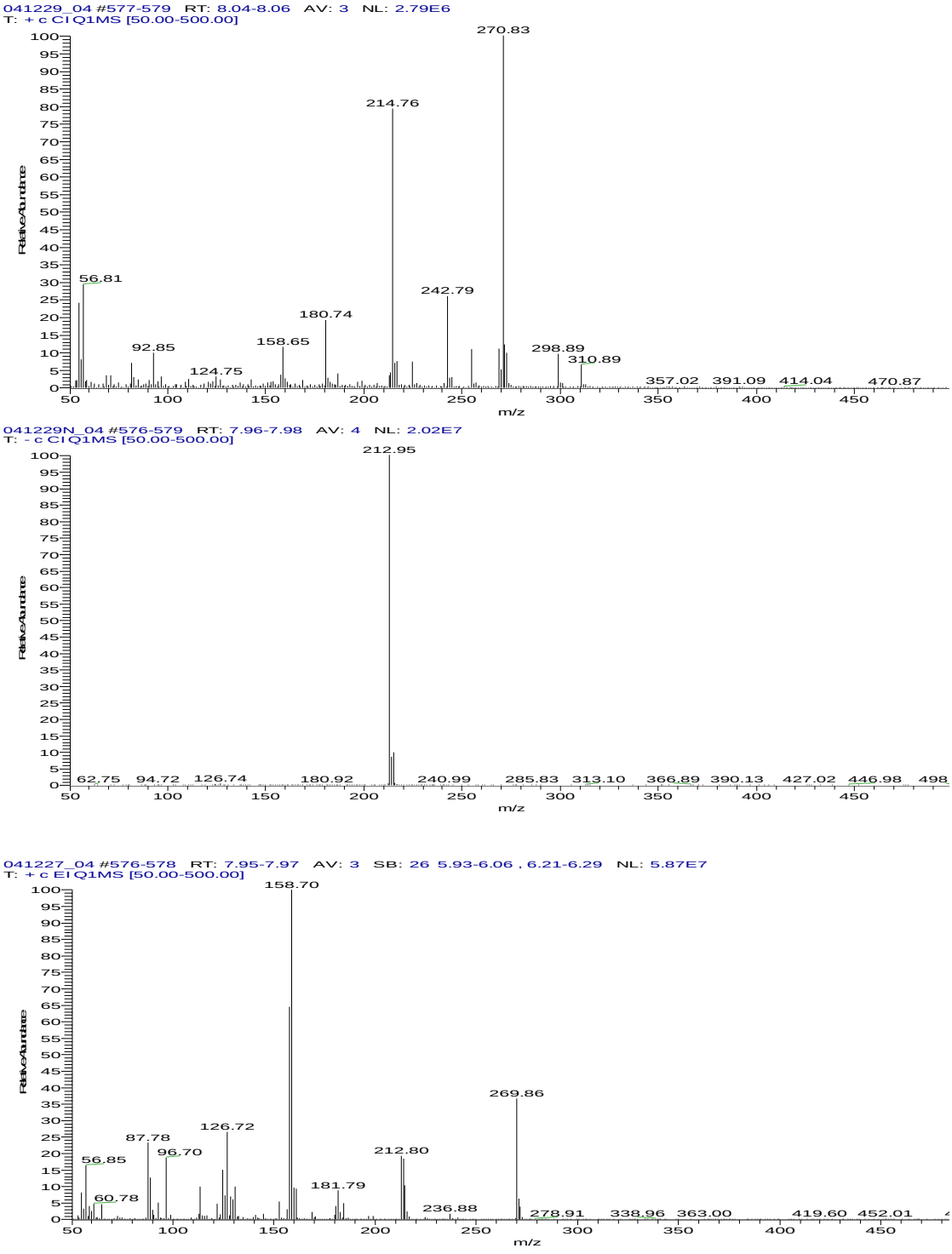


Figure 24 Cadusafos - Positive CI, Negative CI, EI spectra

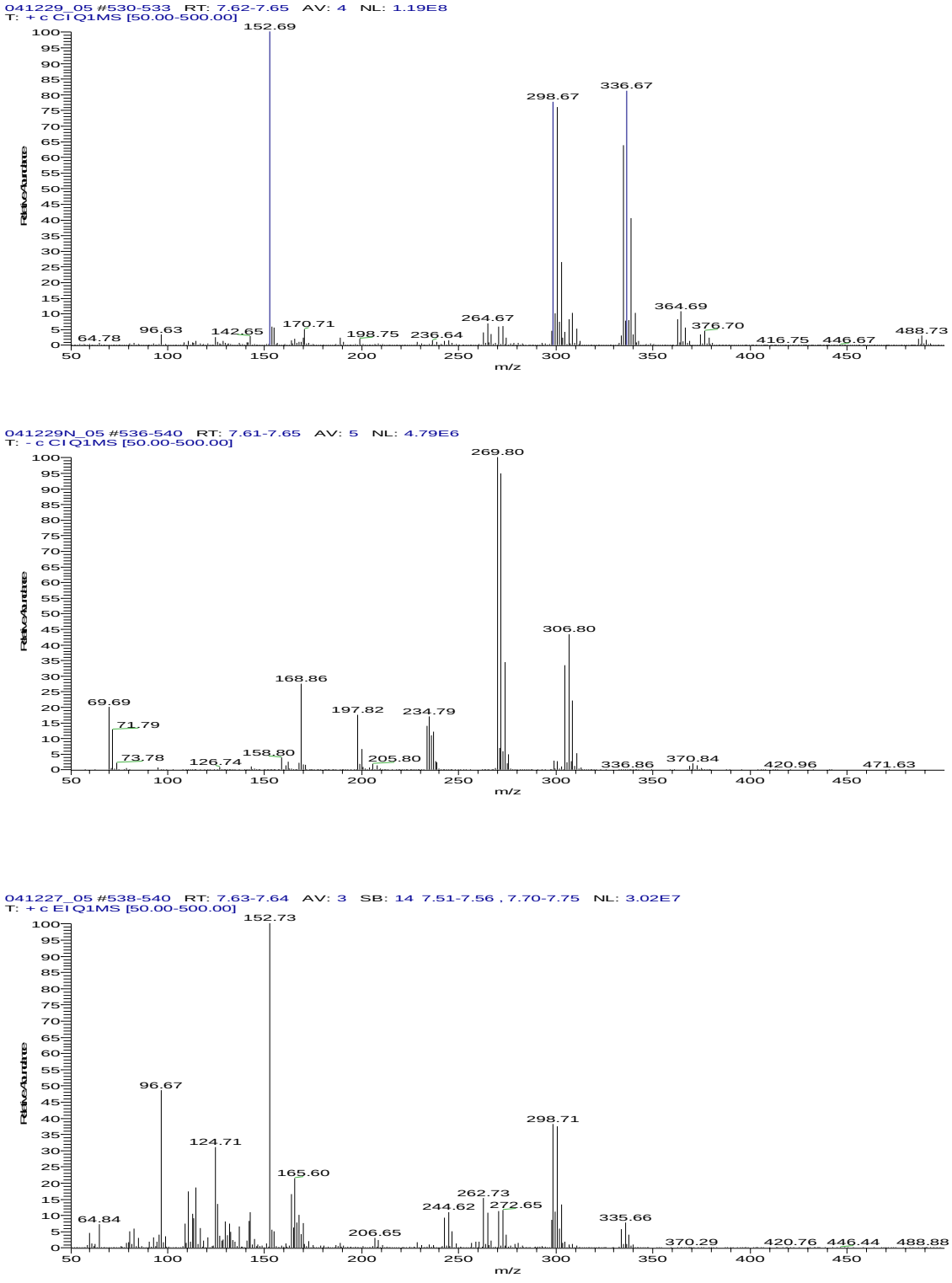


Figure 25 Chlorethoxyfos - Positive CI, Negative CI, EI spectra

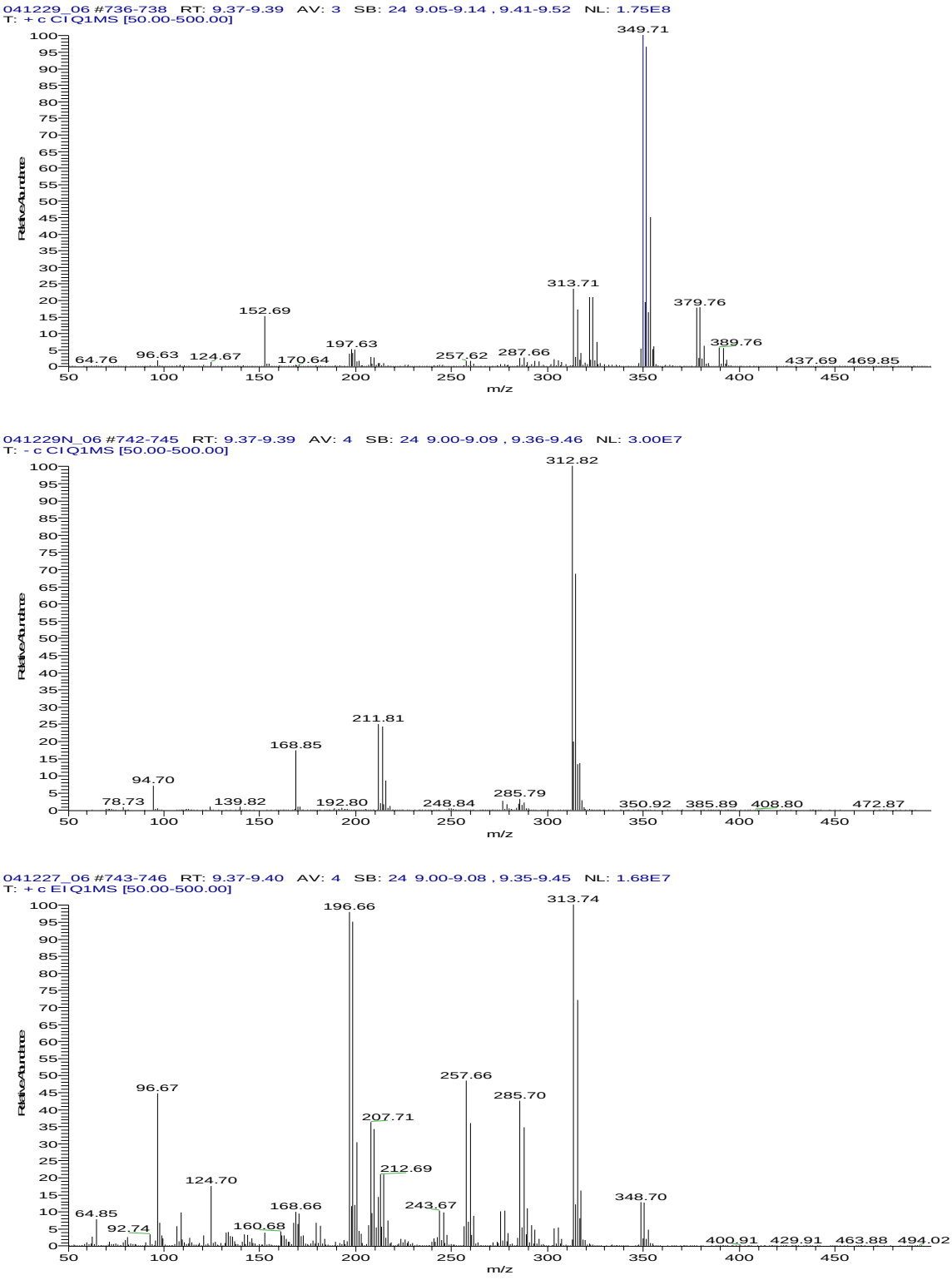


Figure 26 Chlorpirifos - Positive CI, Negative CI, EI spectra

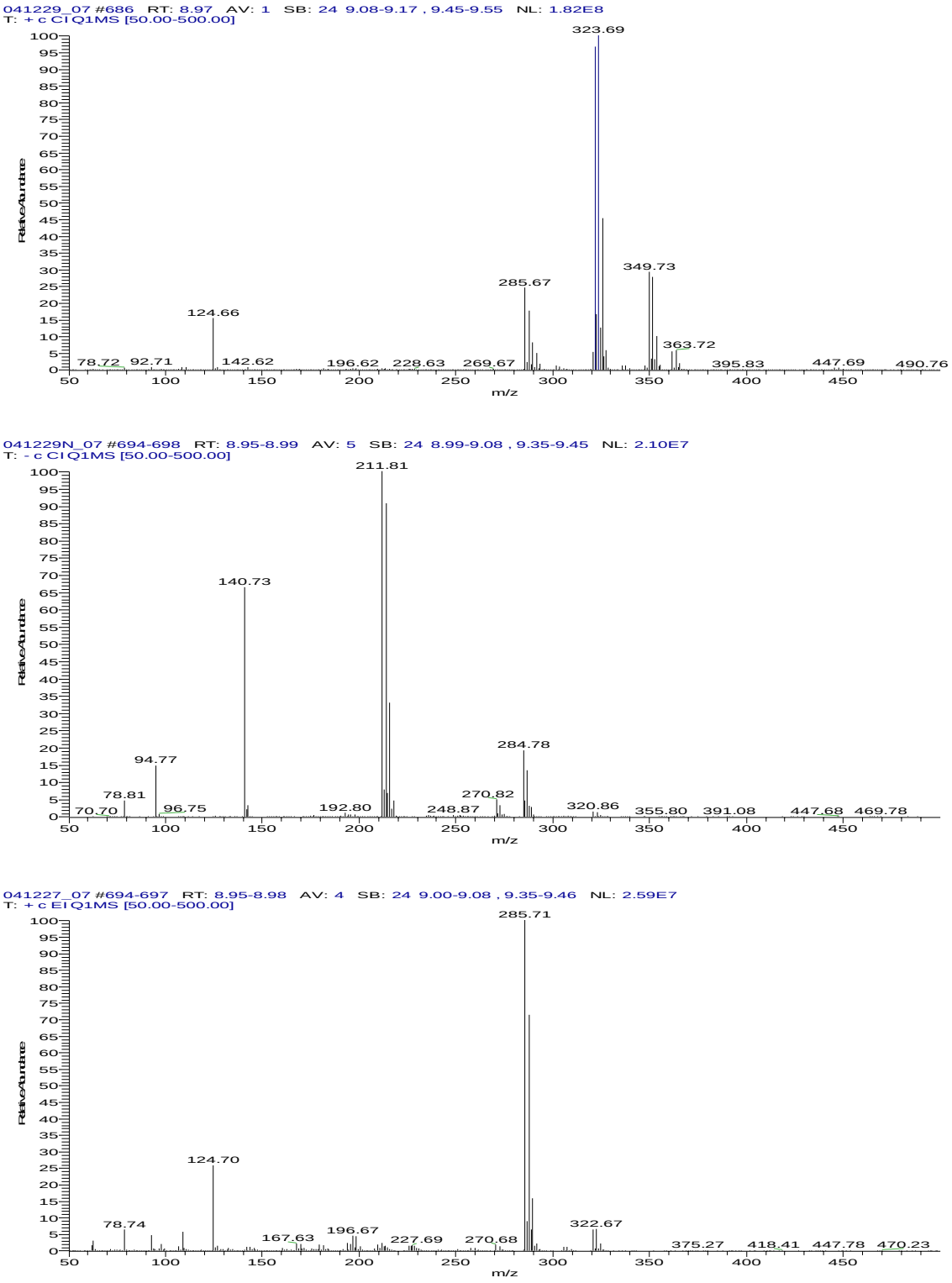


Figure 27 Chlorpyrofos Methyl- Positive CI, Negative CI, EI spectra

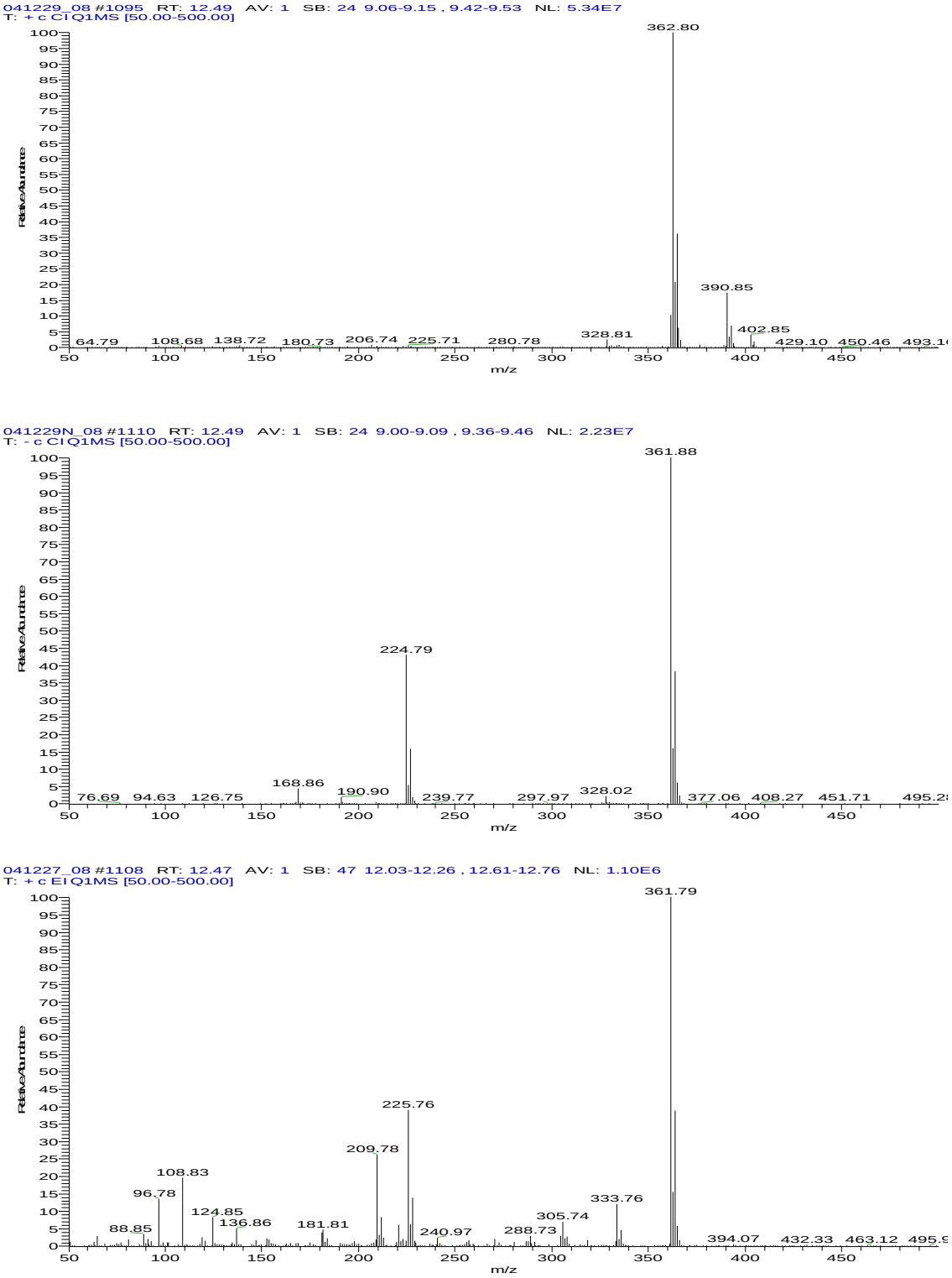


Figure 28 Coumaphos - Positive CI, Negative CI, EI spectra

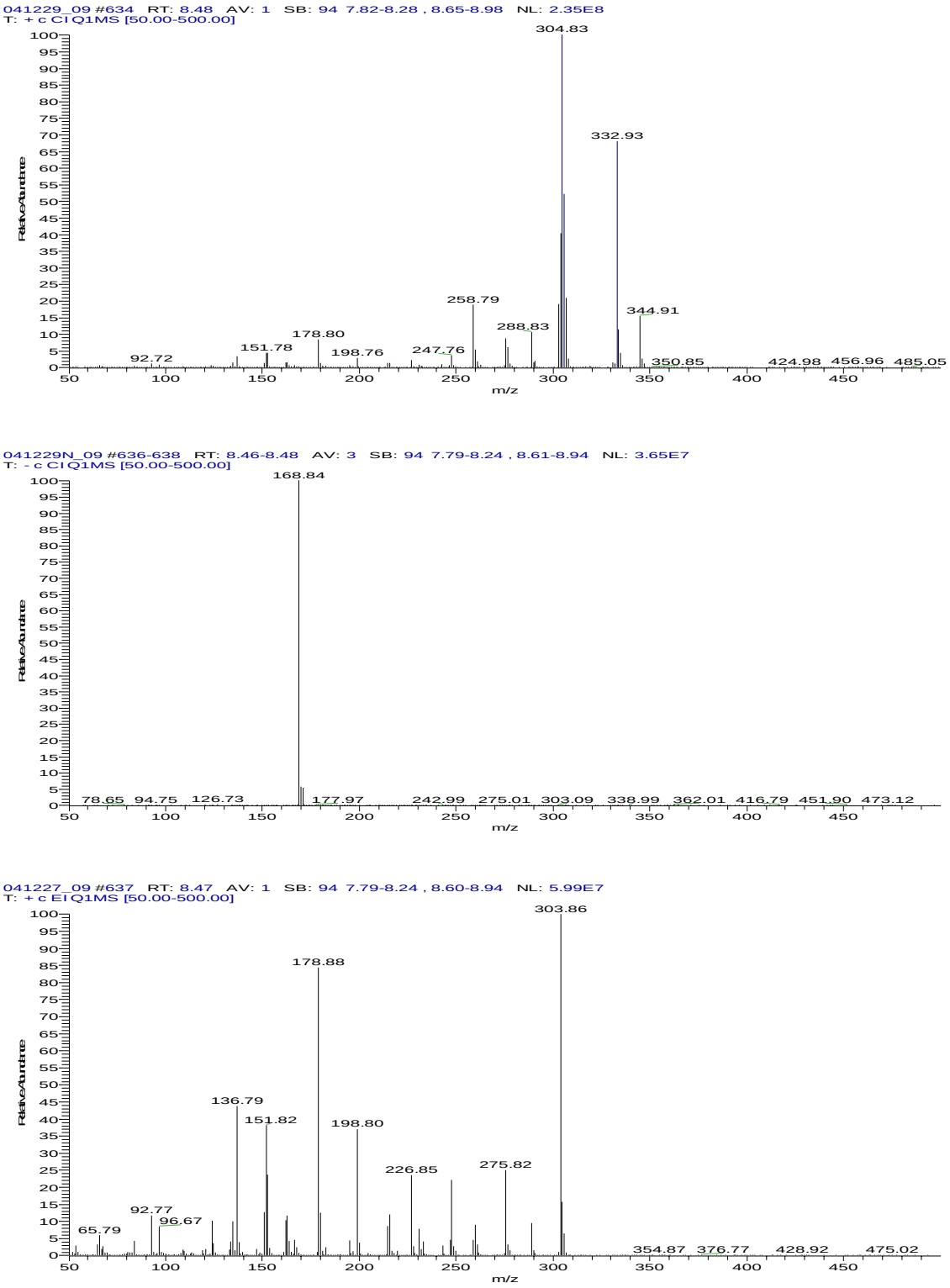


Figure 29 Diazinon - Positive CI, Negative CI, EI spectra

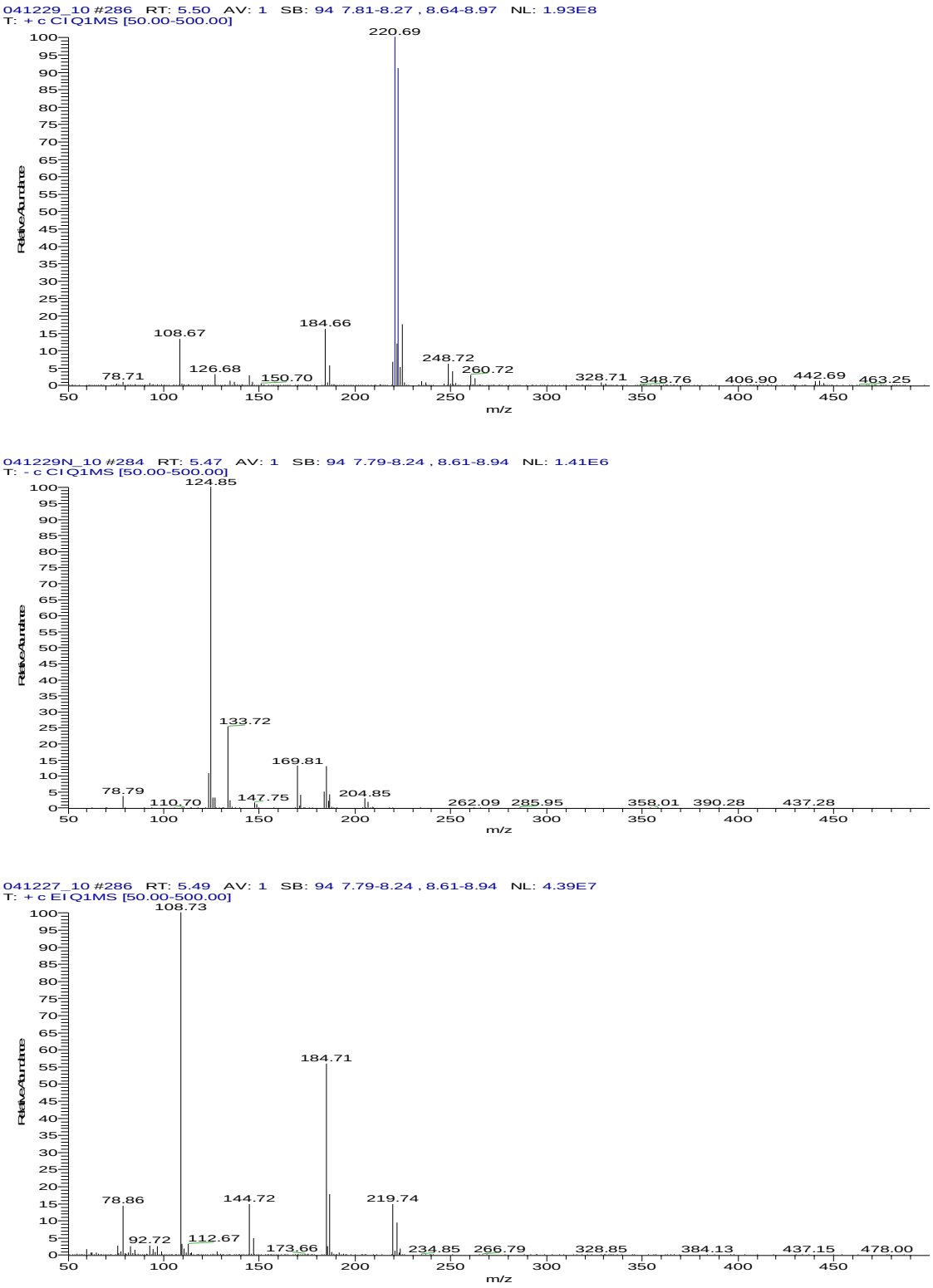


Figure 30 Dichlorvos - Positive CI, Negative CI, EI spectra

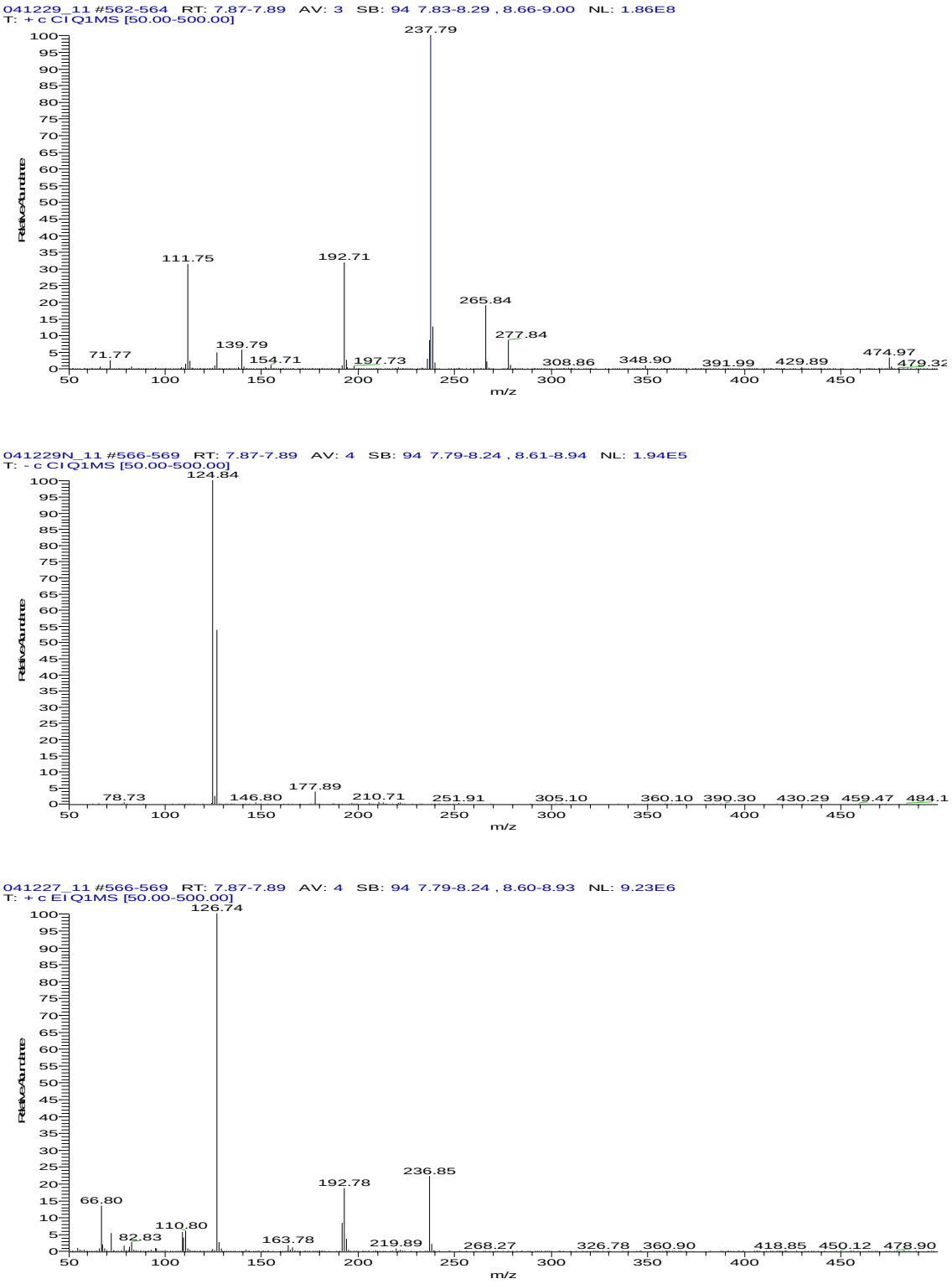


Figure 31 Dichrotophos - Positive CI, Negative CI, EI spectra

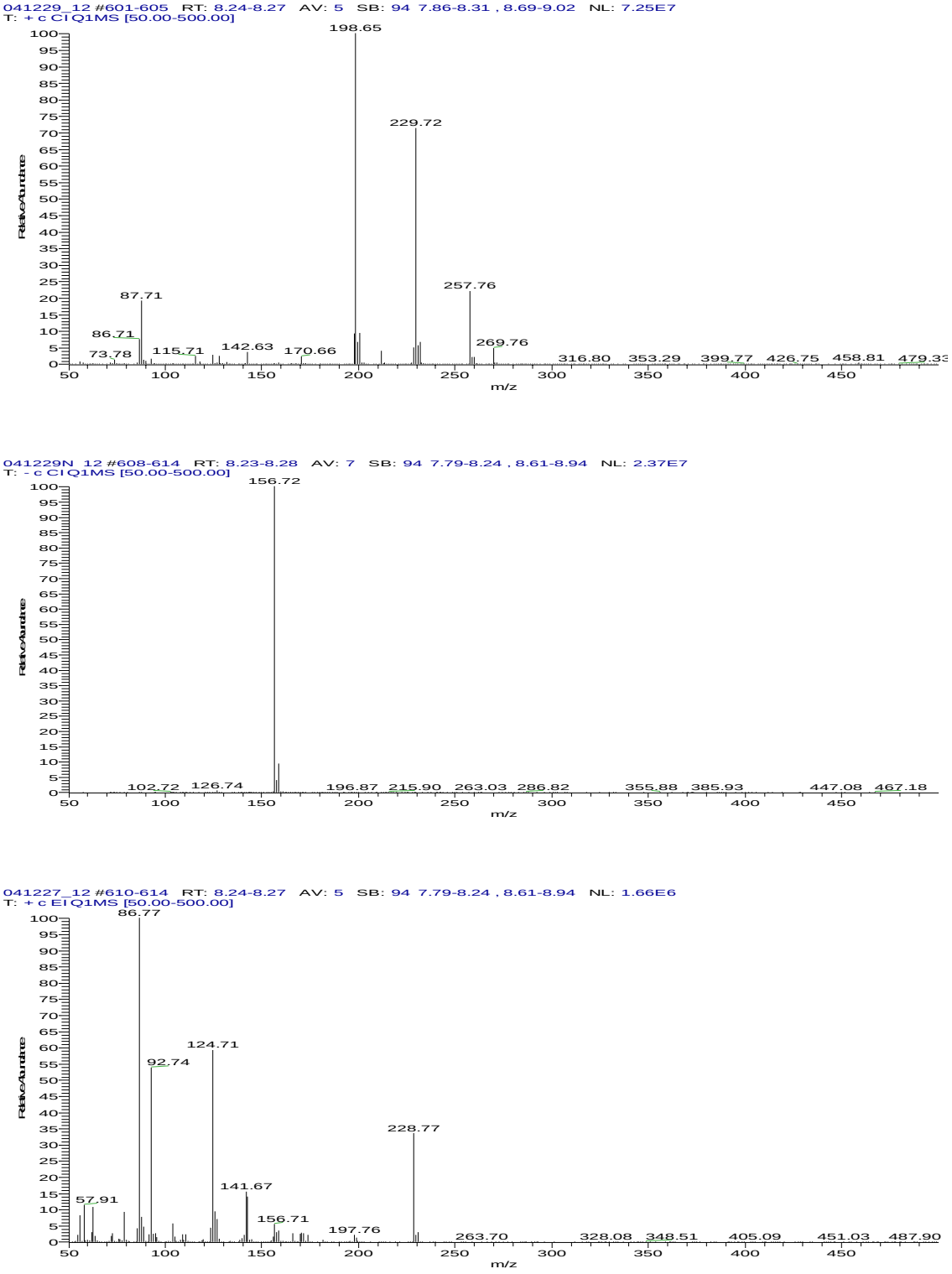


Figure 32 Dimethoate - Positive CI, Negative CI, EI spectra

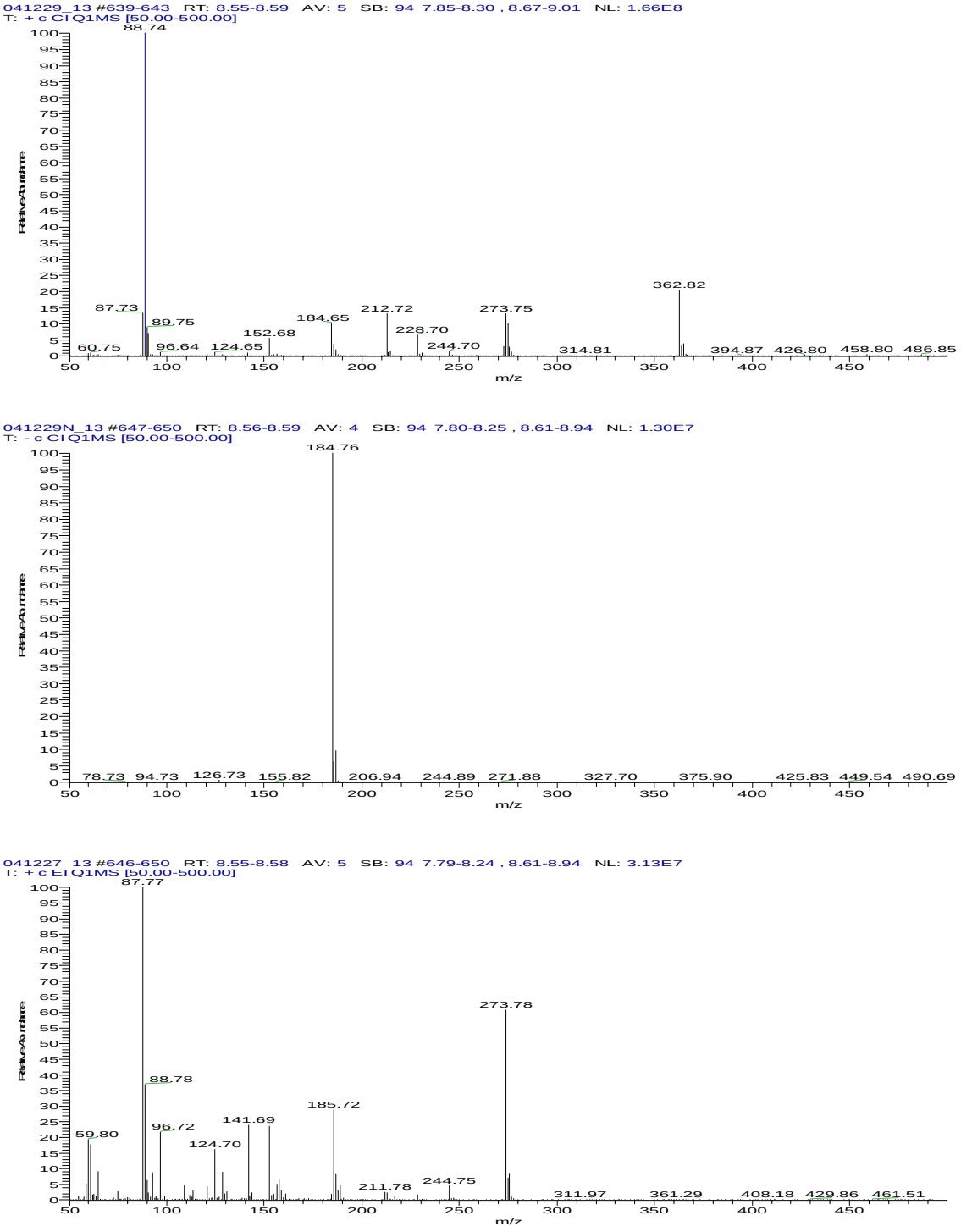


Figure 33 Disulfoton - Positive CI, Negative CI, EI spectra

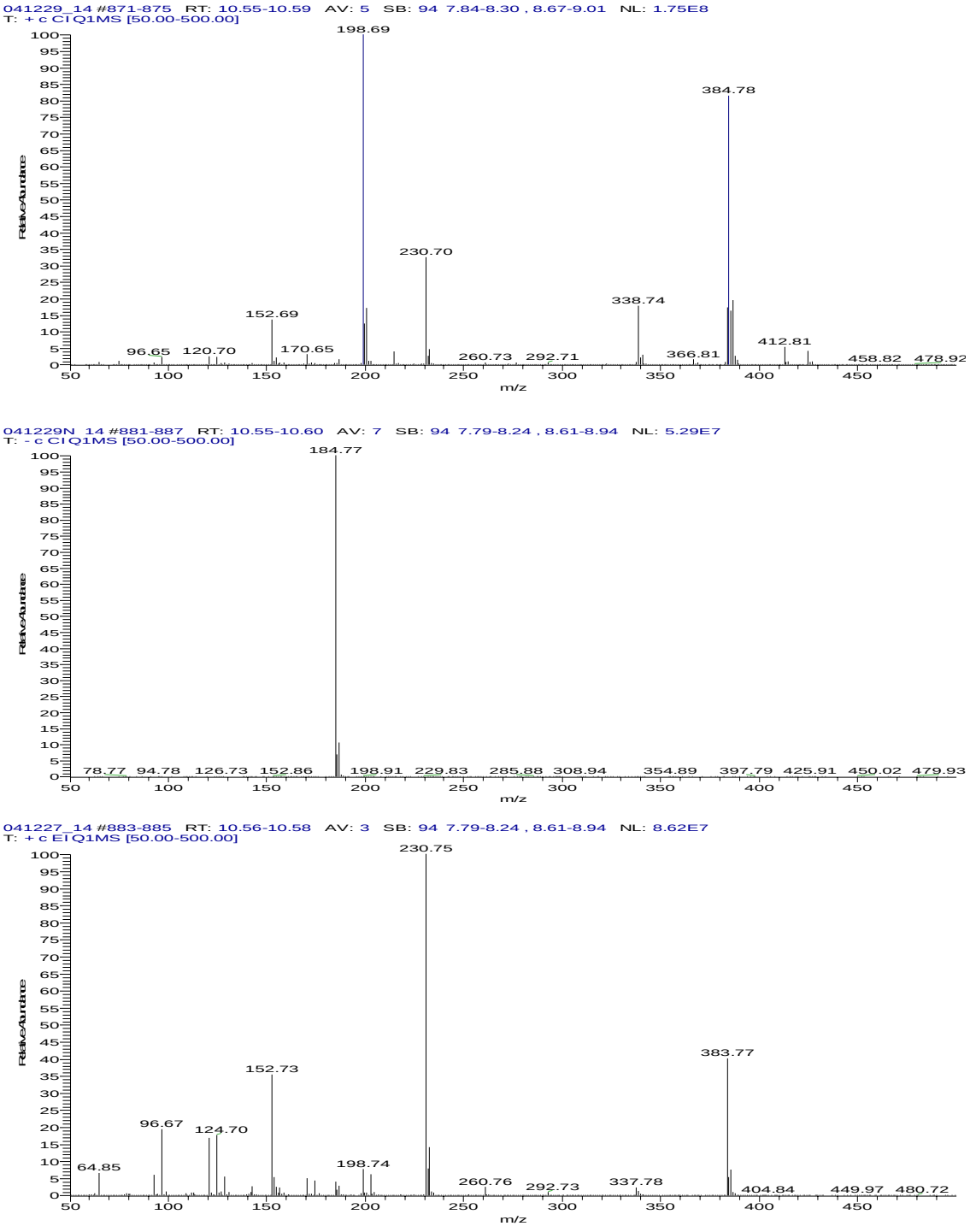


Figure 34 Ethion - Positive CI, Negative CI, EI spectra

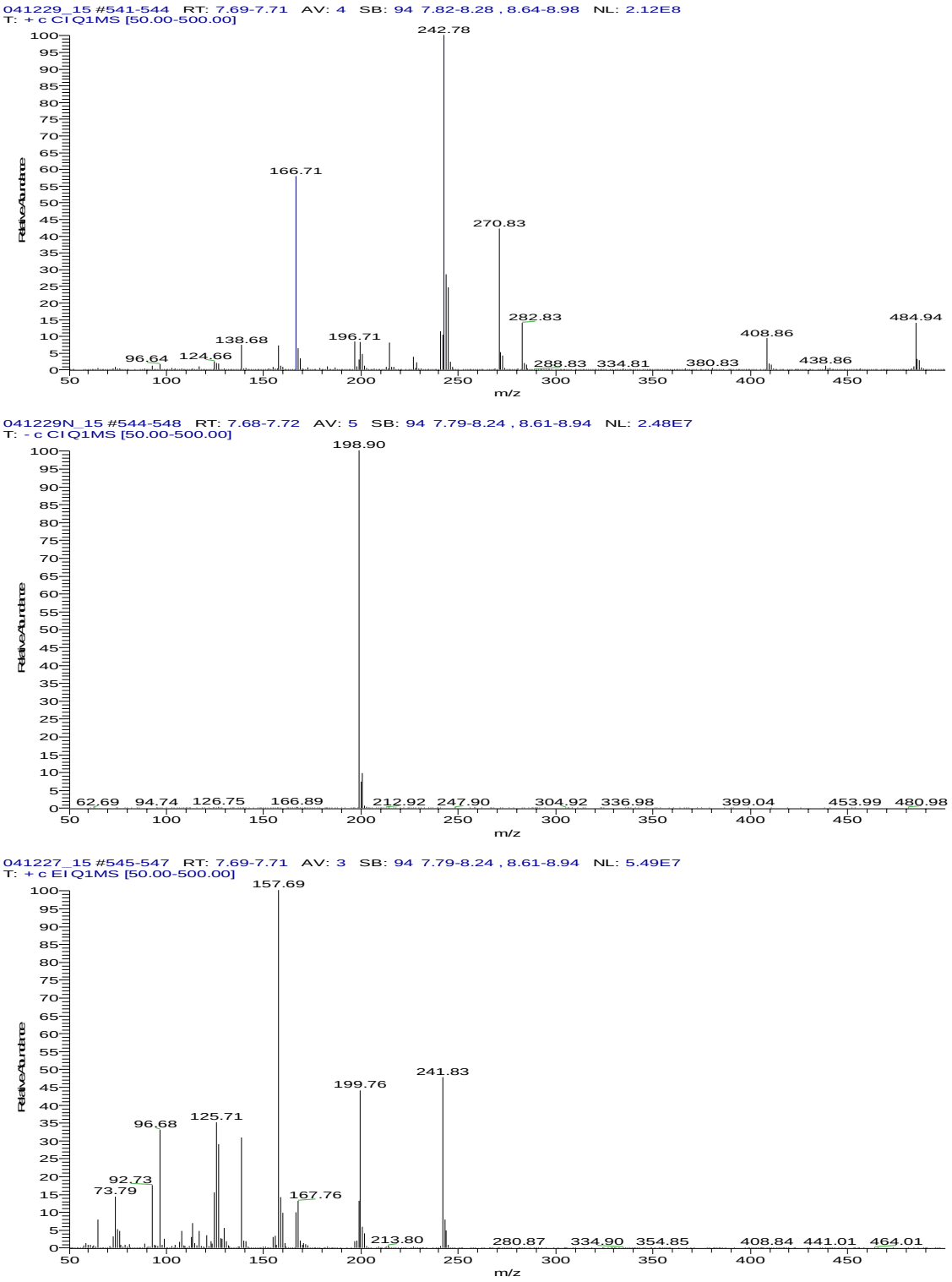


Figure 35 Ethoprop - Positive CI, Negative CI, EI spectra

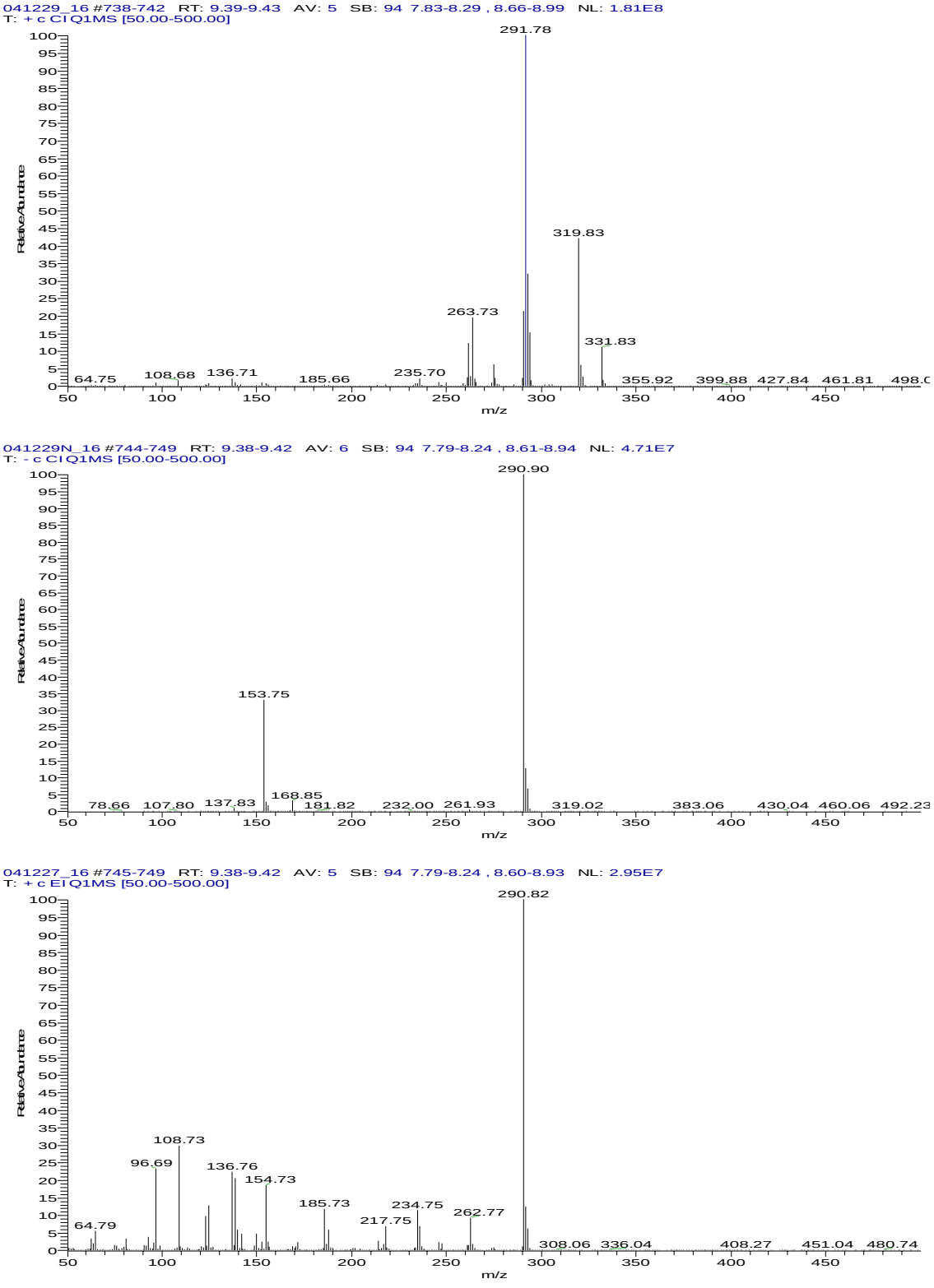


Figure 36 Ethyl Parathion - Positive CI, Negative CI, EI spectra

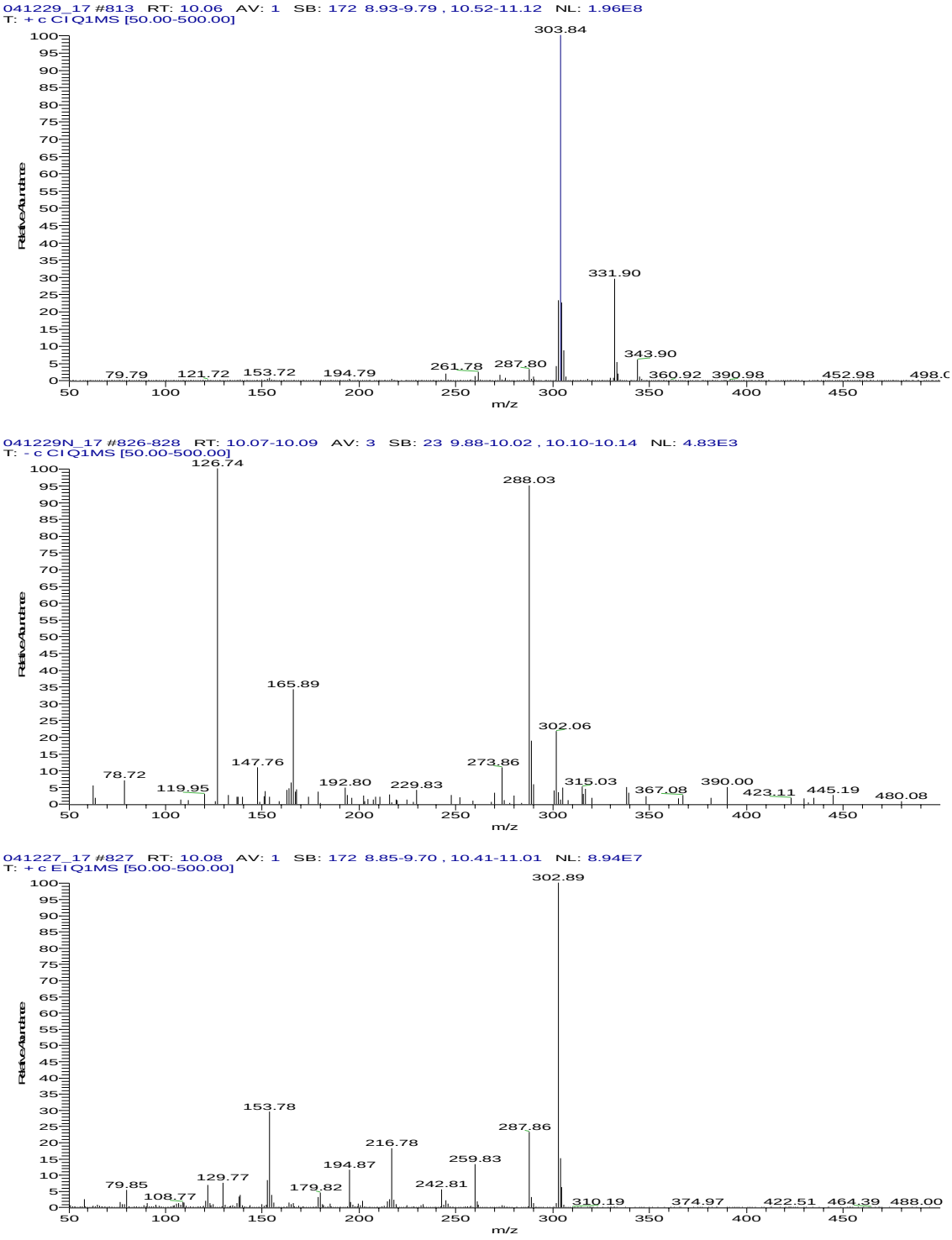


Figure 37 Fenamiphos - Positive CI, Negative CI, EI spectra

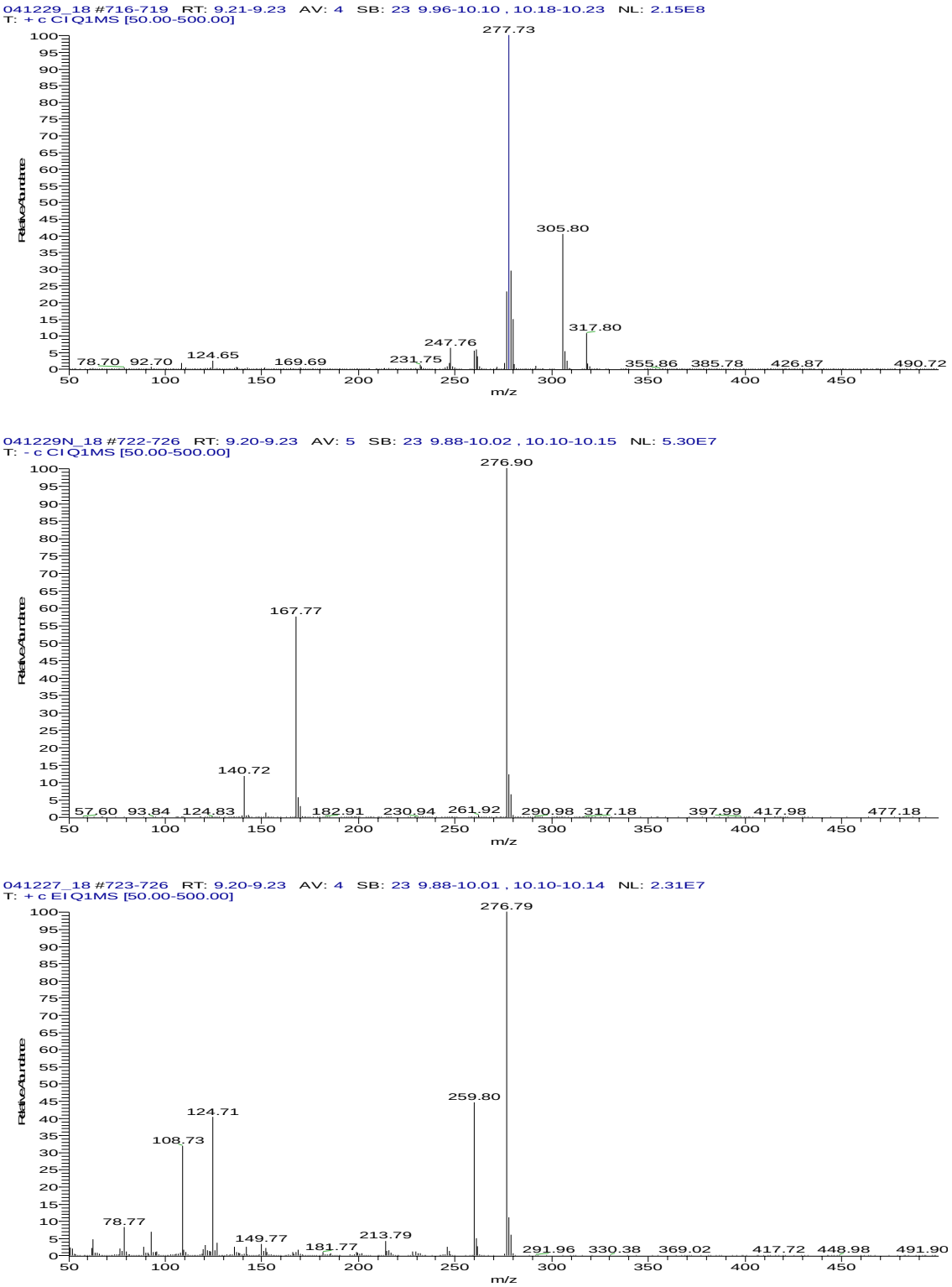


Figure 38 Fenitrothion - Positive CI, Negative CI, EI spectra

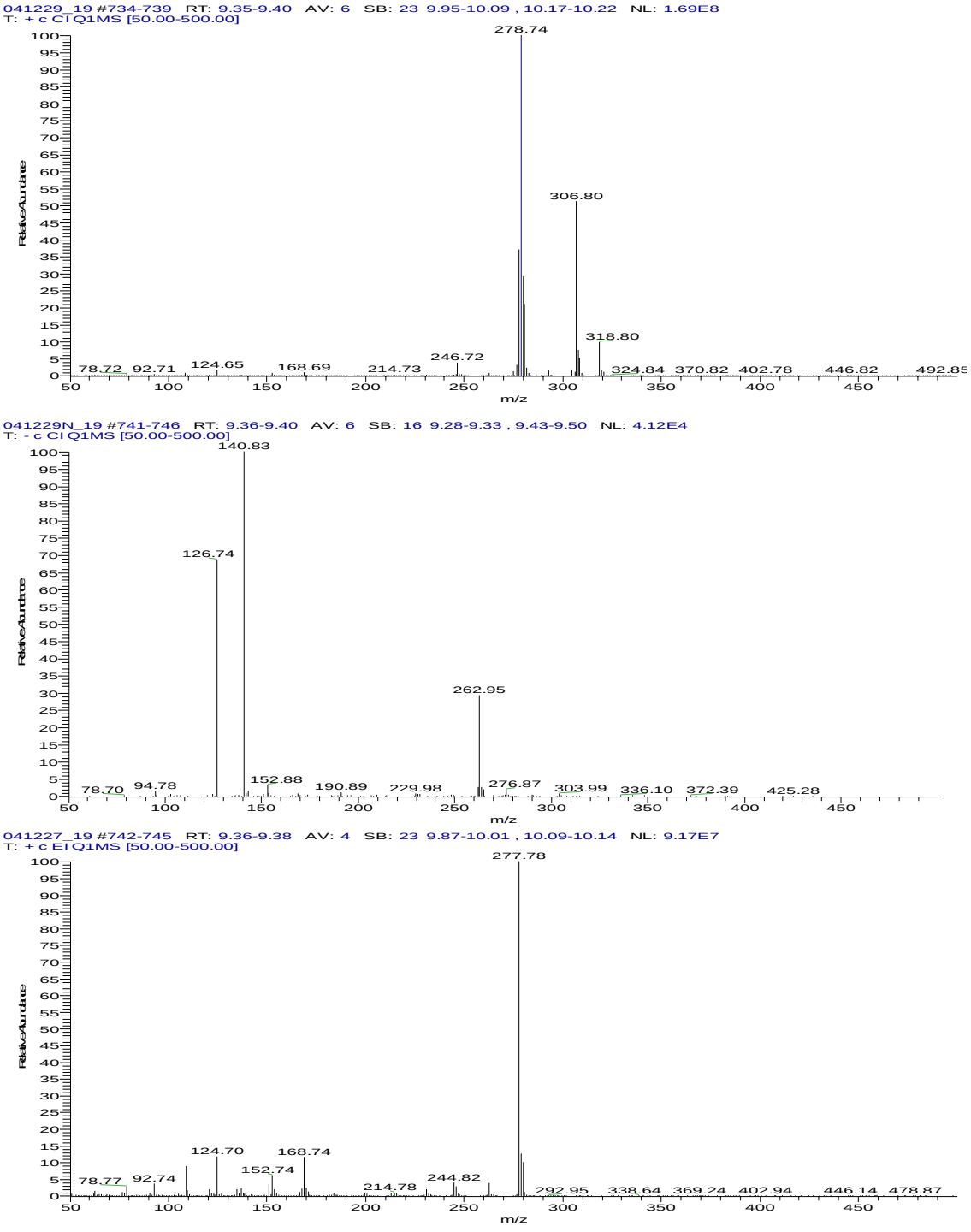


Figure 39 Fenthion - Positive CI, Negative CI, EI spectra

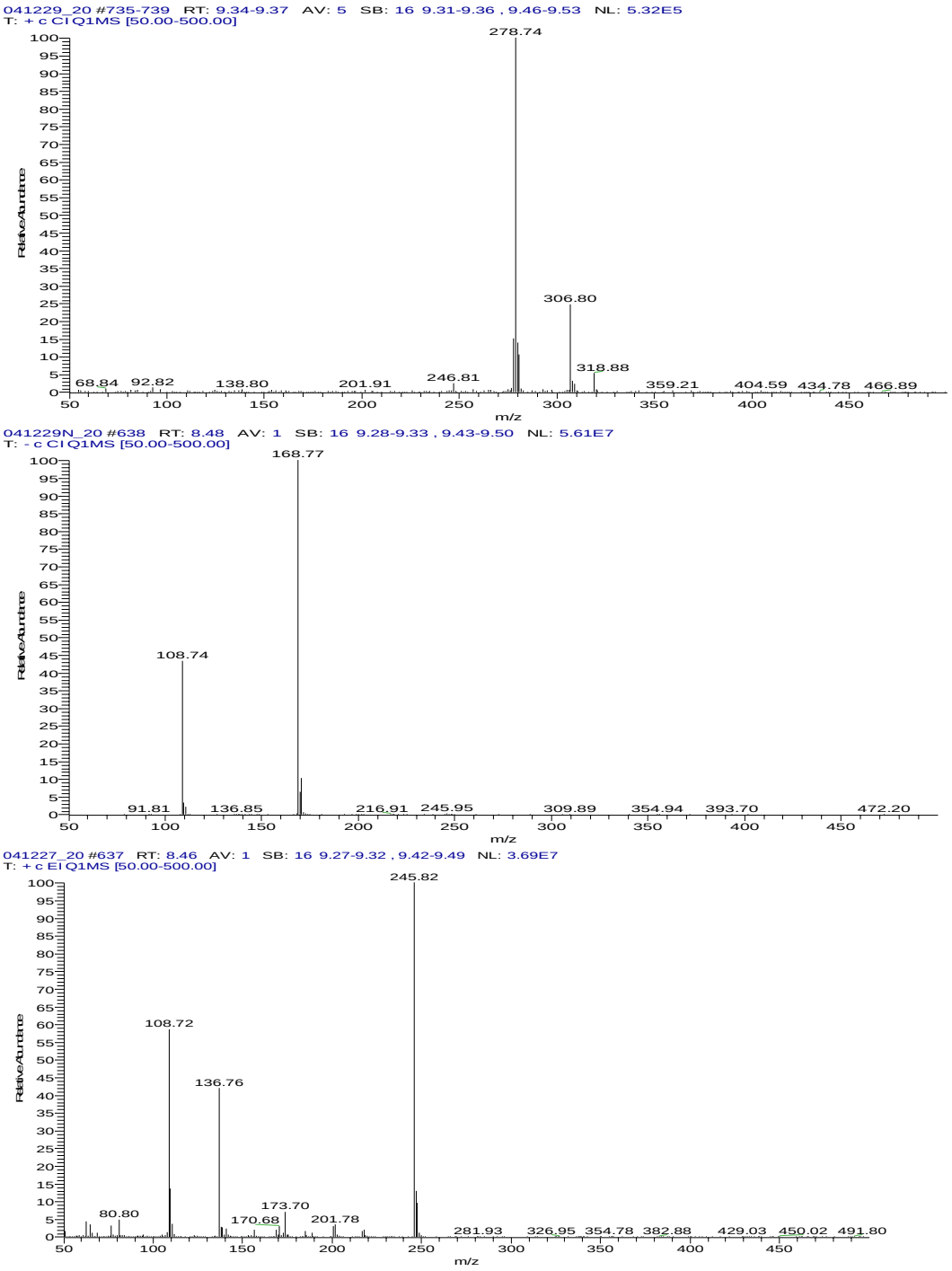


Figure 40 Fonofos- Positive CI, Negative CI, EI spectra

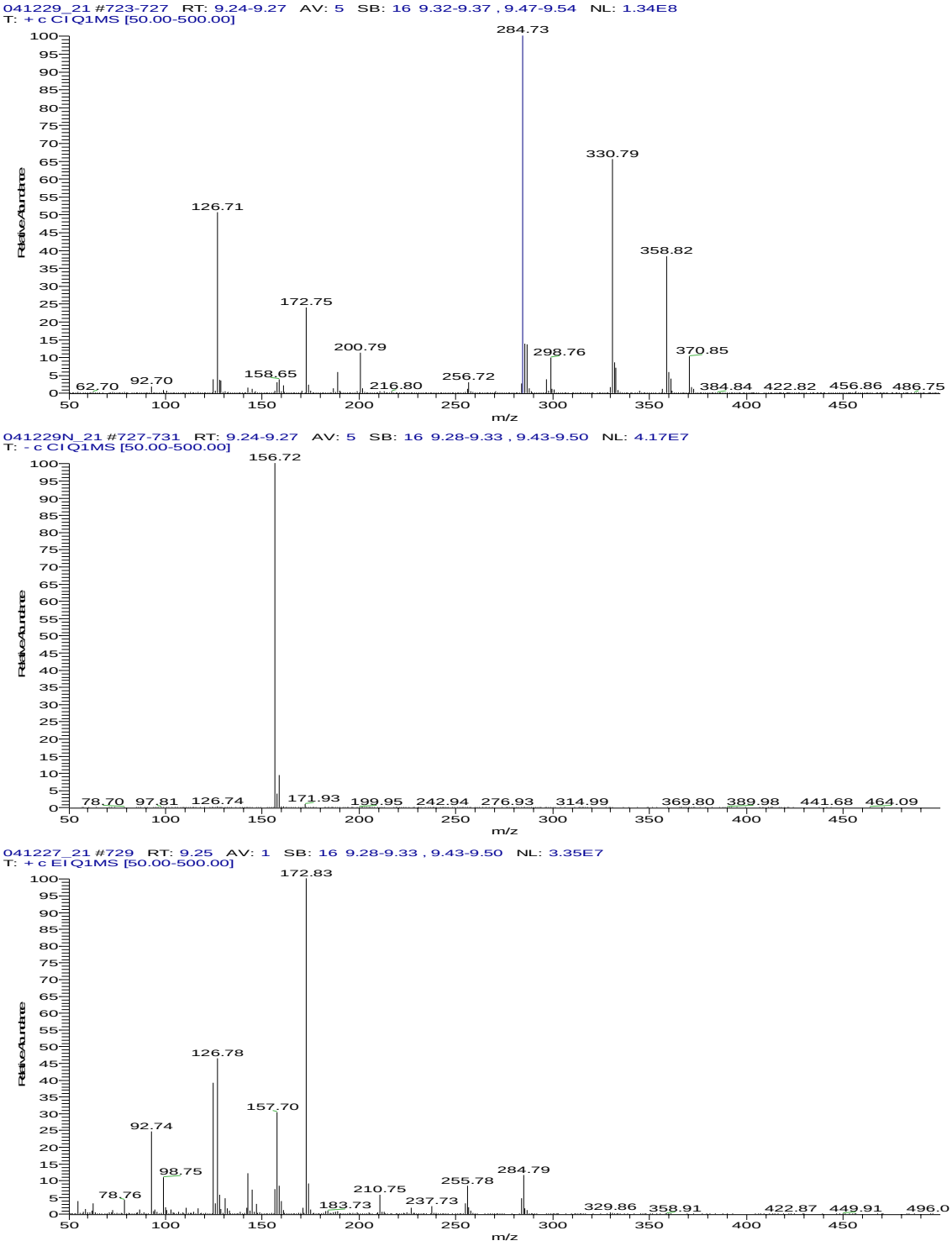


Figure 41 Malathion - Positive CI, Negative CI, EI spectra

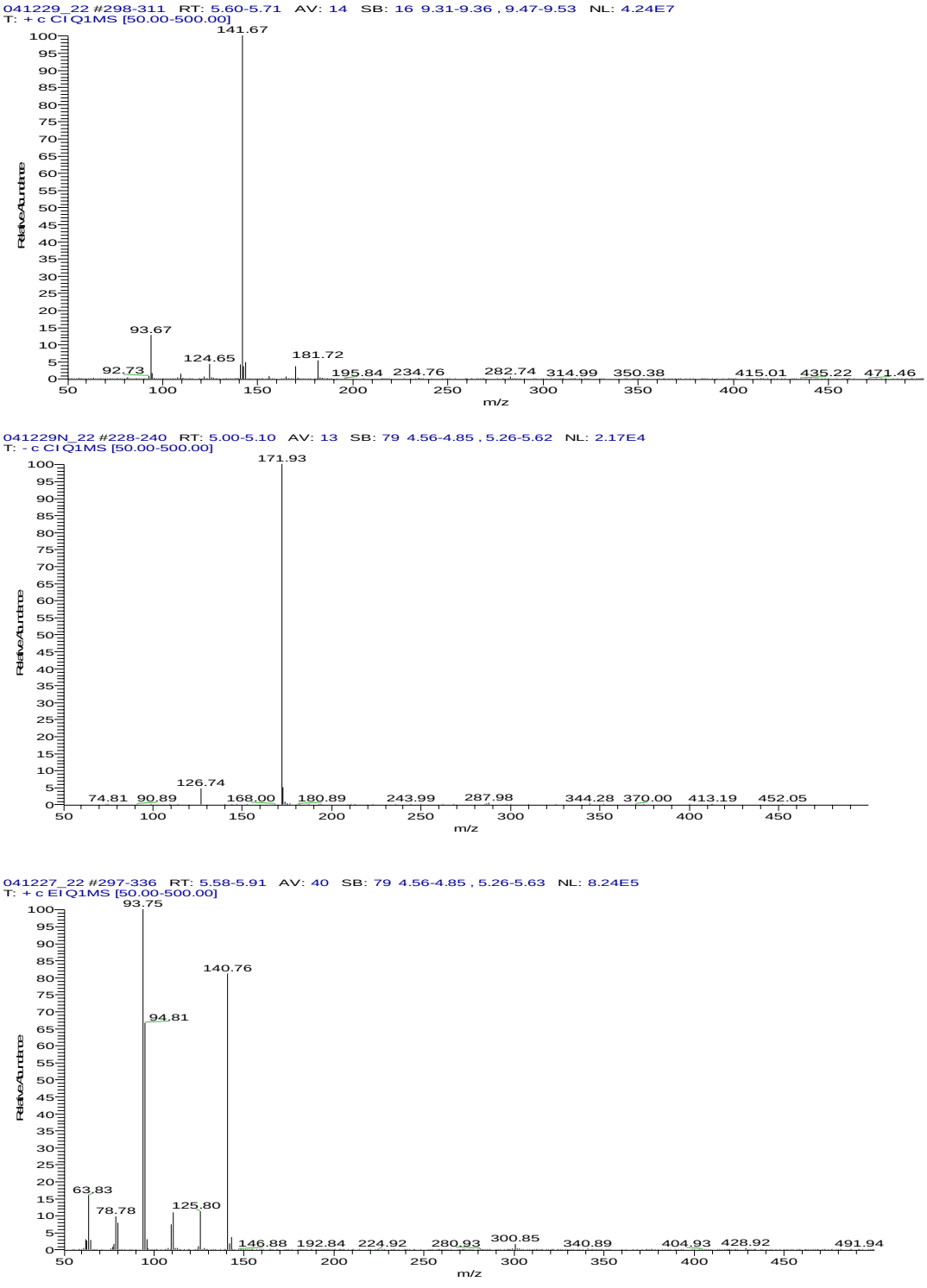


Figure 42 Methamidophos- Positive CI, Negative CI, EI spectra

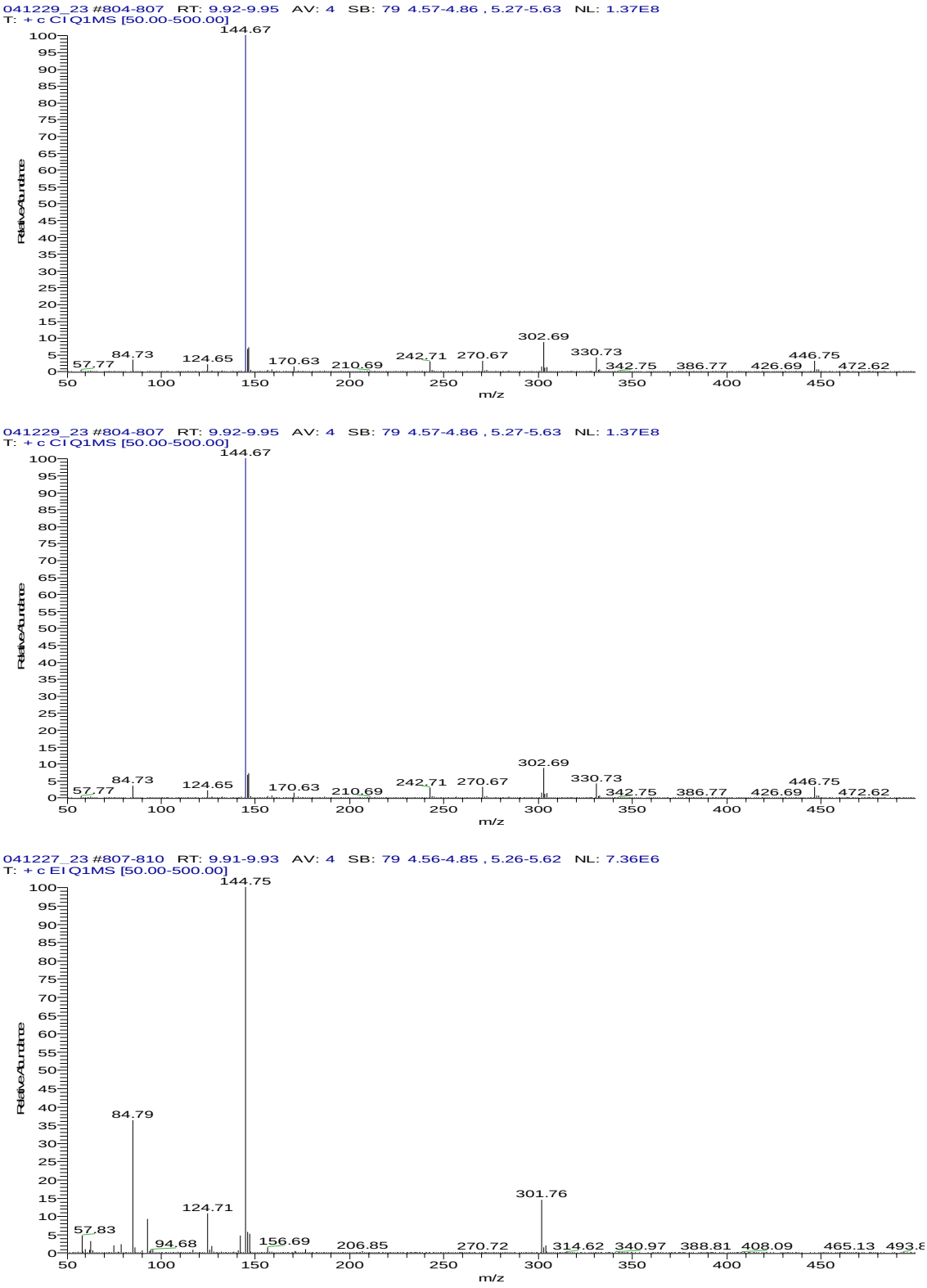


Figure 43 Methidathion - Positive CI, Negative CI, EI spectra

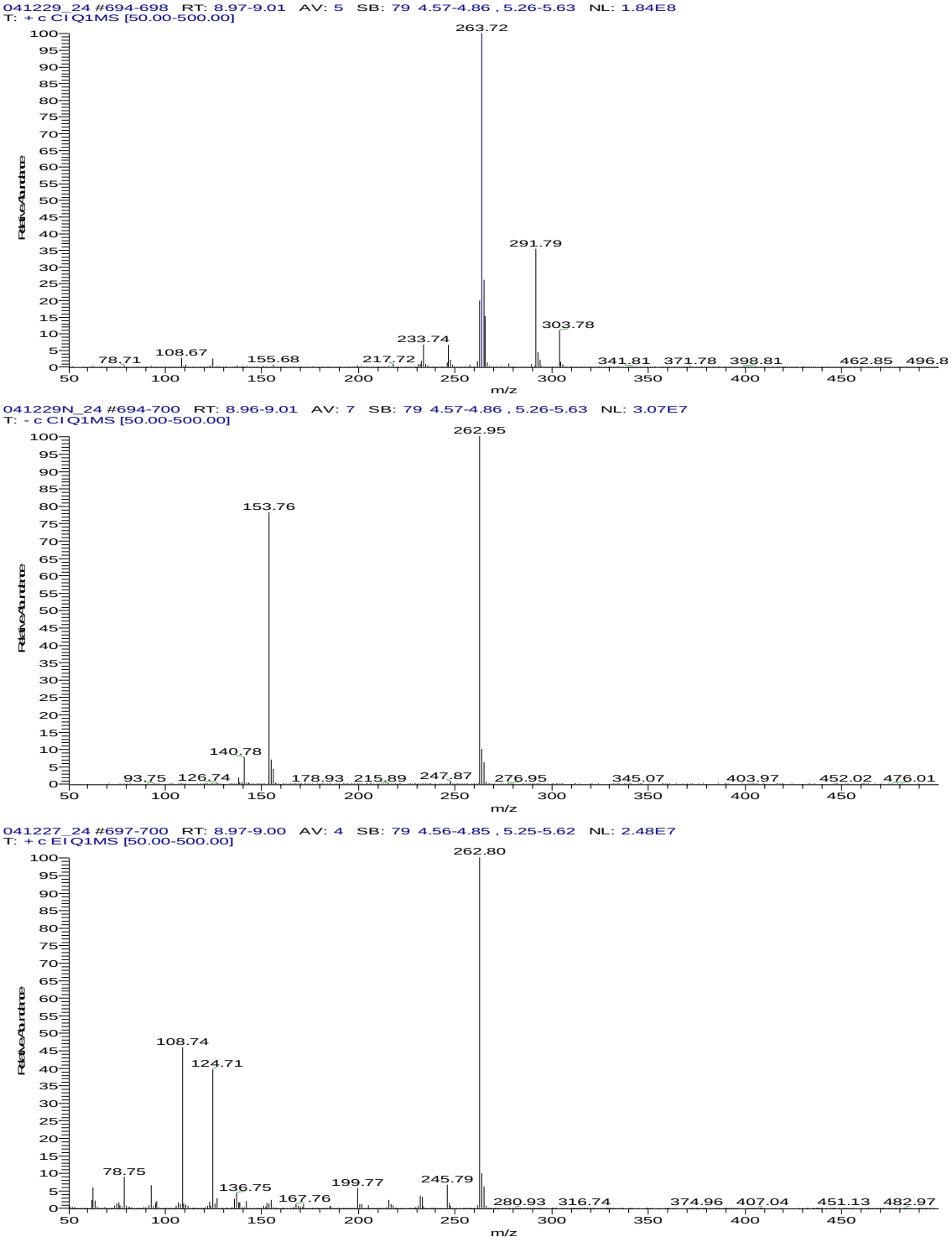


Figure 44 Methyl parathion - Positive CI, Negative CI, EI spectra

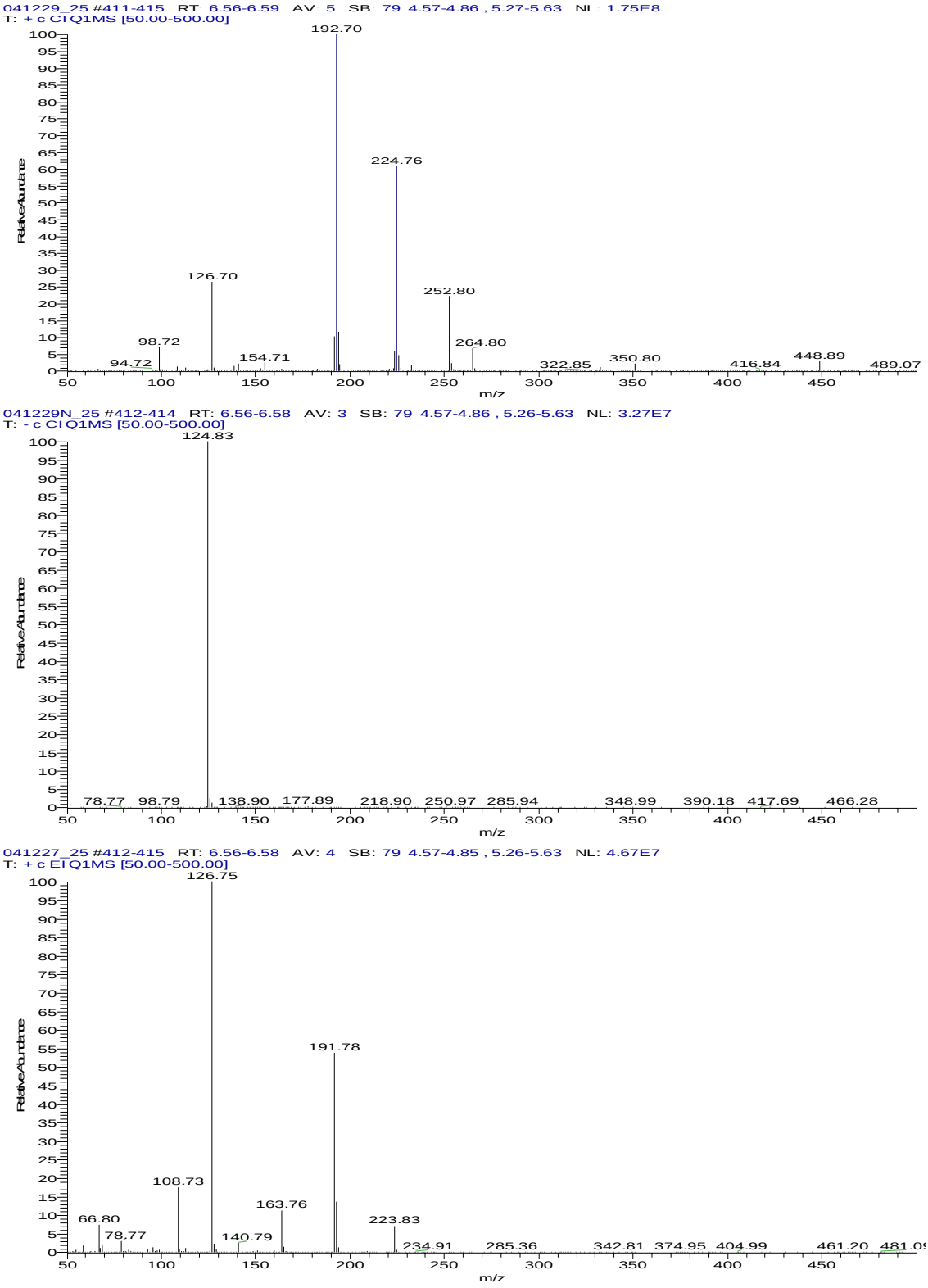


Figure 45 Mevinphos - Positive CI, Negative CI, EI spectra

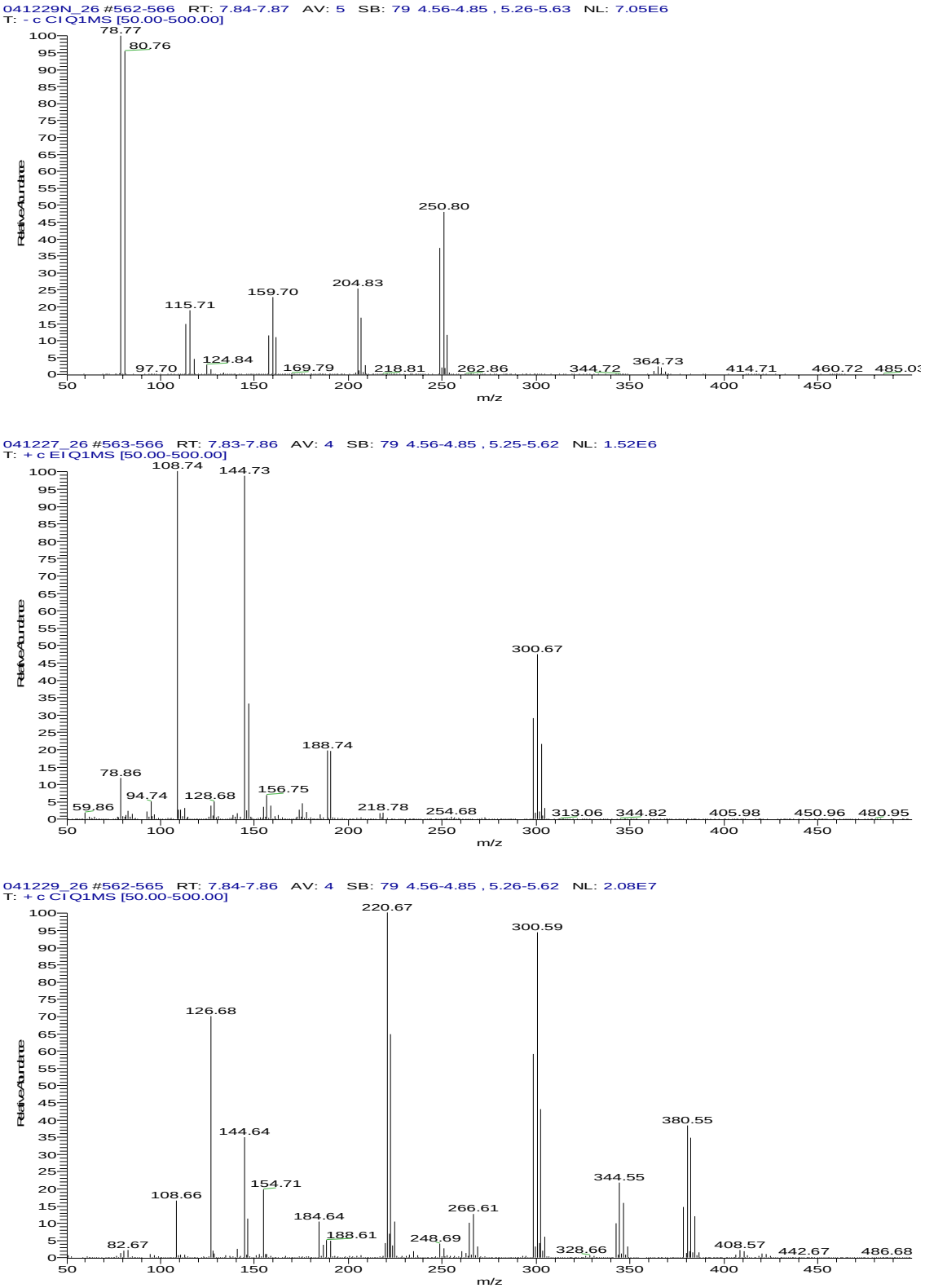


Figure 46 Naled - Positive CI, Negative CI, EI spectra

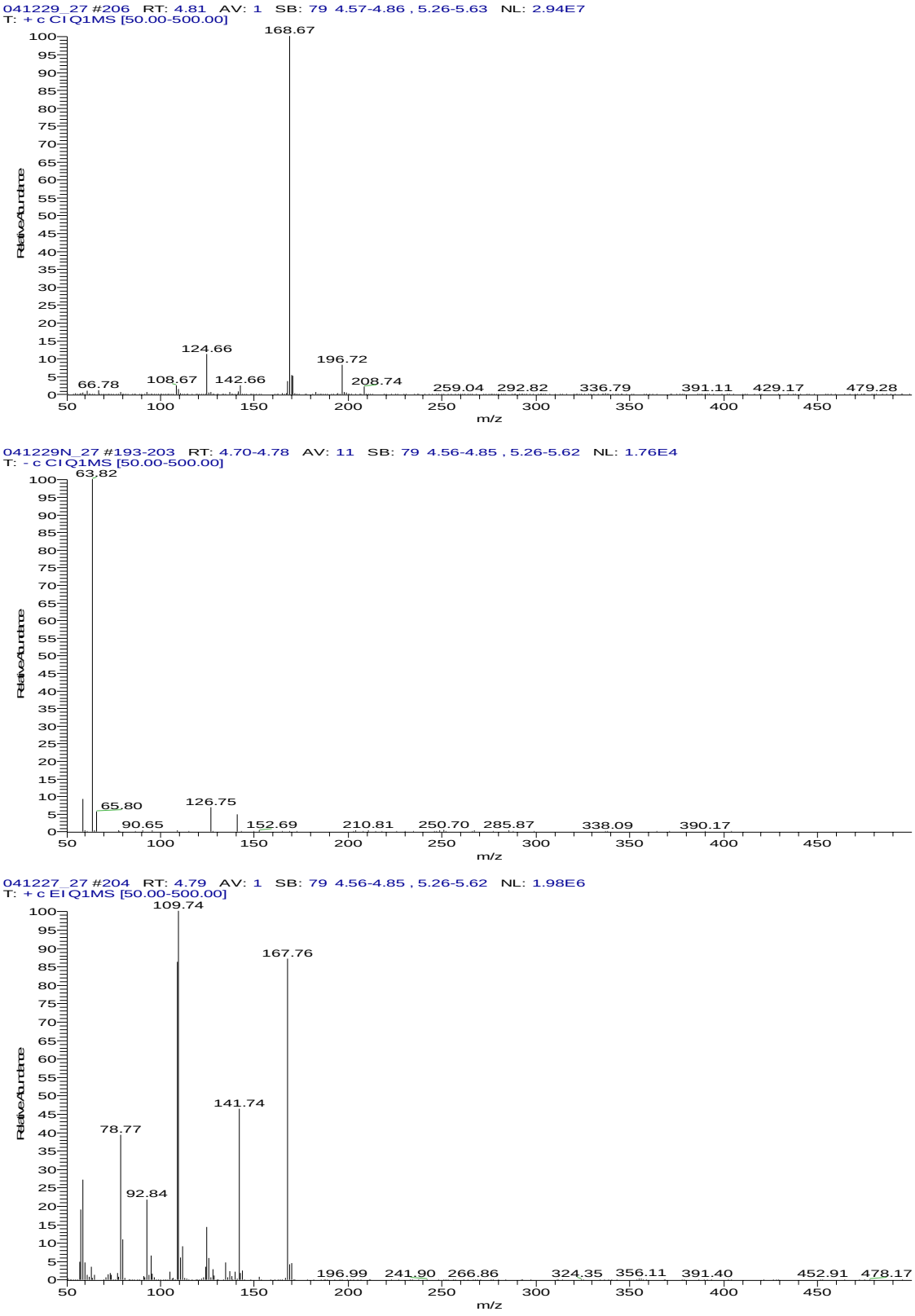


Figure 47 Oxydemethon methyl - Positive CI, Negative CI, EI spectra

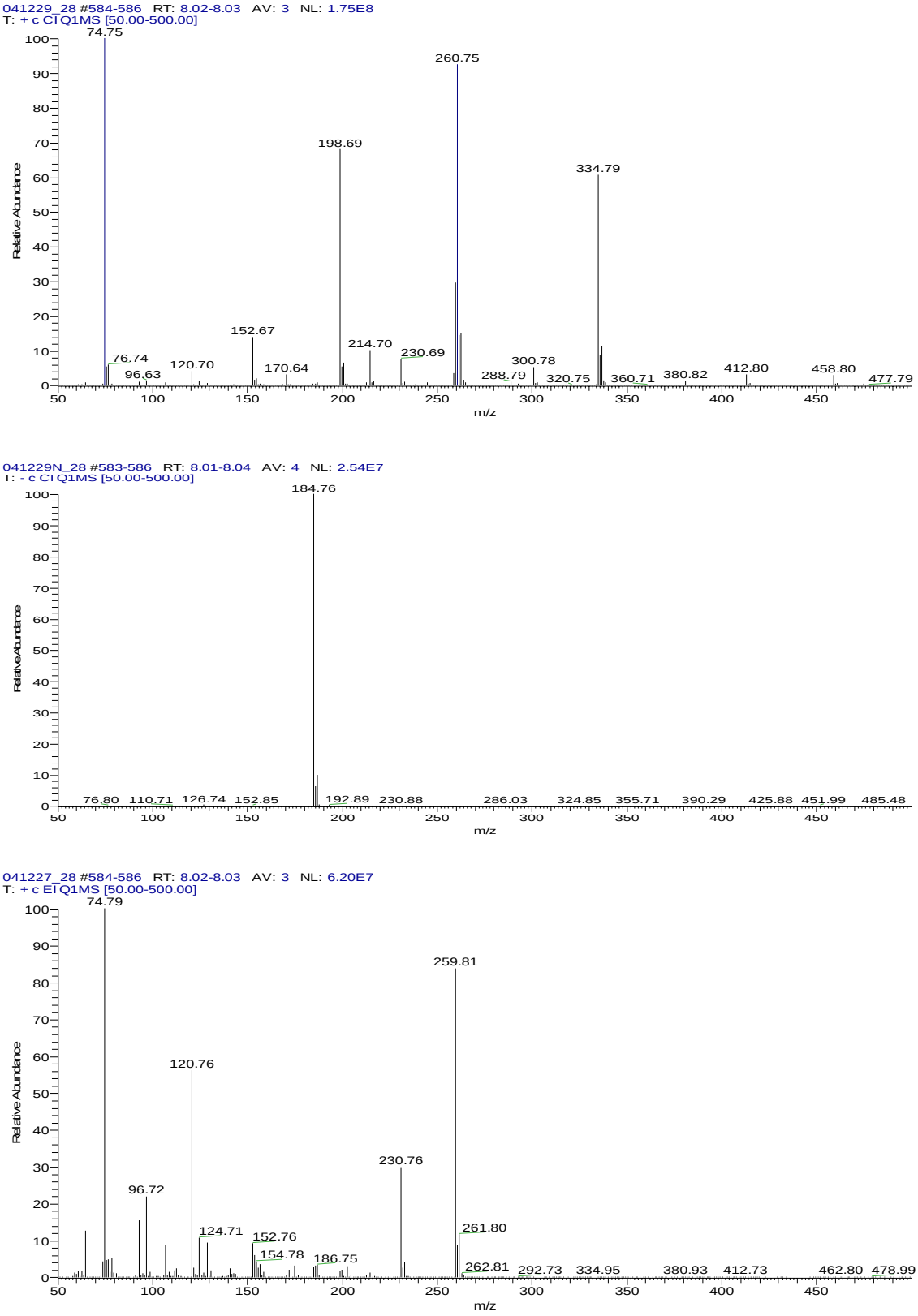


Figure 48 Phorate - Positive CI, Negative CI, EI spectra

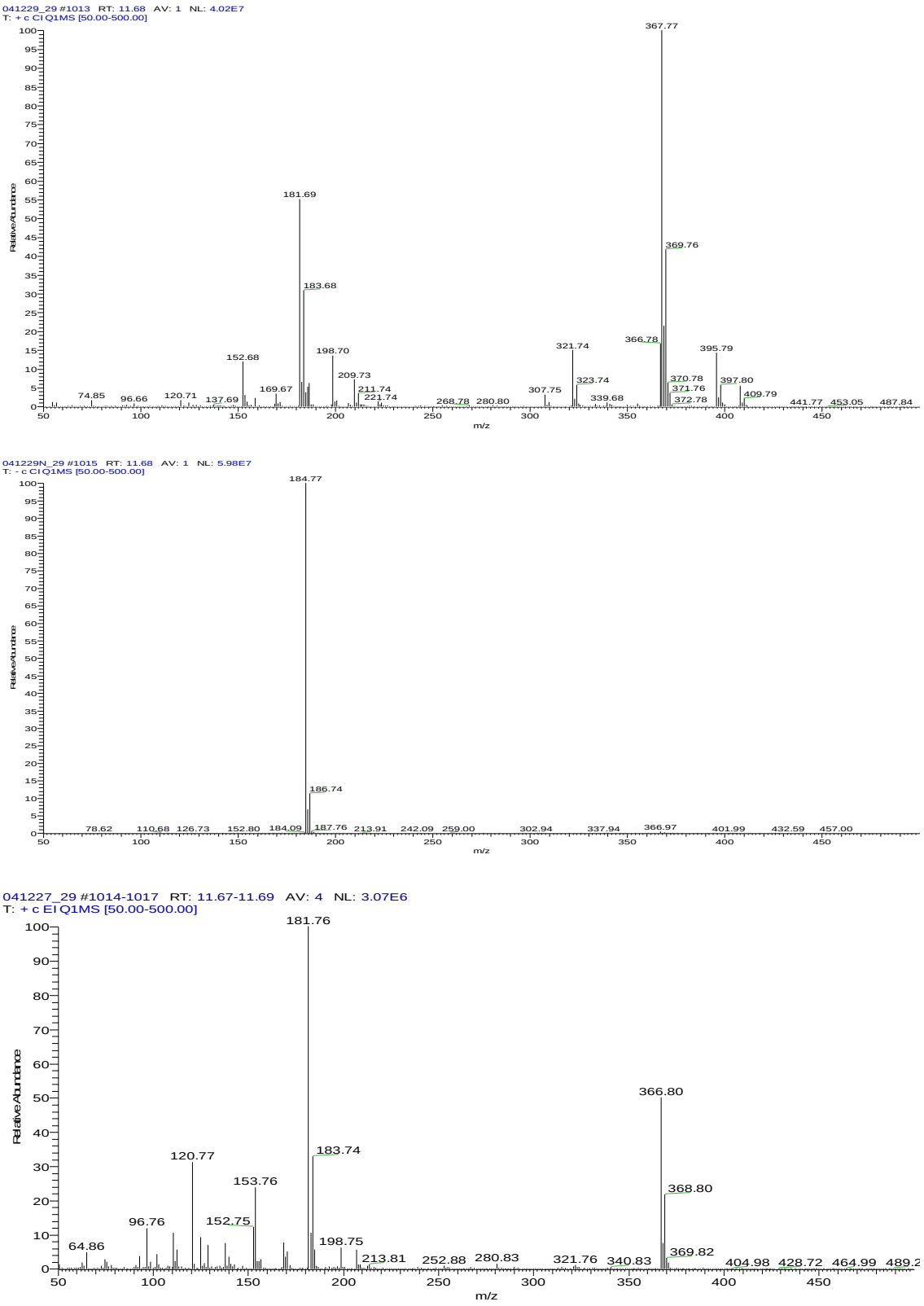


Figure 49 Phosalone - Positive CI, Negative CI, EI spectra

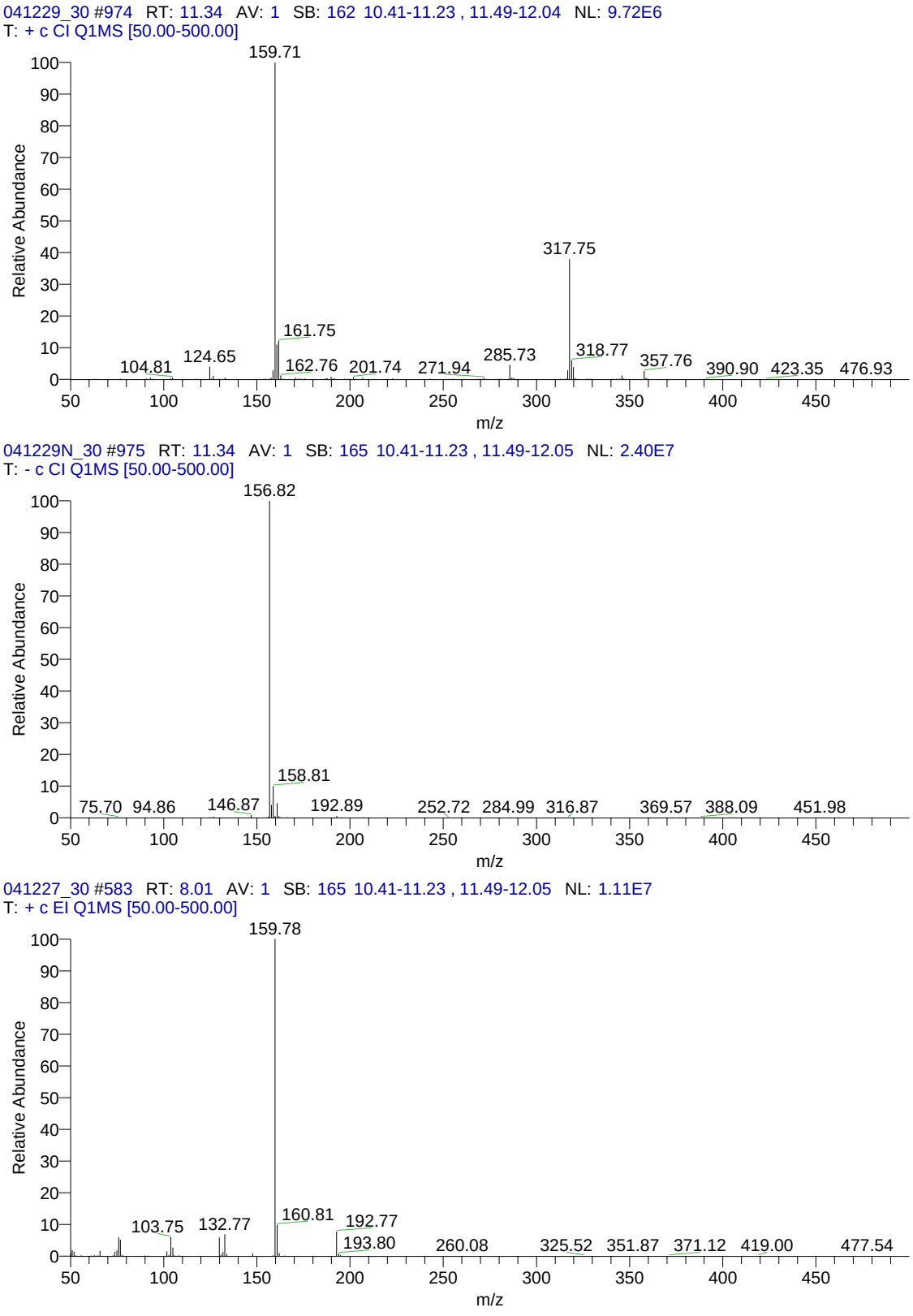


Figure 50 Phosmet - Positive CI, Negative CI, EI spectra

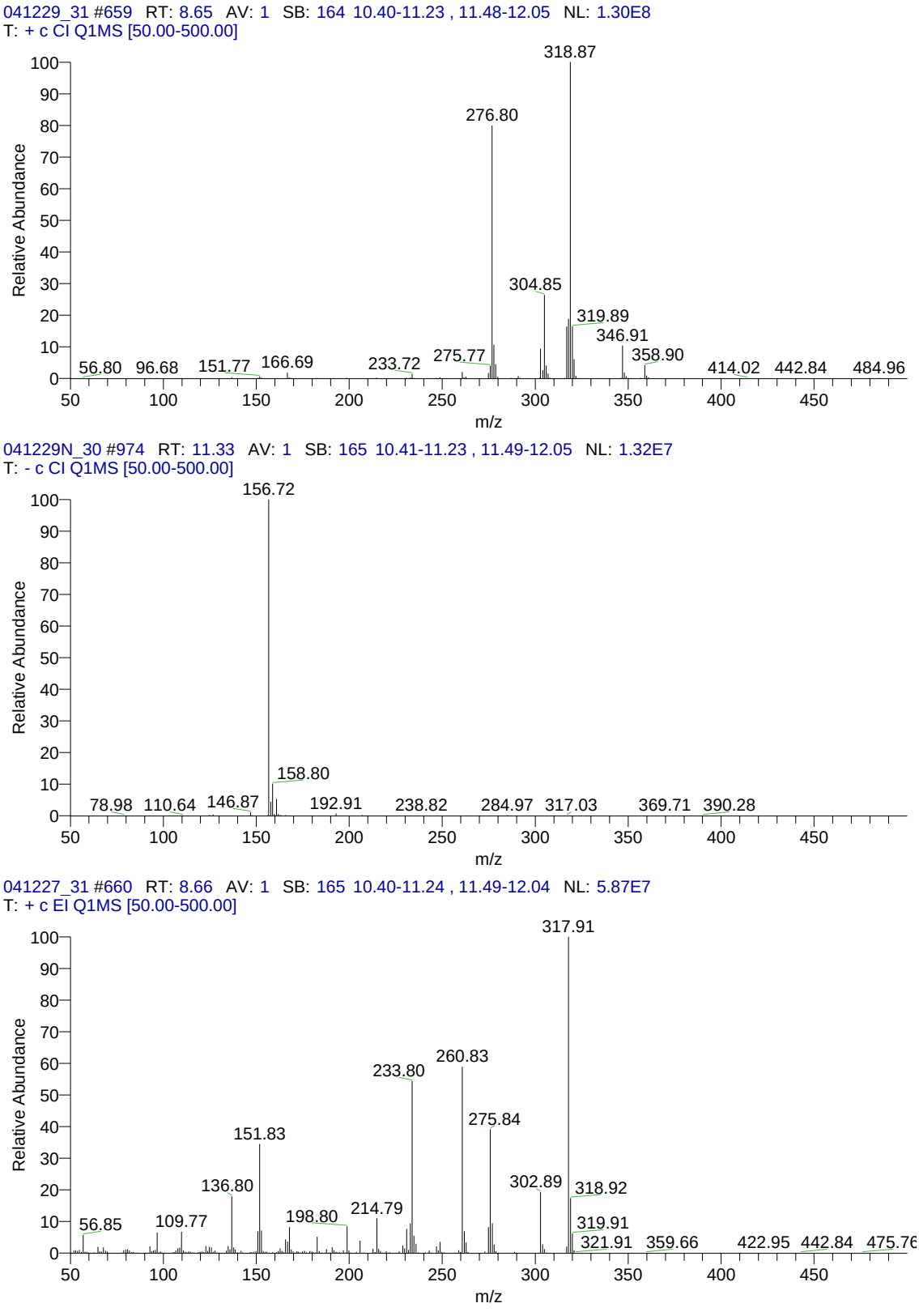


Figure 51 Phostebupirim - Positive CI, Negative CI, EI spectra

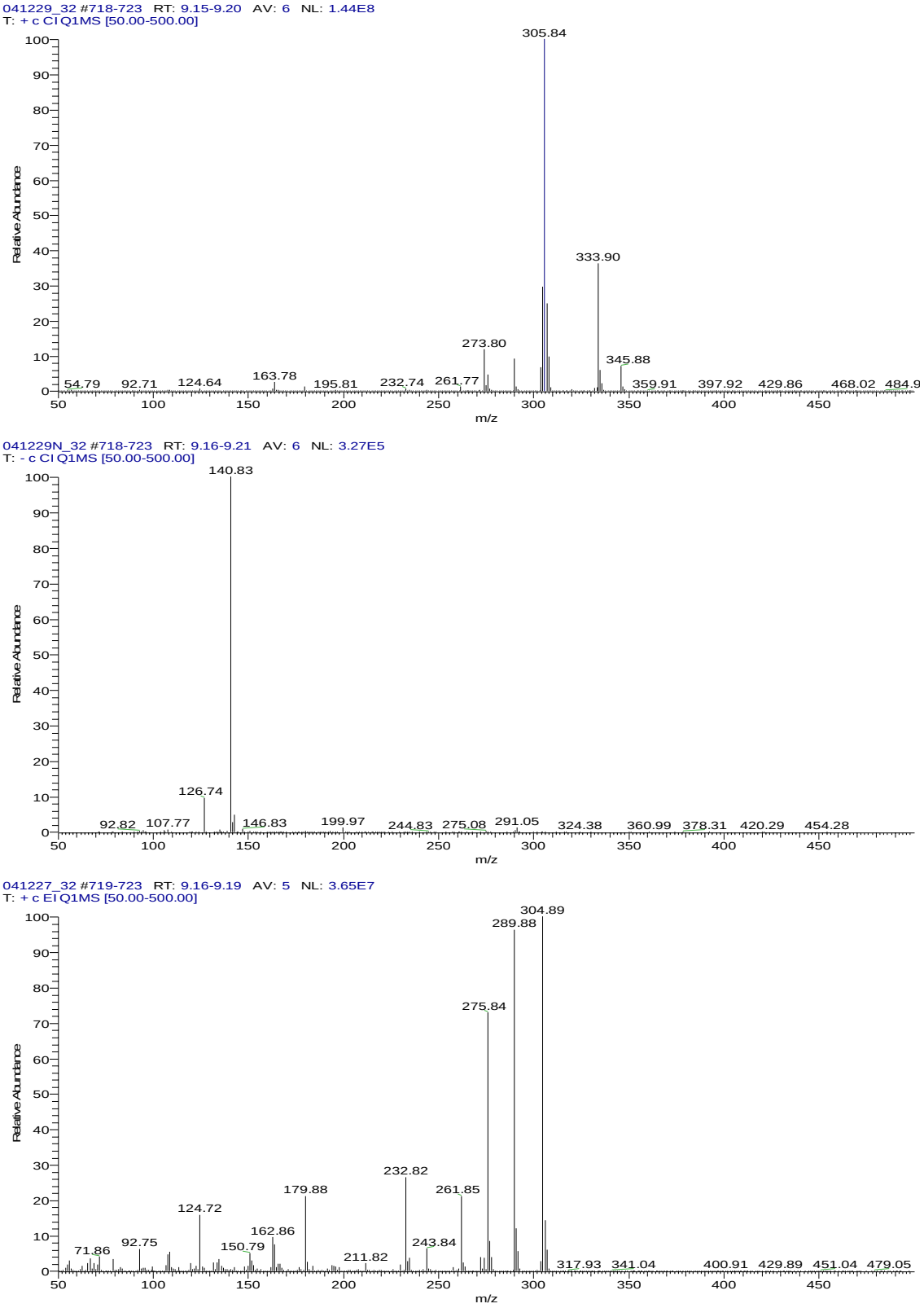


Figure 52 Pirimphos methyl - Positive CI, Negative CI, EI spectra

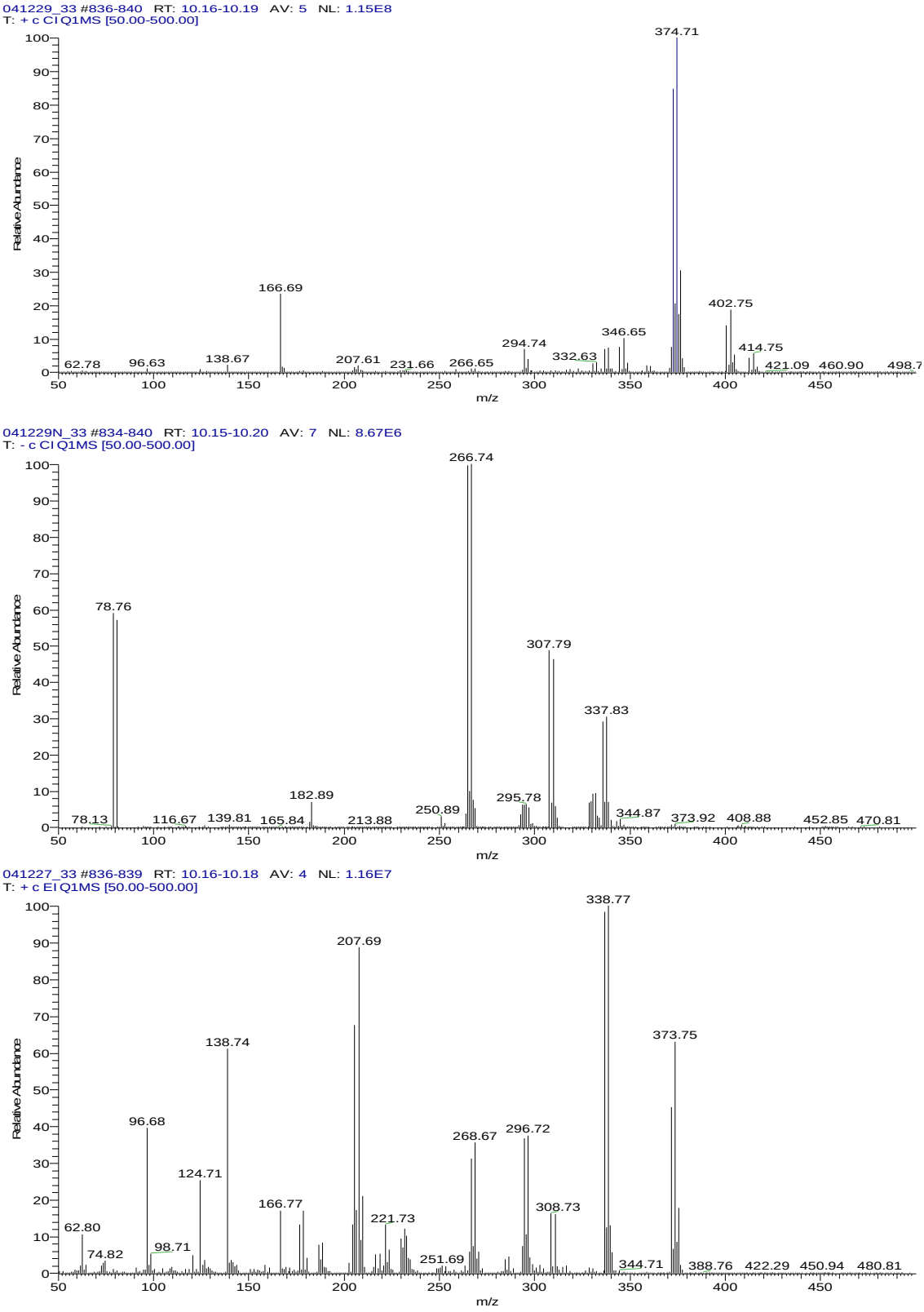


Figure 53 Profenofos - Positive CI, Negative CI, EI spectra

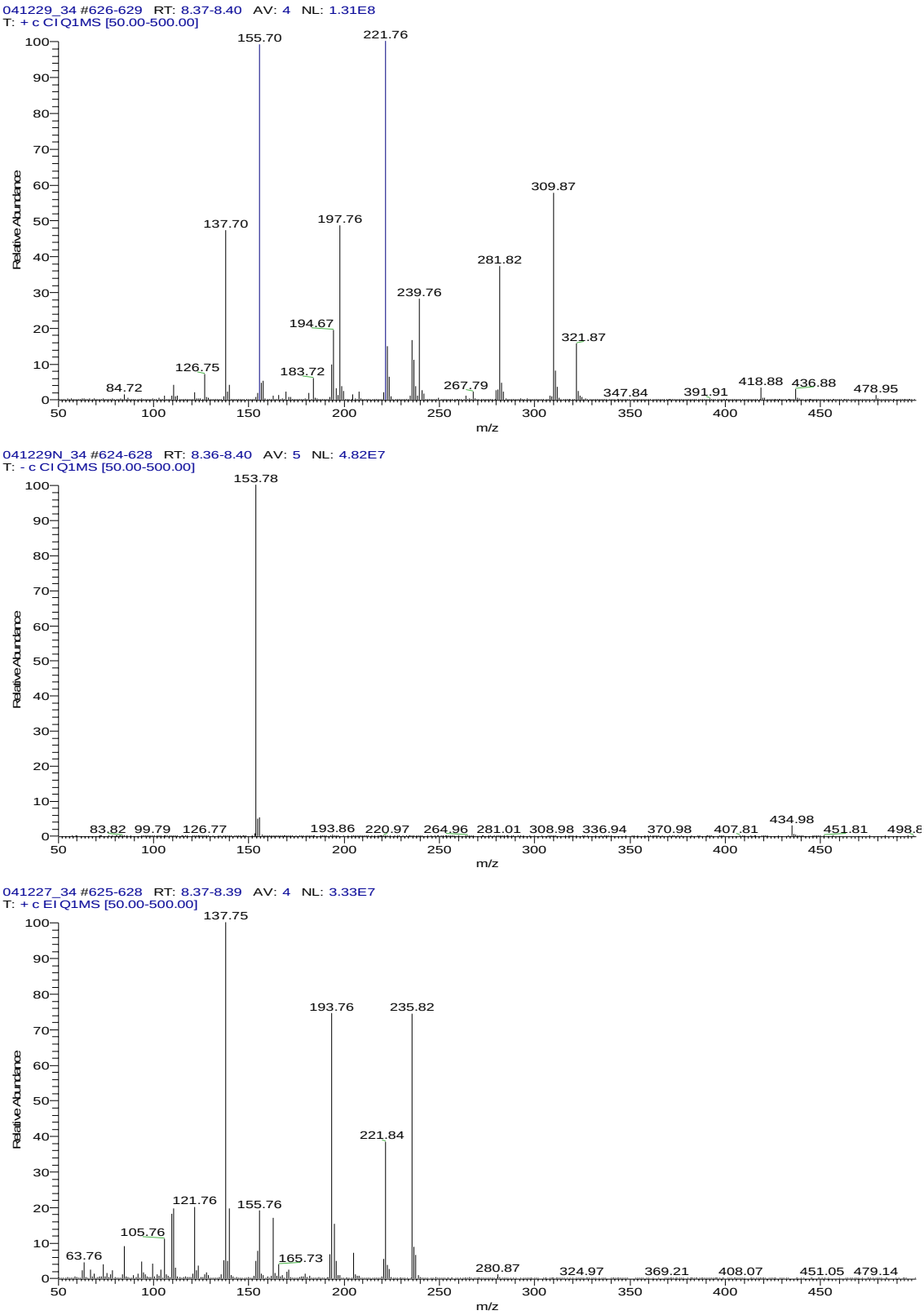


Figure 54 Propetamphos - Positive CI, Negative CI, EI spectra

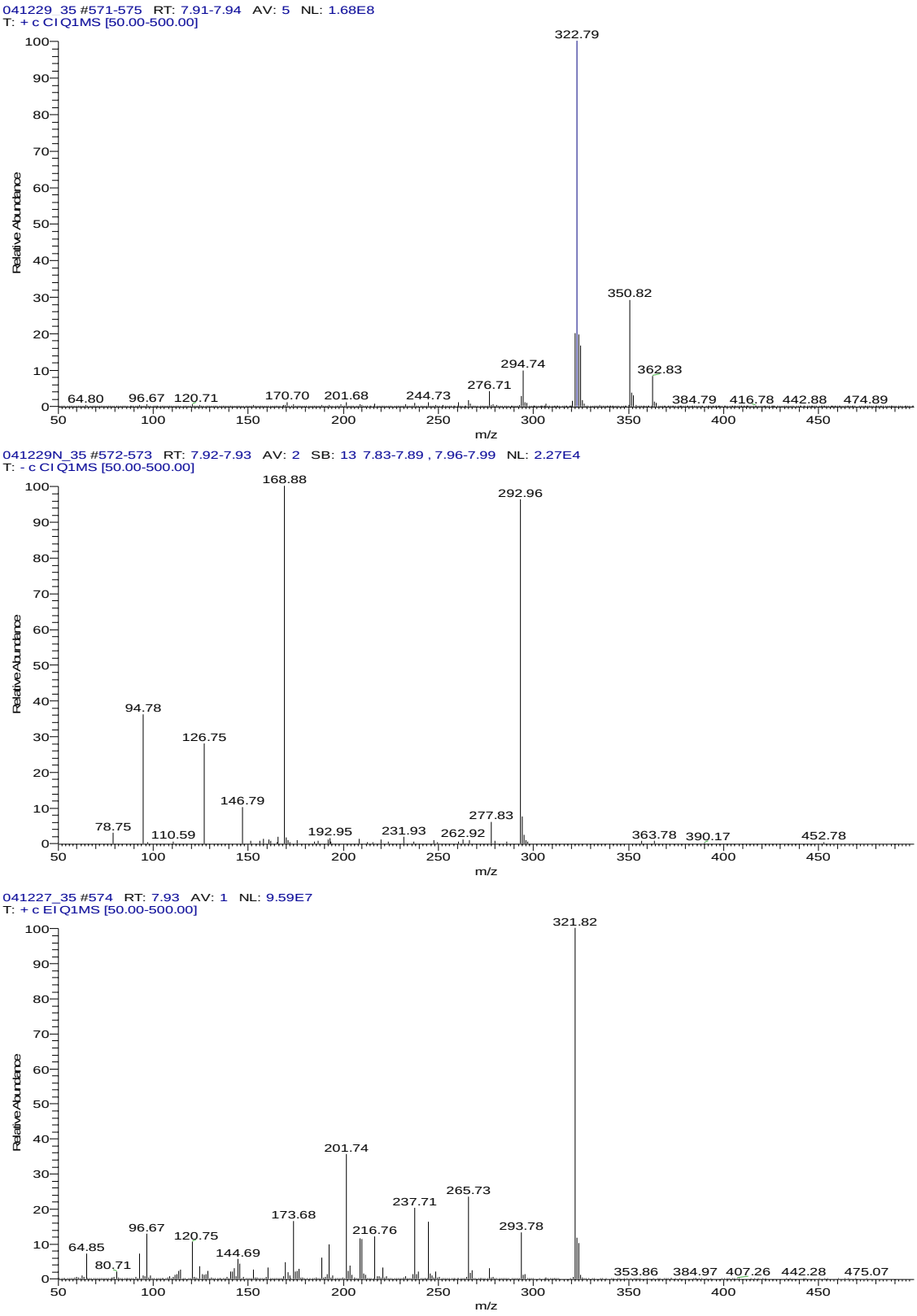


Figure 55 Sulfotepp - Positive CI, Negative CI, EI spectra

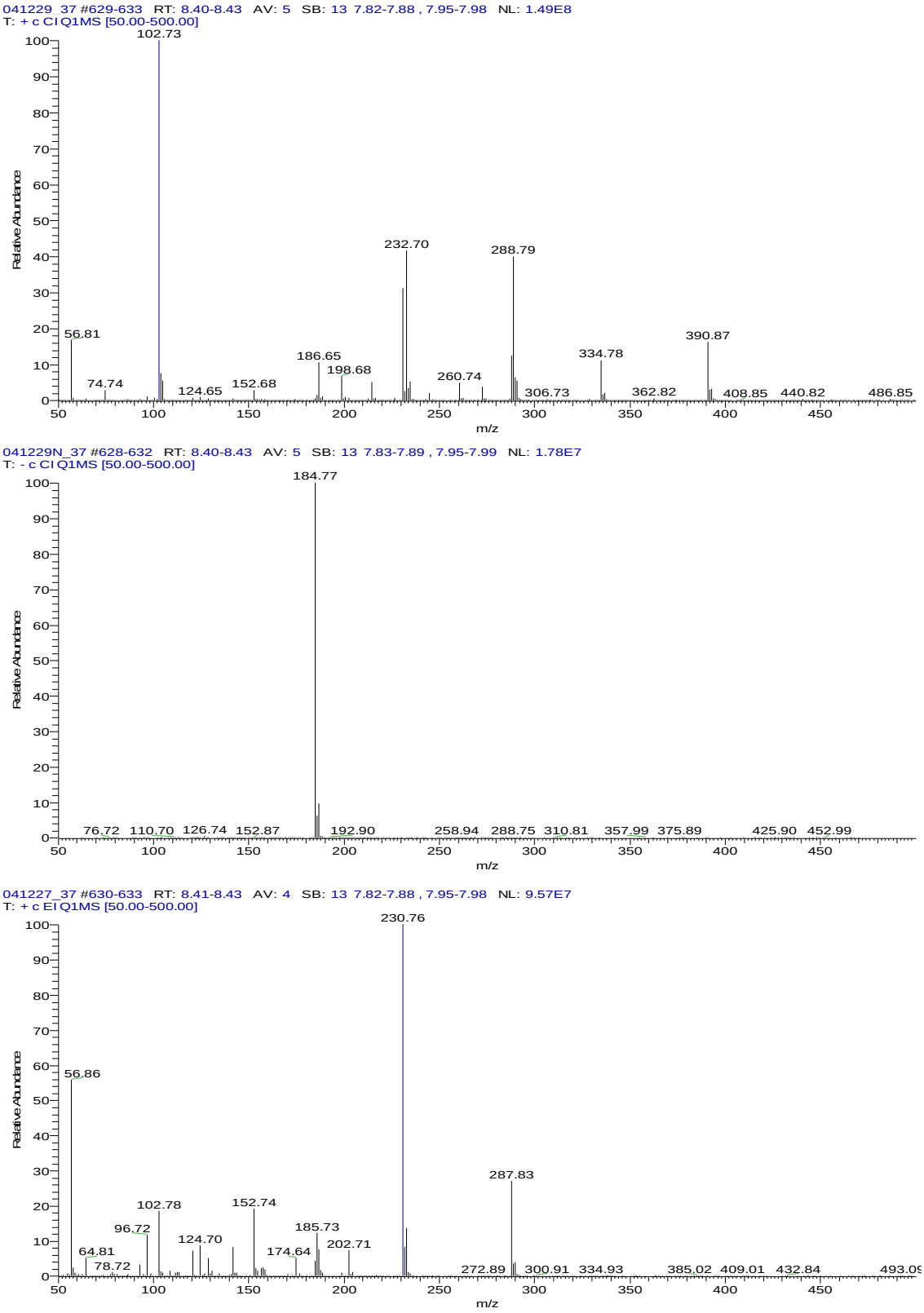


Figure 56 Terbufos - Positive CI, Negative CI, EI spectra

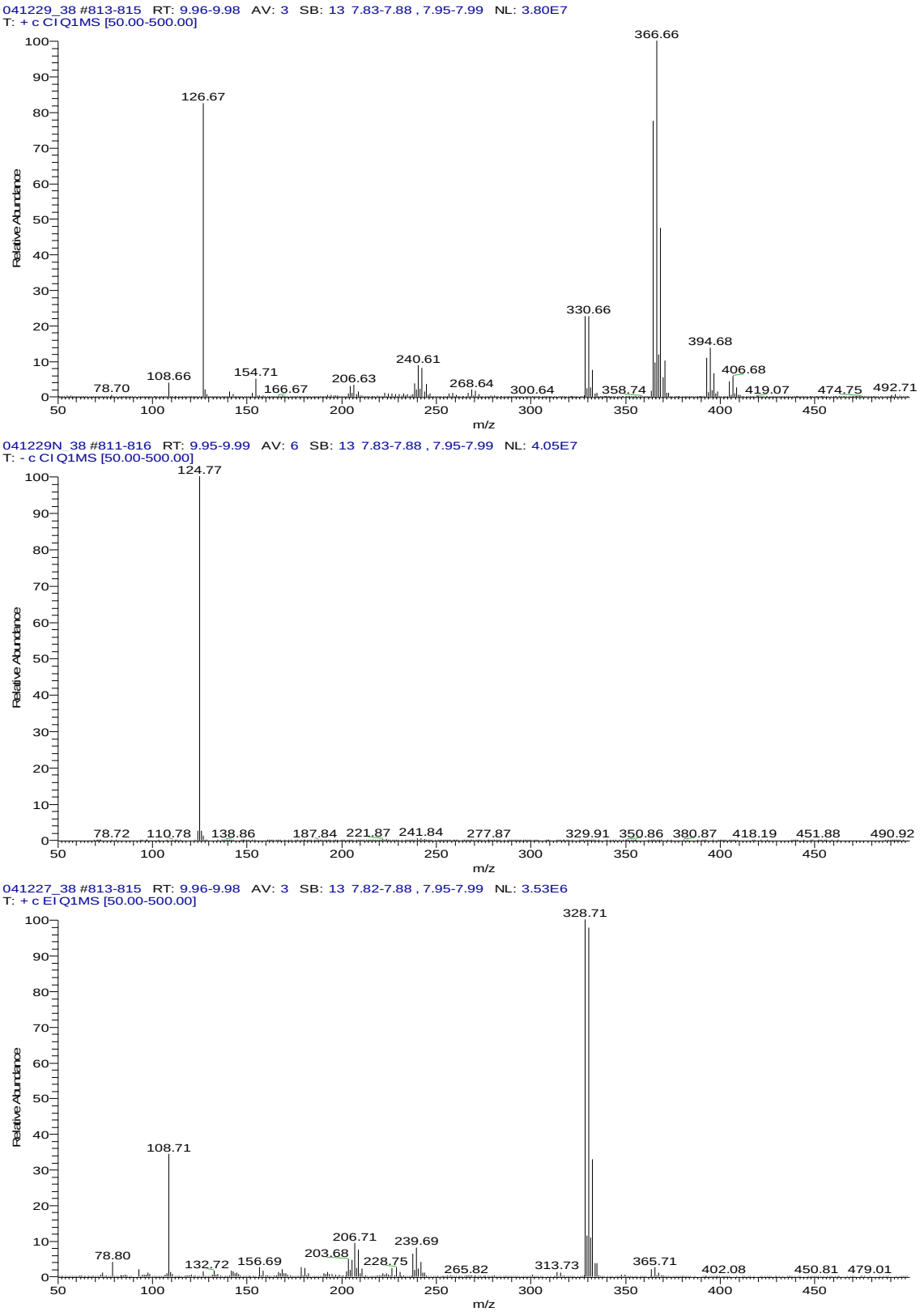


Figure 57 Tetrachlorvinphos - Positive CI, Negative CI, EI spectra

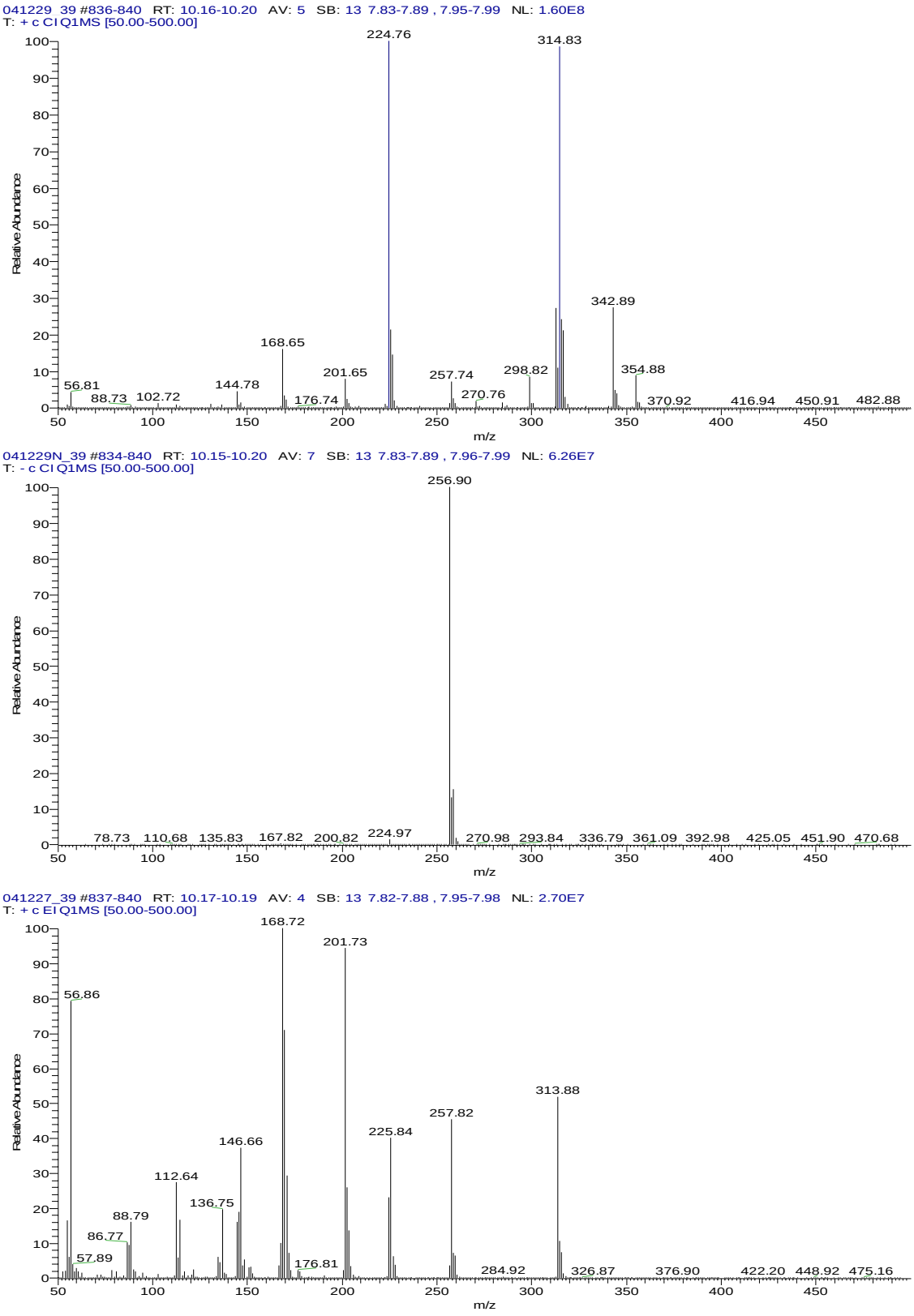


Figure 58 Tribufos - Positive CI, Negative CI, EI spectra

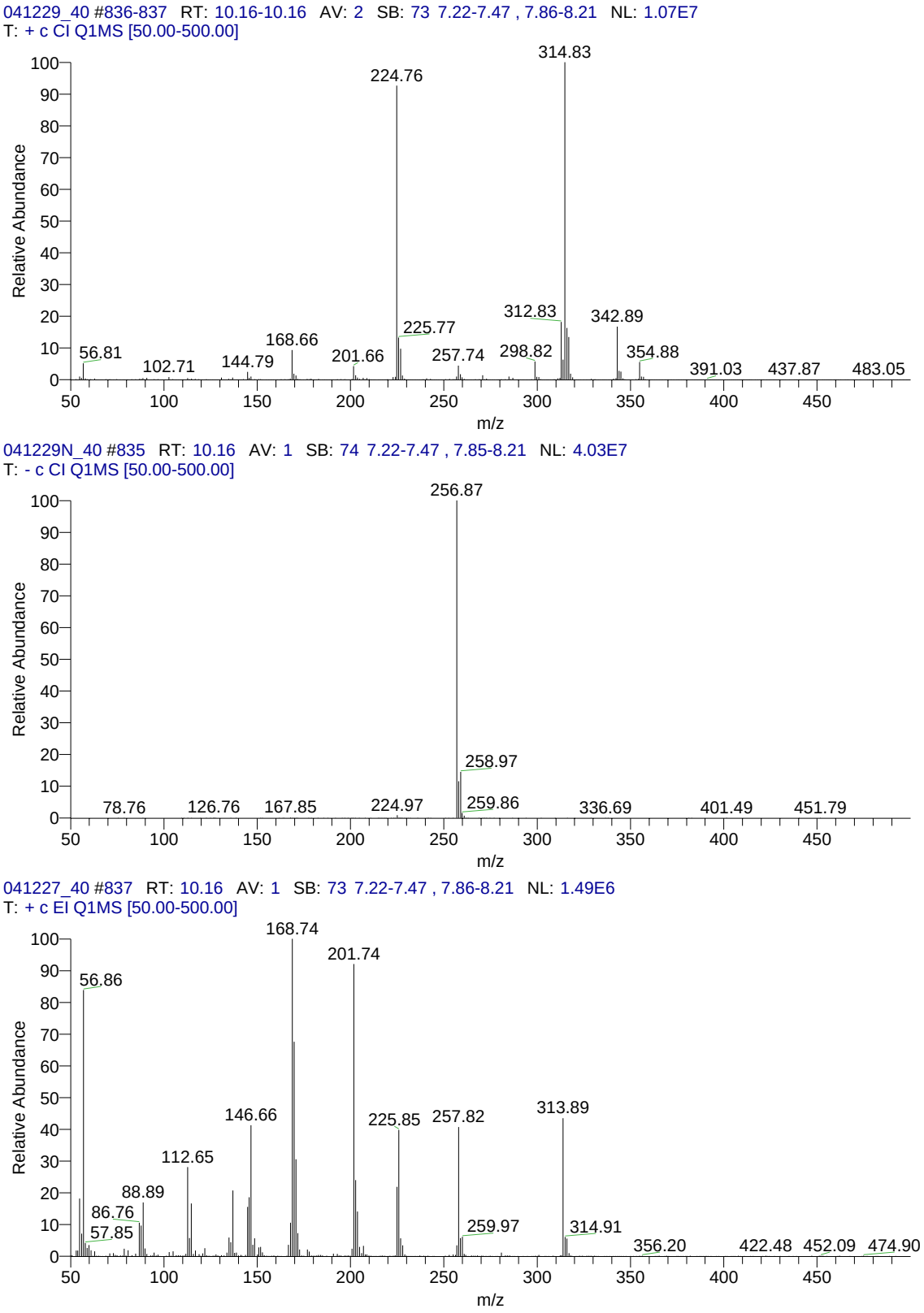


Figure 59 Trichlorfon - Positive CI, Negative CI, EI spectra

CHAPTER 3: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY

Many of the analytes are thermally labile, highly polar or otherwise unsuitable for GC separations. Examples are methamidophos, acephate, and azinphos methyl. These compounds are the candidates for HPLC separations and form the core of the LC group of compounds. Other analytes were added because after preliminary experiments it was determined that they likely have a better LOD in an LC-MS-MS method.

In the HPLC-MS-MS system two ionization modes were available: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), positive or negative polarity for each. The feasibility of ionization modes and polarizations were tested by injecting concentrated (approximately 5 μg/mL samples into a rapid HPLC eluent gradient and observing full scan spectra, using only a single quadropole of the mass spectrometer. This process was repeated for all four combinations of ionization modes in both polarities. All forty of the analytes were tested, because at this point it was unclear weather or not all OP pesticides can be analyzed solely on a HPLC-MS-MS system.

As it is apparent by looking at the ionization tables (Table 13) that more compounds ionize in APCI mode than in ESI mode. The reason is that ESI ionization takes place in the solution phase, while the APCI ionization happens in the plasma surrounding the charged needle. When one has to choose between positive and negative ionization, - especially when studying biological, dirty samples – negative ionization has an advantage. If the analyte of interest is ionizing in the negative mode, the background is often lower. The reason is that in the majority of cases positive ionization requires

addition of a proton, while negative ionization needs the removal of one. It is easier to add a proton from some external source than find an already existing proton to lose.

No.	OP	Mono isotopic Mass	Ions (m/z), nominal amu ESI Positive	ESI Negative	APCI Positive	APCI Negative
1	Acephate	183.012	184	182	184	168
2	Azinphos-methyl	317.006	318		318	157
3	Bensulide	397.061	398	396 **442**	**356**, 398	213
4	Cadusafos*	270.088	271, **541**	426	**271**, 541	213
5	Chlorethoxyfos	333.892				305, **307,** 309
6	Chlorpyrifos	348.926			350	**313,** 315, 317
7	Chlorpyrifos methyl	320.895	**115, 143,**	306 **308**	321	**212,** 214 216
8	Coumaphos	362.014	363		363	**225,** 208, 333
9	Diazinon	304.101	305	**169** 215	305	**169,** 215
10	Dichlorvos (DDVP)	219.946	**262** 264	**205** 207	**262,** 253	**205,** 207
11	Dicrotophos	237.077	475	223 **291** 709	**270**, 283	171
12	Dimethoate	230.000	**230,** 459	274	230	157
13	Disulfoton	274.028	305	185	363	185
14	Ethion	383.988	385		385	185
15	Ethoprop	242.560	485	213	**243,** 485	199
16	Ethyl parathion	291.033	173, **243**, 305		**262**	**262,** 154, 138
17	Fenamiphos	271.134			304, 363, **607**	247
18	Fenitrothion	277.017	305 **115**	262 383	124, **165**, 248, 289	168
19	Fenthion	278.020	342	263	279, **311**, 320	263
20	Fonofos**	246.030	**247,** 173	137	247	169
21	Malathion	330.036	331	**315**	331	157

Table 13 Table of LC-MS ions in APCI and ESI mode, positive or negative ionization Bolded ions ara the most aboundant
(Cont. next page)

No.	OP	Mono isotopic Mass	Ion Masses ESI Positive	ESI Negative	APCI Positive	APCI Negative
22	Methamidophos	141.001	142**, 283**	141 **187**	**283,** 174, 142	
23	Methidathion	301.962	**303** 366	248 **287**	303	157
24	Methyl parathion	263.002	115	**277** 138 248 325	**151**, 234, 275	**248,** 154
25	Mevinphos*	224.045	225 449	**209** 255	**225**, 257	**171,** 125, 237
26	Naled	377.783	420 422 424	363 **365 367**	411, **413**, 426	363, **365,** 367
27	Oxydemeton methyl	246.015	247	231	247	141
28	Phorate	260.013	335 115 143	**185** 231 355	261, **335**	185
29	Phosalone*	366.986	368	167	368	185
30	Phosmet	316.995			**318**	157
31	Phostebupirim	318.117	319		319	275
32	Pirimiphos methyl	305.096	318, 381	290	306	**141,** 290 187
33	Profenofos	371.935	306	313 315, 317 343 **345** 347	373 405, **407**	343, **345,** 347
34	Propetamphos	281.085	282		282…**327**	154, **280,** 200
35	Sulfotepp	322.023			323	169, **293,** 155
36	Temephos	465.990		451	467	**451,** 141
37	Terbufos	288.044	115 143	185	**245**, 289	185
38	Tetrachlorvinphos	363.899	365 406,**408**,410	349 351 353… **373**	365, 397..**399**…	**171,** 125
39	Tribufos (DEF)	314.096	315		**315,** 347, 629	257
40	Trichlorfon	255.923	257… 515	301 **303** 305	**143,** 152, 221, 257, 289	

Table 13 Table of LC-MS ions in ESI and APCI mode, positive or negative Bolded ions ara the most aboundant (Weak) ionization (cont. from previous page)

Liquid Chromatography: HPLC

After deciding an ionization mode the next step is to develop the analytical separation part of the method

In the literature the commonly used column type for the analytical separation of OP pesticides is the reversed phase C-18. [26-28] For this reason we started with variants of C-18 columns: betasil C18, Aquasil C18. Hydrophilic – hydrophobic properties of our compounds of interest vary widely as it was observed in the introduction. Compounds like acephate and methamidophos are extremely water soluble, do not retain very well on most packing. Very lipophilic, oily OP pesticides, such as Tribufos on the other hand; have very good retention.

Name	Manufacturer	Length [mm]	Diam. [mm]	Particle size [µm]	Carbon Load	Pore Size	End-cap		
Aquasil C18	Thermo	100	4.6	5	12%	100 A	Yes	High purity,base deactivated	C18
Betasil C8	Thermo	100	4.6	5	12%	100 A	Yes	High purity,base deactivated	C8
Betasil C18	Thermo	100	4.6, & 2.1	5	20%	100 A	Yes	High purity,base deactivated	C18
Betasil Phenyl	Thermo	100	4.6	5	11%	100 A	Yes	High purity,base deactivated	
Merck Chromolit RP-e	Merck Life Sciences	100	4.6	5	17%	120 A	Yes	High purity silica gel	C18
Discovery HS F5	Supelco	100	4.6	3	12%	120 A	Yes	silica gel, high purity, spherical particle platform	Pentafluorophenyl propyl
Fluorphase RP	Thermo	50	2.1	5	10%	100 A	Yes	High purity,base deactivated	perfluorinated C6
Prism RP	Thermo	50	2.1	5	12%	100 A	Yes	High purity,base deactivated	Urea+ C12 chain
Zorbax SB C3	Agilent	100	4.6	5	4%	80 A	No		Diisopropyl propyl

Table 14 HPLC columns tested

Using the C-18 type columns , it became apparent that when applying linear – or close to linear – elution curves, that the compounds separate into two groups: early and late eluting compounds. Many of the experiments listed in tables 15-17 were attempts to further resolve these two elution groups to their components. While it is not absolutely necessary when using tandem mass spectrometer as detector, resolving peaks can help by eliminating possible interferents and, ion competition. If many analytes elute very closely, it can complicate the instrument method setup. Since the MS has to spend a finite amount of time on each transition, in the presence of many overlapping peaks the number of data points for each peak may be limited.

An other aspect of the overlapping peaks is the signal to noise ratio. The detector works in ion counting mode, so when the time to collect ion counts is limited – background being the same- the signal to noise ratio worsens. So even in with the resolving power of tandem mass spectrometry, good chromatographic resolution provides an advantage.

Selection of eluents.

Before developing the analytical separations part of the method, the detection was decided to be APCI, positive ionization. The selection of ionization mode has a major influence on the on the selection of solvents, sizing of the column and picking the right flow rate. The two most often used combination of eluents are: water-methanol and water-acetonitrile.

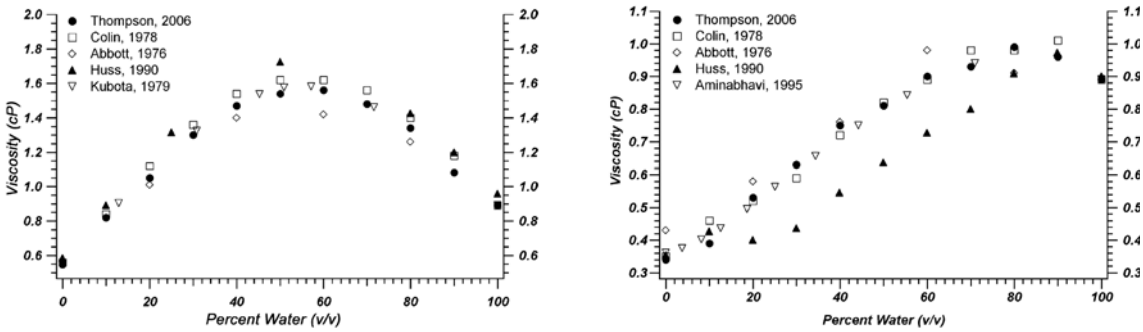


Figure 60 Viscosity profiles of Methanol - Water and Acetonitrile - Water blends, according to Thompson [34]

One of the practical differences between the two pairs is that the water - methanol gradient has a viscosity maximum while the water – acetonitrile mixture does not.

Acetonitrile and methanol gives different retention times.

Another difference between the two solvents is the propensity to form adducts. From previous work we saw that both solvents tend to form adducts with many of the analytes. Polarity of ionization makes a difference: acetonitrile is more likely to form adducts in positive ionization while methanol adducts are favored in negative ionization.

The TSQ Quantum-s APCI probe can be operated between .2 and 2 mL/min, the recommended optimum being approximately 1mL/min. This flow rate will work well with a 4.6 mm internal diameter column. Smaller diameter columns may be – and were – tried, but the increased pressure leads to leaks and sometimes clogging. In this method real life samples, with varying degree of purity have to be run reliably. The 4.6mm internal diameter, 100mm length combined with 1.0 mL/min flow rate proved to be the most useful combination.

No.	OP	Width: Length:	Betasil C18 2.1mm 100mm	Betasil C18 4.6mm 100mm	Betasil Phenyl 4.6mm MeOH		4.6mm ACN
1	Acephate		8.1	7.94	6.15	5.62	
2	Azinphos-methyl		21.83	19.35	20.53	17.11	
3	Bensulide		24.2	21.4	21.72	19.08	
4	Cadusafos*		25.64	22.39	21.05	17.59	
5	Chlorethoxyfos		26.88	-	N/F	N/F	E/B
6	Chlorpyrifos		27.41	23.5	22.53	28.06	E/B
7	Chlorpyrifos methyl		25.86	22.57	21.67	27.61	E/B
8	Coumaphos		24.85	21.88	22.24	19.15	
9	Diazinon		24.8	21.83	21.18	17.92	
10	Dichlorvos (DDVP)		19.08	16.72	15.67	N/F	E/B
11	Dicrotophos		14.25	12.13	13.68	9.94	
12	Dimethoate		15.71	14.29	14.16	11.68	
13	Disulfoton		28.94	22.43	21.66	N/F	
14	Ethion		26.97	23.19	23.1	20.58	
15	Ethoprop		23.72	21.08	19.86	16.28	
16	Ethyl parathion		24.46	21.62	21.09	N/F	E/B
17	Fenamiphos		24.19	21.42	20.77	16.5	
18	Fenitrothion		23.36	20.76	20.64	N/F	E/B
19	Fenthion		24.83	21.9	21.82	N/F	E/B
20	Fonofos**		24.91	21.98	21.25	18.85	
21	Malathion		22.97	20.41	20.57	17.89	
22	Methamidophos		4.08	6.43	3.67	3.47	
23	Methidathion		21.66	19.18	19.75	16.86	
24	Methyl parathion		22.46	19.98	19.66	N/F	E/B
25	Mevinphos*		16.97	15.14	15.18	11.72	
26	Naled		21.52	18.96	18.47	N/F	
27	Oxydemeton methyl		12.79	10.67	12.34	10.22	split
28	Phorate		25.4	22.27	21.29	N/F	E/B
29	Phosalone*		25.26	22.17	22.12	N/F	E/B
30	Phosmet		29.03	N/F	N/F	N/F	E/B
31	Phostebupirim		26.79	23.13	22.04	19.27	
32	Pirimiphos methyl		24.72	21.71	20.69	17.56	
33	Profenofos		26.4	22.86	22	N/F	E/B
34	Propetamphos		23.21	20.64	19.87	17.68	
35	Sulfotepp		24.56	21.67	21.31	19.11	
36	Temephos		26.73	22.98	24.04	N/F	E/B
37	Terbufos		26.75	23.09	22.15	N/F	
38	Tetrachlorvinphos		24.28	21.51	21.03	17.53	
39	Tribufos (DEF)		28.58	24.31	23.17	20.08	
40	Trichlorfon		19.09	16.73	15.64	N/F	E/B

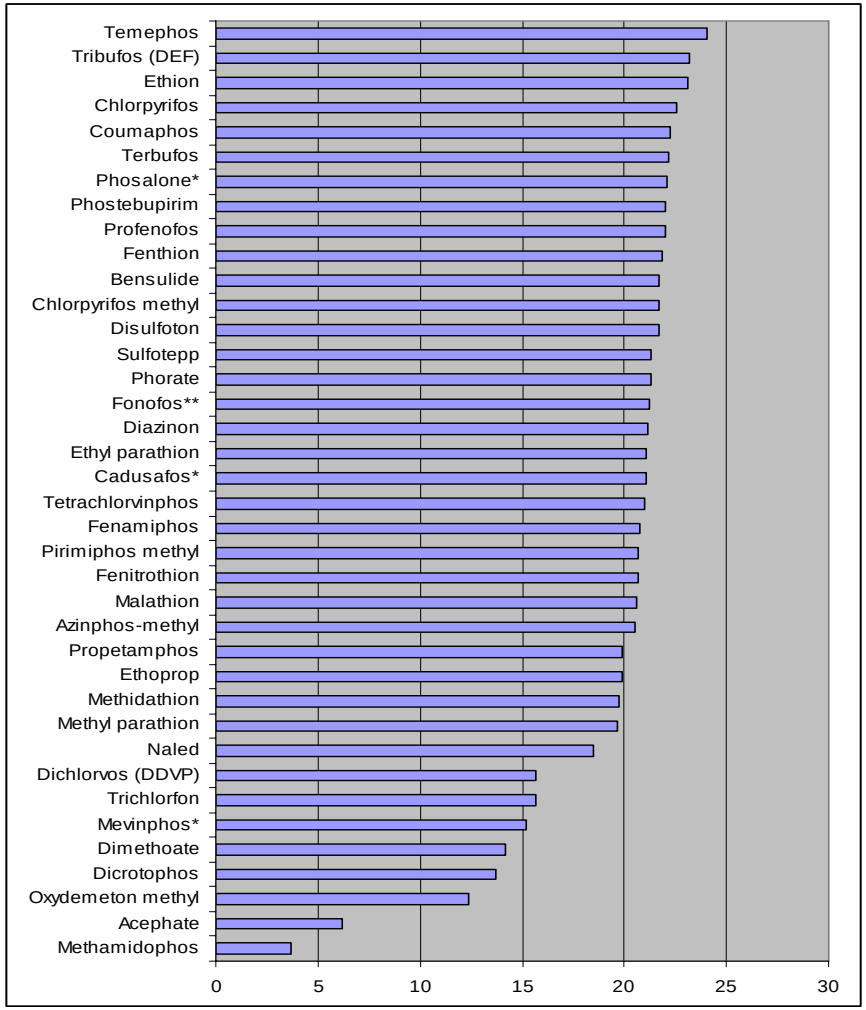
Table 15 Retenetion times of OP pesticides on Betasil C18 and Phenyl Columns (N/F: not found, E/B: extreme peak broadening)

No.	OP	Width: / Length:	Aquasil C18 4.6mm 100 mm		Chromolith RP 18e 4.6mm 100 mm		Zorbax SBC3 4.6mm 100 mm	
1	Acephate		8.35		7.33		7.53	poor peakshape
2	Azinphos-methyl		20.12		18.42		19.43	
3	Bensulide		21.56		21.19		21.19	
4	Cadusafos*		22.76		22.51		22.19	
5	Chlorethoxyfos		20.1	Poor peak	N/F		N/F	
6	Chlorpyrifos		23.63		23.79		22.65	
7	Chlorpyrifos methyl		22.77	Poor peak	22.61		21.63	
8	Coumaphos		22.57		21.8		22.29	
9	Diazinon		22.32		21.67		21.75	
10	Dichlorvos (DDVP)		16.77		15.79		16.24	
11	Dicrotophos		13.8		11.73		14.13	
12	Dimethoate		14.12		12.42		14	
13	Disulfoton		22.47	Bad tailing	22.39	Smearing	21.4	
14	Ethion		23.55		23.59		22.58	
15	Ethoprop		21.45		20.39		20.63	
16	Ethyl parathion		21.86		21.25		20.97	
17	Fenamiphos		21.79		21.05		20.93	
18	Fenitrothion		21.04		19.91		20.15	
19	Fenthion		22.23		21.66		21.01	
20	Fonofos**		22.1		21.65		20.92	
21	Malathion		20.45		19.52		20.01	
22	Methamidophos		6.84		4.24	Excessive Peak broadening	4.03	
23	Methidathion		19.26		18.08		18.86	
24	Methyl parathion		20.06		18.88		19.21	
25	Mevinphos*		15.26		13.96		15.91	
26	Naled		*		18.06		N/F	
27	Oxydemeton methyl		12.79		10.39		12.38	
28	Phorate		22.35		22.14		21.13	
29	Phosalone*		22.41		22.17		21.64	
30	Phosmet		N/F		N/F		N/F	
31	Phostebupirim		23.26		23.38		22.48	
32	Pirimiphos methyl		21.87		20.85		20.77	
33	Profenofos		23.31		23.14		22.29	
34	Propetamphos		20.64		19.79		20.07	
35	Sulfotepp		21.71		21.43		20.86	
36	Temephos		23.47		23.55		22.78	
37	Terbufos		23.17		23.33		22.23	
38	Tetrachlorvinphos		21.88		21.15		20.9	
39	Tribufos (DEF)		24.31		24.51		23.6	
40	Trichlorfon		15.27		14.17		15.88	

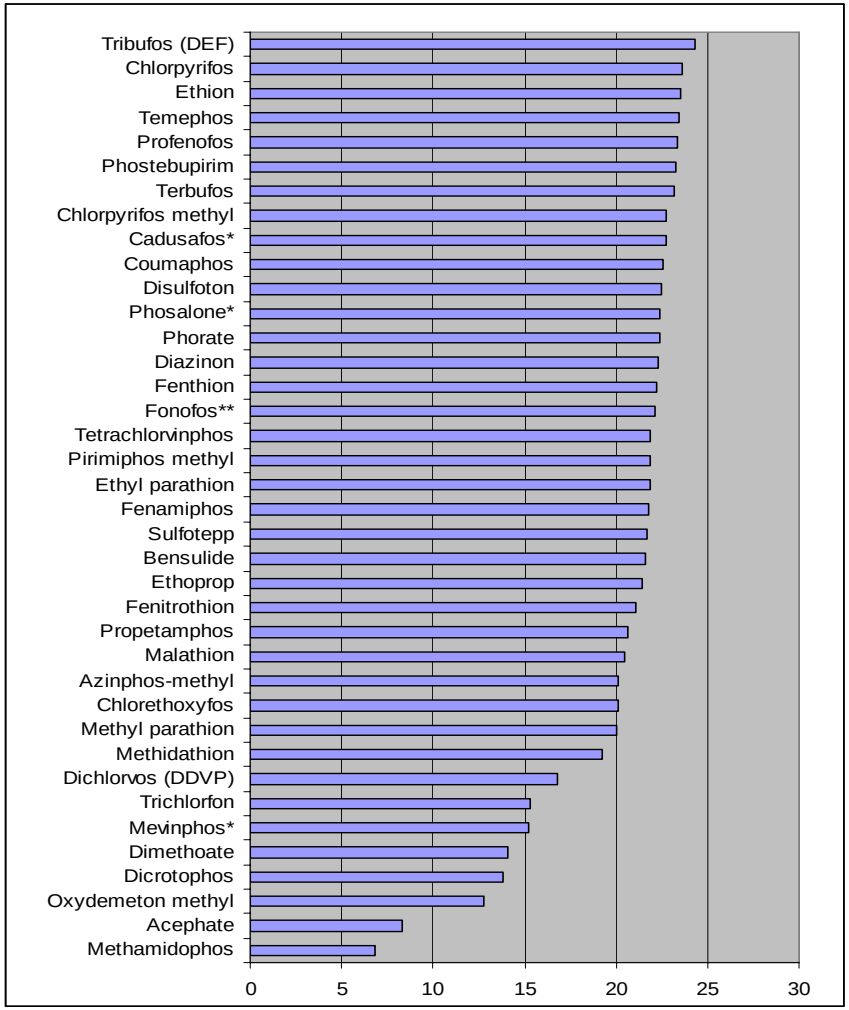
Table 16 Retenetion times of OP pesticides on Aquasil C18 and Chromolit RP 18e, Zorbax SBC3

No.	OP	Width: Length:	Discovery HS F5 3µm 4.6mm 100 mm		Hypercarb 4.6mm 100 mm		Varian C18 4.6mm 250mm	
1	Acephate		7.61		3.22		12.74	
2	Azinphos-methyl		20.61		N/F	E/B	23.99	
3	Bensulide		22.21		22.62	tailing	24.65	
4	Cadusafos*		21.7		18.73		26.17	
5	Chlorethoxyfos		N/F		N/F	E/B	N/F	
6	Chlorpyrifos		22.85		N/F	E/B	27.53	
7	Chlorpyrifos methyl		22.12		N/F	E/B	N/F	E/B
8	Coumaphos		23.83		N/F	E/B	25.62	
9	Diazinon		22.4		21.05		25.77	
10	Dichlorvos (DDVP)		16.8		N/F	E/B	21.64	
11	Dicrotophos		13.39		9.33		16.69	
12	Dimethoate		14.86		10.46		18.43	
13	Disulfoton		N/F		N/F		26.21	
14	Ethion		23.31		25.26	tailing	26.8	
15	Ethoprop		19.98		15.63		24.98	
16	Ethyl parathion		22.75		N/F	E/B	25.28	
17	Fenamiphos		20.97		N/F	E/B	24.88	
18	Fenitrothion		22.1		N/F	E/B	24.7	
19	Fenthion		21.99		N/F	E/B	25.77	
20	Fonofos**		22.02		8.23		25.92	
21	Malathion		21.27		18.7		24.23	
22	Methamidophos		4.28		1.43		12	
23	Methidathion		20.4		22.03		23.74	
24	Methyl parathion		21.57		N/F	E/B	24.13	
25	Mevinphos*		15.59		N/F	E/B	19.41	
26	Naled		18.55		N/F		N/F	
27	Oxydemeton methyl		11.57		8.23		15.35	
28	Phorate		22.02	tailing	N/F		26.9	
29	Phosalone*		22.57		N/F		25.66	
30	Phosmet		N/F		N/F	E/B	N/F	
31	Phostebupirim		22.73		20.45		27	
32	Pirimiphos methyl		26.26		N/F	E/B	25.89	
33	Profenofos		22.25		N/F	E/B	26.72	
34	Propetamphos		20.6		17.76		24.26	
35	Sulfotepp		21.92		17.32		25.21	
36	Temephos		23.59		N/F	E/B	26.44	
37	Terbufos		22.96		N/F		26.87	
38	Tetrachlorvinphos		21.19		N/F	E/B	25.09	
39	Tribufos (DEF)		23.01		N/F		28.5	
40	Trichlorfon		16.82		N/F	E/B	19.38	

Table 17 Retenetion times of OP pesticides on Discovery HS F5, Hyper carb and Varian C18 columns

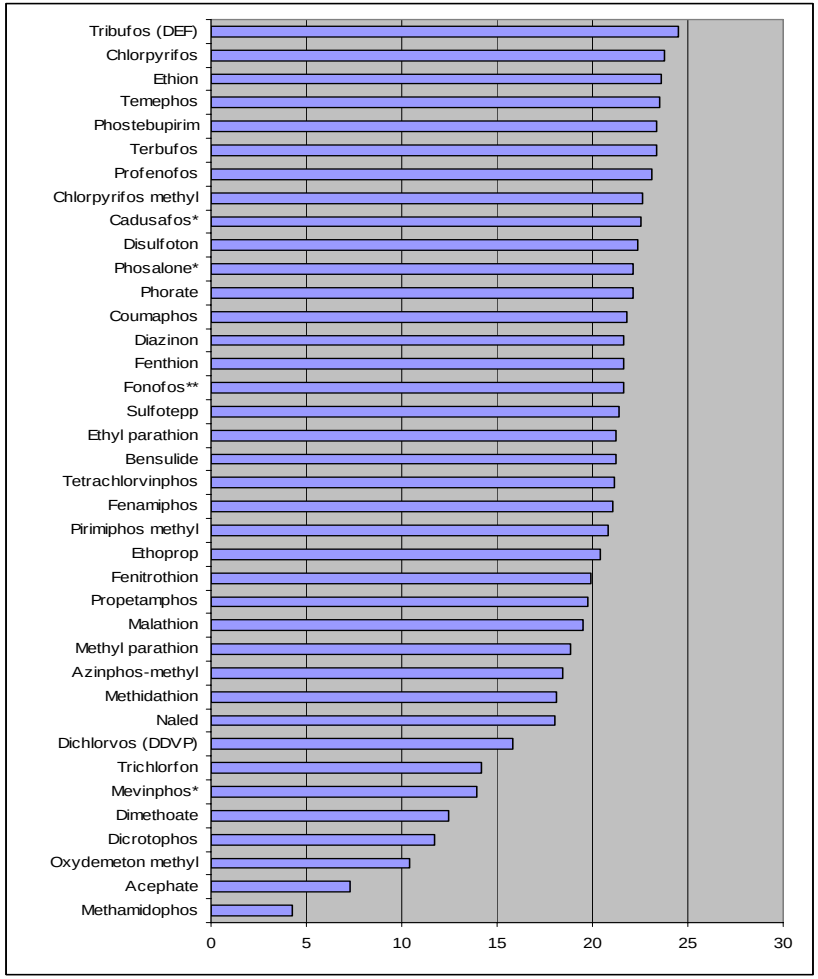


Betasil C18

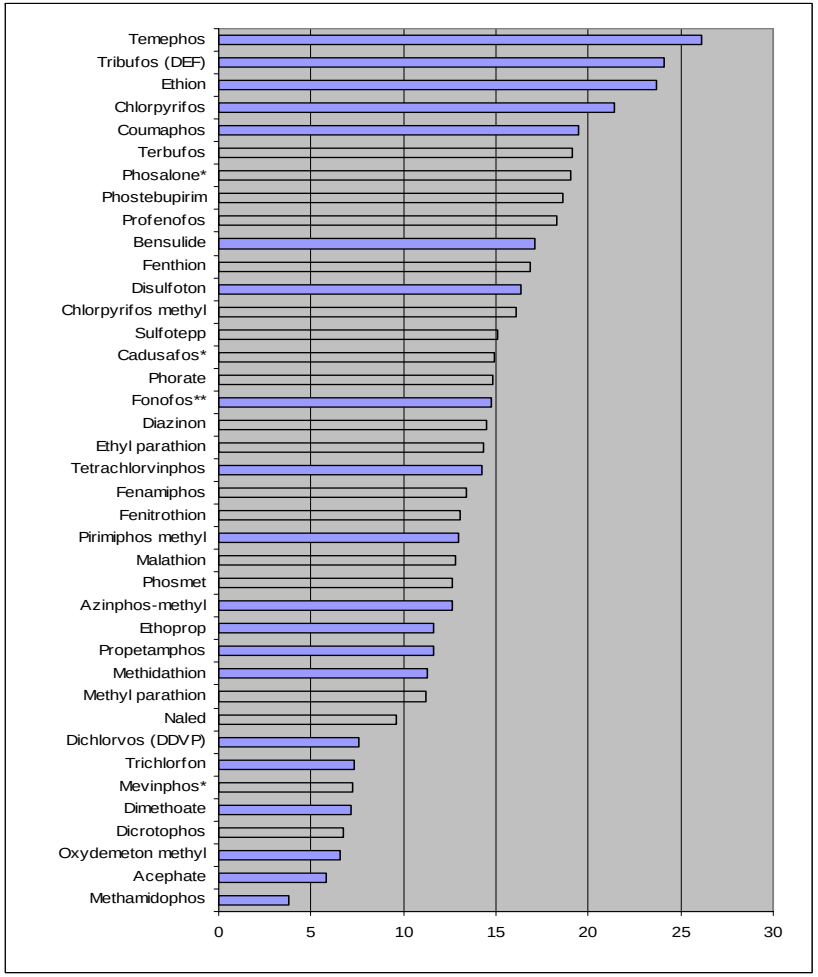


Betasil Phenyl

Figure 61 Betasi C18 and Betasil Phenyl retention profile using a linear water methanol gradient



Aquasil C18



Betasil Phenyl, final gradient (GC group is grayed out)

Figure 62 Aquasil C18 retention profile using a linear water methanol gradient and Betasil Phenyl using the final, non lina=ear solvent gradient

Alternative to the one column HPLC separation

For sake of simplicity, as mentioned earlier, a single column, the 4.6X100mm Betasil Phenyl column was selected. This column did not resolve all the compounds in the late eluting group, but with the modified gradient gave sufficient separation. The reason for this is the same as the core difficulty of our problem: we have to deal with a very large group of compounds. It is difficult if not impossible to find one single column that will separate all the analytes in an even manner.

An alternative is to use two columns and create a “two dimensional system”, where one column separates the early eluting group but not the late eluting ones. This late group then directed on a different type of column, where it is separated with a more organic rich gradient. It would be recommended to use Aquasil C18 or Betasil Phenyl for first column and Discovery HF S5 as a second column.

This system can be achieved with two properly timed six port valves. As it is illustrated in Figure 63

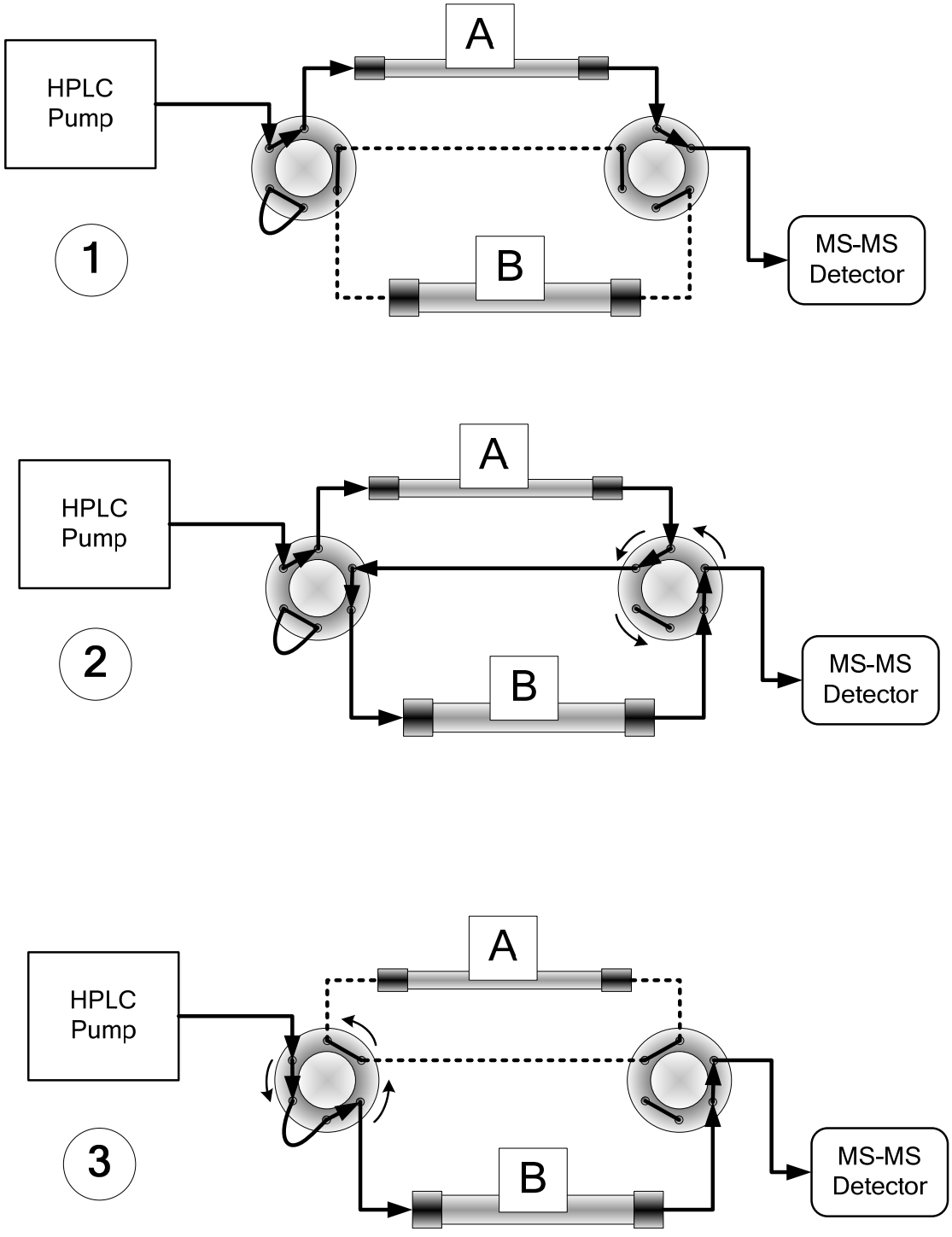


Figure 63 Two column HPLC system. 1: Column A separates the first analyte group. 2: The group of unresolved analytes pass column A and are loaded onto column B. 3: This second group of analytes are gradient eluted and separated on column B.

CHAPTER 3 APPENDIX

Appendix 3A APCI Source Temperature Studies

Because many of the OP pesticides known to be thermally labile, an investigation was undertaken to study the effect of source temperature on on signal strength.

In APCI, source temperature has two components:

- Evaporator temperature is the temperature of the porcelain tube where the LC solvent stream enters in the source. This is where the liquid eluent starts to evaporate and forms an aerosol with the nitrogen sheet gas. Evaporator temperature may be raised up to 600°C.
- Capillary or "ion transfer tube" temperature may be adjusted between 0 and 400°. The capillary is the narrow ion guide that separates the atmospheric part of the APCI source from the first stage of vacuum. Its temperature is important optimization factor because this is where the last traces of water is dried off, or where the dehydratation of the ions is completed.

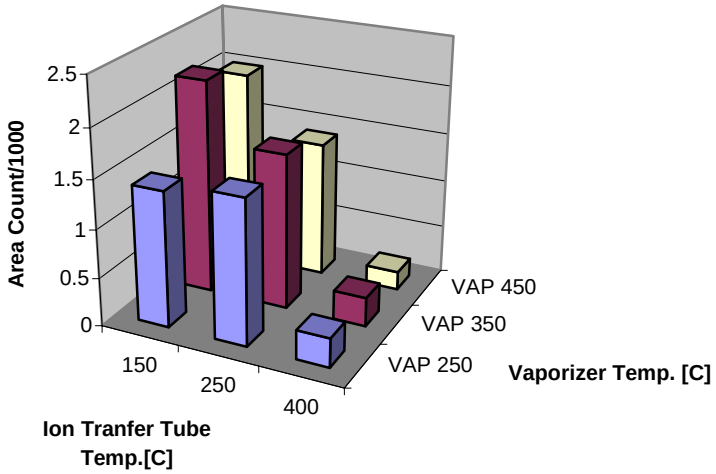
  Too low temperature will result in incomplete drying, too high temperature causes thermal decomposition.

While both temperatures may be adjusted, neither one of them is suitable to change during a run. A pair of compromise temperatures have to be found to accommodate all the analytes.

The following set of charts illustrates the effect of these temperature combinations for all analytes. 350 °C for evaporator temperature and 250°C capillary temperature was found to be the best combination for the analytes selected for HPLC MS-MS.

12 **Dimethoate**

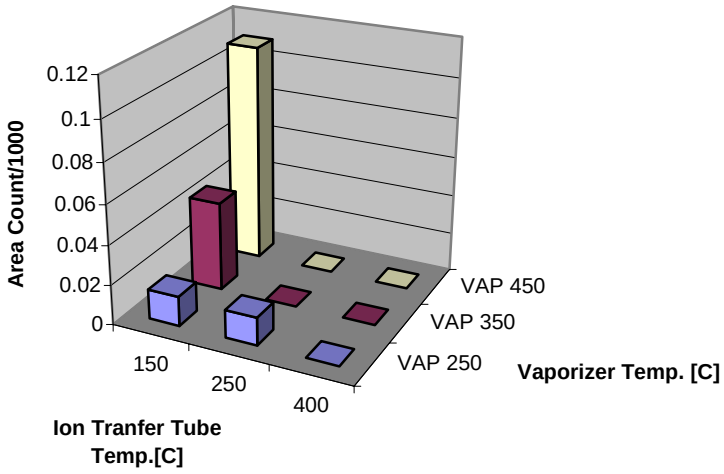
		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	1.4	1.5	0.3
Vaporizer	**VAP 350**	2.2	1.6	0.3
	VAP 450	2	1.4	0.19



Vaporizer - Heated Capillary Temperature Effect

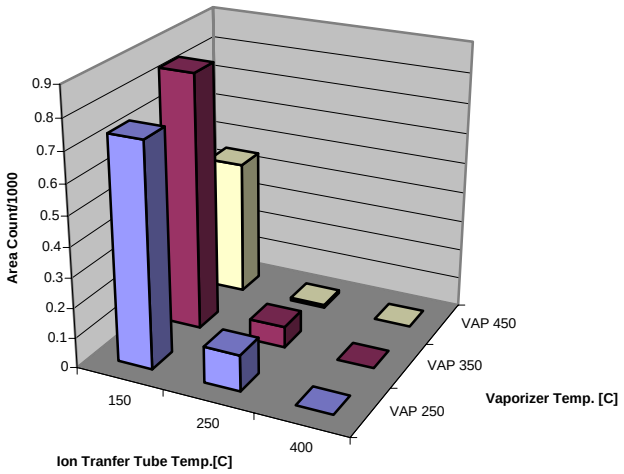
13 **Disulfoton**

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	0.015	0.014	0
Vaporizer	**VAP 350**	0.045	0	0
	VAP 450	0.11	0	0



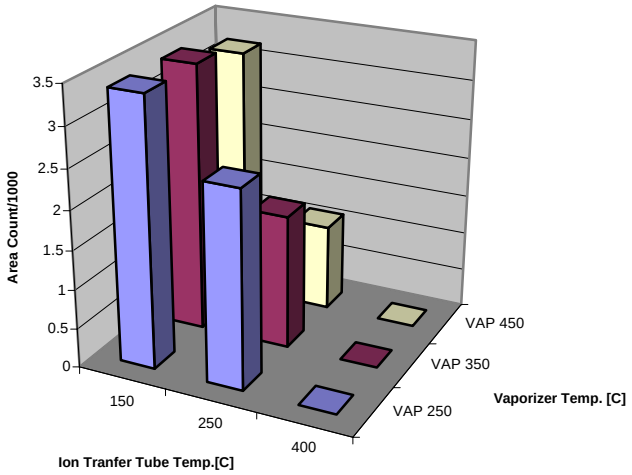
3 **Bensulide**

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	0.74	0.12	0
Vaporizer	**VAP 350**	0.85	0.07	0
	VAP 450	0.45	0.01	0



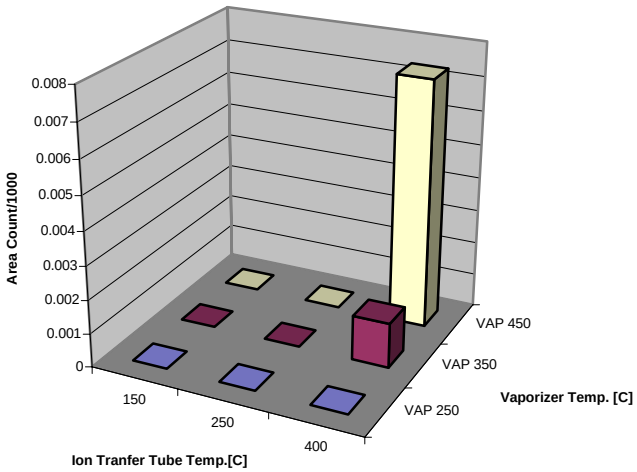
4 **Cadusafos**

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	3.4	2.5	0
Vaporizer	**VAP 350**	3.4	1.7	0
	VAP 450	3.2	1.1	0



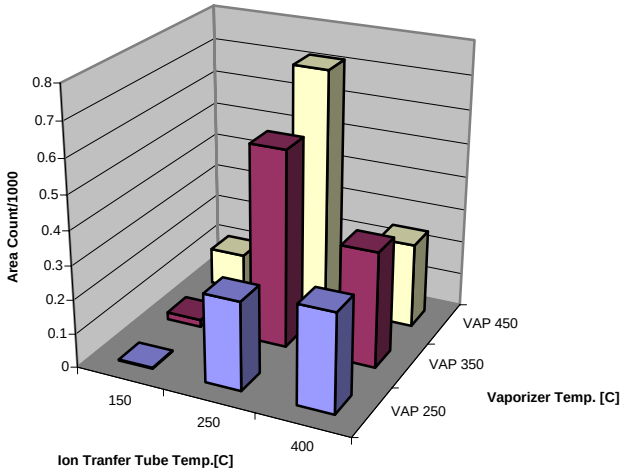
5 Clorethoxyfos

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	0	0	0
Vaporizer	**VAP 350**	0	0	0.001327
	VAP 450	0	0	0.007349



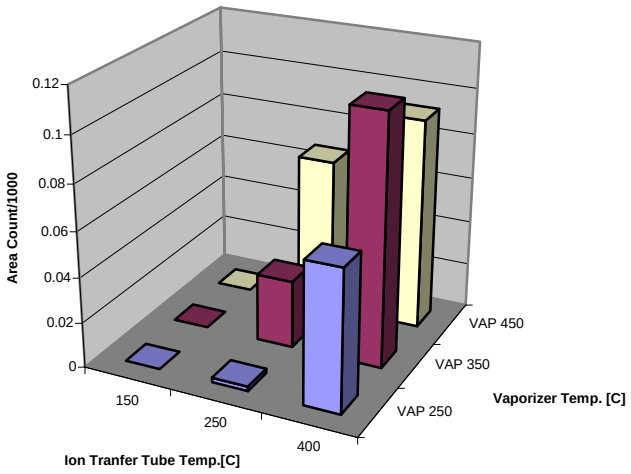
6 Clorpyrifos

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	0.003	0.26	0.29
Vaporizer	**VAP 350**	0.022	0.58	0.34
	VAP 450	0.11	0.72	0.25



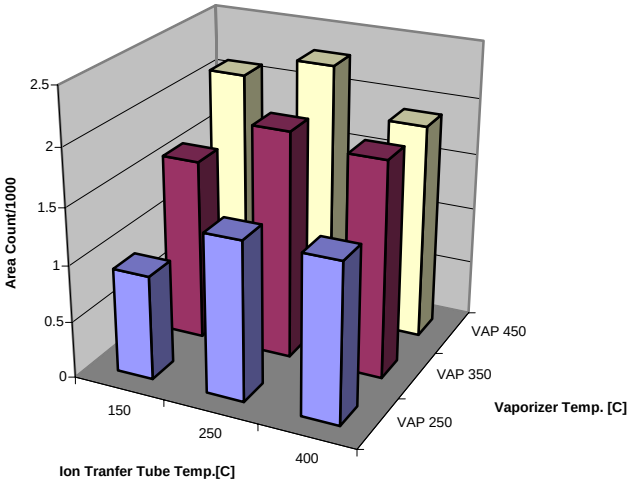
7 Clorpyrifos -Me

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	0	0.002	0.062
Vaporizer	**VAP 350**	0	0.03	0.11
	VAP 450	0	0.068	0.093



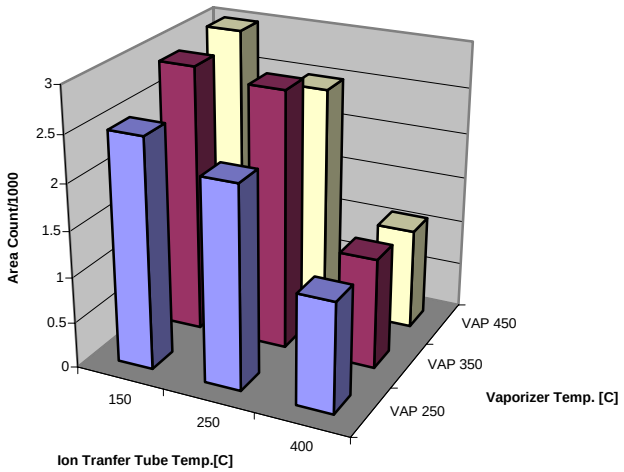
8 Coumaphos

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	0.92	1.4	1.4
Vaporizer	**VAP 350**	1.6	2	1.9
	VAP 450	2.1	2.3	1.9



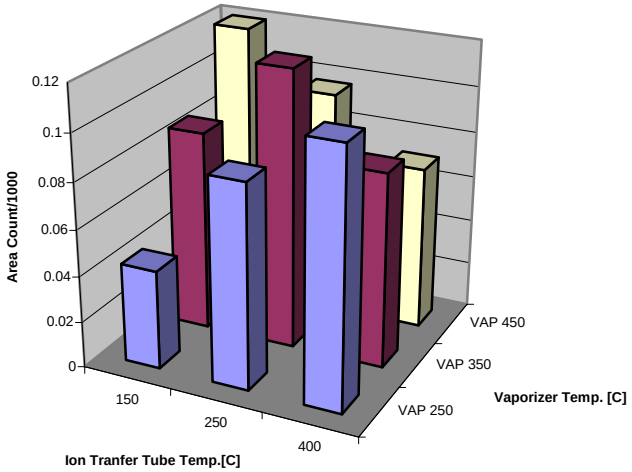
9 **Diazinon**

	Area Count/1000	Capillary 150	250	400
	VAP 250	2.5	2.2	1.2
Vaporizer	VAP 350	2.9	2.8	1.2
	VAP 450	3	2.5	1.1



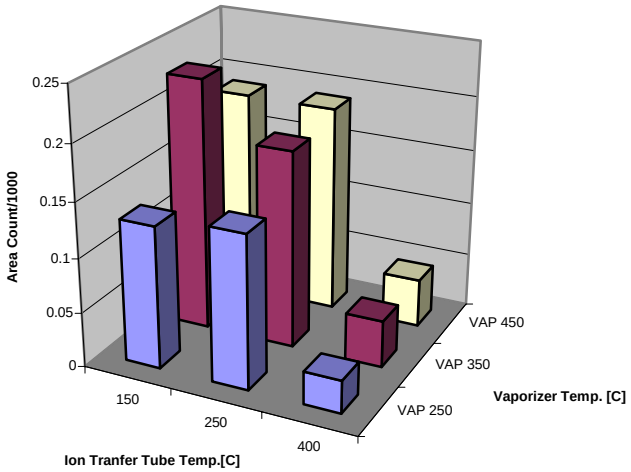
10 **Dichlorvos**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0.043	0.088	0.11
Vaporizer	VAP 350	0.087	0.12	0.084
	VAP 450	0.12	0.097	0.071



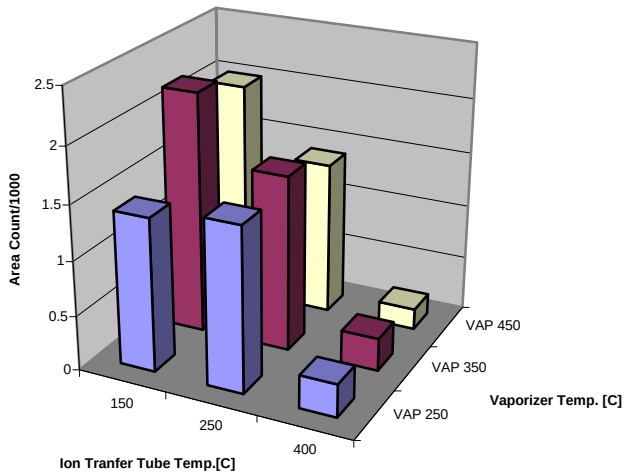
11 **Dichrotophos**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0.13	0.14	0.03
Vaporizer	VAP 350	0.23	0.18	0.043
	VAP 450	0.19	0.19	0.044



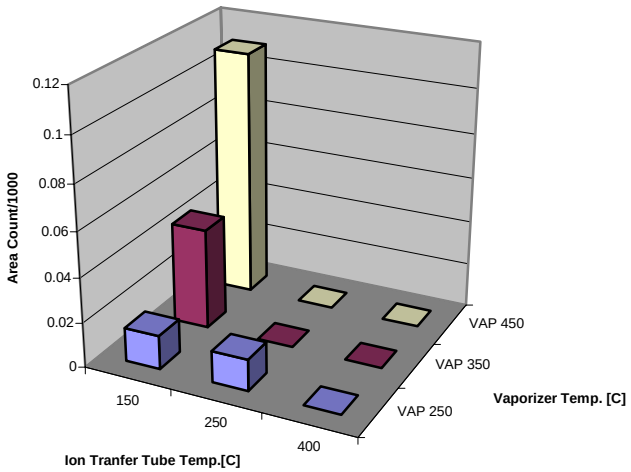
12 **Dimethoate**

	Area Count/1000	Capillary 150	250	400
	VAP 250	1.4	1.5	0.3
Vaporizer	VAP 350	2.2	1.6	0.3
	VAP 450	2	1.4	0.19



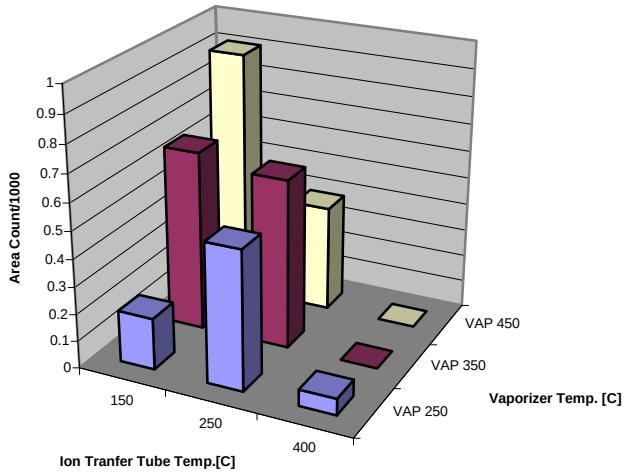
13 **Disulfoton**

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	0.015	0.014	0
Vaporizer	**VAP 350**	0.045	0	0
	VAP 450	0.11	0	0



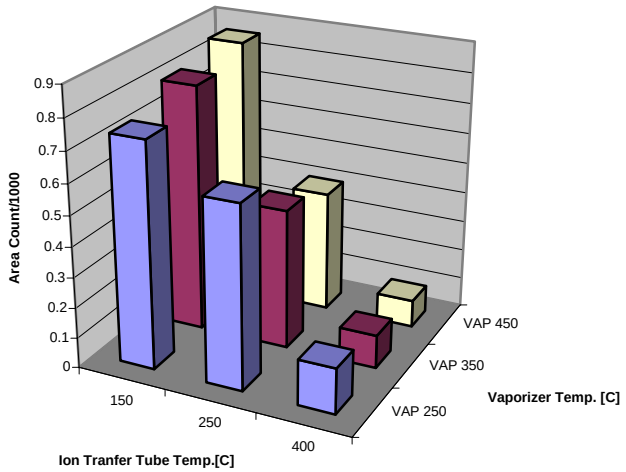
14 **Ethion**

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	0.19	0.51	0.06
Vaporizer	**VAP 350**	0.66	0.62	0
	VAP 450	0.91	0.39	0



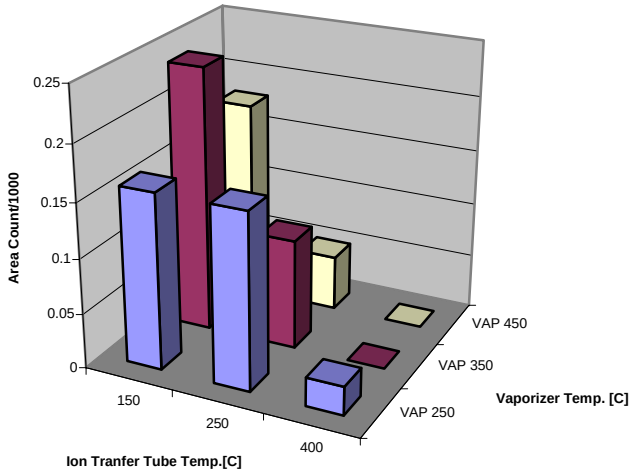
15 **Ethoprop**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0.74	0.6	0.15
Vaporizer	VAP 350	0.81	0.46	0.11
	VAP 450	0.86	0.4	0.09



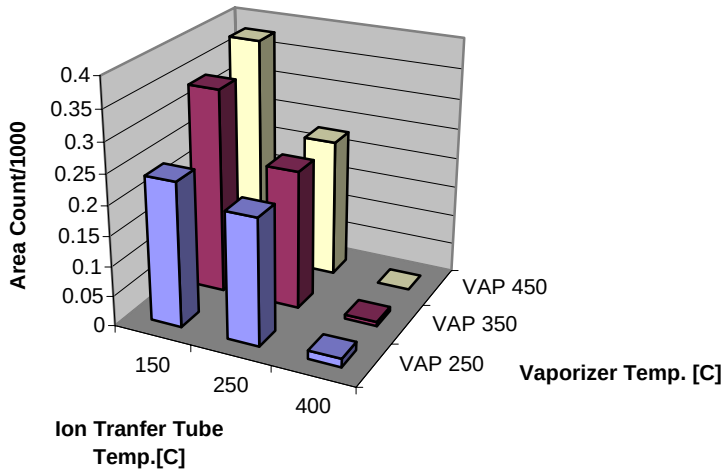
16 **Ethyl Parathion**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0.16	0.16	0.026
Vaporizer	VAP 350	0.24	0.1	0
	VAP 450	0.18	0.05	0



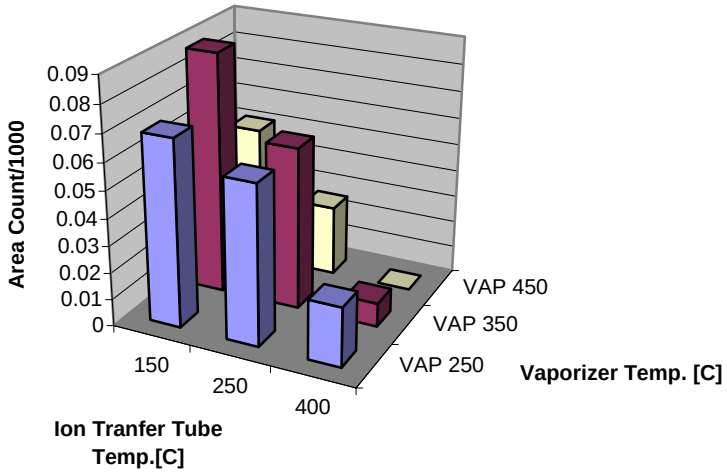
17 **Fenamiphos**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0.24	0.21	0.014
Vaporizer	VAP 350	0.34	0.23	0.007
	VAP 450	0.38	0.23	0



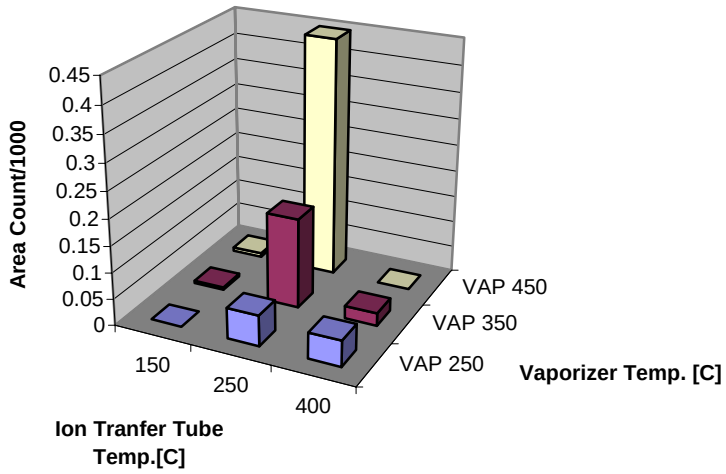
18 **Fenitrothion**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0.069	0.059	0.022
Vaporizer	VAP 350	0.089	0.06	0.0087
	VAP 450	0.051	0.026	0



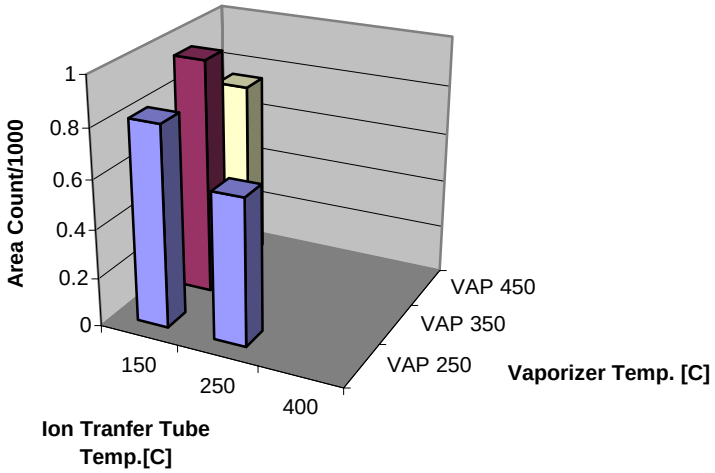
19 **Fenthion**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0	0.058	0.049
Vaporizer	VAP 350	0.005	0.17	0.024
	VAP 450	0.007	0.45	0



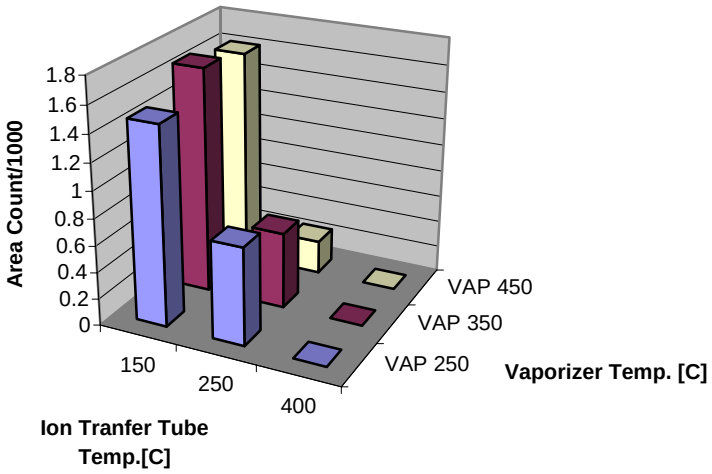
20 **Fonofos**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0.82	0.6	
Vaporizer	VAP 350	0.96		
	VAP 450	0.75		



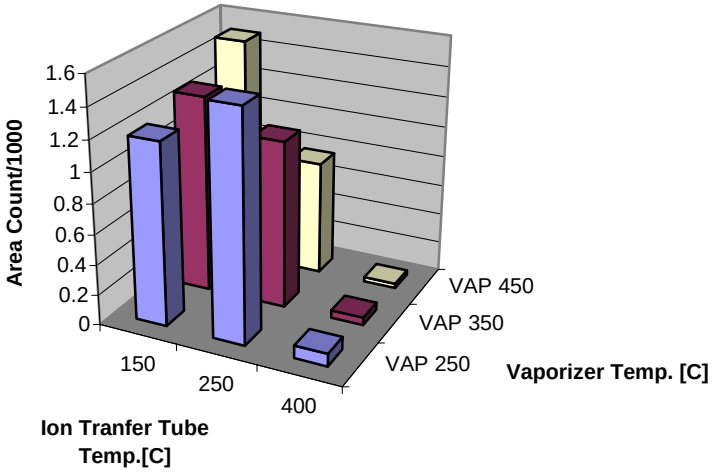
21 **Malathion**

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	1.48	0.73	0
Vaporizer	**VAP 350**	1.68	0.57	0
	VAP 450	1.61	0.25	0



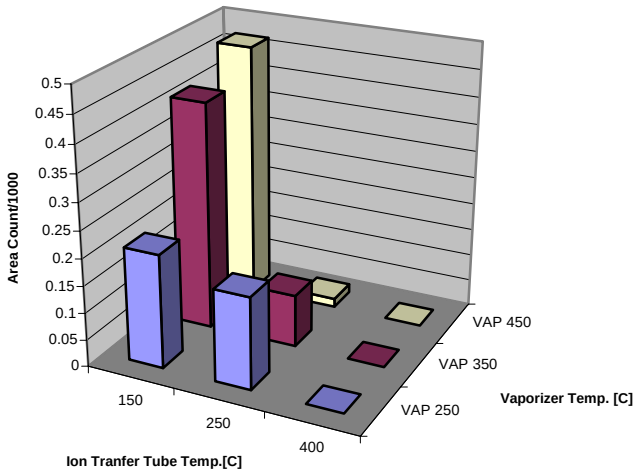
22 **MMP**

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	1.2	1.5	0.084
Vaporizer	**VAP 350**	1.3	1.1	0.05
	VAP 450	1.5	0.76	0.03



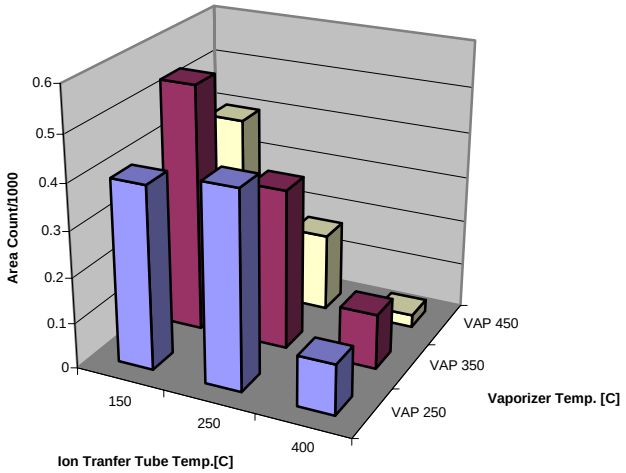
23 **Methidathion**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0.21	0.17	0
Vaporizer	VAP 350	0.42	0.097	0
	VAP 450	0.47	0.016	0

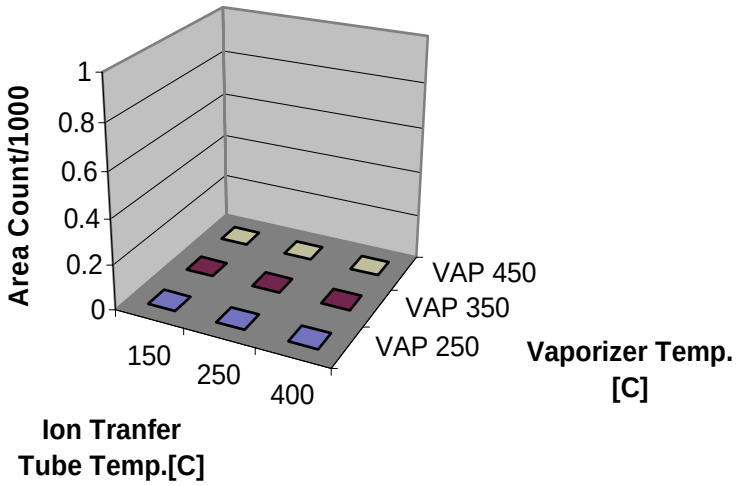


24 **Me-Parathion**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0.4	0.43	0.11
Vaporizer	VAP 350	0.54	0.35	0.12
	VAP 450	0.4	0.17	0.027



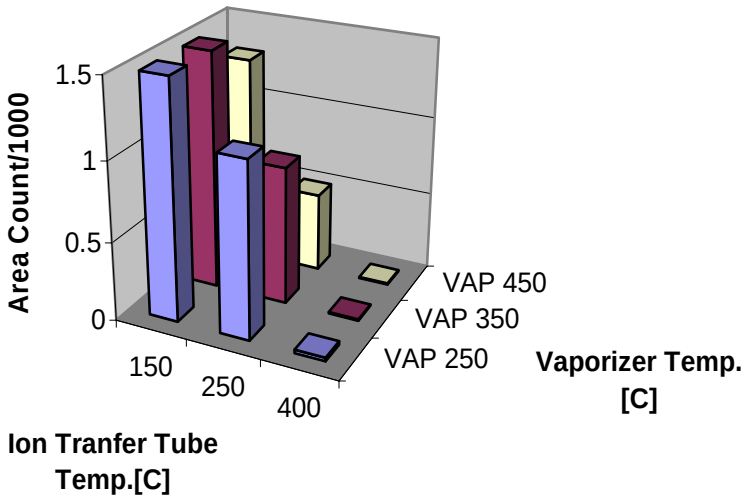
Area Count/1000		150	250	400
	VAP 250	0	0	0
Vaporizer	VAP 350	0	0	0
	VAP 450	0	0	0



Vaporizer - Heated Capillary Temperature Effect

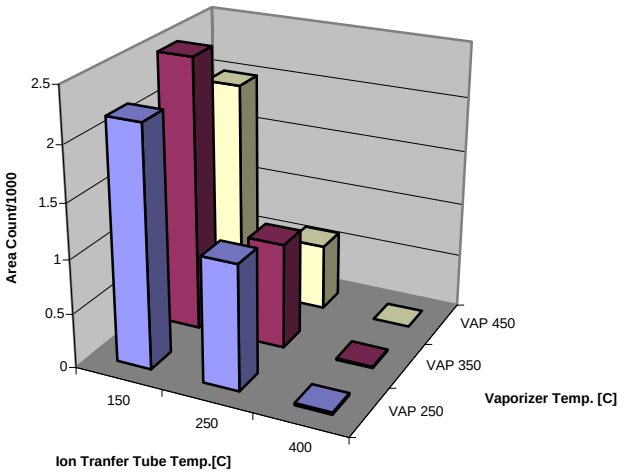
31 **Phostebupyrim**

Area Count/1000		150	250	400
	VAP 250	1.5	1.11	0.021
Vaporizer	VAP 350	1.5	0.87	0.0092
	VAP 450	1.3	0.5	0.008



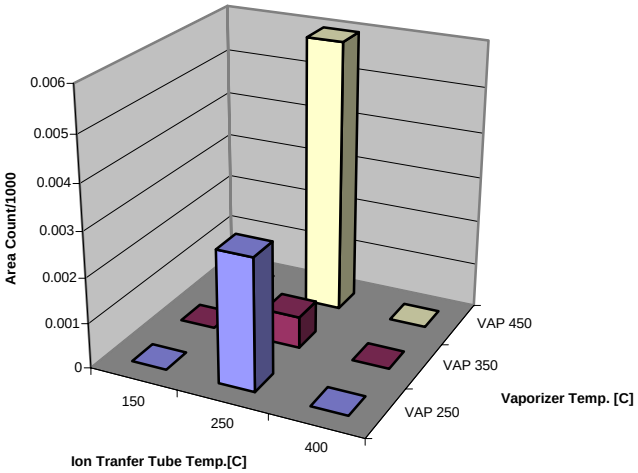
27 **Oxydemethon-Me**

	Area Count/1000	Capillary 150	250	400
	VAP 250	2.2	1.15	0.022
Vaporizer	VAP 350	2.5	0.97	0.014
	VAP 450	2	0.61	0



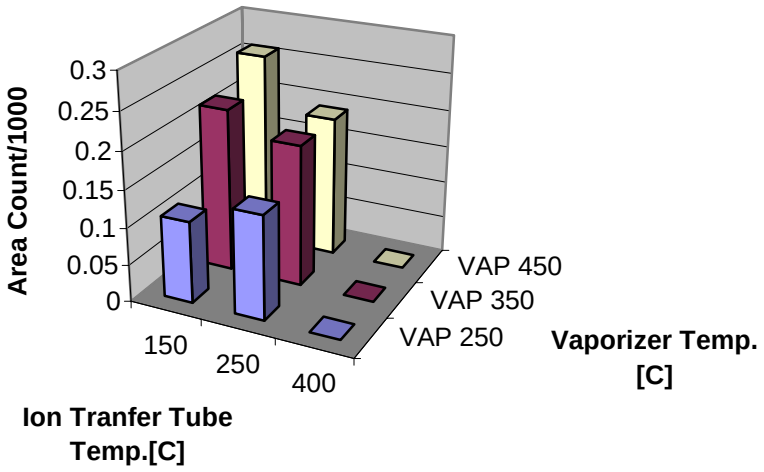
28 **Phorate**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0	0.0029	0
Vaporizer	VAP 350	0	0.0007	0
	VAP 450	0	0.006	0



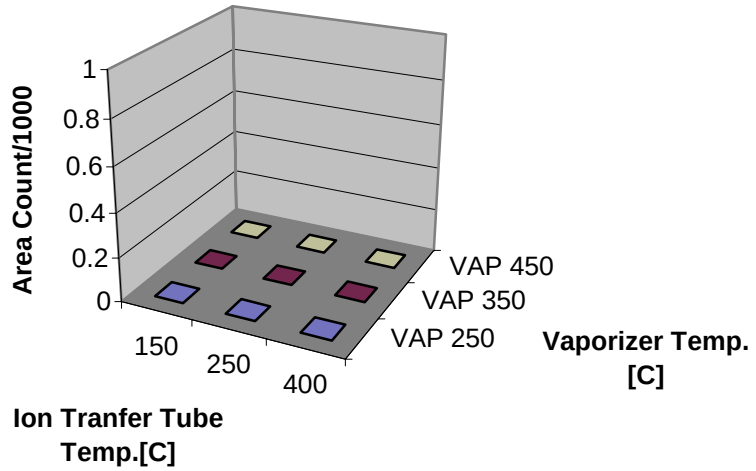
29 **Phosalone**

Area Count/1000		Capillary 150	250	400
Vaporizer	VAP 250	0.11	0.14	0
	VAP 350	0.22	0.19	0
	VAP 450	0.26	0.19	0



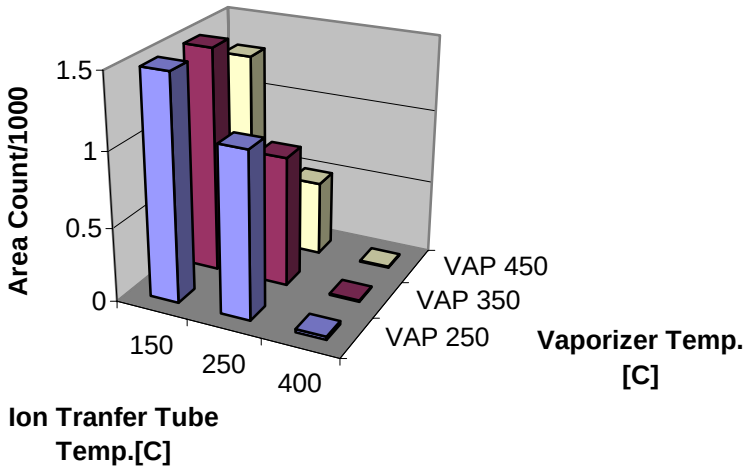
30 **Phosmet**

Area Count/1000		Capillary 150	250	400
Vaporizer	VAP 250	0	0	0
	VAP 350	0	0	0
	VAP 450	0	0	0



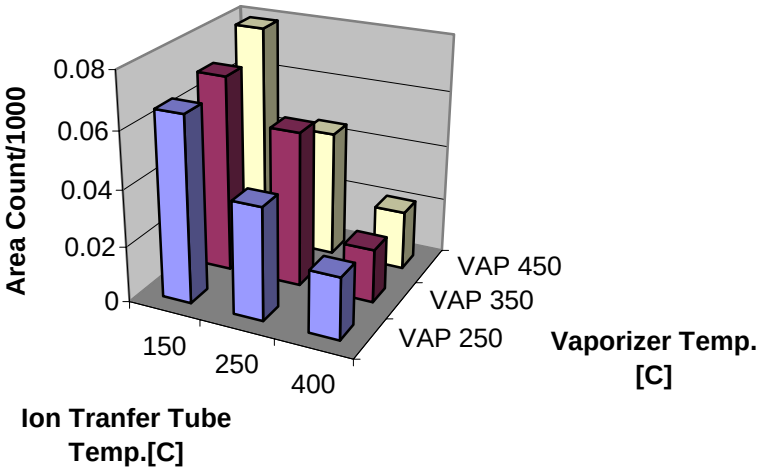
31 **Phostebupyrim**

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	1.5	1.11	0.021
Vaporizer	**VAP 350**	1.5	0.87	0.0092
	VAP 450	1.3	0.5	0.008



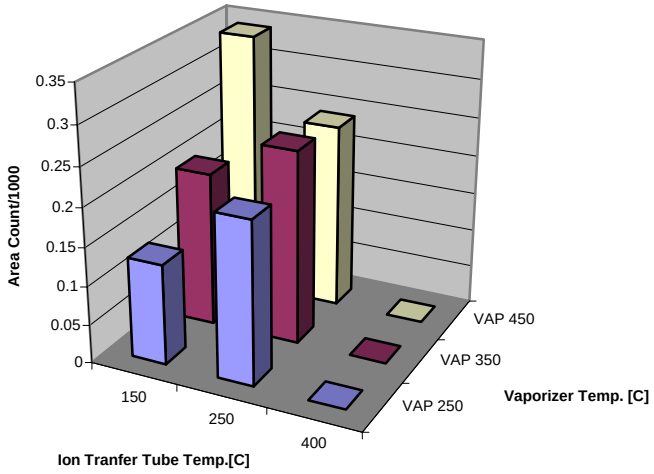
32 **Pirimiphos - Me**

		Capillary		
	Area Count/1000	**150**	**250**	**400**
	VAP 250	0.066	0.04	0.022
Vaporizer	**VAP 350**	0.07	0.055	0.019
	VAP 450	0.079	0.045	0.021



38 **Tetrachlorvinphos**

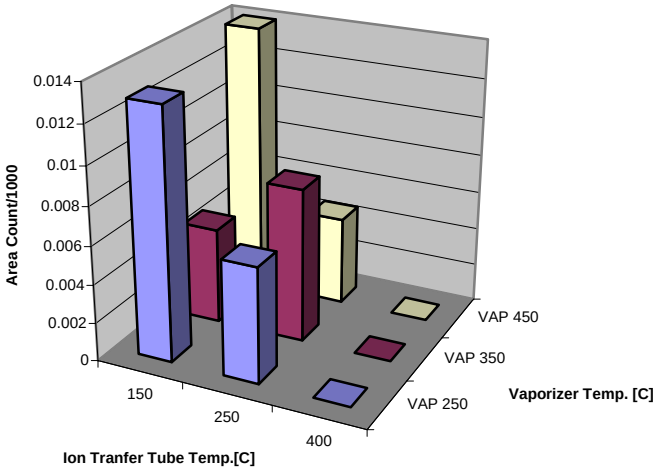
Area Count/1000		Capillary 150	250	400
Vaporizer	VAP 250	0.13	0.21	0
	VAP 350	0.2	0.25	0
	VAP 450	0.34	0.24	0



Vaporizer - Heated Capillary Temperature Effect

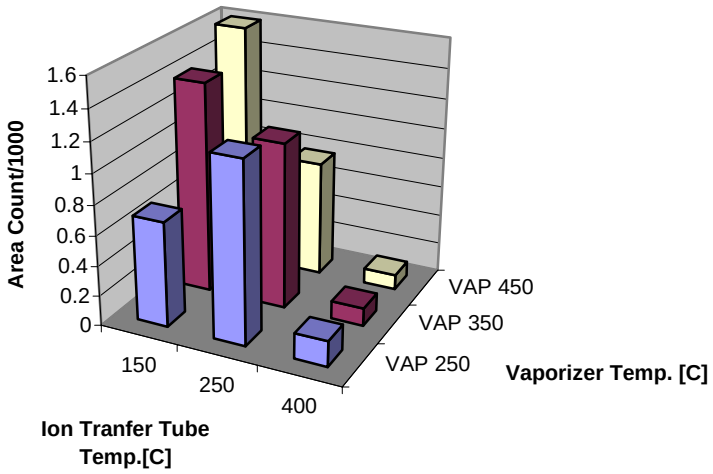
39 **Tribufos**

Area Count/1000		Capillary 150	250	400
Vaporizer	VAP 250	0.013	0.006	0
	VAP 350	0.005	0.008	0
	VAP 450	0.014	0.0046	0



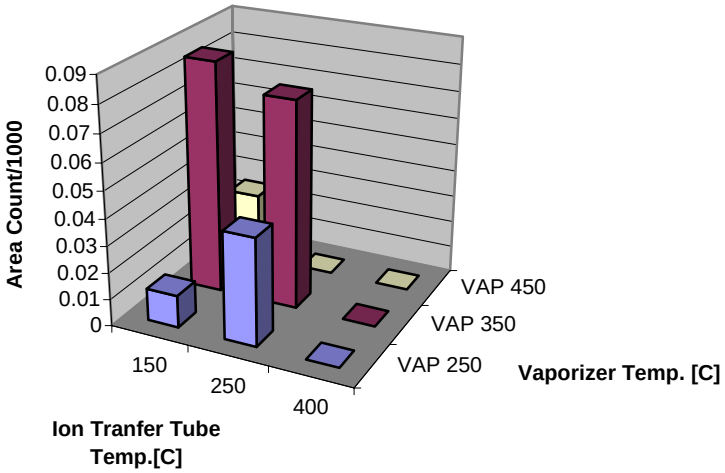
35 **Sulfotepp**

Area Count/1000		Capillary 150	250	400
Vaporizer	VAP 250	0.7	1.2	0.17
	VAP 350	1.4	1.1	0.12
	VAP 450	1.6	0.77	0.099



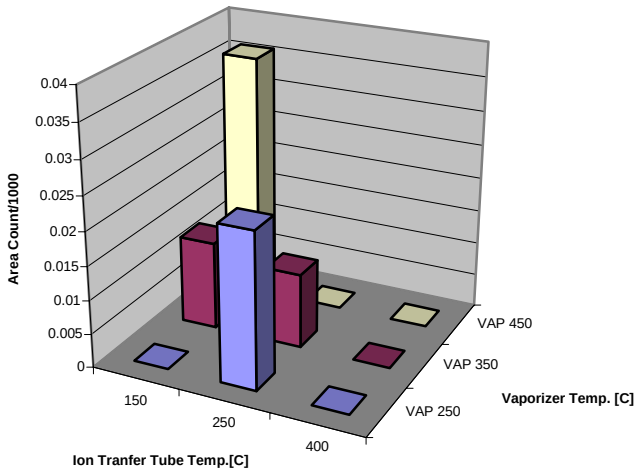
36 **Temephos**

Area Count/1000		Capillary 150	250	400
Vaporizer	VAP 250	0.012	0.04	0
	VAP 350	0.086	0.077	0
	VAP 450	0.025	0	0



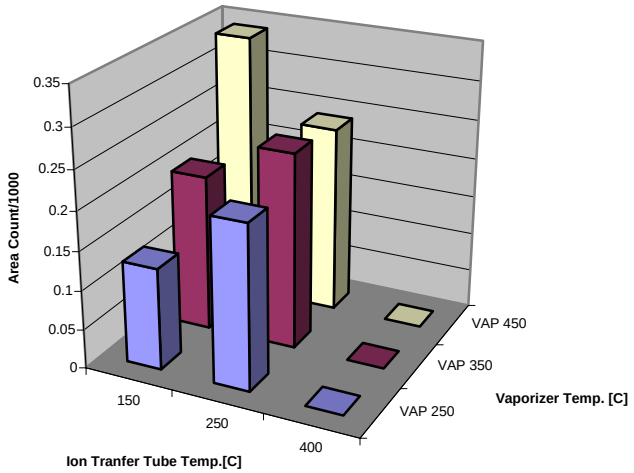
37 **Terbufos**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0	0.023	0
Vaporizer	VAP 350	0.013	0.011	0
	VAP 450	0.036	0	0



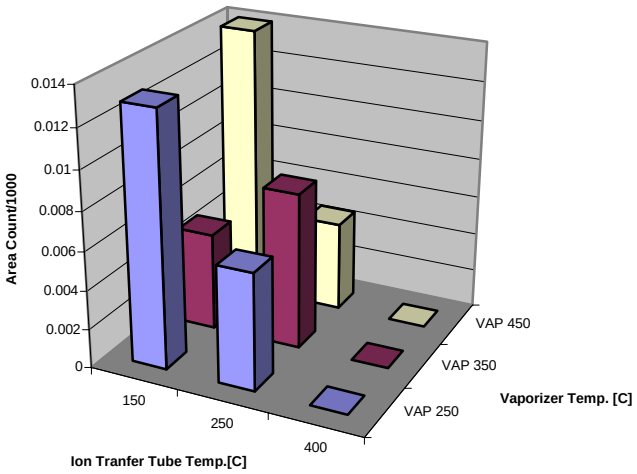
38 **Tetrachlorvinphos**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0.13	0.21	0
Vaporizer	VAP 350	0.2	0.25	0
	VAP 450	0.34	0.24	0



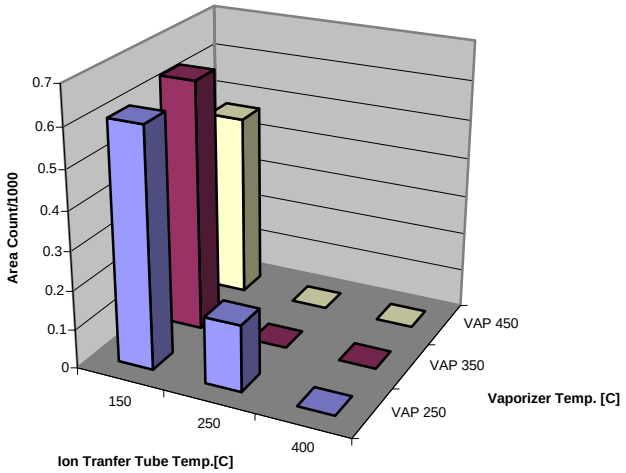
39 **Tribufos**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0.013	0.006	0
Vaporizer	VAP 350	0.005	0.008	0
	VAP 450	0.014	0.0046	0



40 **Trichlorfon**

	Area Count/1000	Capillary 150	250	400
	VAP 250	0.61	0.17	0
Vaporizer	VAP 350	0.64	0	0
	VAP 450	0.47	0	0



Appendix 3B : Applied Solvent Gradient curves for testing HPLC columns and

This section lists the solvent gradients and their reference codes used while developing the HPLC portion of the analytical separations.

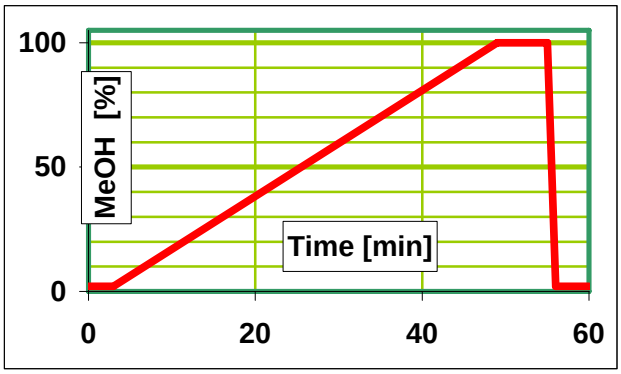
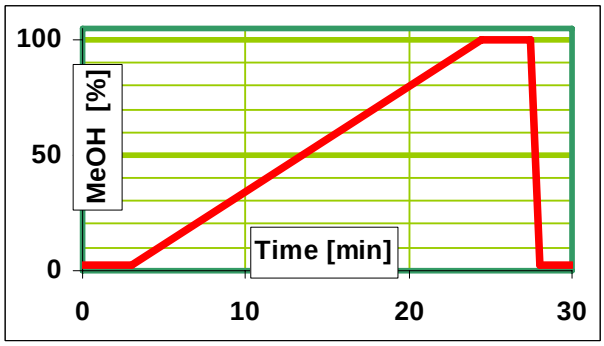
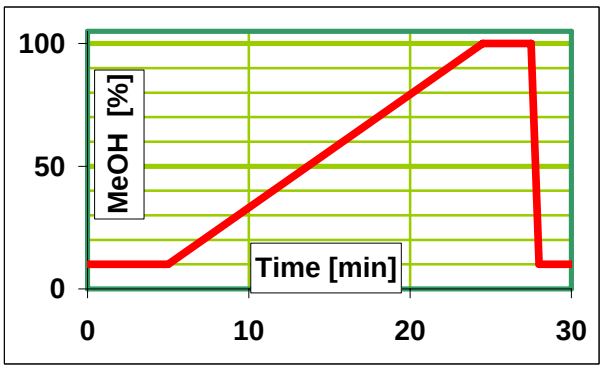
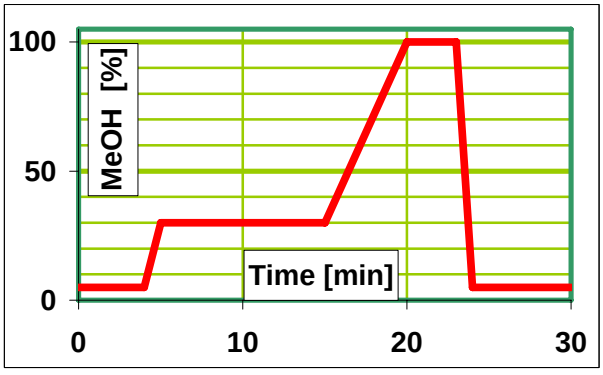
code	Gradient		Graph
11_1 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	2	
	3	2	
	49	100	
	55	100	
	56	2	
	60	2	
11_2, **11_3** 1 ,mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	2	
	3	2	
	24.5	100	
	27.5	100	
	28	2	
	30	2	
11_4 0.2mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	10	
	5	10	
	24.5	100	
	27.5	100	
	28	10	
	30	10	
11_5 0.2mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	5	
	4	5	
	5	30	
	15	30	
	20	100	
	23	100	
	24	5	
	30	5	

Table 18 List of elution curves applied while optimizing LC separations

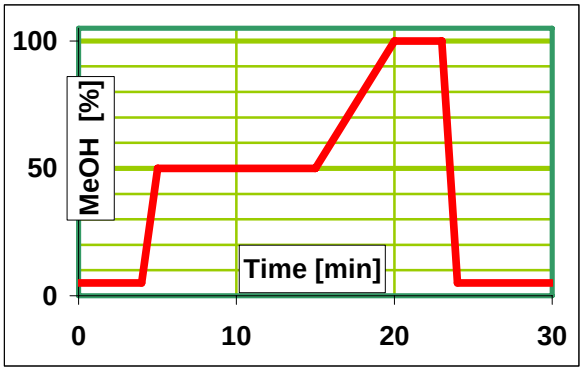
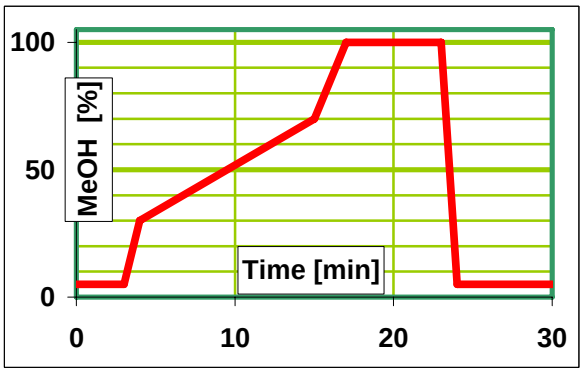
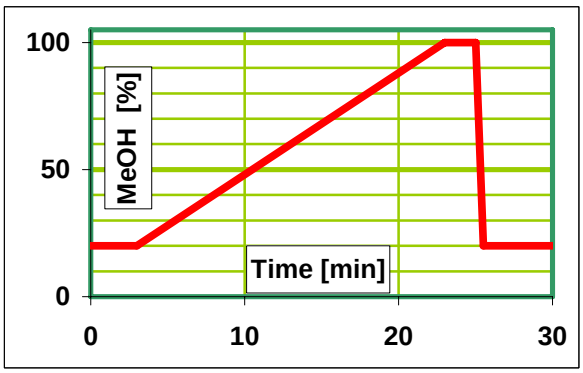
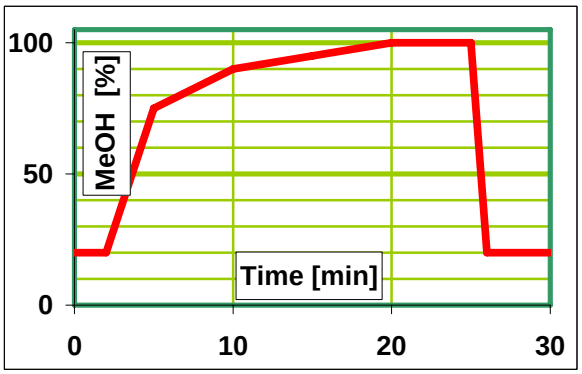
code	Gradient		Graph
11_6 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	5	
	4	5	
	5	50	
	15	50	
	20	100	
	23	100	
	24	5	
	30	5	
11_7 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	5	
	3	5	
	4	30	
	15	70	
	17	100	
	23	100	
	24	5	
	30	5	
11_8 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	20	
	3	20	
	23	100	
	25	100	
	25.5	20	
	30	20	
11_9 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	20	
	2	20	
	5	75	
	10	90	
	15	95	
	20	100	
	25	100	
	26	20	
	30	20	

Table Table 18 (cont) List of elution curves applied while optimizing LC separations

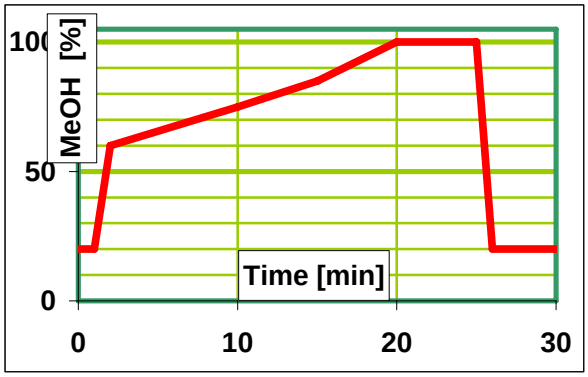
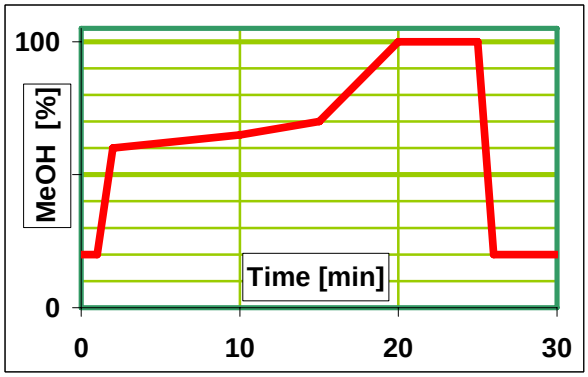
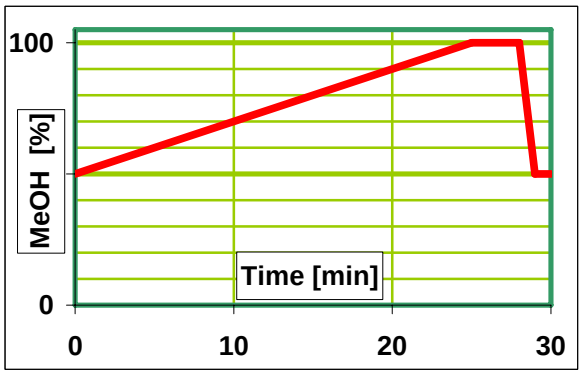
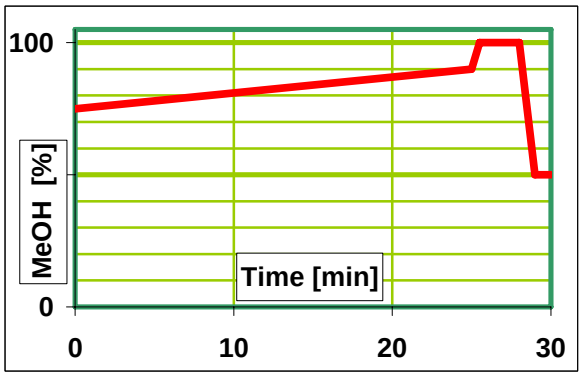
code	Gradient		Graph
11-10 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	20	
	1	20	
	2	60	
	10	75	
	15	85	
	20	100	
	25	100	
	26	20	
	30	20	
11_11 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	20	
	1	20	
	2	60	
	10	65	
	15	70	
	20	100	
	25	100	
	26	20	
	30	20	
11_12 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	50	
	25	100	
	28	100	
	29	50	
	30	50	
11_13 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	75	
	25	90	
	25.5	100	
	28	100	
	29	50	
	30	50	

Table Table 18 (cont) List of elution curves applied while optimizing LC separations

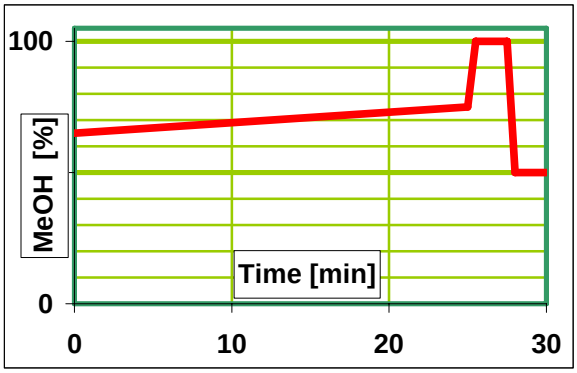
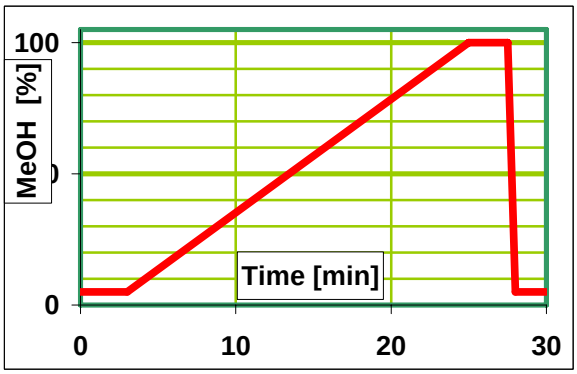
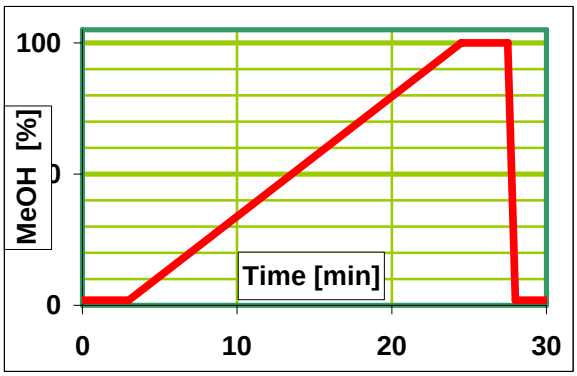
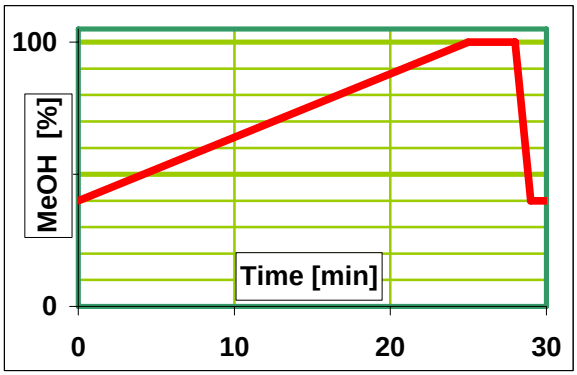
code	Gradient	Graph
11_14 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 / 65 25 / 75 25.5 / 100 27.5 / 100 28 / 50 30 / 50	
11_15 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 / 5 3 / 5 25 / 100 27.5 / 100 28 / 5 30 / 5	
11_16 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 / 2 3 / 2 24.5 / 100 27.5 / 100 28 / 2 30 / 2	
11_17 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 / 40 25 / 100 28 / 100 29 / 40 30 / 40	

Table Table 18 (cont) List of elution curves applied while optimizing LC separations

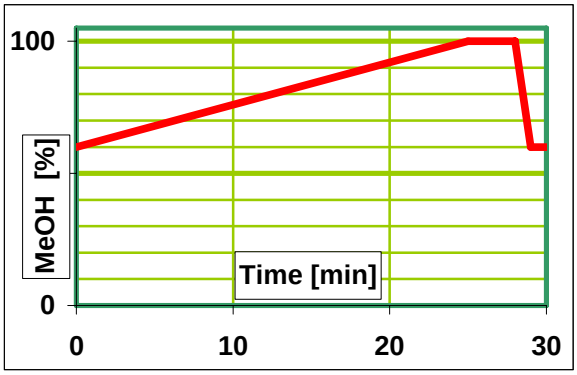
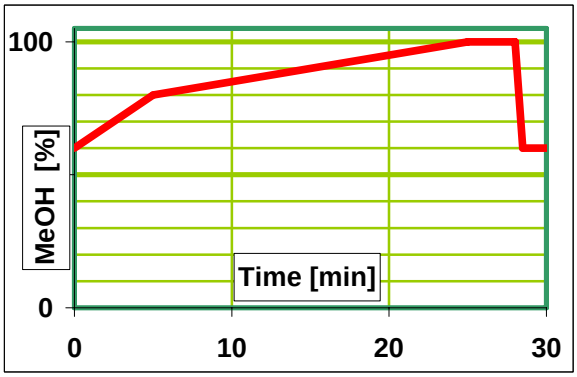
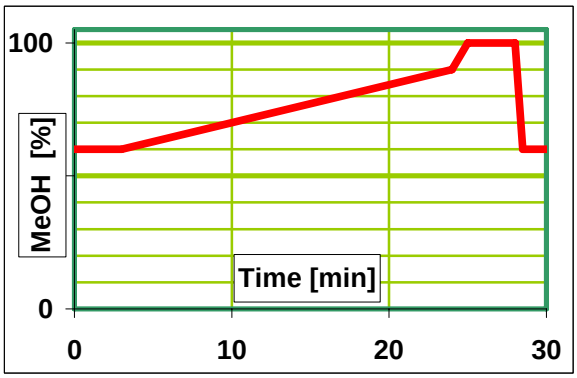
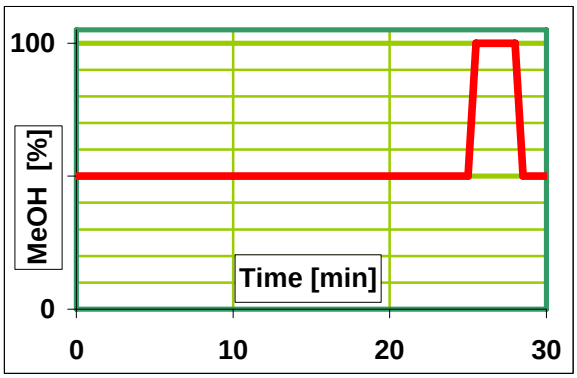
code	Gradient		Graph
11_18 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	60	
	25	100	
	28	100	
	29	60	
	30	60	
11_19 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	60	
	5	80	
	25	100	
	28	100	
	28.5	60	
	30	60	
11_20 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	60	
	3	60	
	24	90	
	25	100	
	28	100	
	28.5	60	
	30	60	
11_21 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	50	
	25	50	
	25.5	100	
	28	100	
	28.5	50	
	30	50	

Table Table 18 (cont) List of elution curves applied while optimizing LC separations

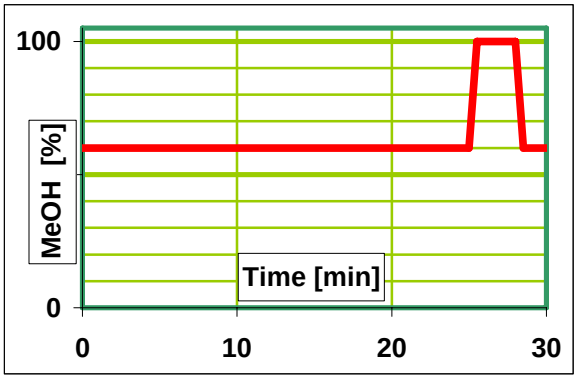
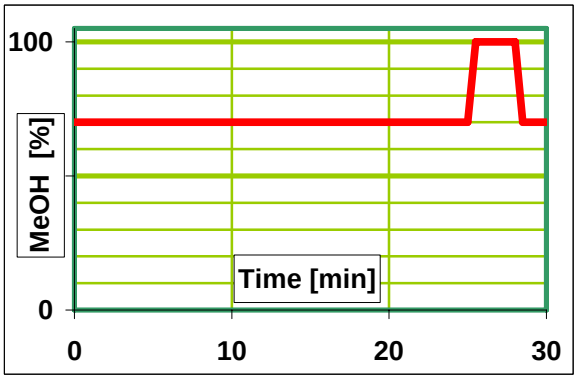
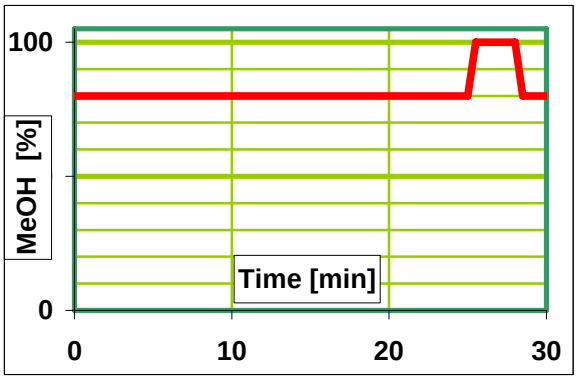
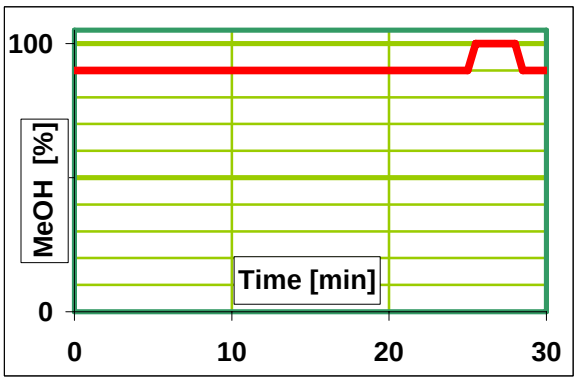
code	Gradient	Graph
11_22 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 60 25 60 25.5 100 28 100 28.5 60 30 60	
11_23 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 70 25 70 25.5 100 28 100 28.5 70 30 70	
11_24 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 80 25 80 25.5 100 28 100 28.5 80 30 80	
11_25 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 90 25 90 25.5 100 28 100 28.5 90 30 90	

Table Table 18 (cont) List of elution curves applied while optimizing LC separations

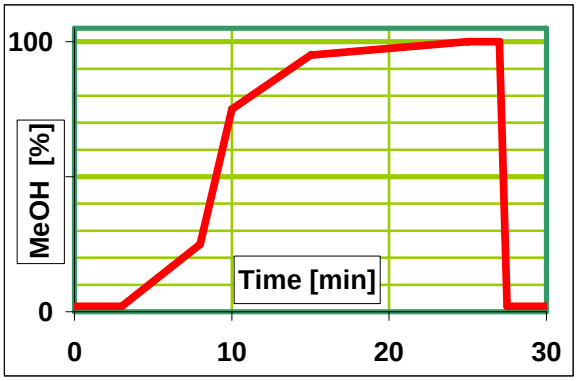
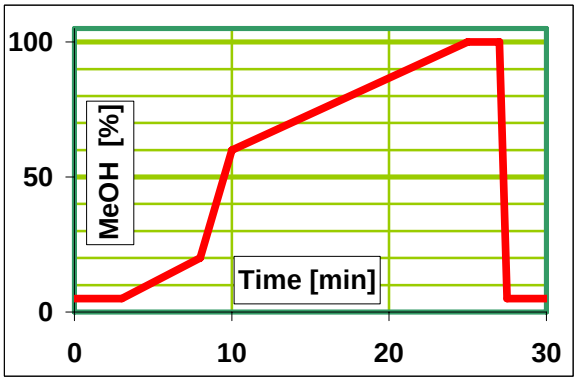
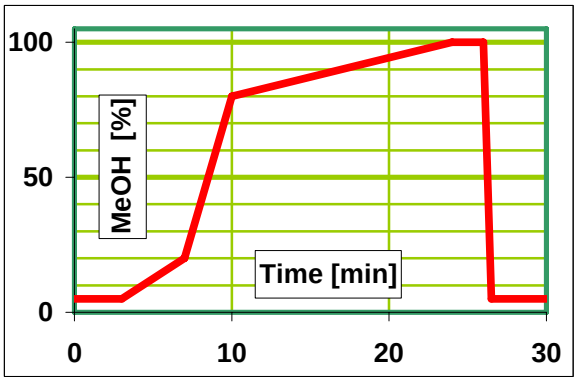
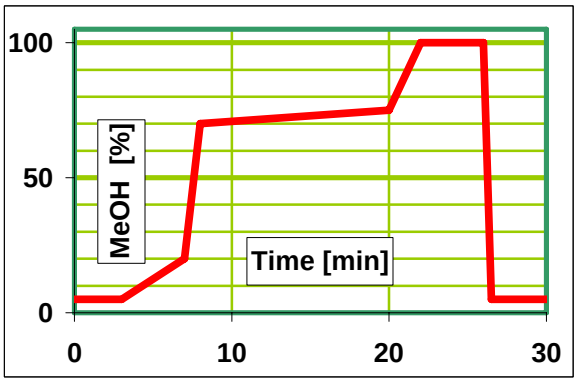
code	Gradient		Graph
12 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	2	
	3	2	
	8	25	
	10	75	
	15	95	
	25	100	
	27	100	
	27.5	2	
	30	2	
13 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	5	
	3	5	
	8	20	
	10	60	
	25	100	
	27	100	
	27.5	5	
	30	5	
14 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	5	
	3	5	
	7	20	
	10	80	
	24	100	
	26	100	
	26.5	5	
	30	5	
15 1 mL/min 0.1% formic acid in both	Time [min]	MeOH [%V/V]	
	0	5	
	3	5	
	7	20	
	8	70	
	20	75	
	22	100	
	26	100	
	26.5	5	
	30	5	

Table Table 18 (cont) List of elution curves applied while optimizing LC separations

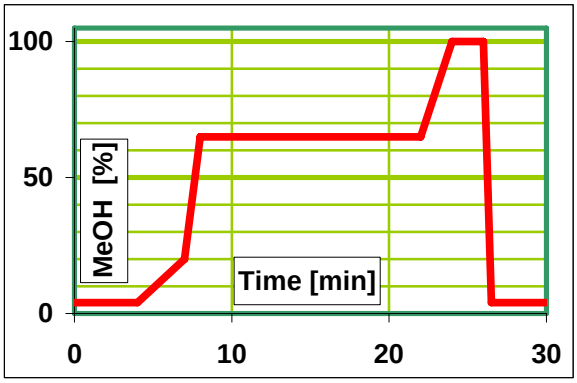
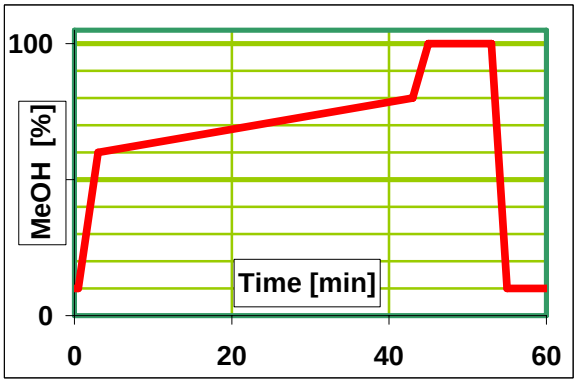
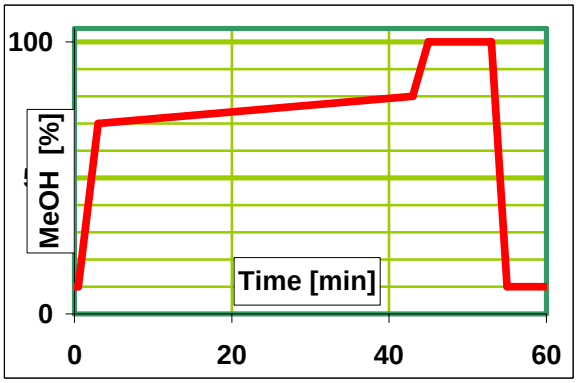
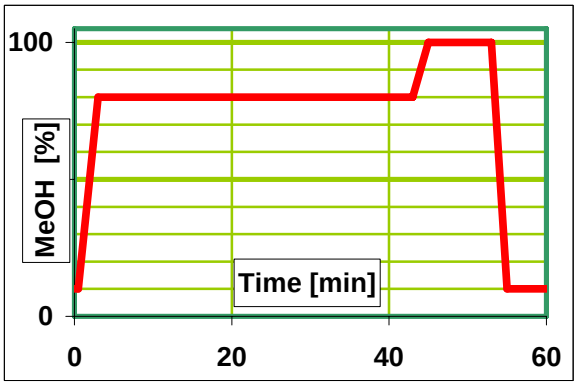
code	Gradient	Graph
16 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 4 4 4 7 20 8 65 22 65 24 100 26 100 26.5 4 30 4	
17_1 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 10 0.5 10 3 60 43 80 45 100 53 100 55 10 60 10	
17_2 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 10 0.5 10 3 70 43 80 45 100 53 100 55 10 60 10	
17_3 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 10 0.5 10 3 80 43 80 45 100 53 100 55 10 60 10	

Table Table 18 (cont) List of elution curves applied while optimizing LC separations

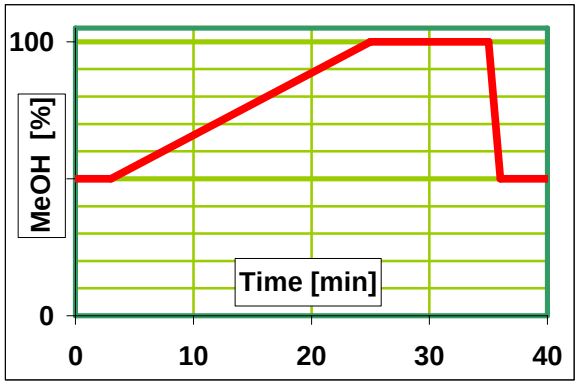
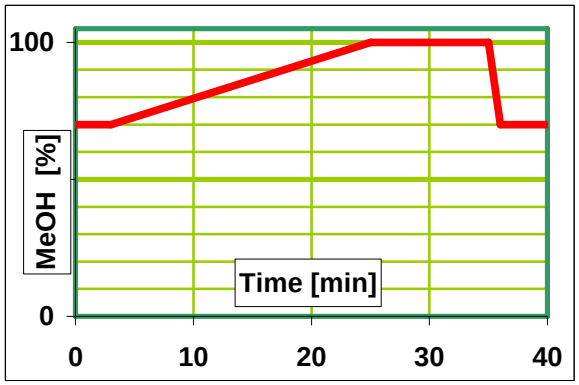
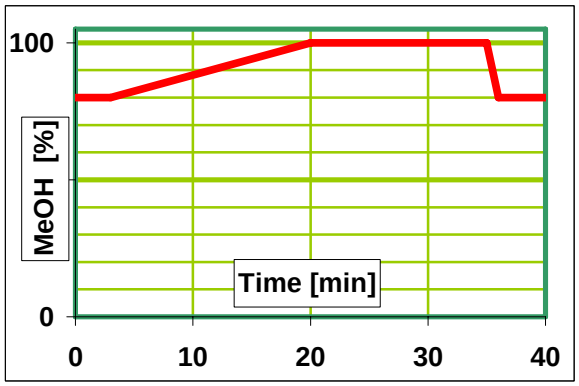
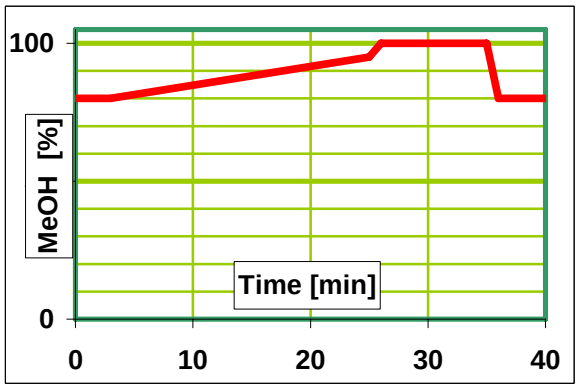
code	Gradient	Graph
18_1 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 50 3 50 25 100 30 100 35 100 36 50 40 50	
18_2 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 70 3 70 25 100 30 100 35 100 36 70 40 70	
18_3 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 80 3 80 20 100 30 100 35 100 36 80 40 80	
18_4 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 80 3 80 25 95 26 100 35 100 36 80 40 80	

Table Table 18 (cont) List of elution curves applied while optimizing LC separations

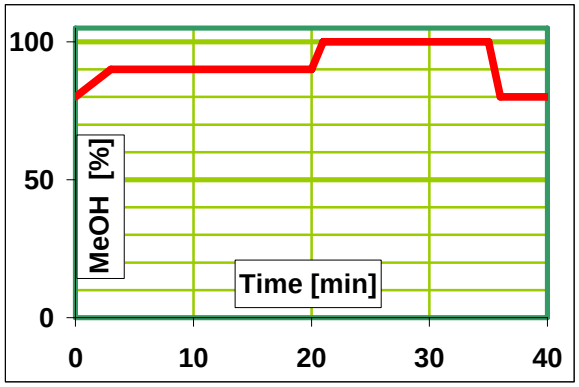
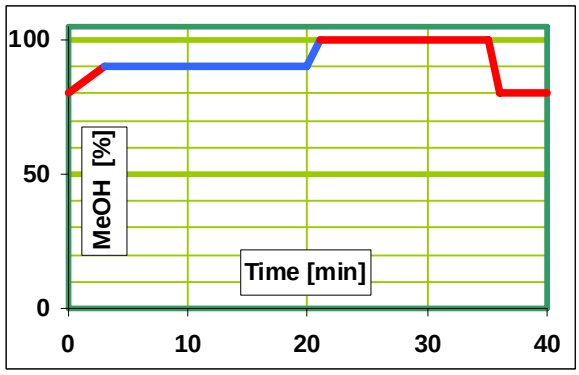
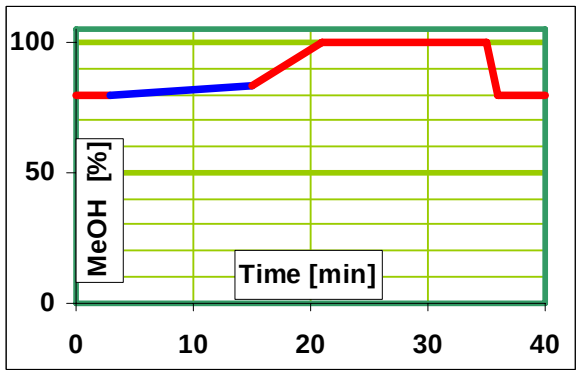
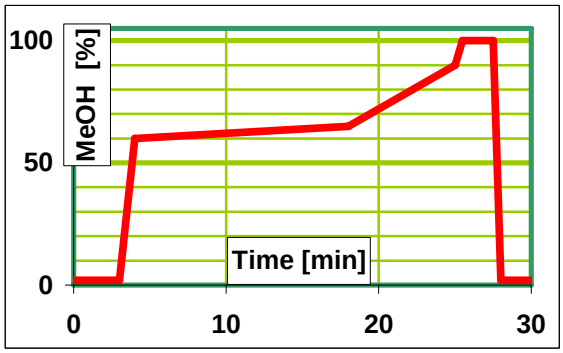
code	Gradient	Graph
18_5 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 80 3 90 20 90 21 100 35 100 36 80 40 80	
18_6 3-21min: .75mL/min Else: 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 80 3 90 20 90 21 100 35 100 36 80 40 80	
18_7 3-15min: Increasing .75 to 1.0 mL/min else: 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 80 3 80 15 83 21 100 35 100 36 80 40 80	
Final 1 mL/min 0.1% formic acid in both	Time [min] / MeOH [%V/V] 0 2 3 2 4 60 18 65 25 90 25.5 100 27.5 100 28 2 30 2	

Table Table 18 (cont) List of elution curves applied while optimizing LC separations

Appendix 3C: ESI and APCI positive and negative full scan spectra

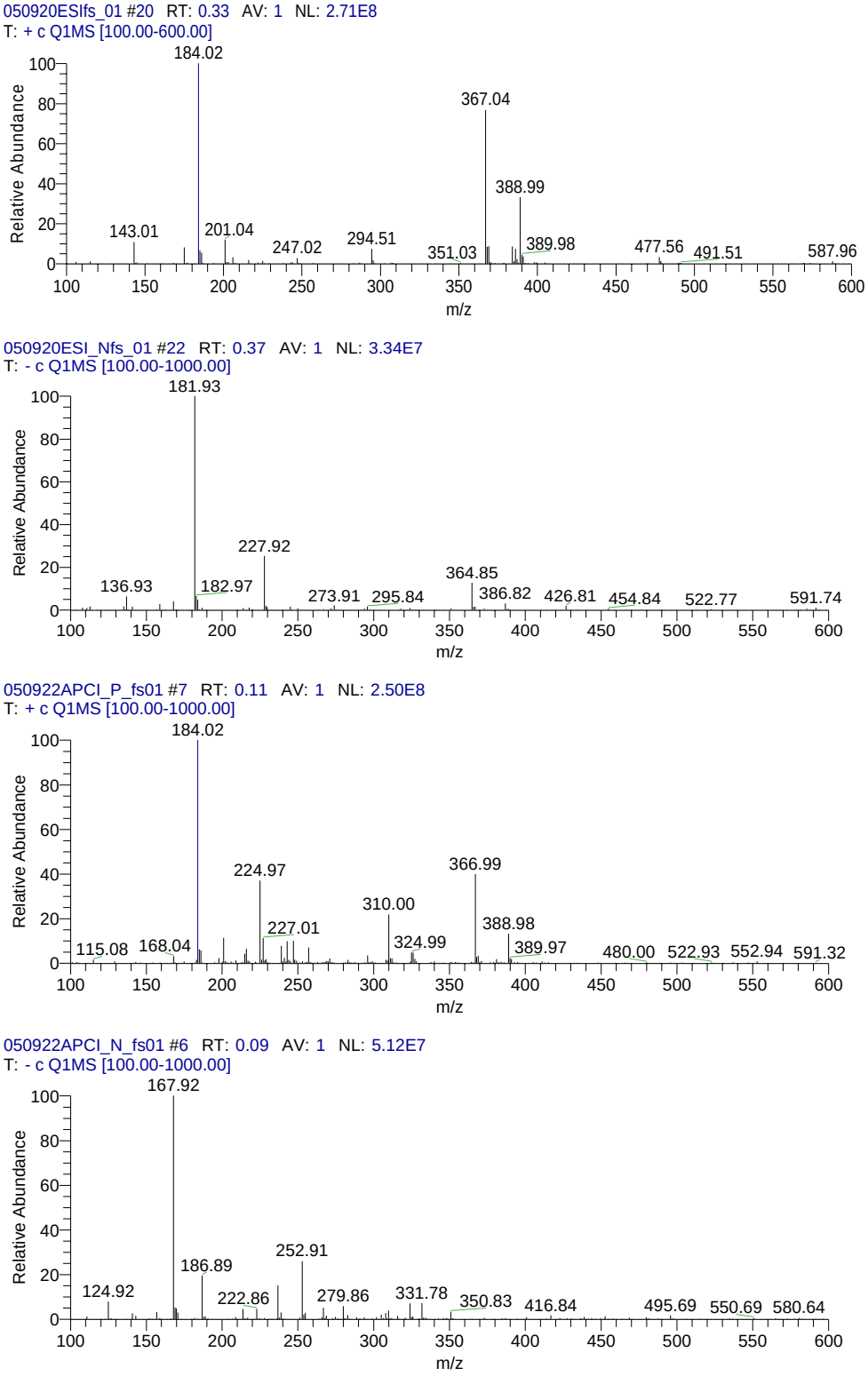
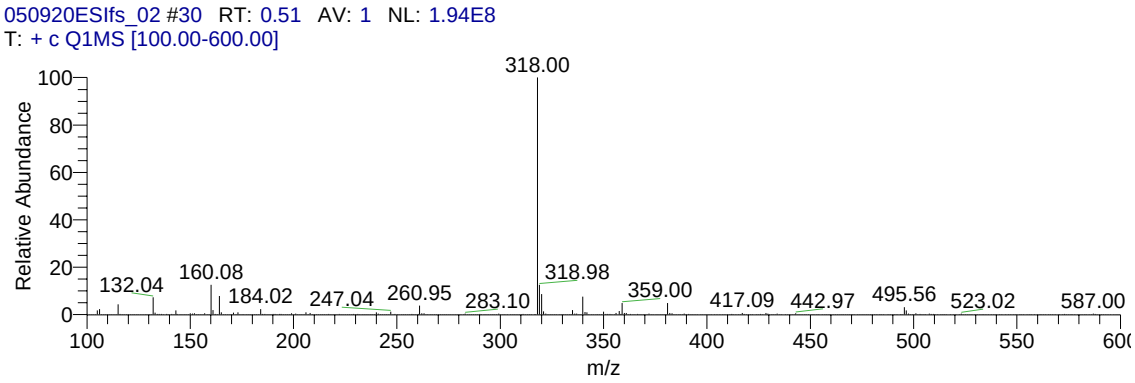


Figure 64 Acephate ESI positive, ESI negative, APCI positive, APCI negative full scan spectra



N/F

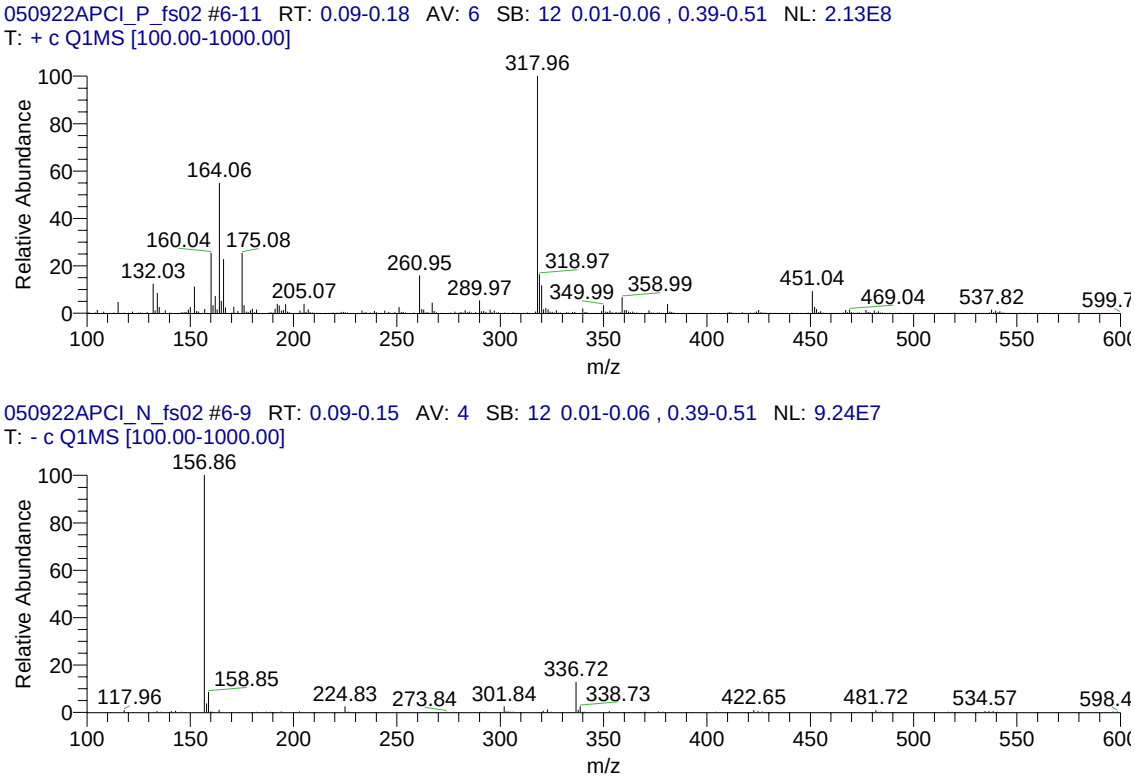


Figure 65 Azinphos Methyl ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

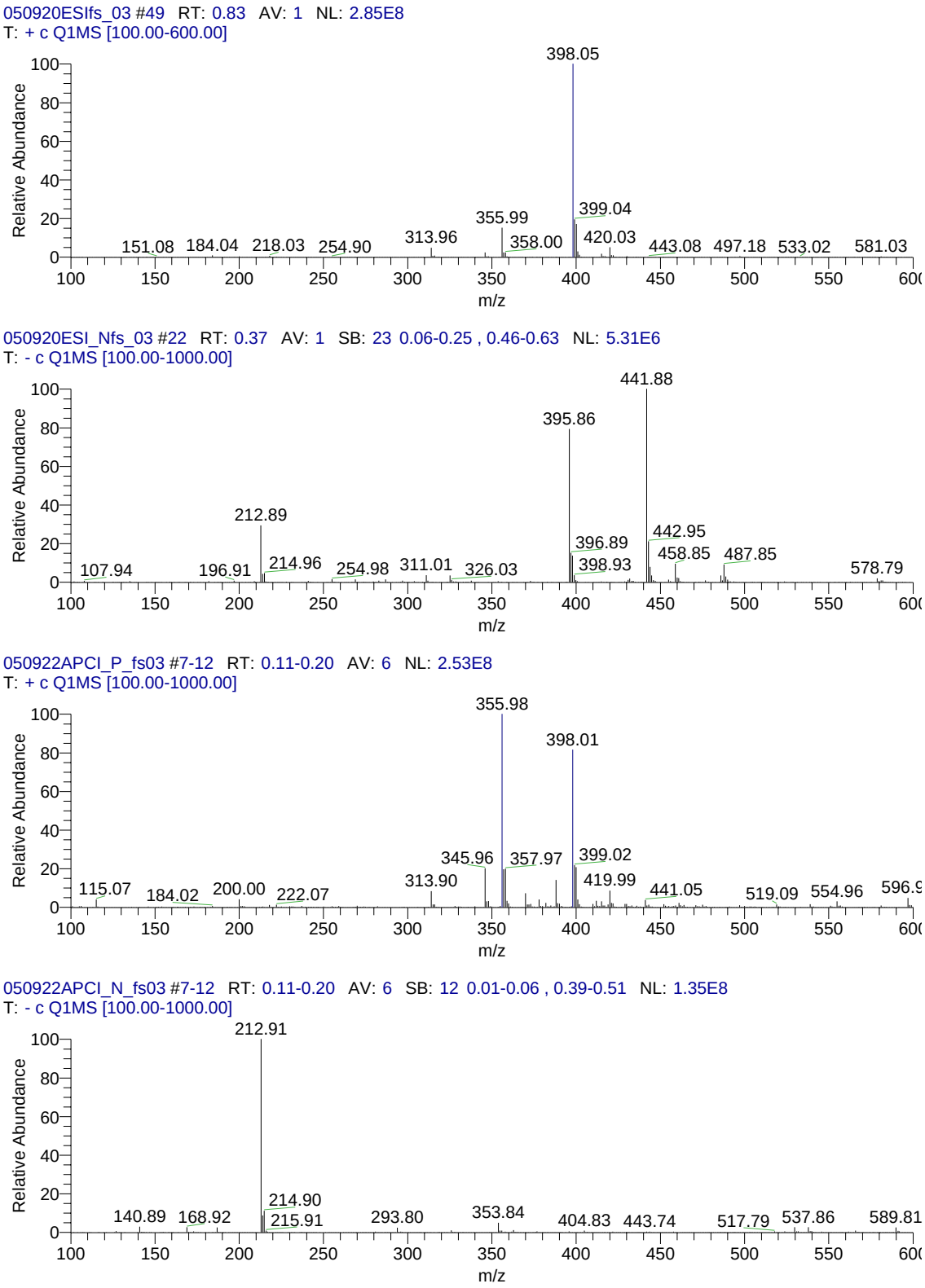


Figure 66 Bensulide ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

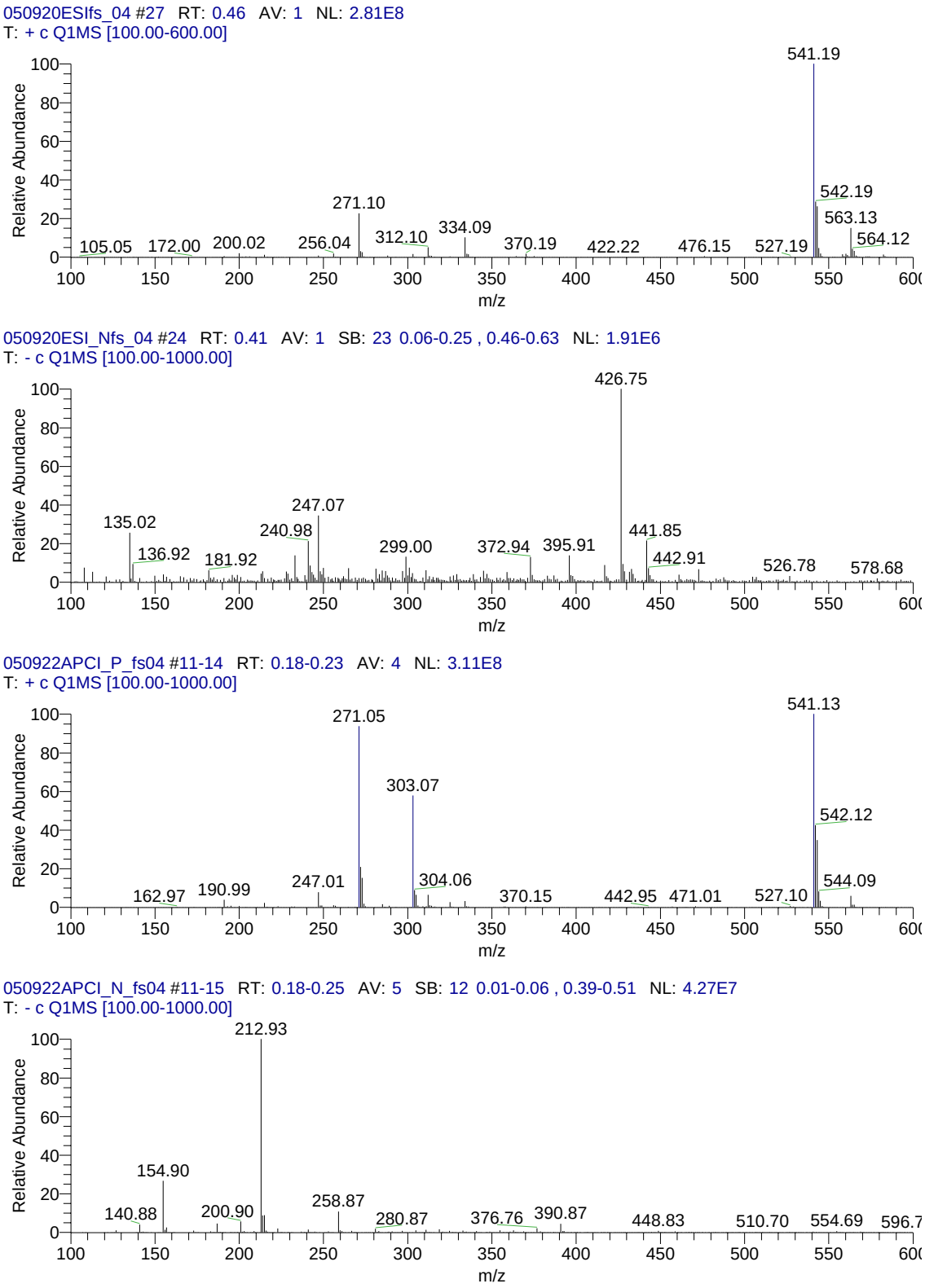


Figure 67 Cadusafos ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

N/F

N/F

N/F

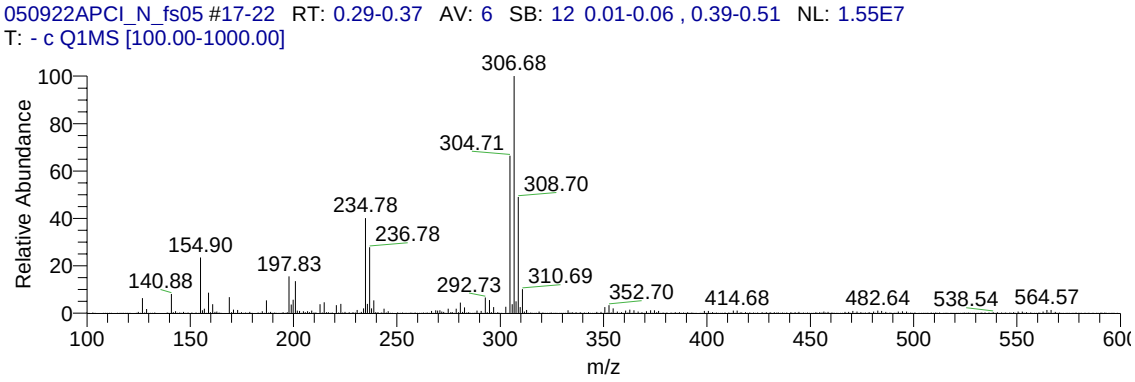


Figure 68 Chlorethoxyfos ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

N/F

N/F

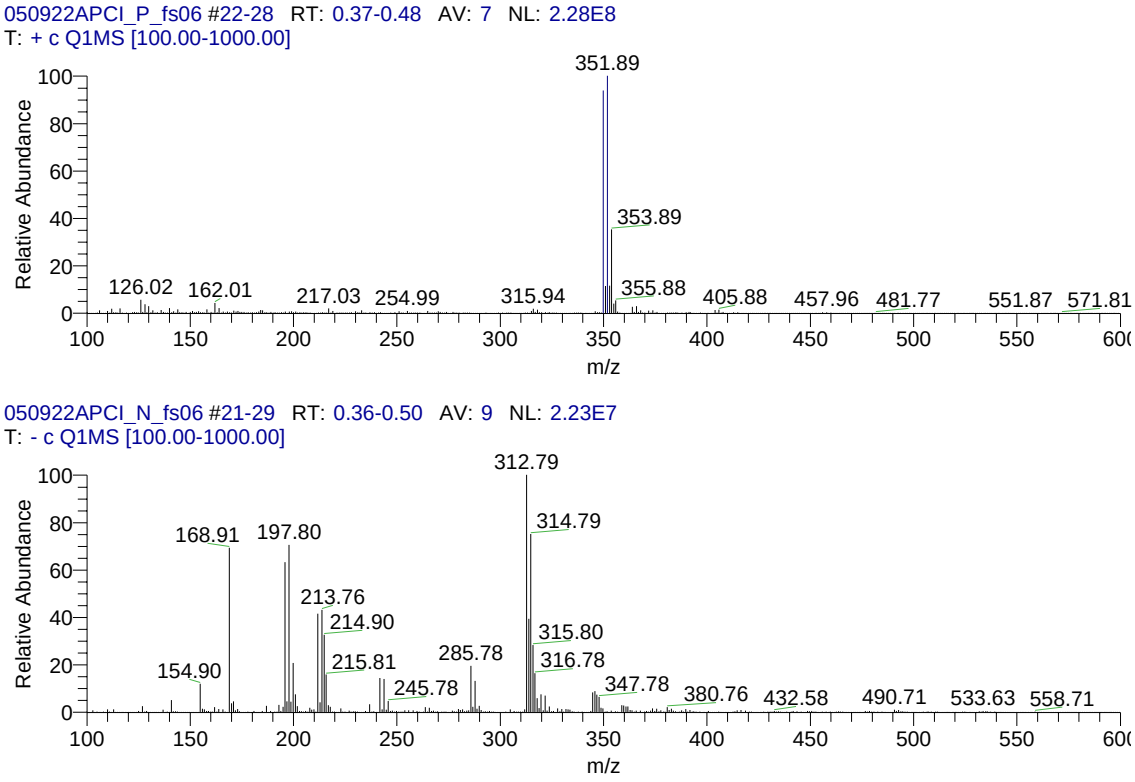


Figure 69 Chlorpyrifos ESI positive (weak), ESI negative (weak), APCI positive, APCI negative full scan spectra

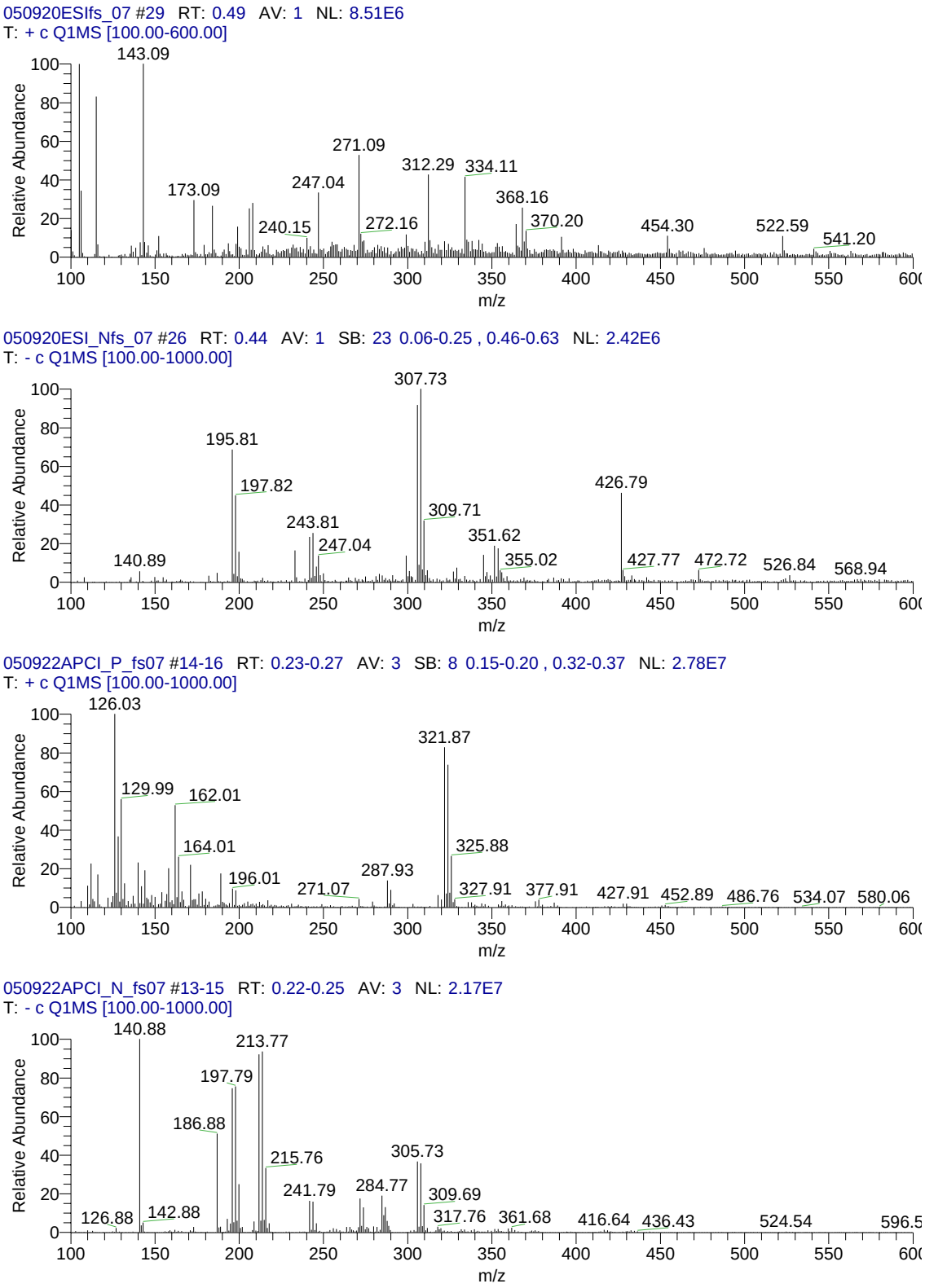


Figure 70 Chlorpyrifos Methyl ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

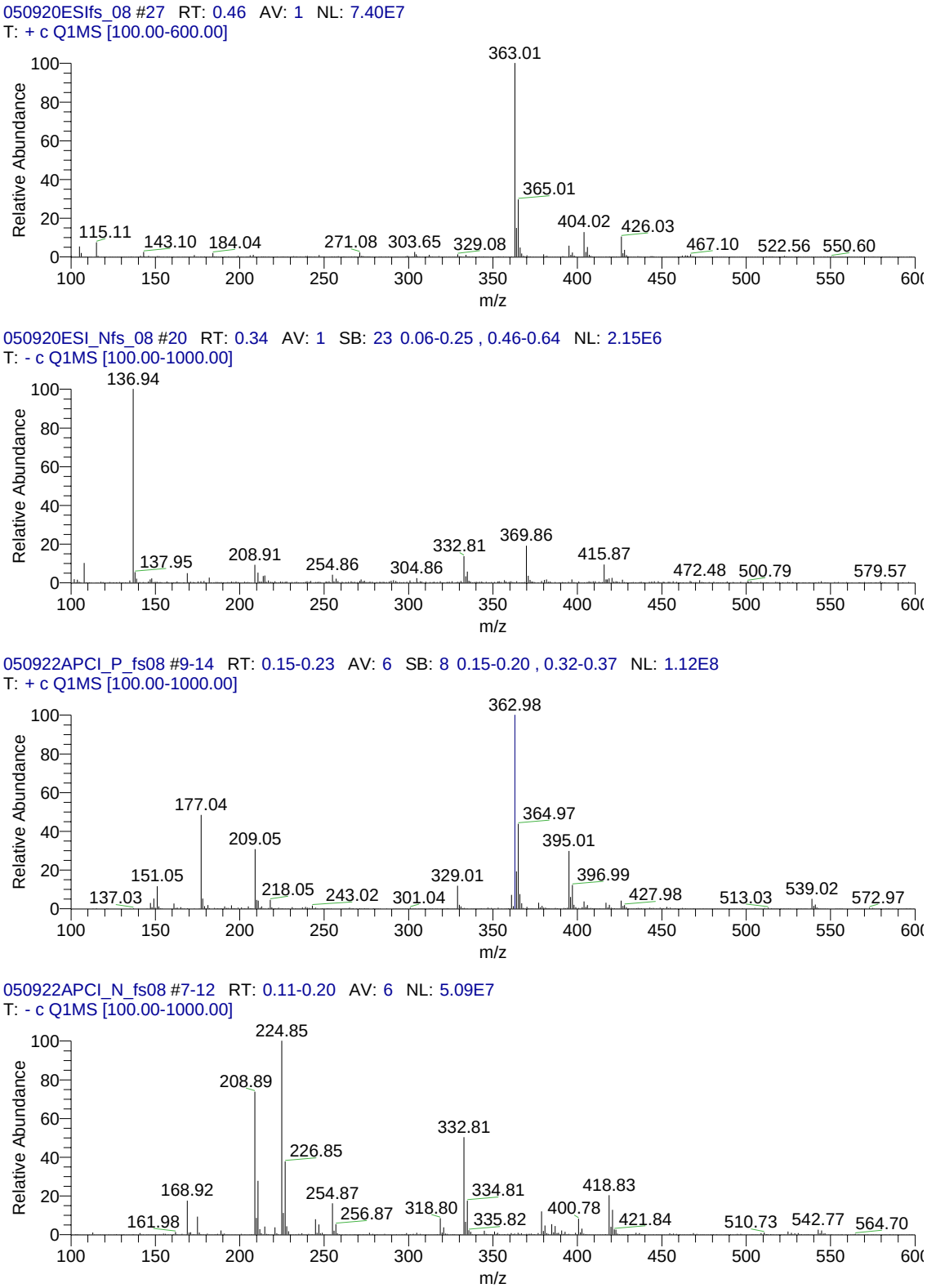


Figure 71 Coumaphos ESI positive, ESI negative (weak), APCI positive, APCI negative full scan spectra

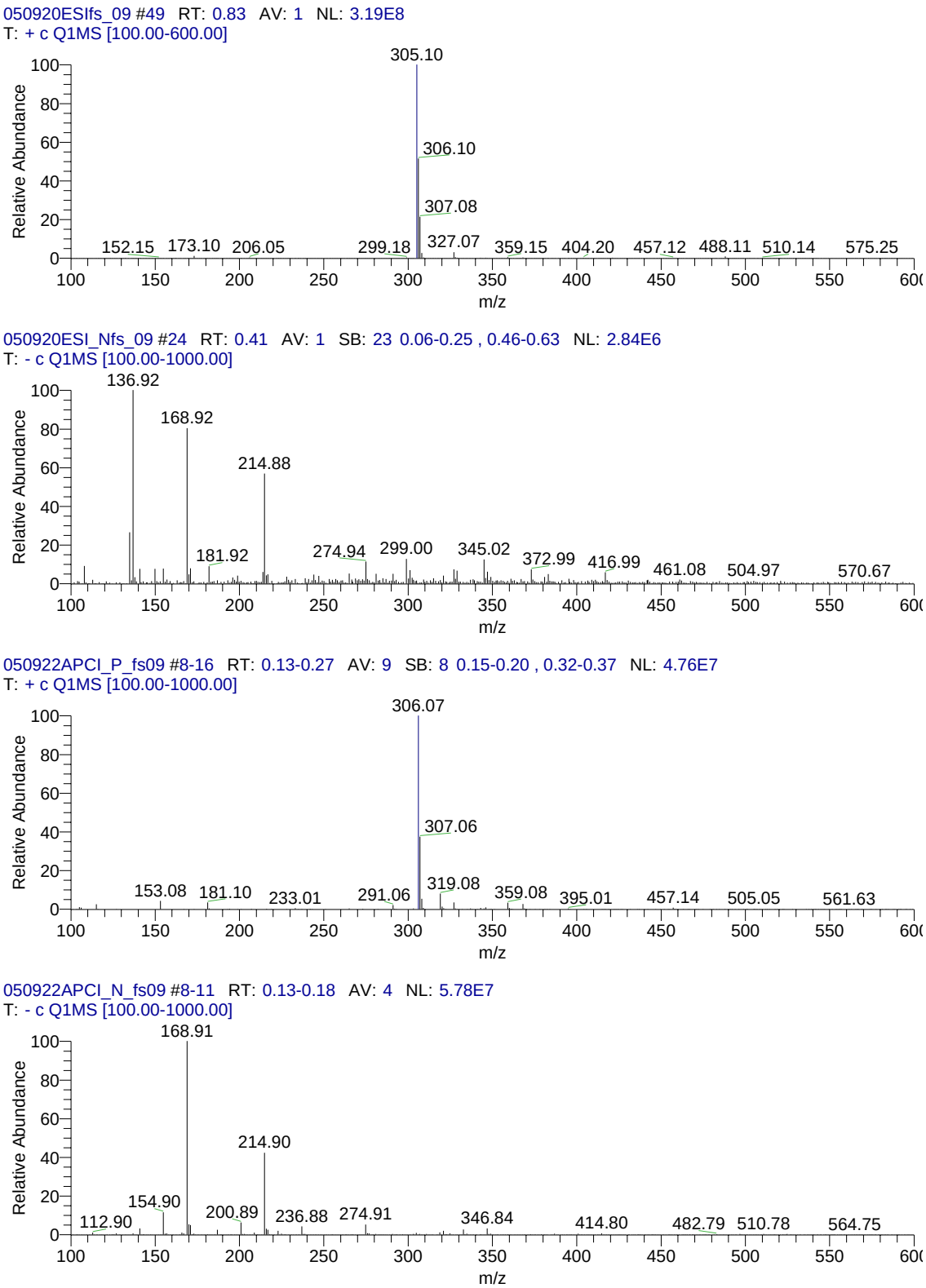


Figure 72 Diazinon ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

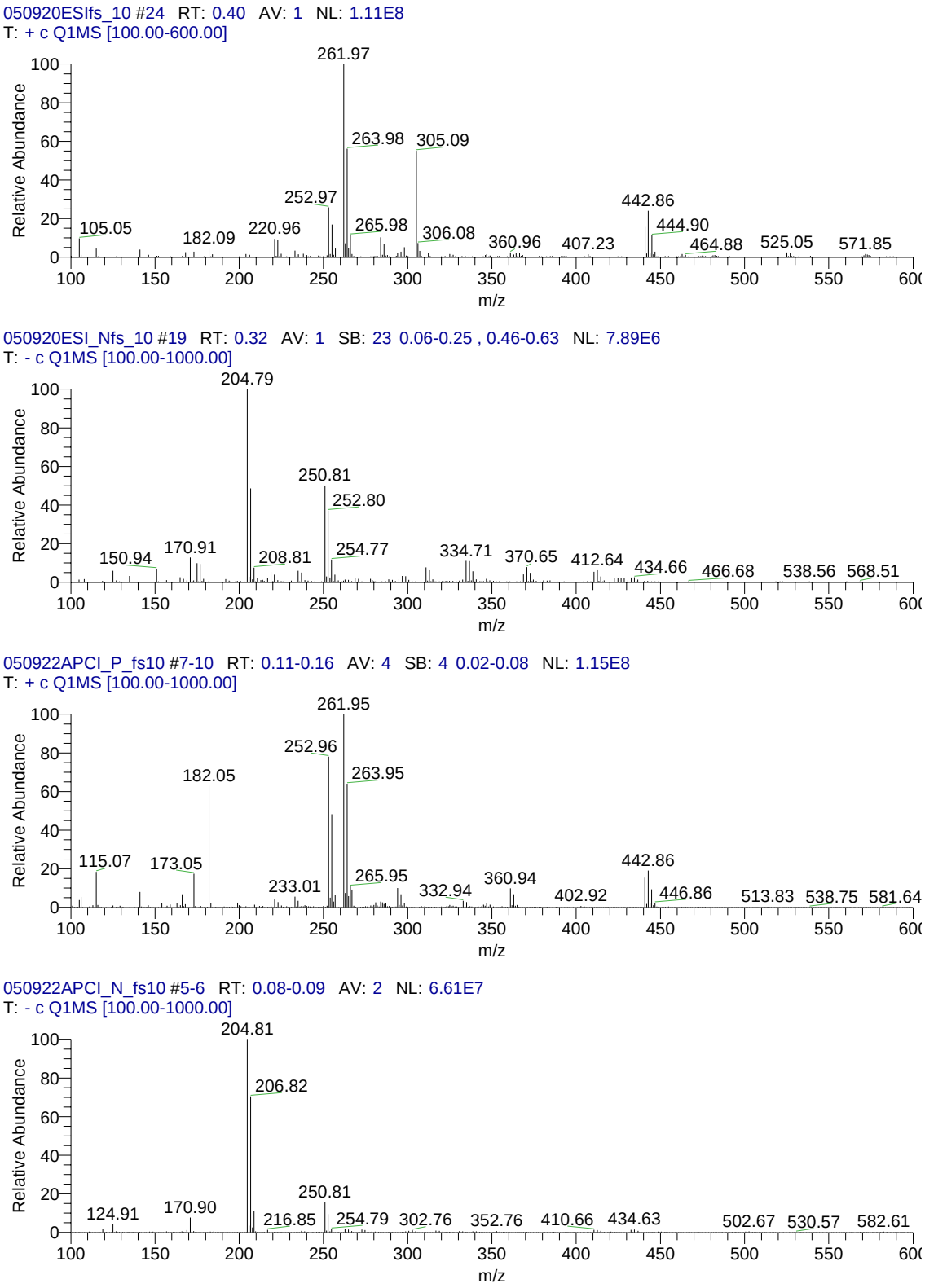


Figure 73 Dichlorvos ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

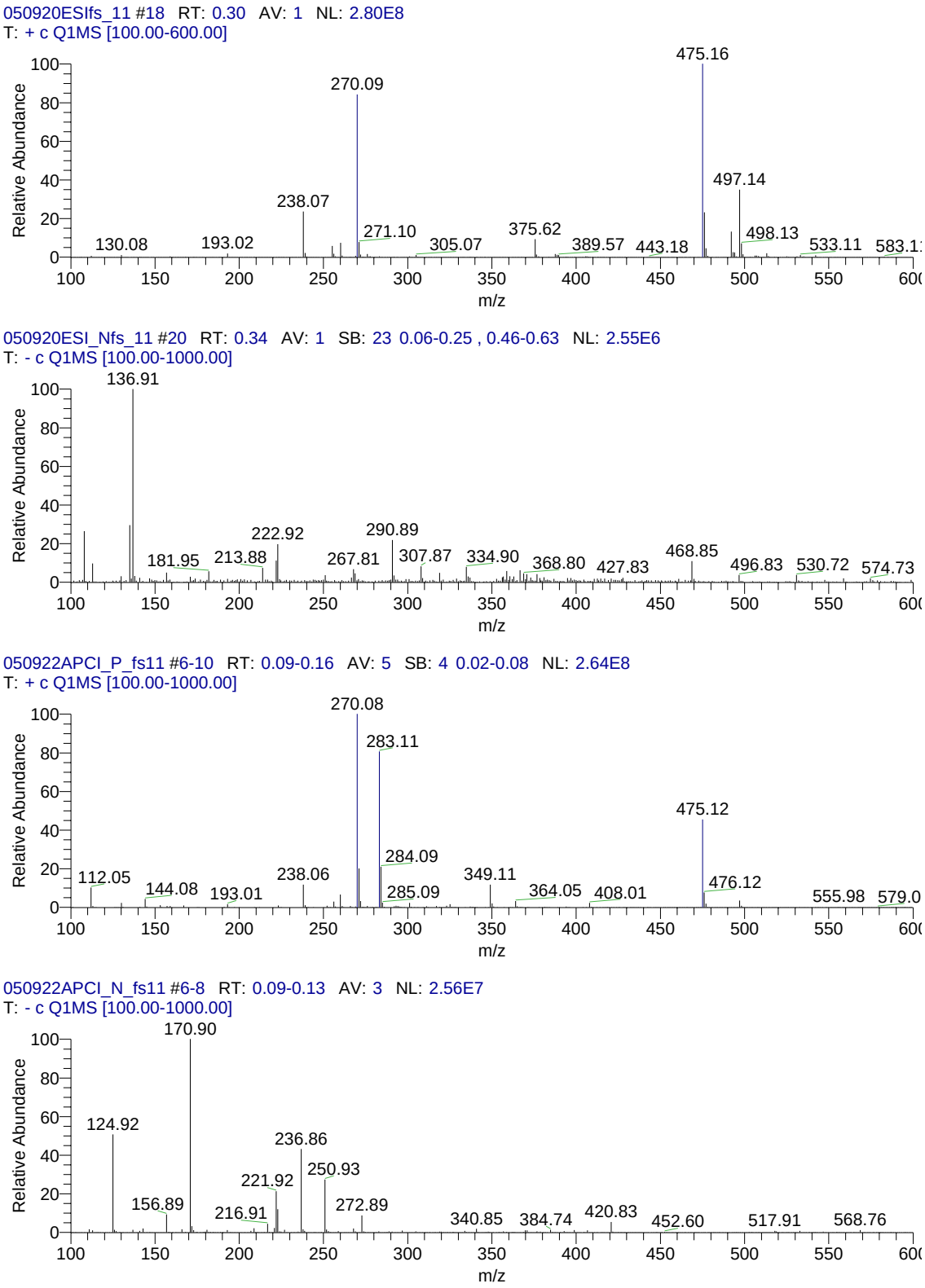


Figure 74 Dicrotophos ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

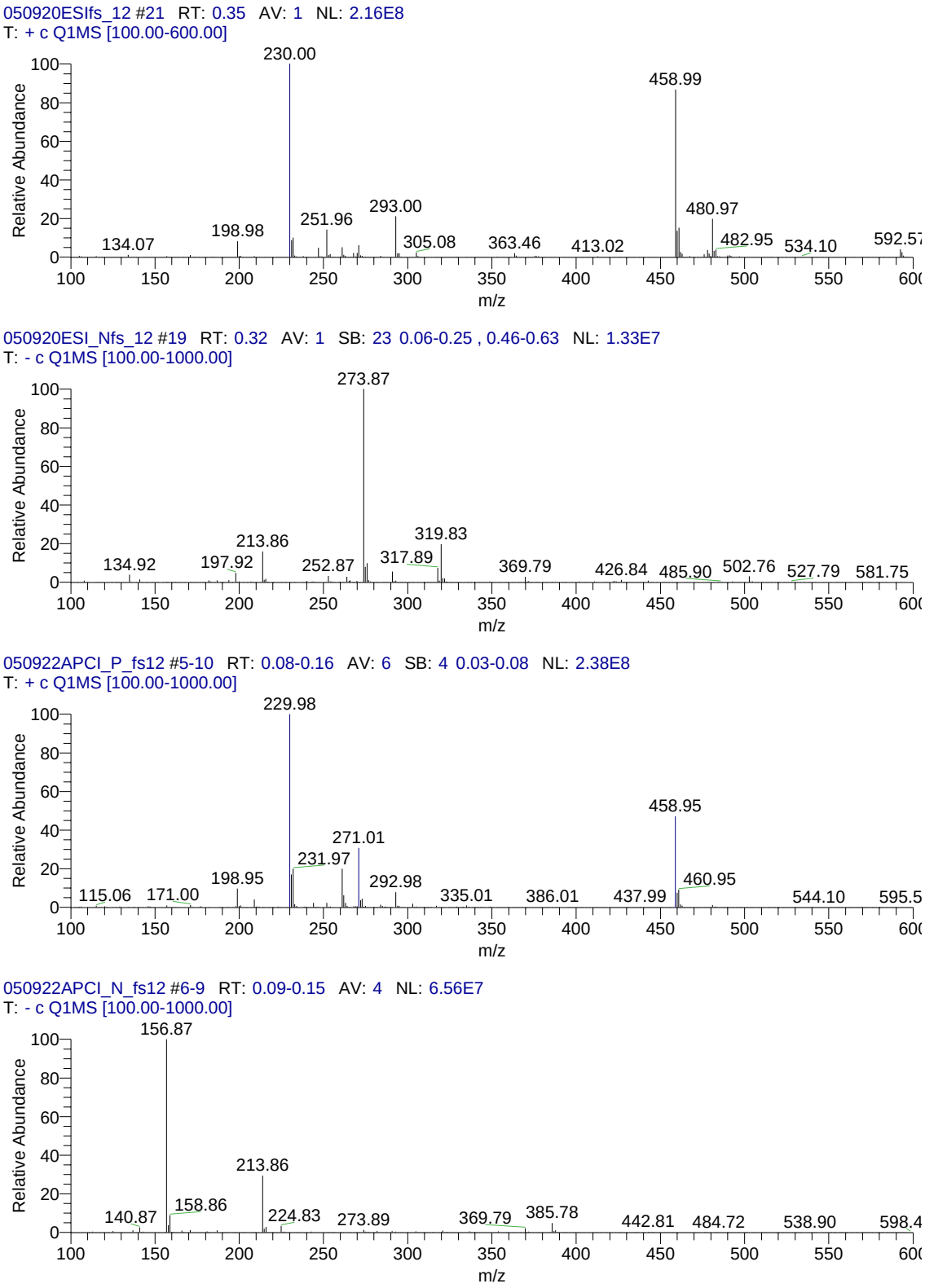


Figure 75 Dimethoate ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

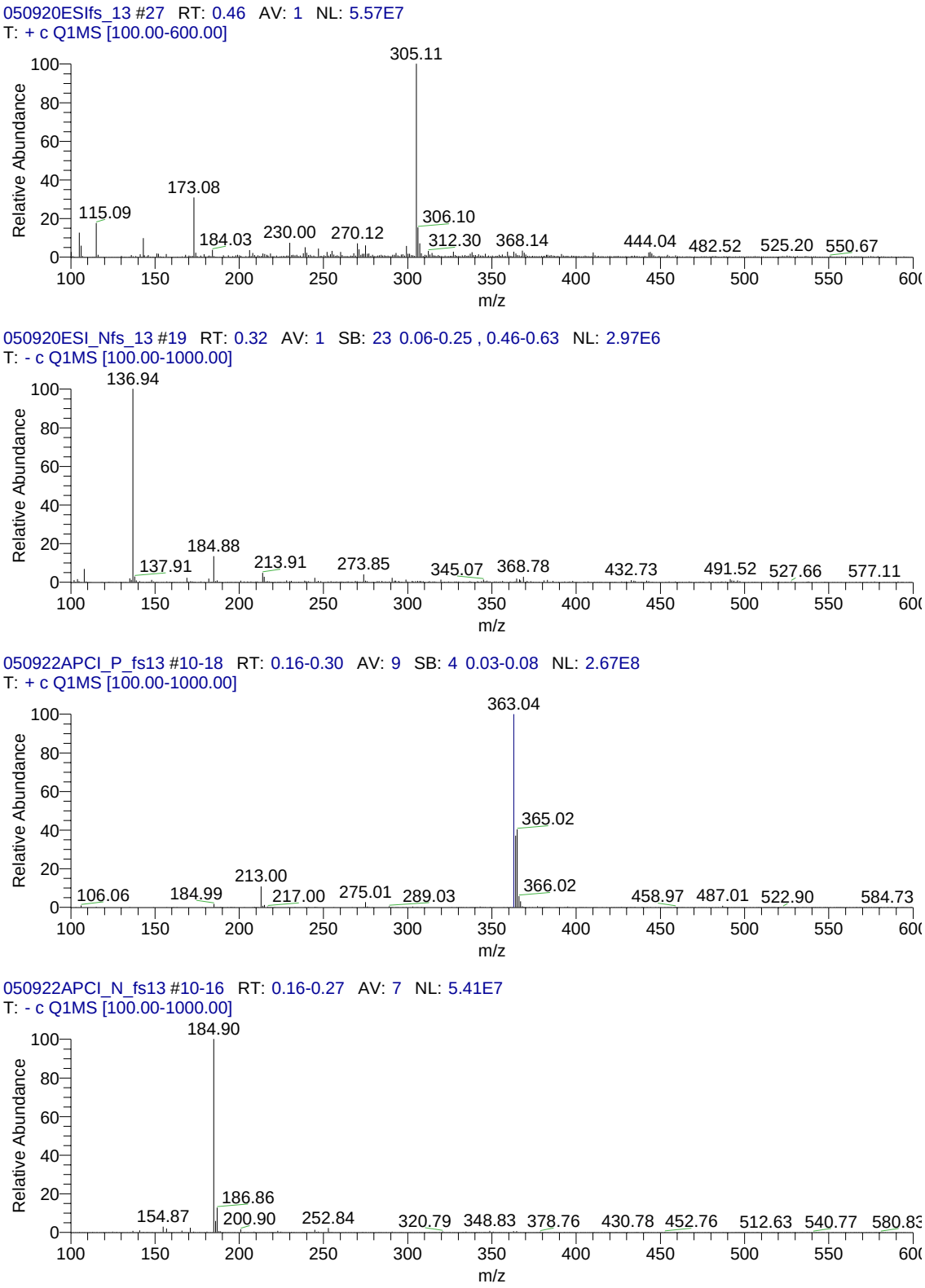
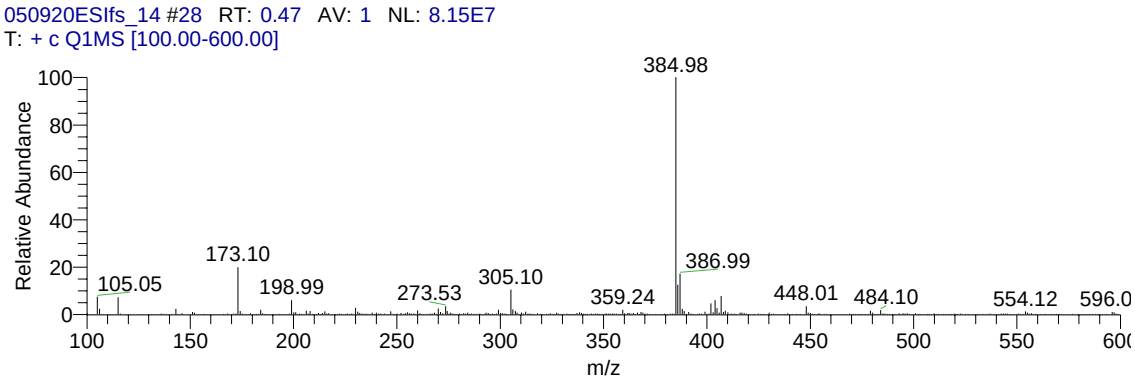


Figure 76 Disulfoton ESI positive, ESI negative (weak), APCI positive, APCI negative full scan spectra



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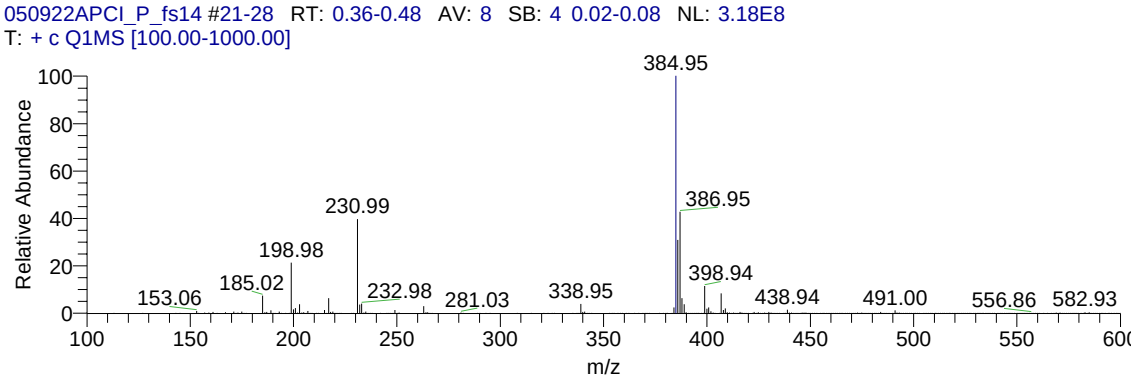


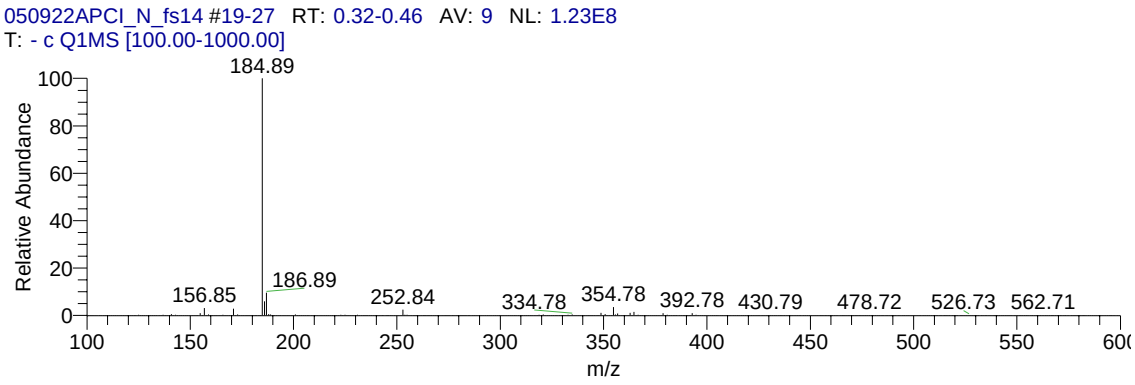


Figure 77 Ethion ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

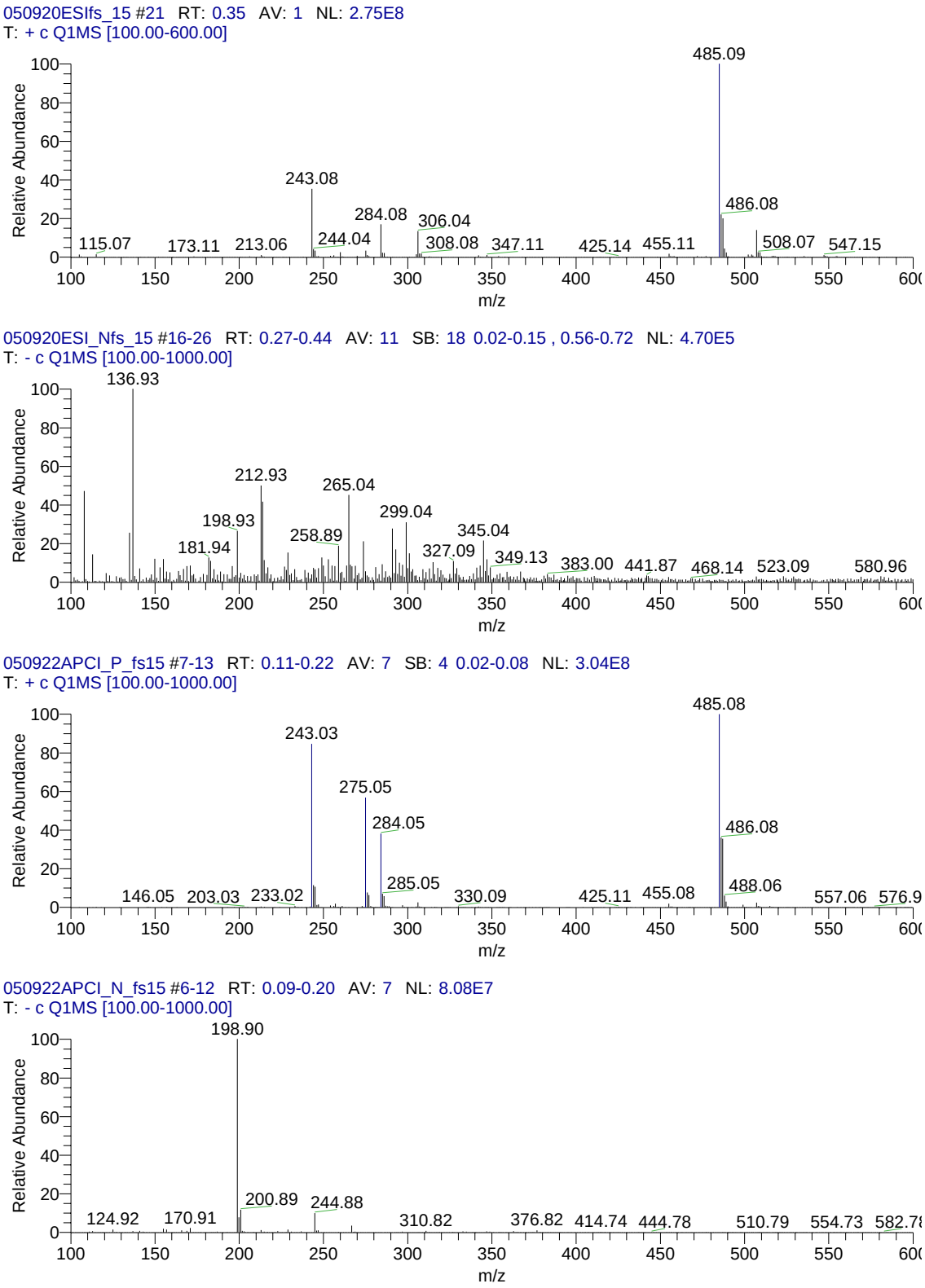


Figure 78 Ethoprop ESI positive, ESI negative (weak), APCI positive, APCI negative full scan spectra

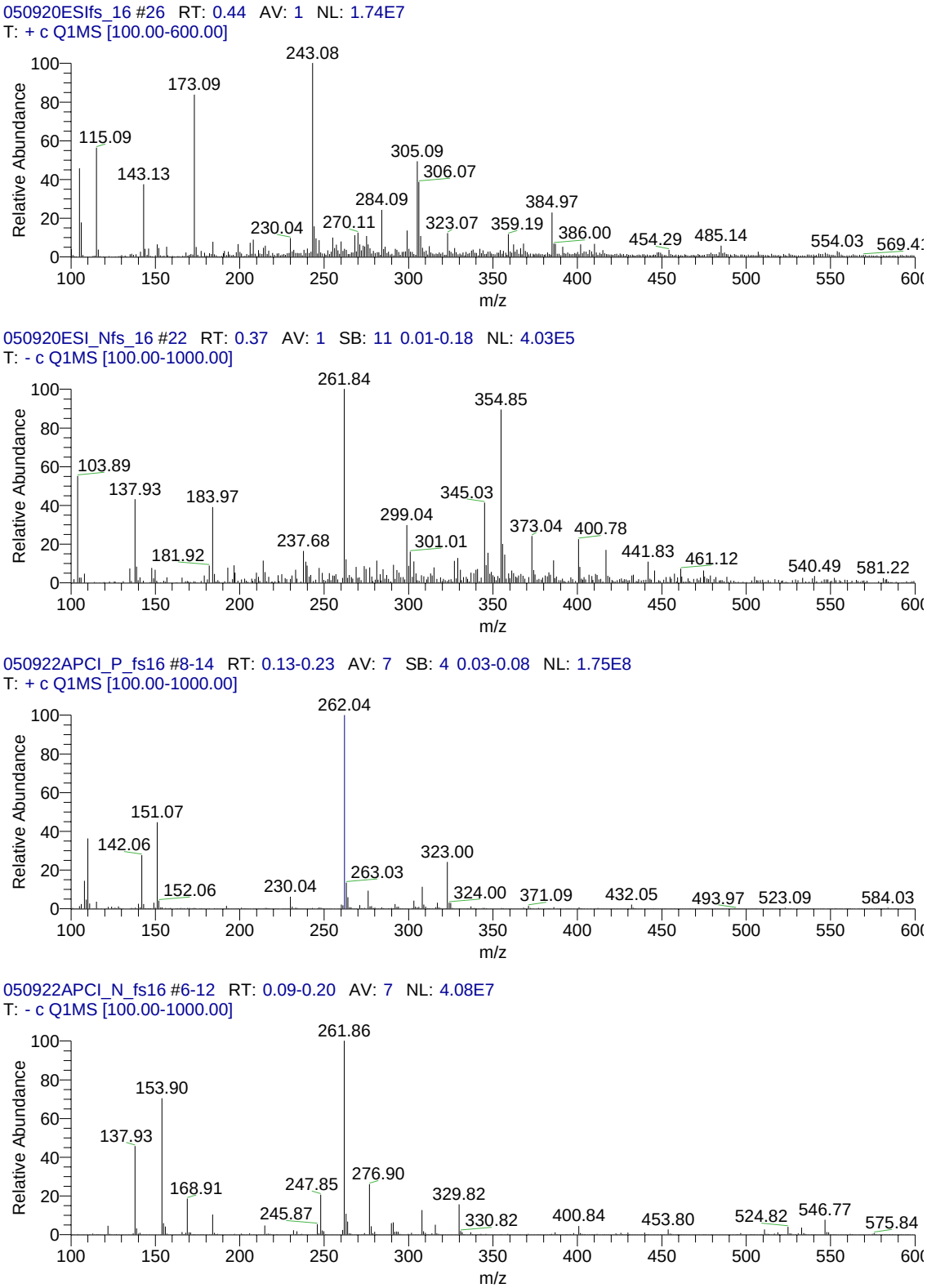


Figure 79 Ethyl parathion ESI positive (weak), ESI negative (weak), APCI positive, APCI negative full scan spectra

N/F

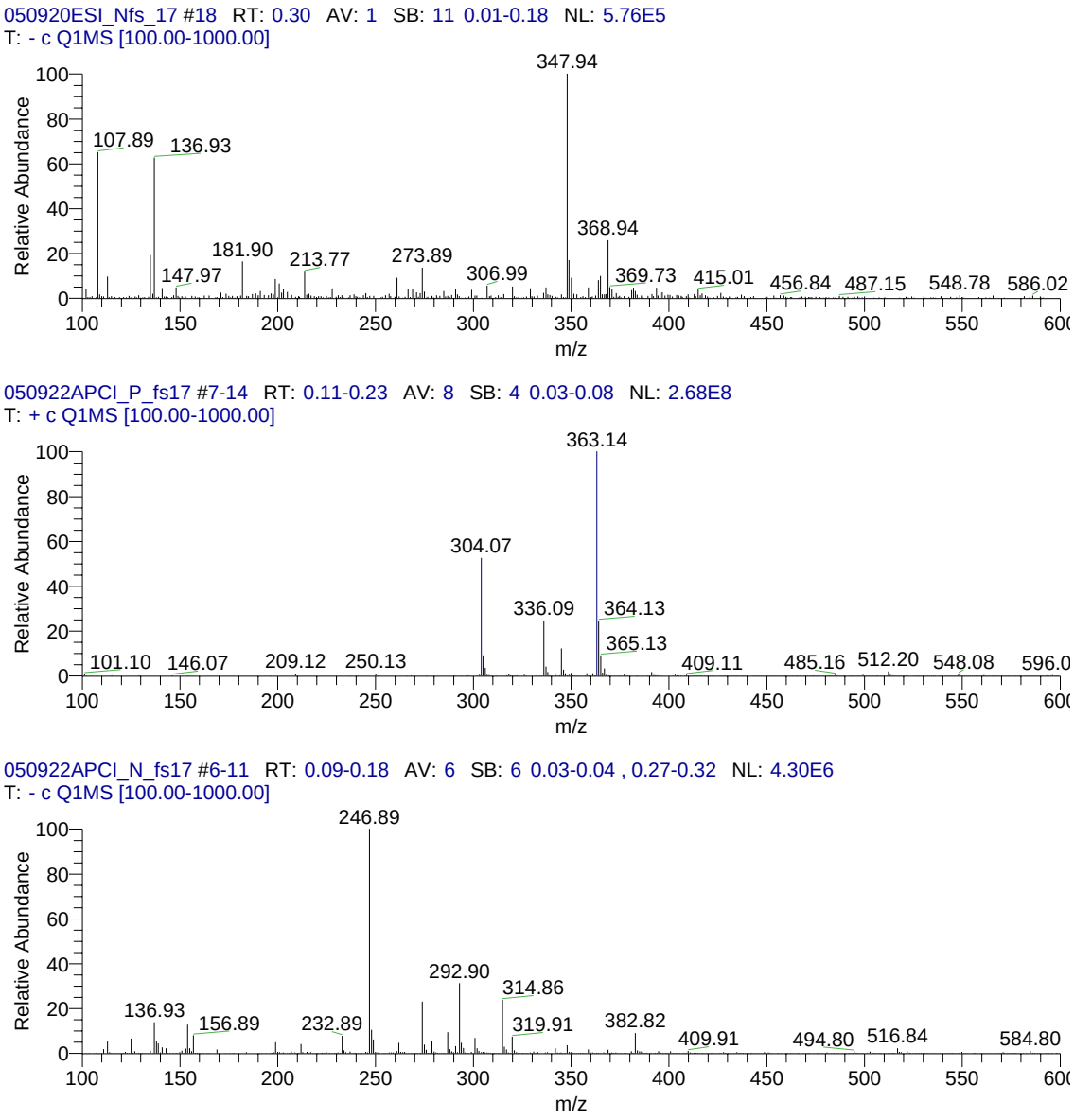


Figure 80 Fenamiphos ESI positive (weak),, ESI negative (weak), APCI positive, APCI negative full scan spectra

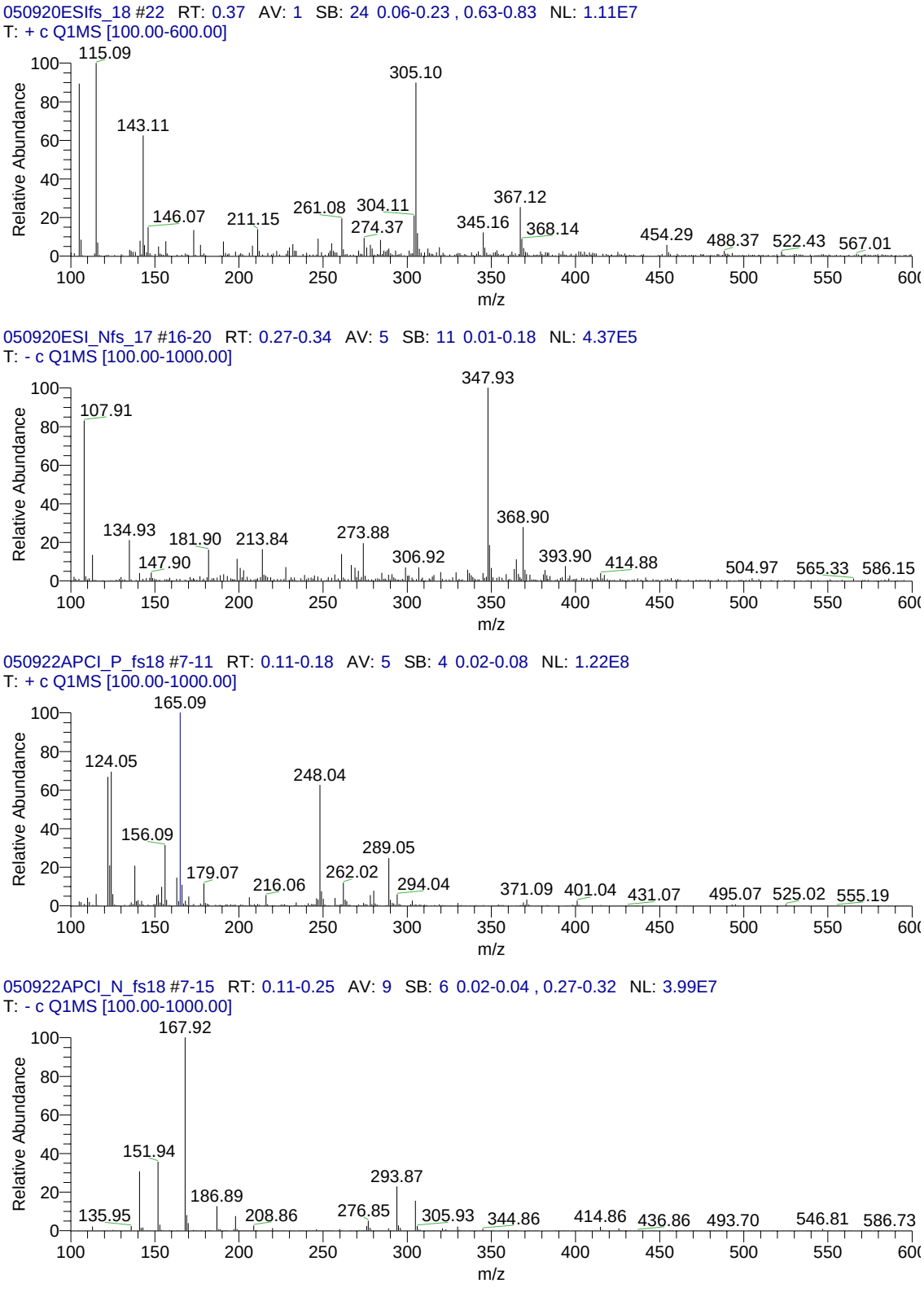


Figure 81 Fenitrothion ESI positive (weak),, ESI negative, APCI positive, APCI negative full scan spectra

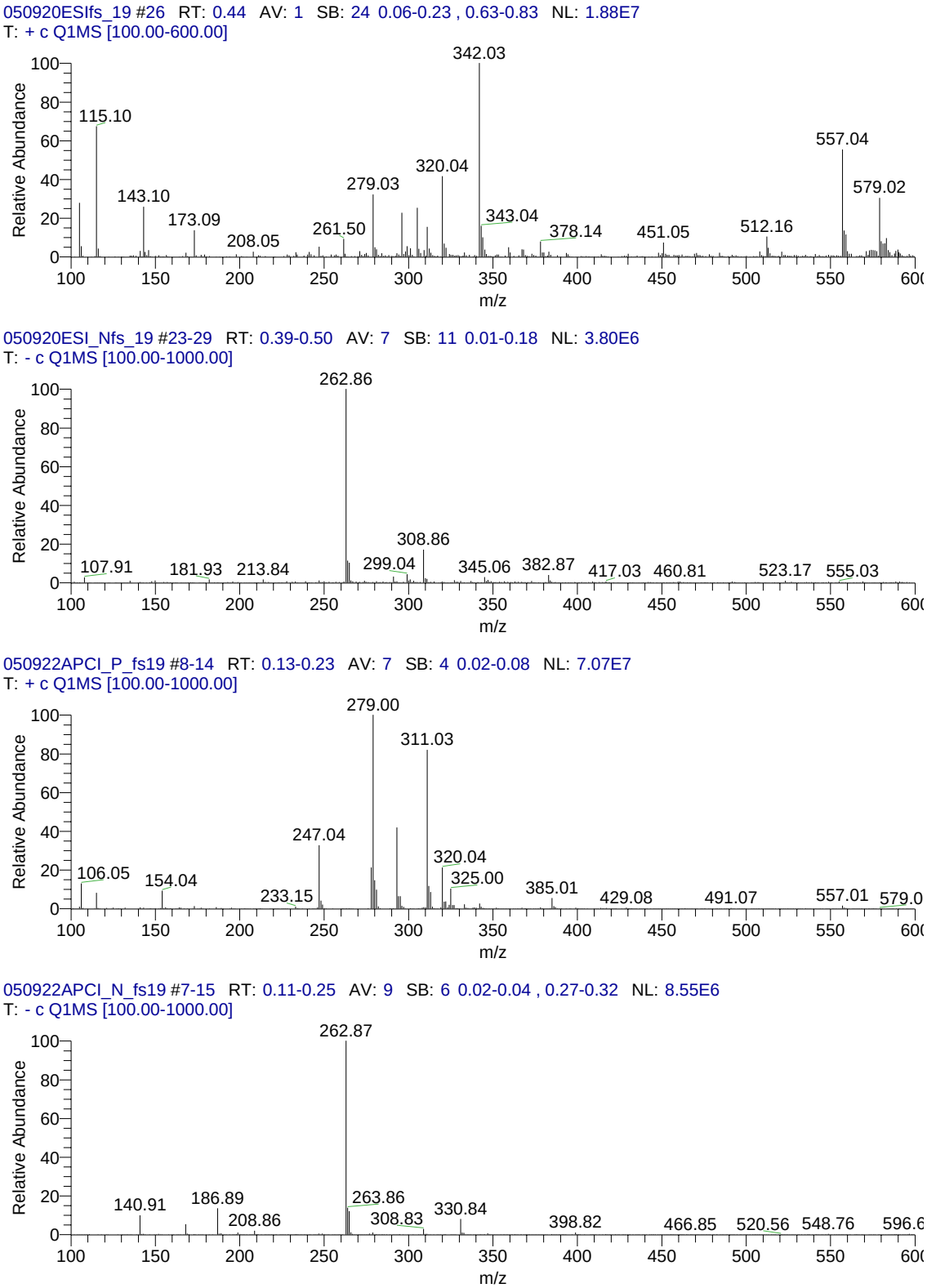


Figure 82 Fenthion ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

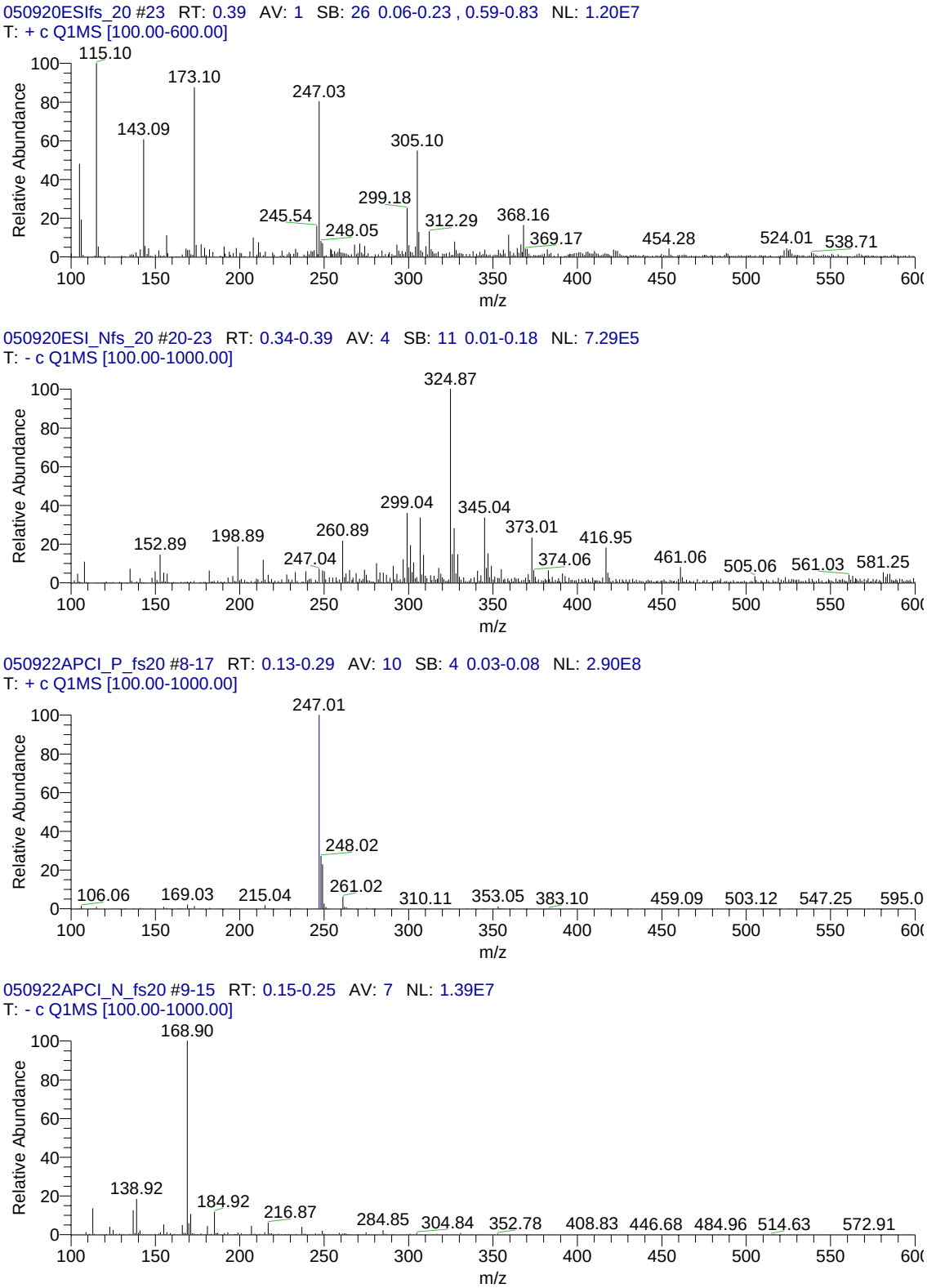


Figure 83 Fonofos ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

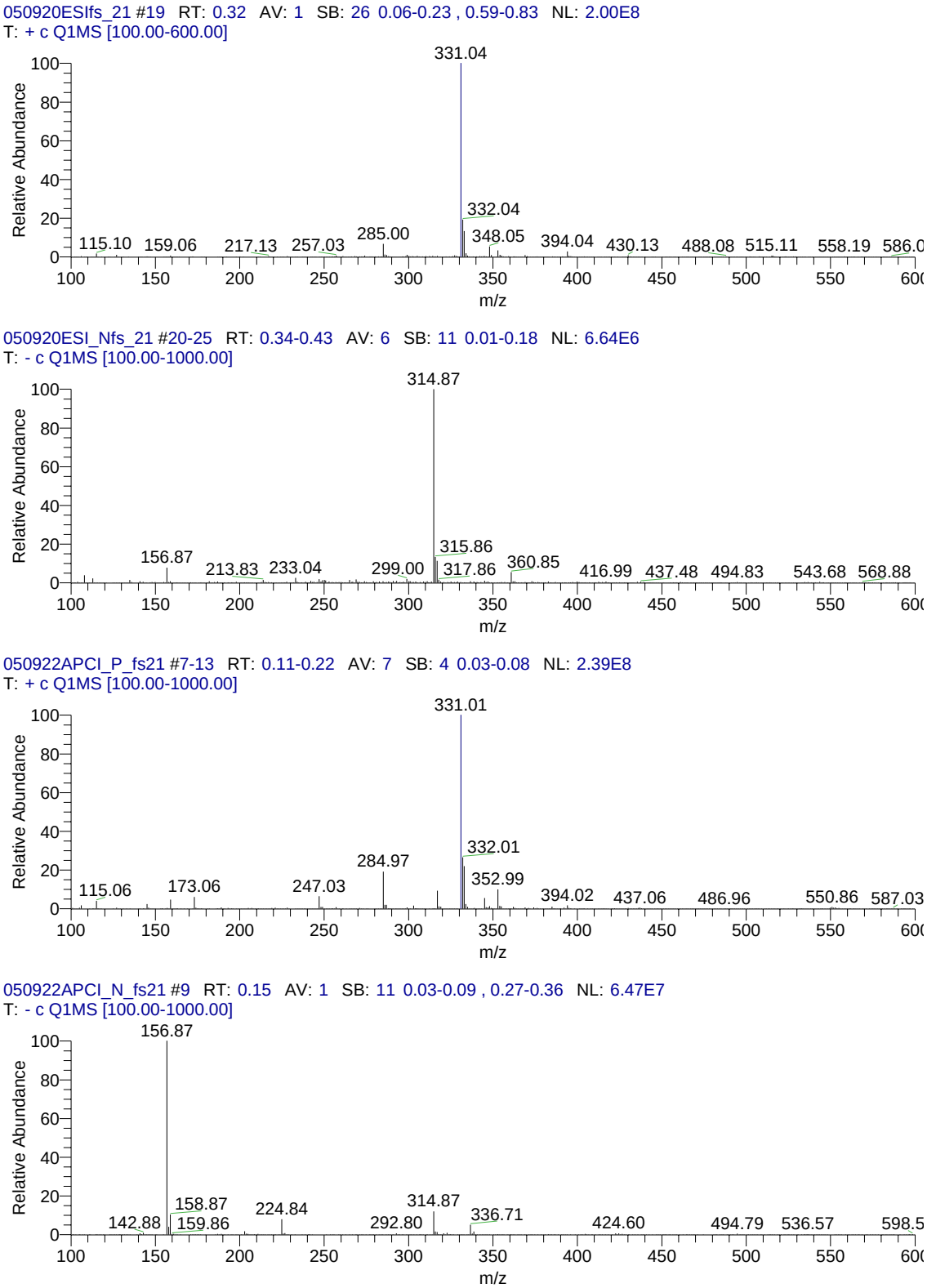
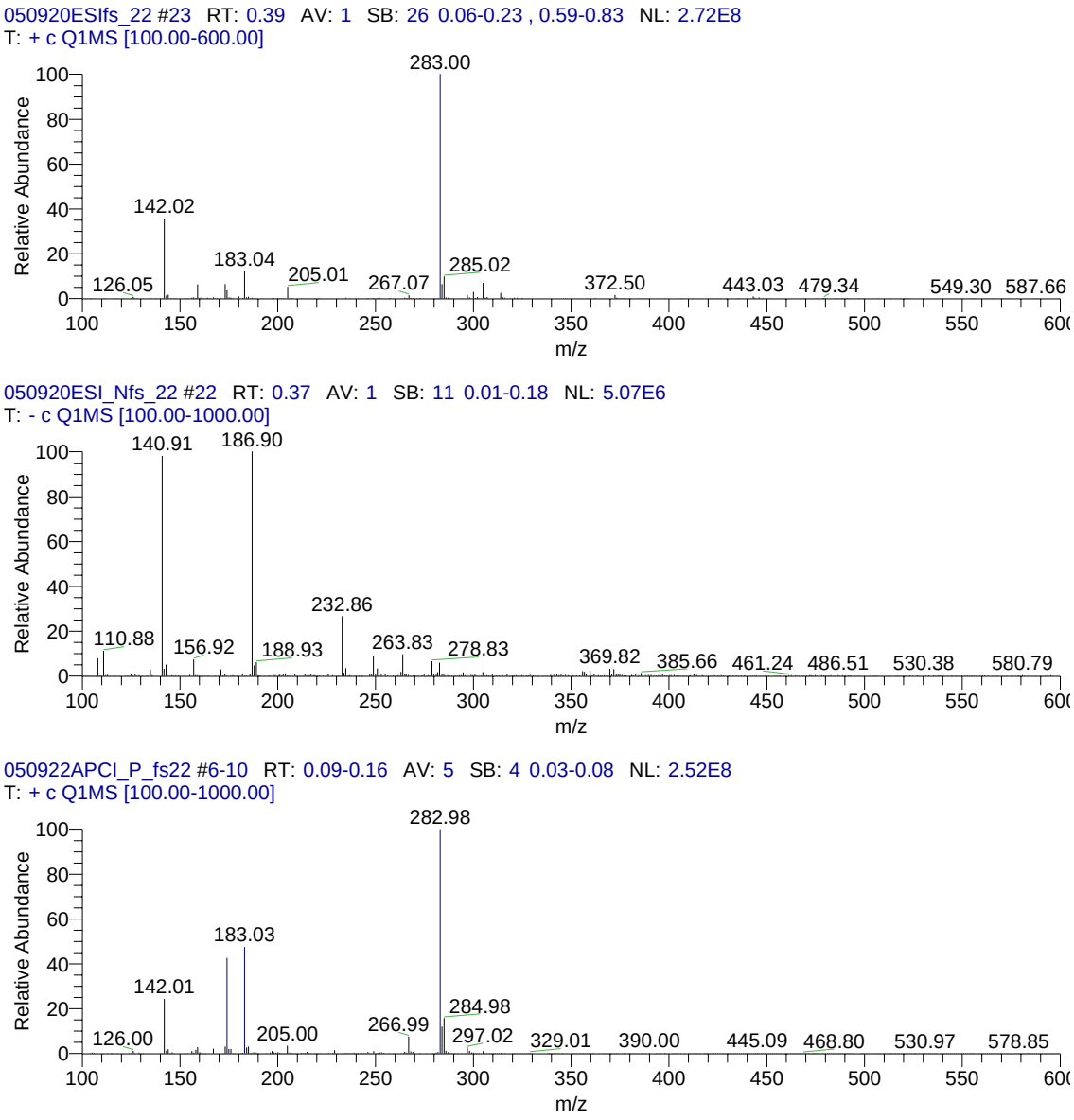


Figure 84 Malathion ESI positive, ESI negative, APCI positive, APCI negative full scan spectra



N/F

Figure 85 Methamidophos ESI positive, ESI negative, APCI positive, APCI negative (weak)full scan spectra

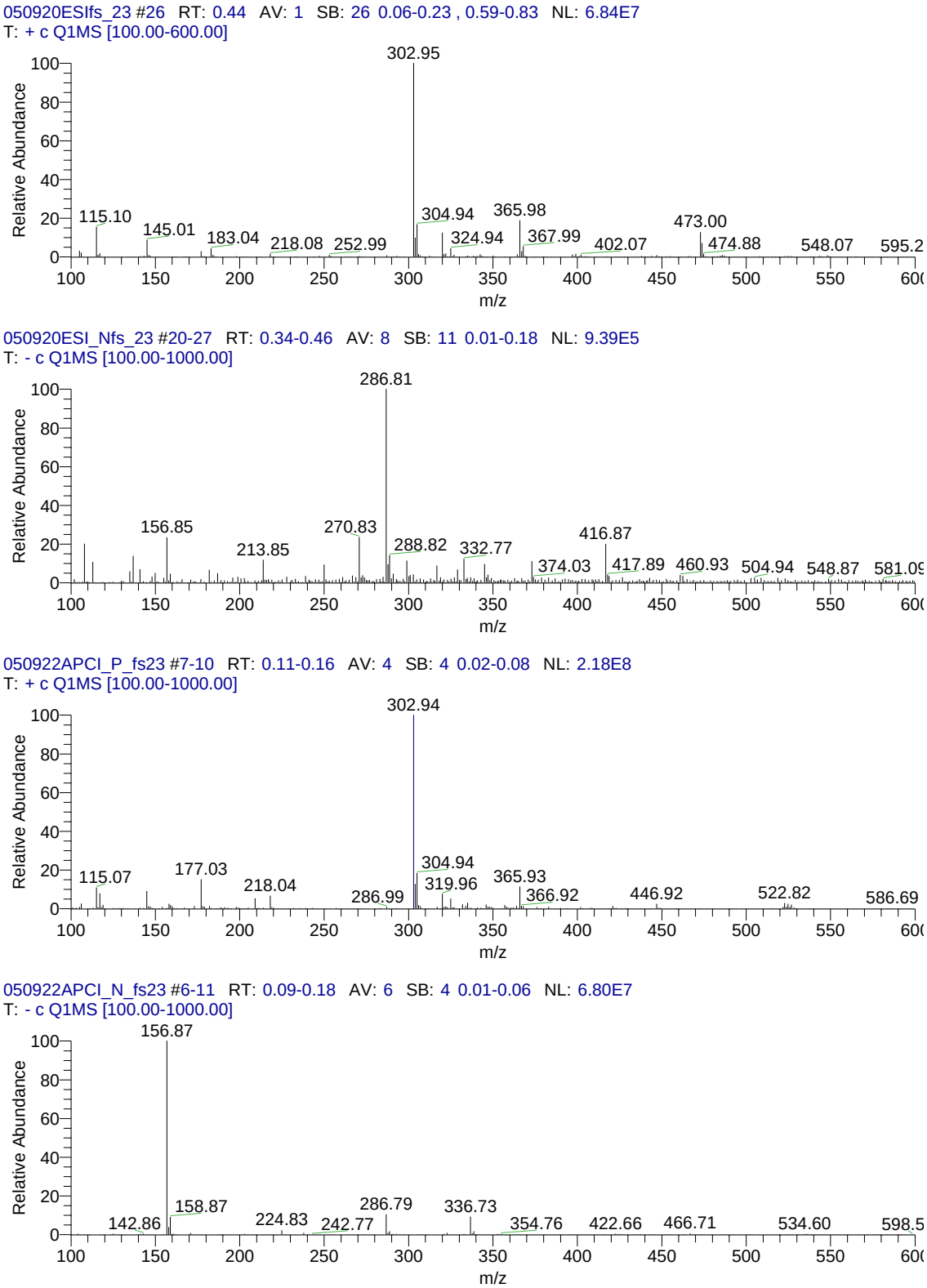


Figure 86 Methidathion ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

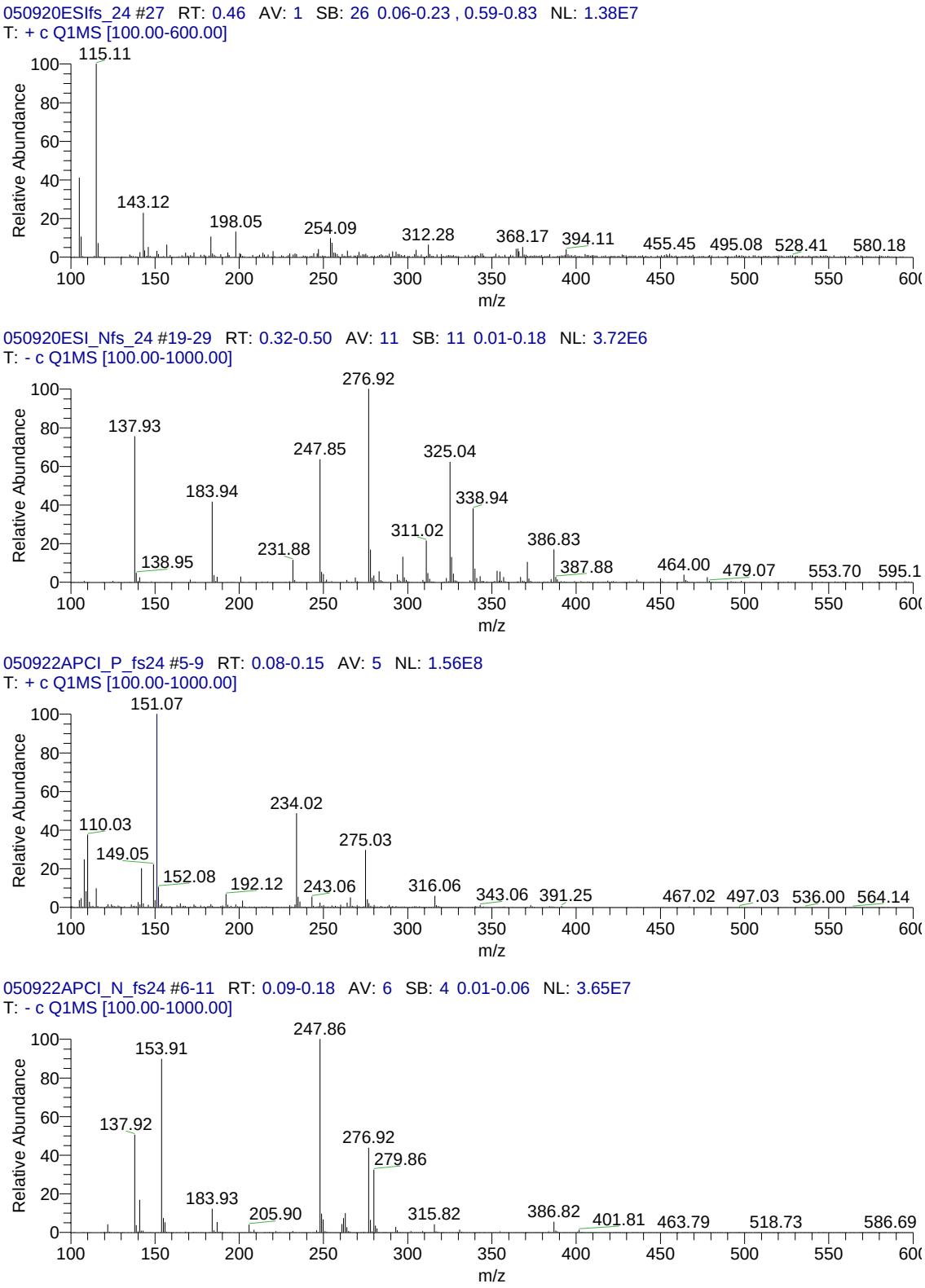


Figure 87 Methyl parathion ESI positive (weak), ESI negative, APCI positive, APCI negative full scan spectra

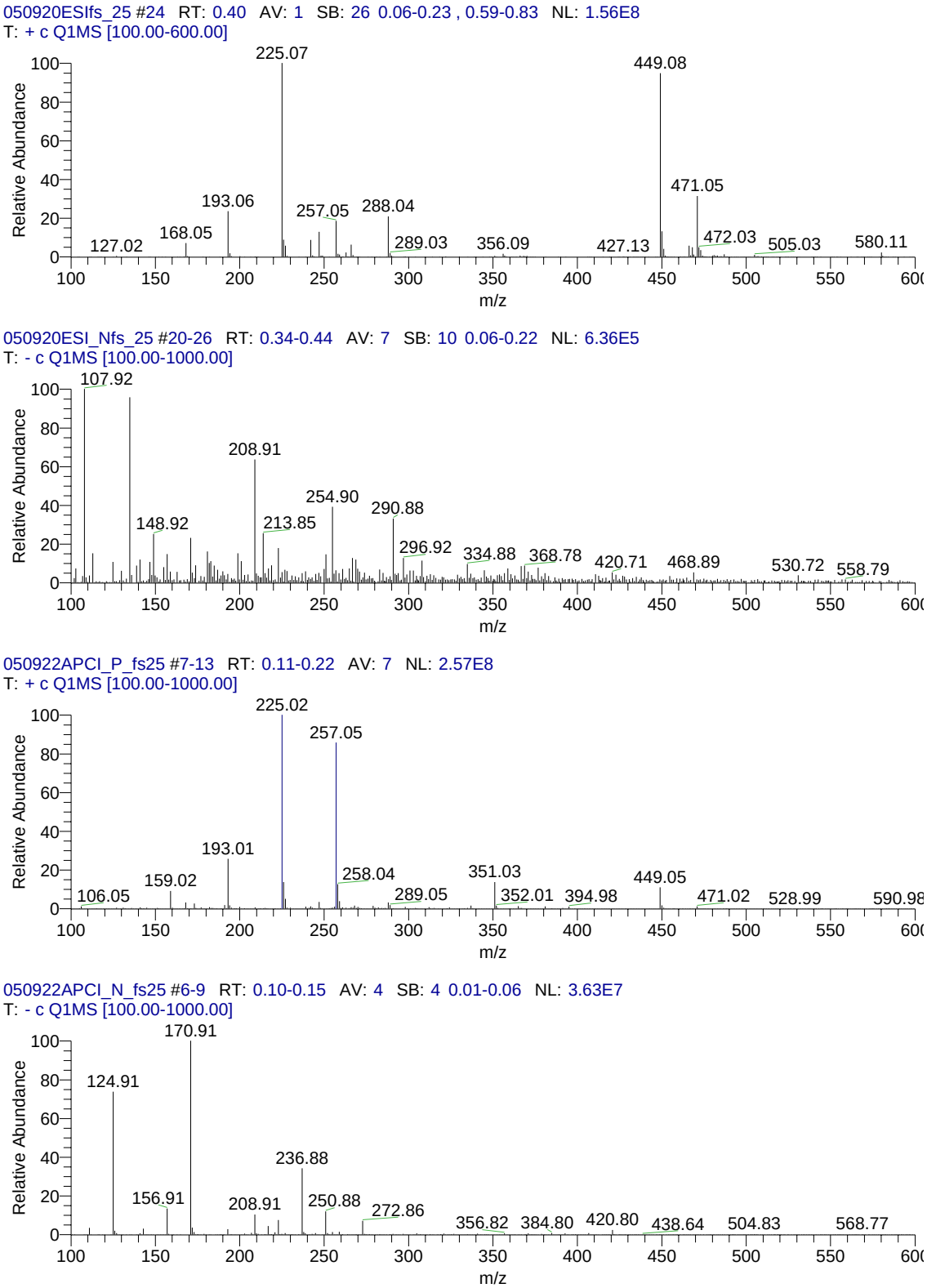


Figure 88 Mevinphos ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

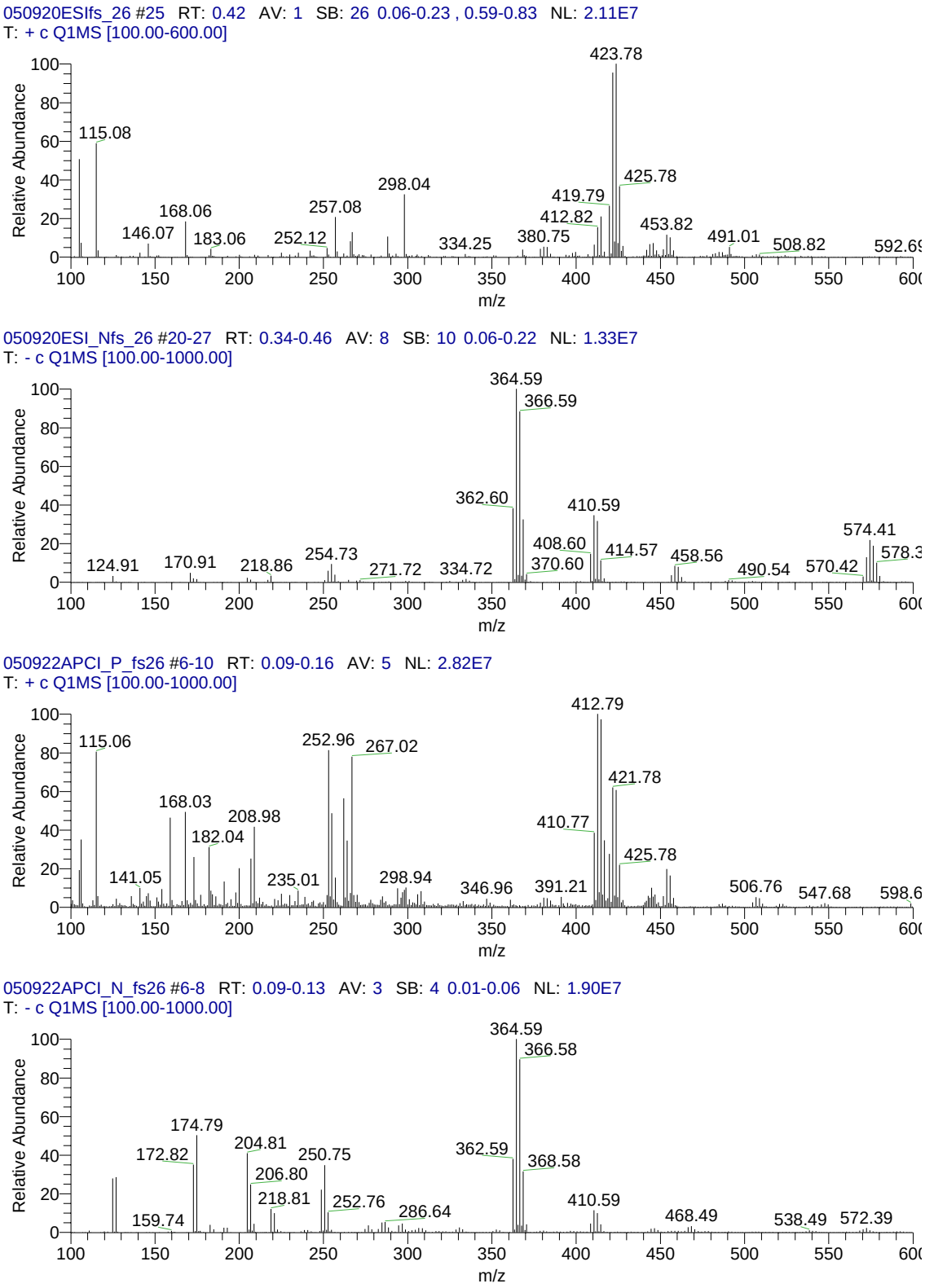


Figure 89 Naled ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

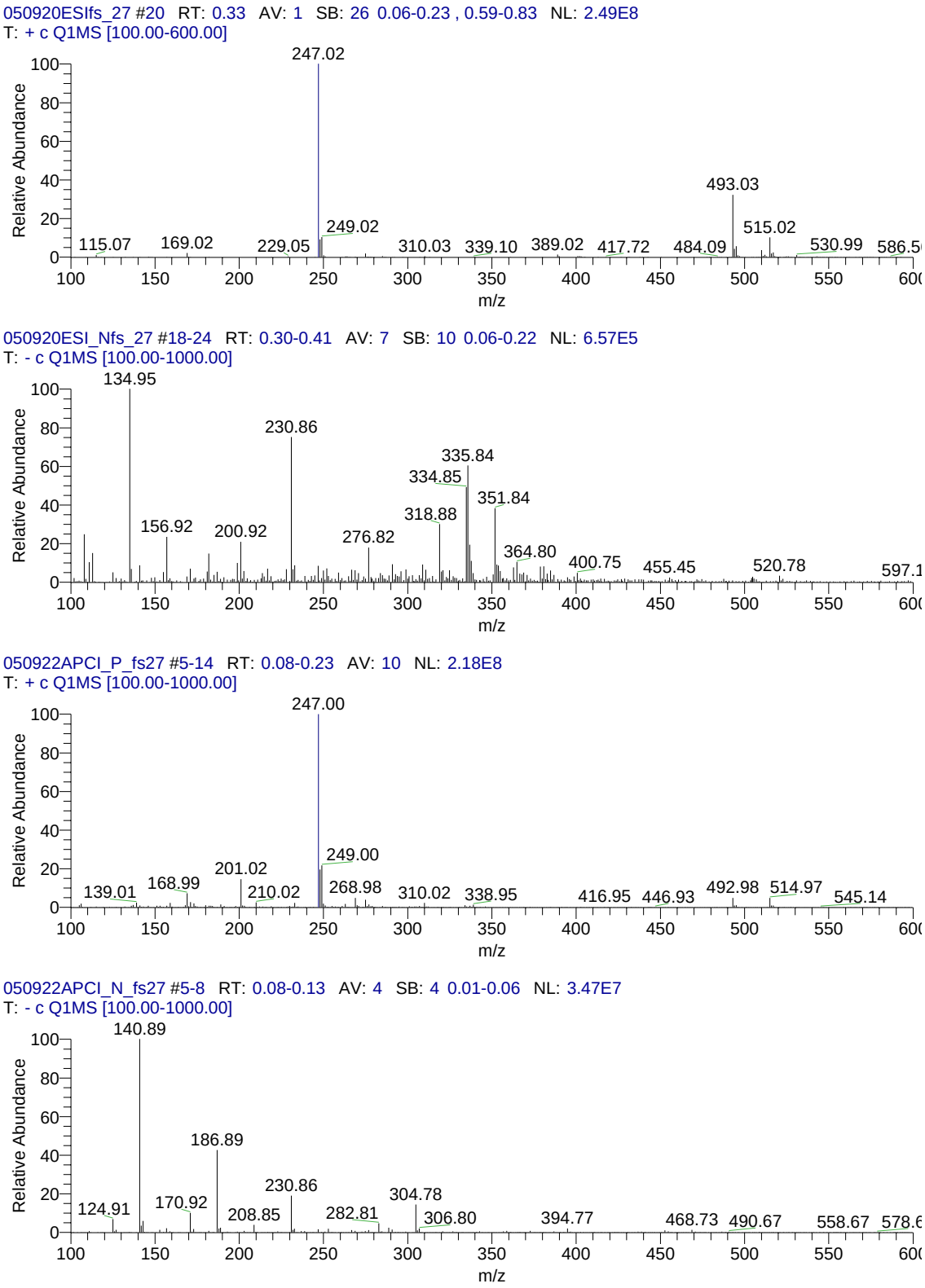


Figure 90 Oxydemethon methyl ESI positive, ESI negative (weak), APCI positive, APCI negative full scan spectra

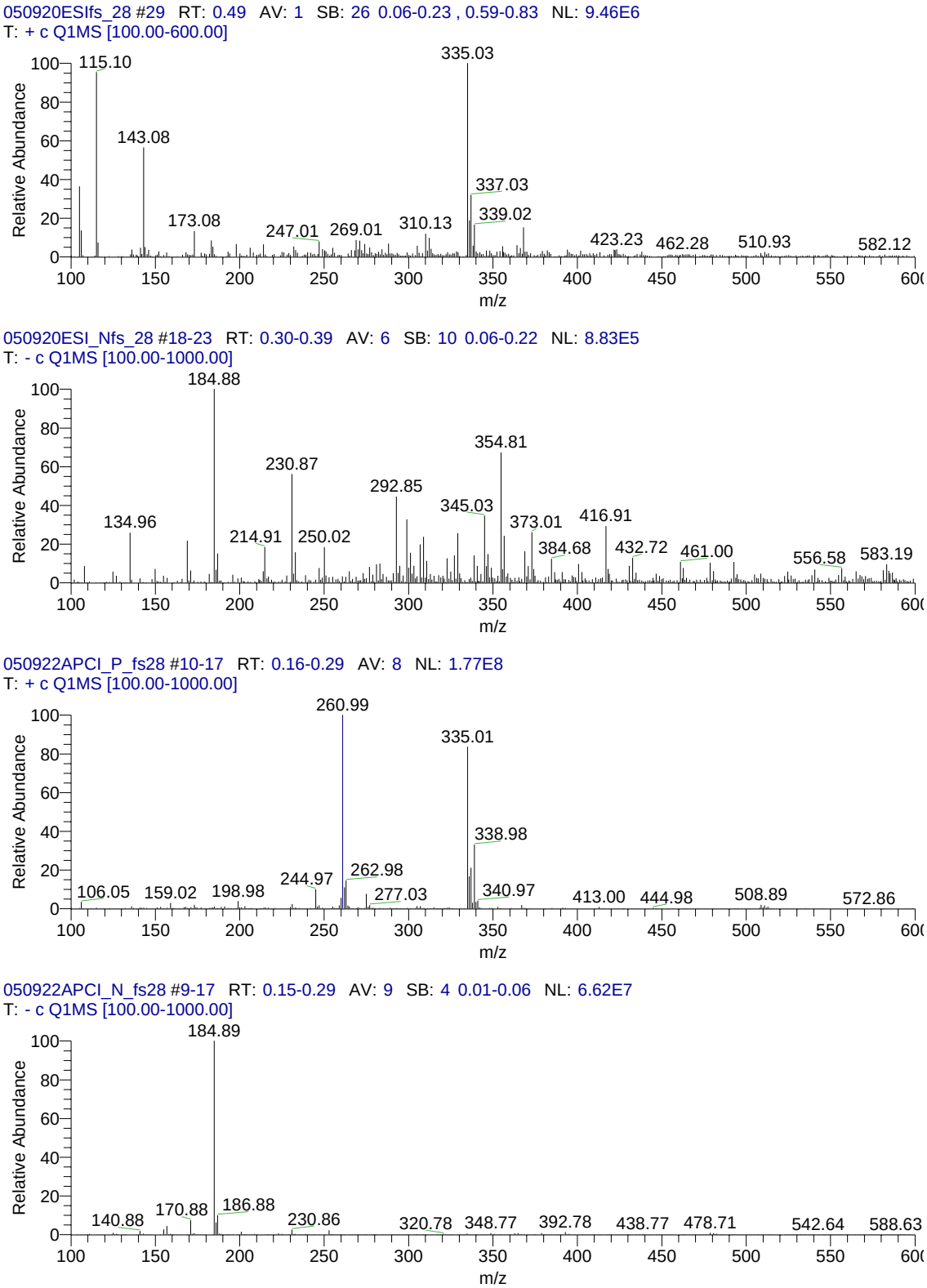


Figure 91 Phorate ESI positive, ESI negative (weak), APCI positive, APCI negative full scan spectra

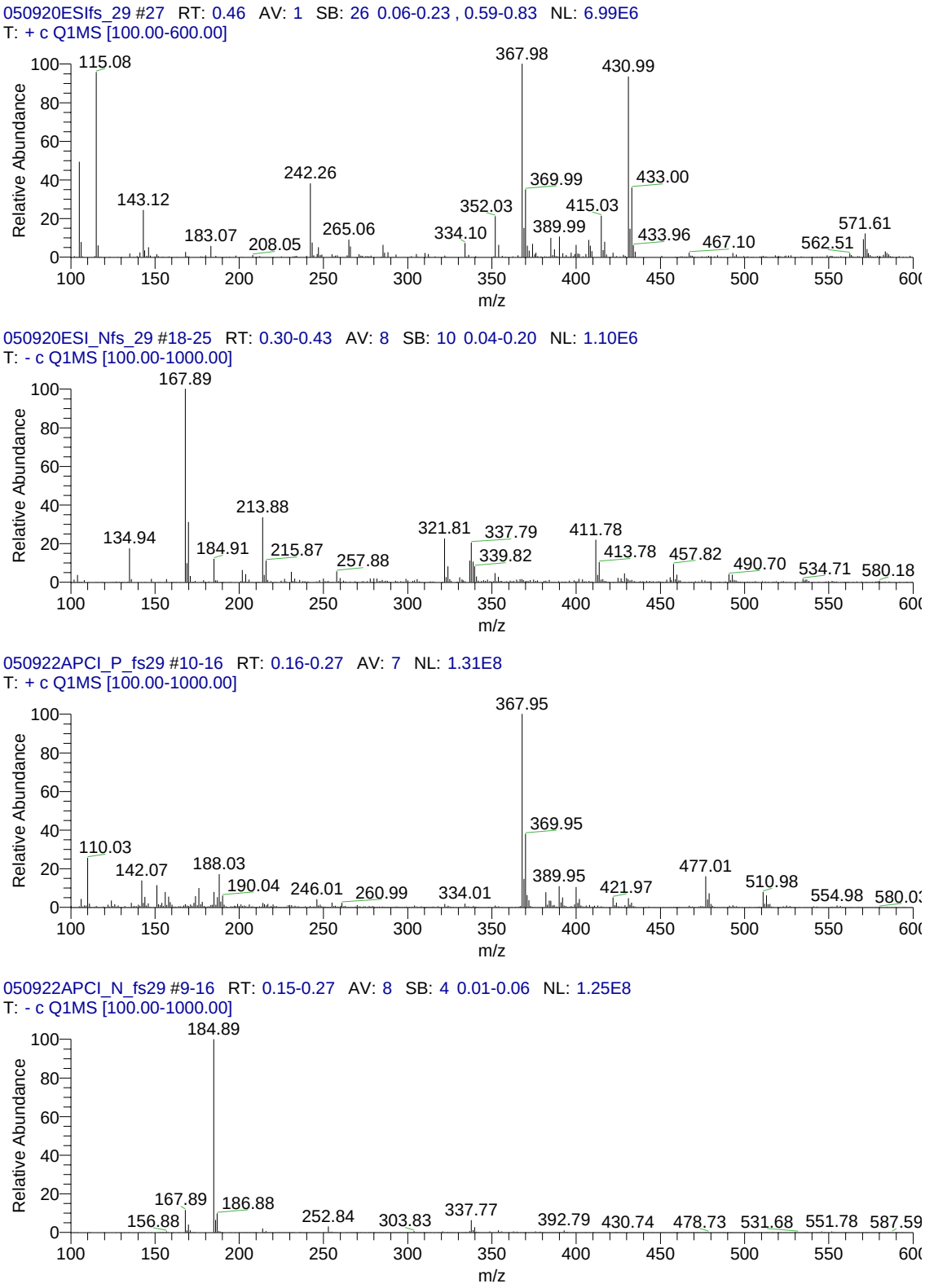


Figure 92 Phosalone ESI positive (weak), ESI negative, APCI positive, APCI negative full scan spectra

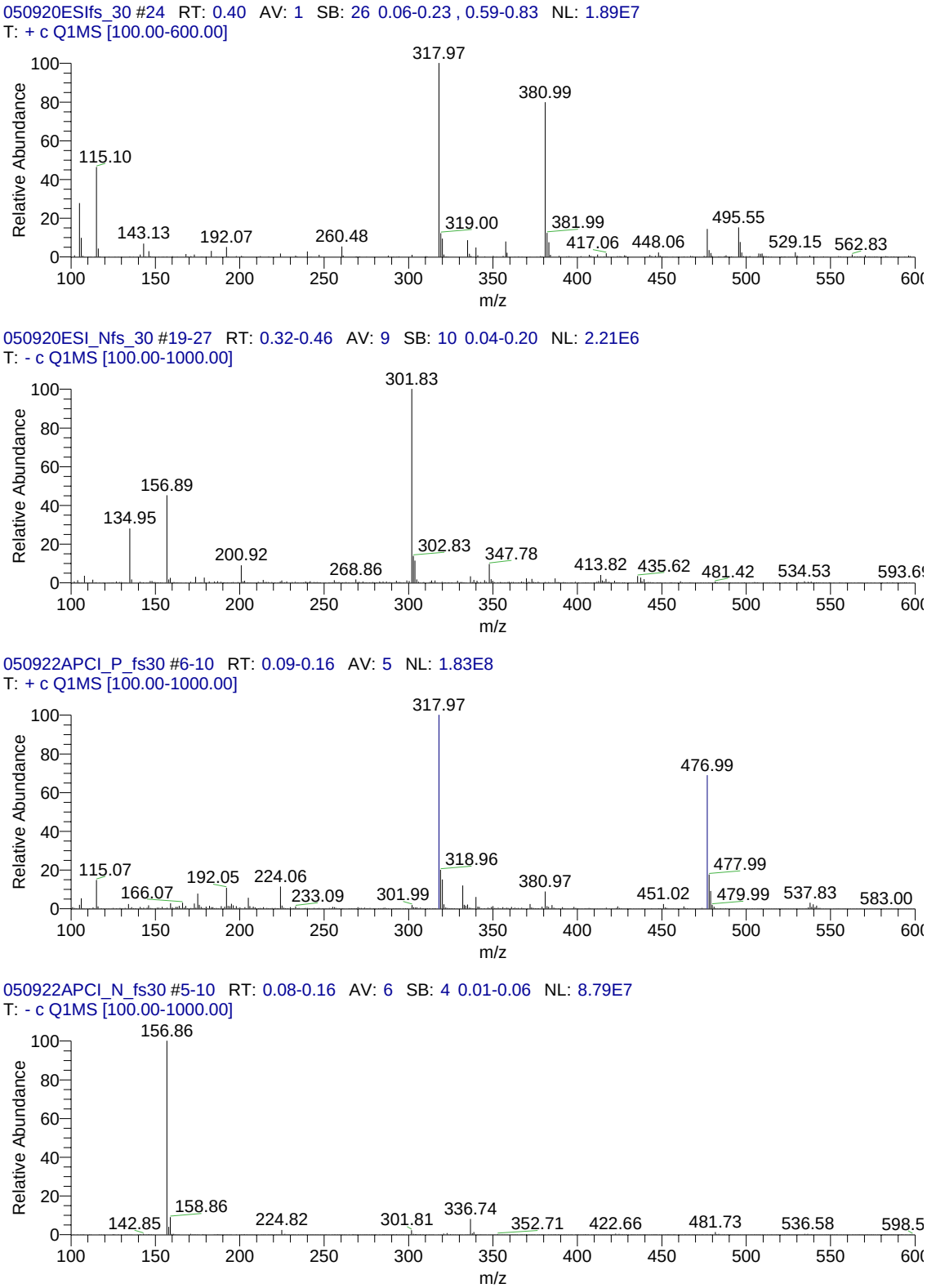


Figure 93 Phosmet ESI positive, ESI negative (weak), APCI positive, APCI negative full scan spectra

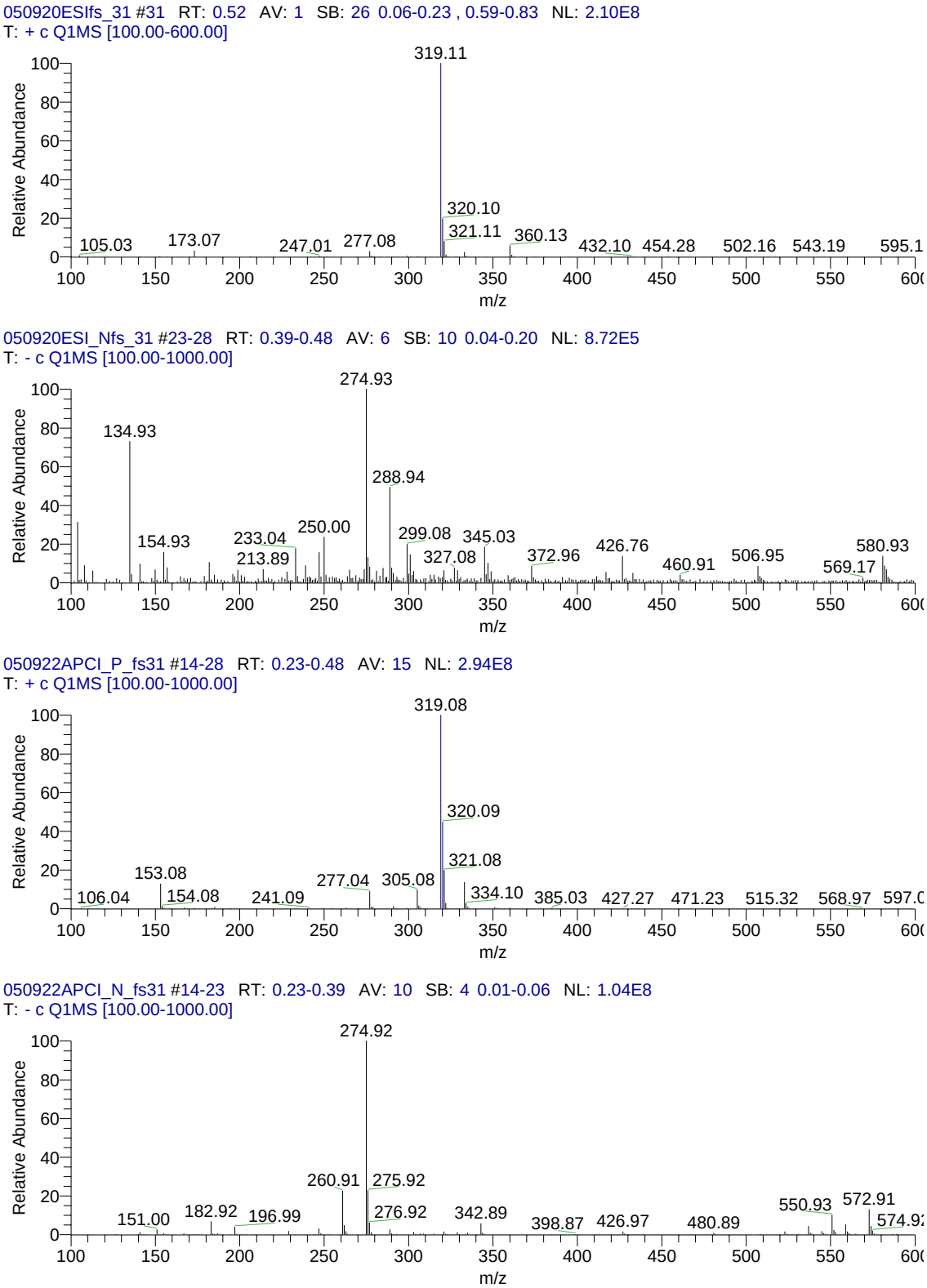


Figure 94 Phostebupirim ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

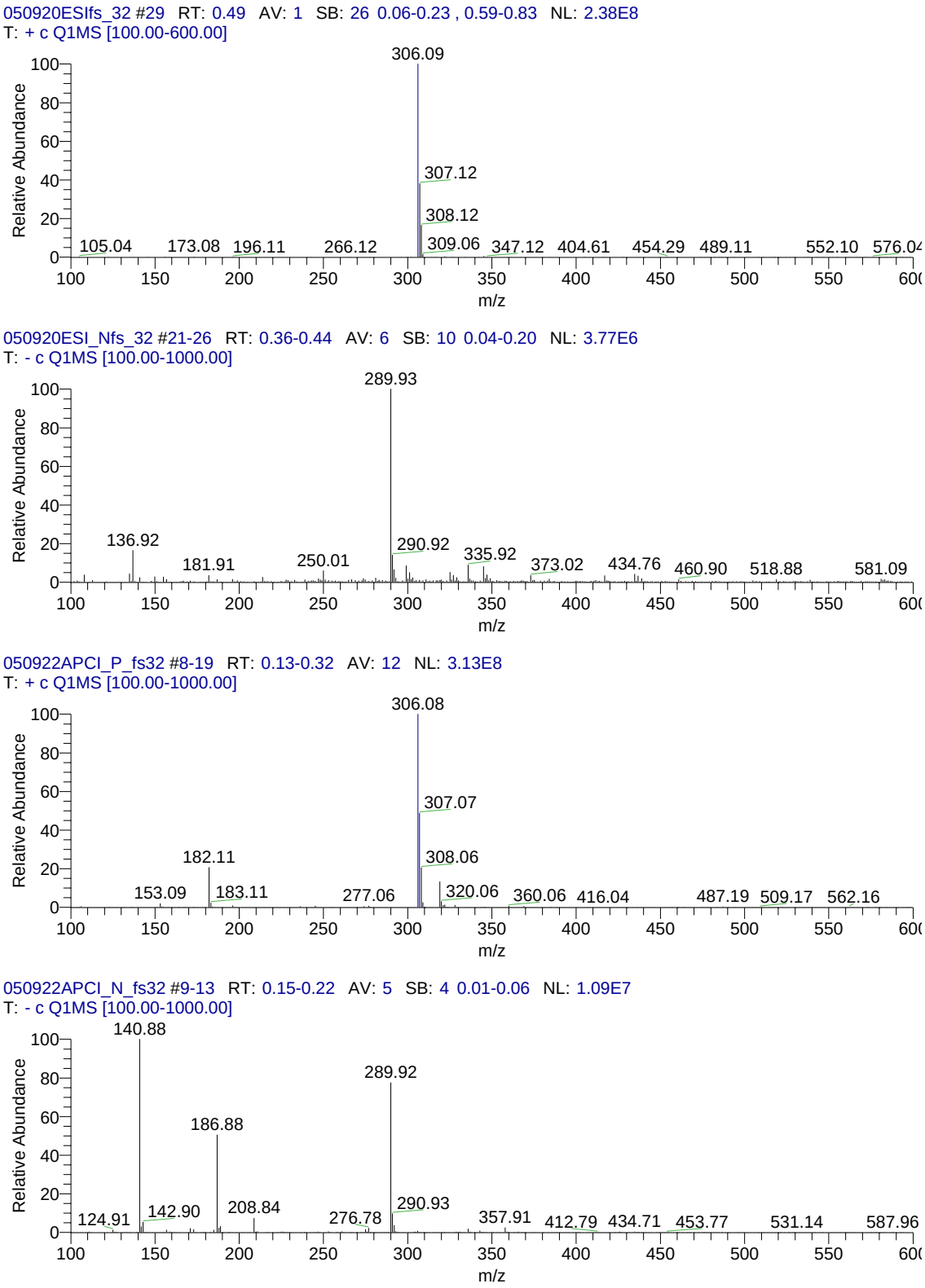


Figure 95 Pirimphos methyl ESI positive, ESI negative, APCI positive, APCI negative (weak) full scan spectra

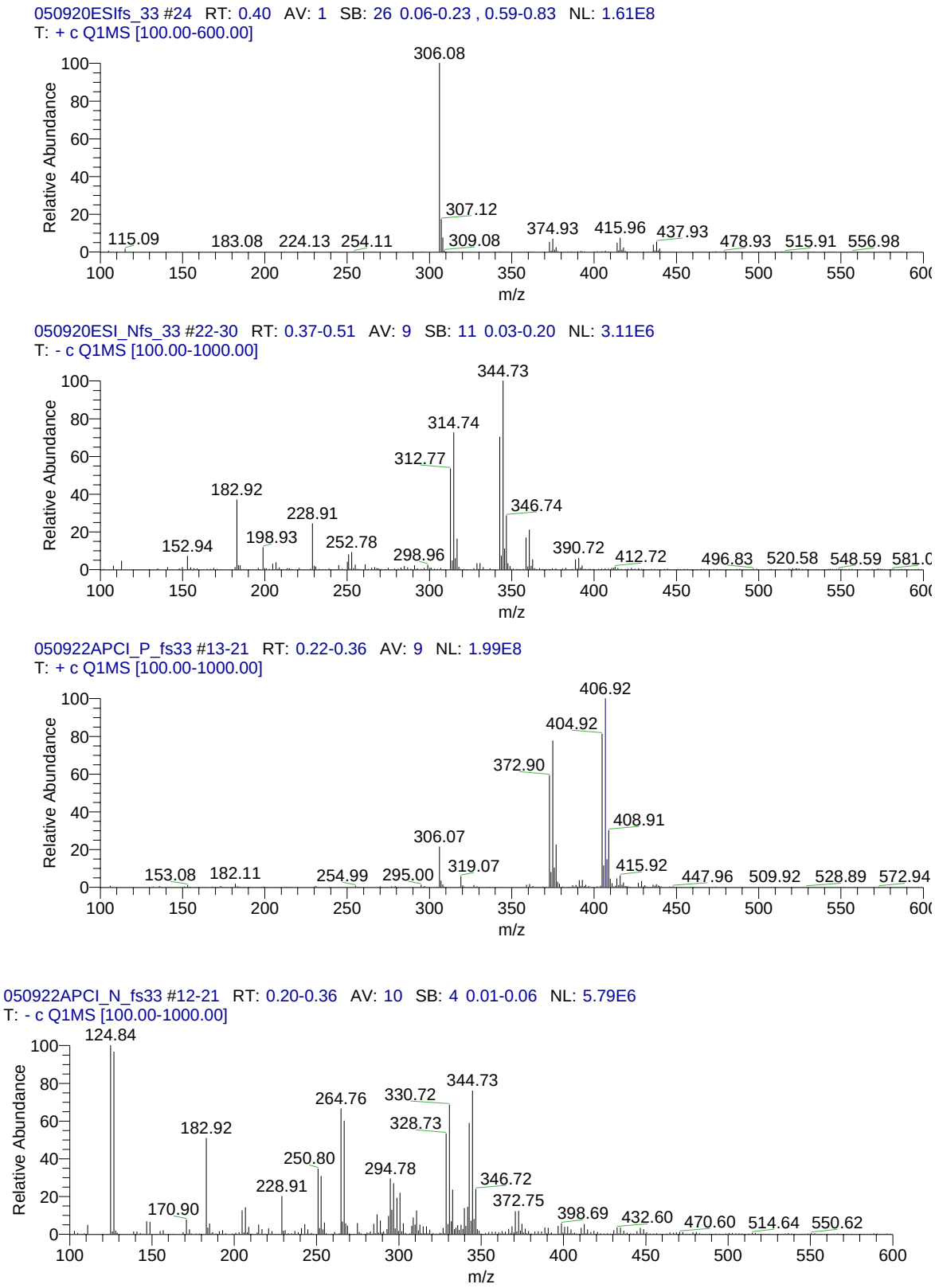


Figure 96 Profenofos ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

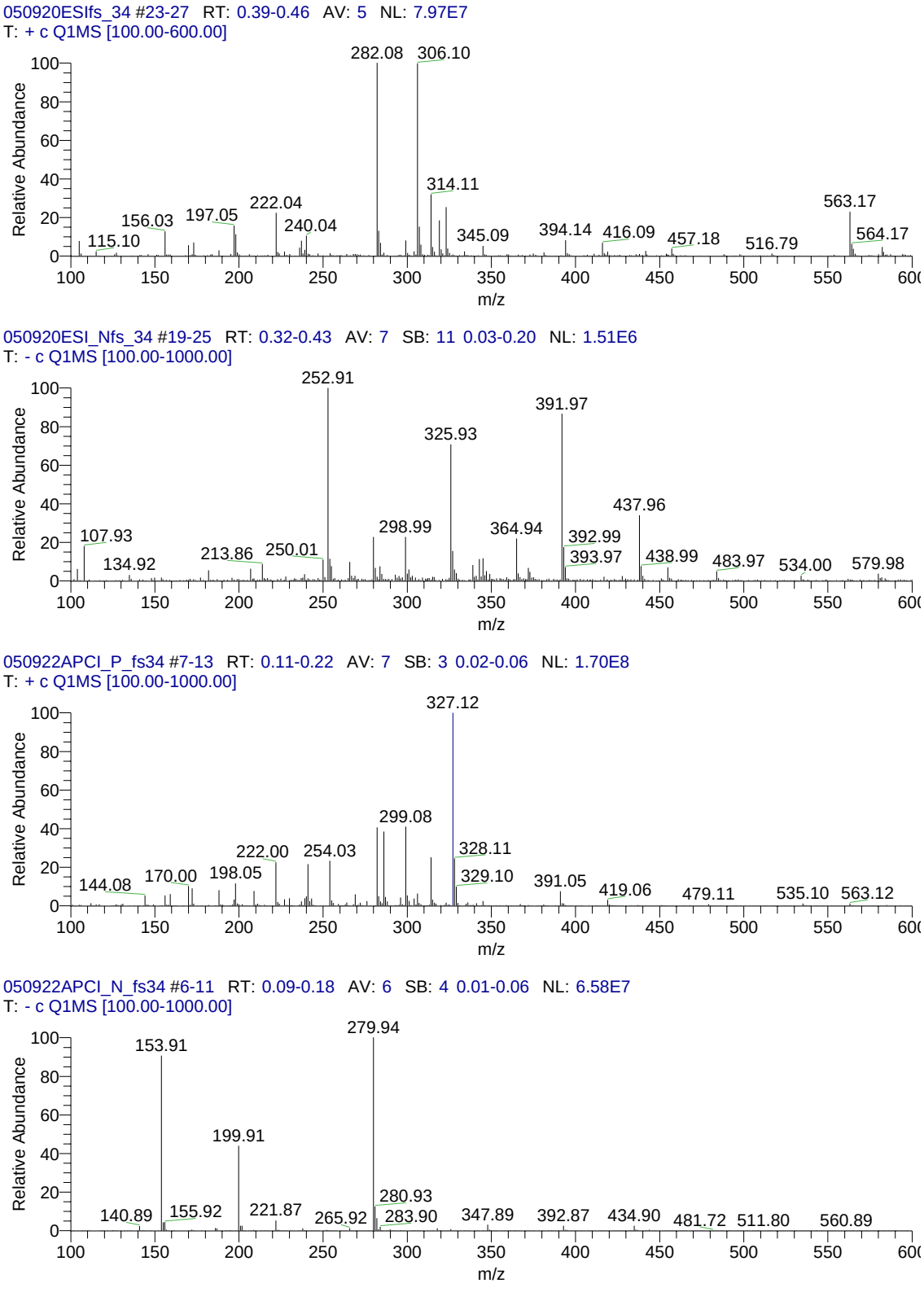


Figure 97 Propetamphos ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

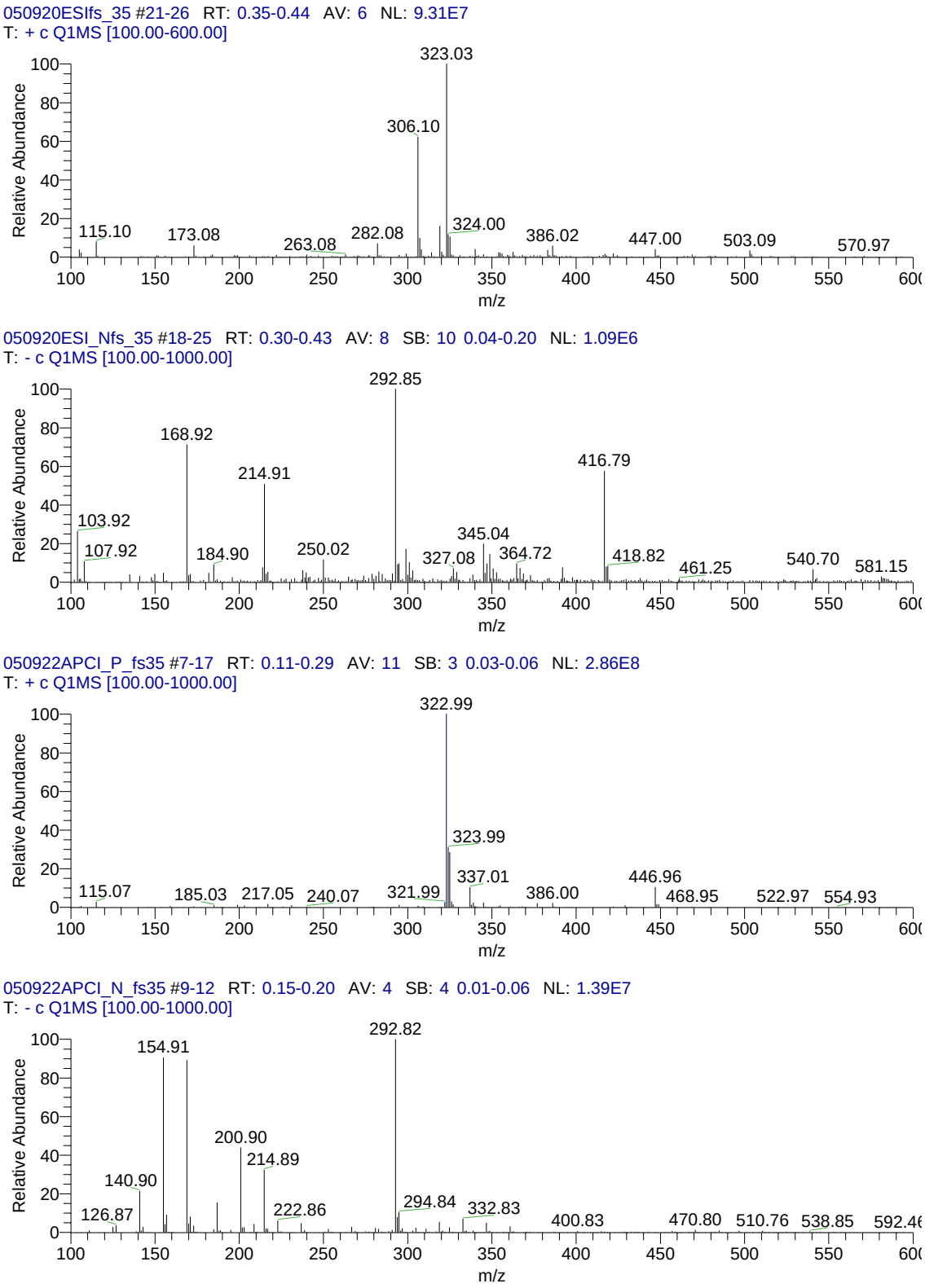


Figure 98 Sulfotepp ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

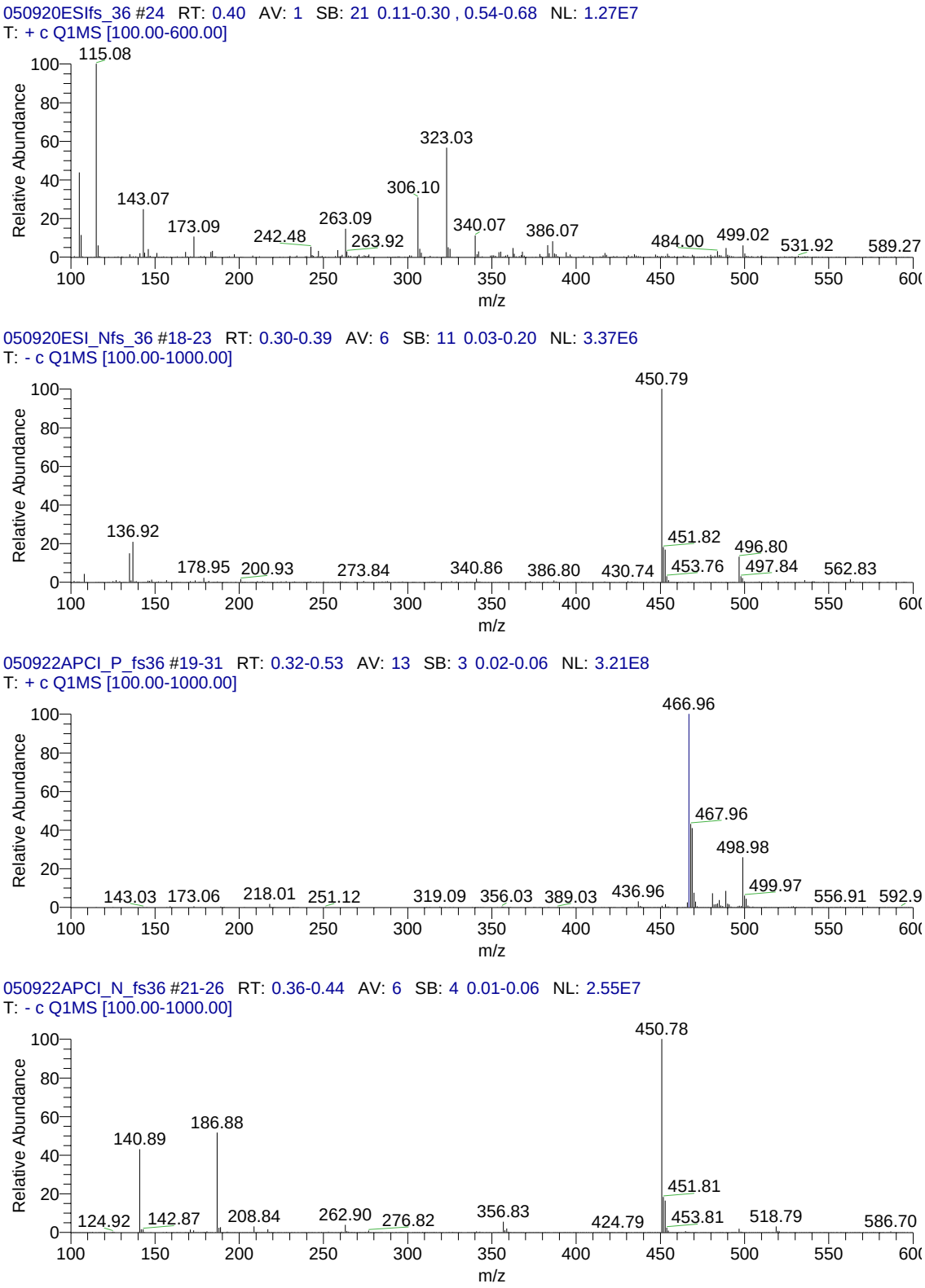


Figure 99 Temephos ESI positive (weak), ESI negative, APCI positive, APCI negative full scan spectra

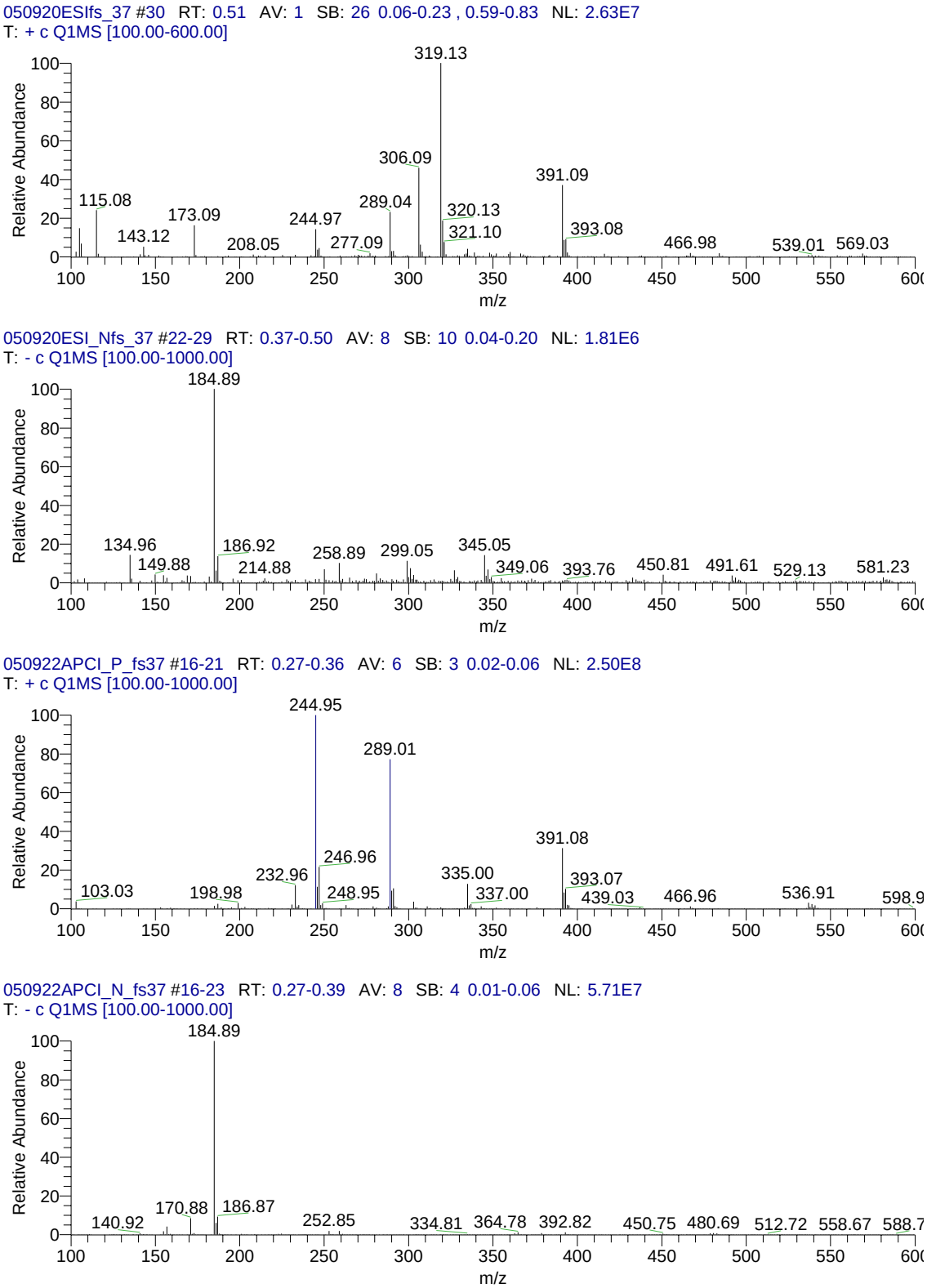


Figure 100 Terbufos ESI positive (weak), ESI negative, APCI positive, APCI negative full scan spectra

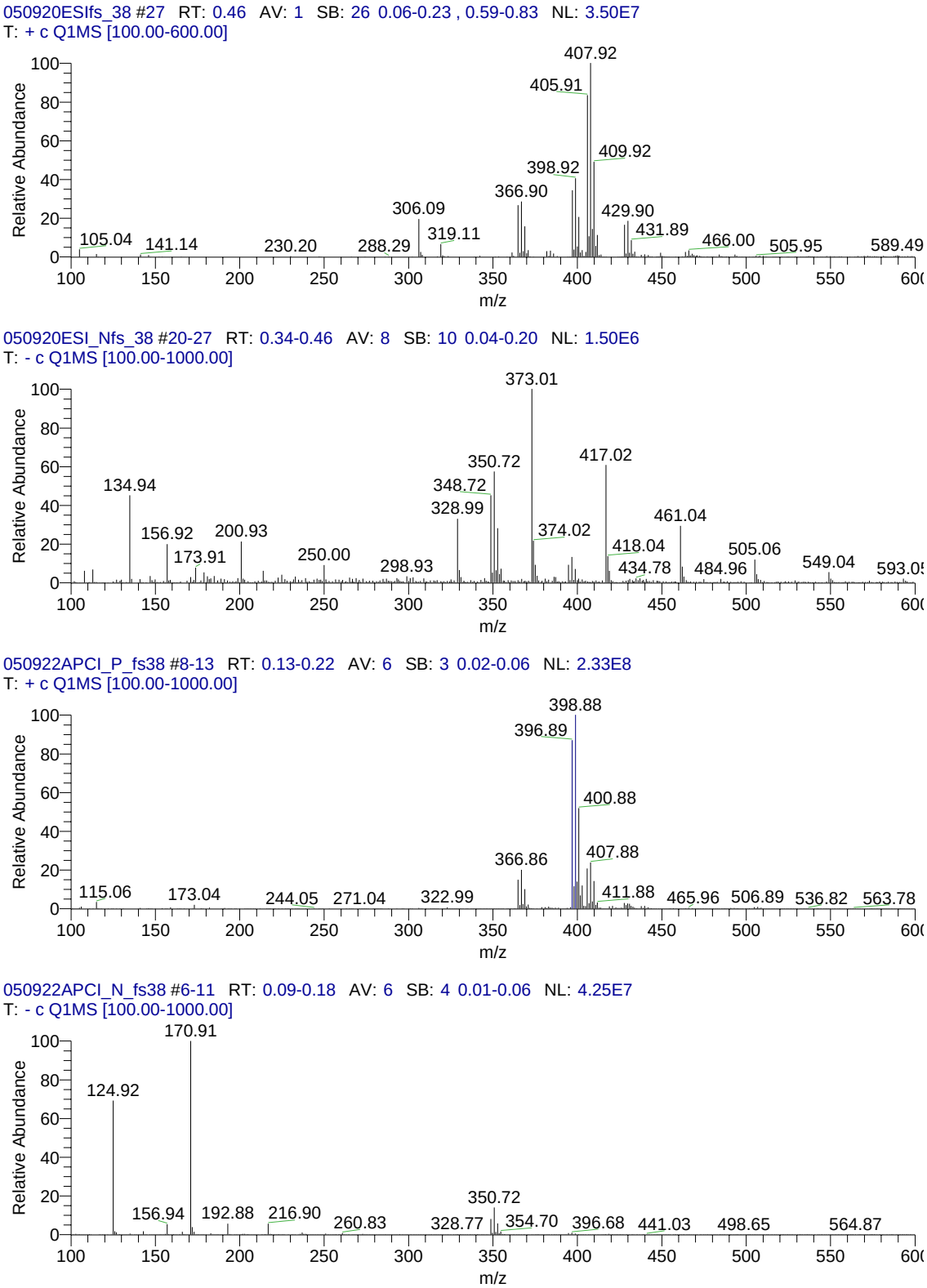
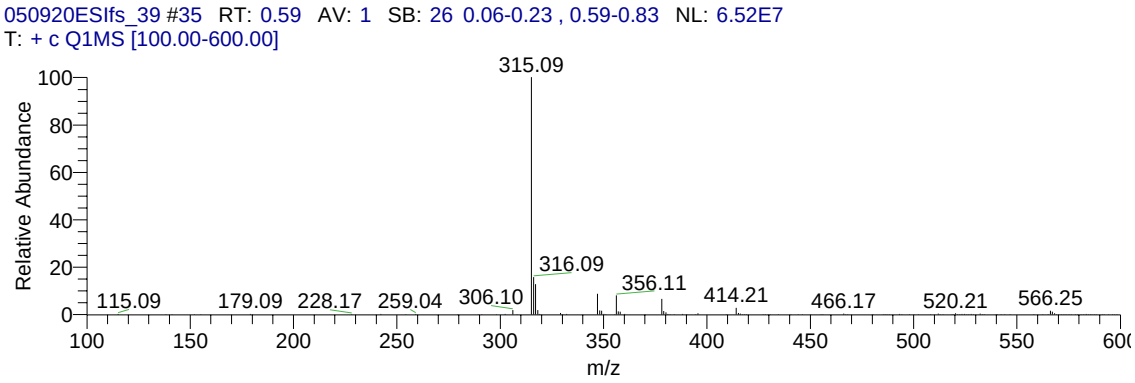


Figure 101 Tetrachlorvinphos ESI positive, ESI negative, APCI positive, APCI negative full scan spectra



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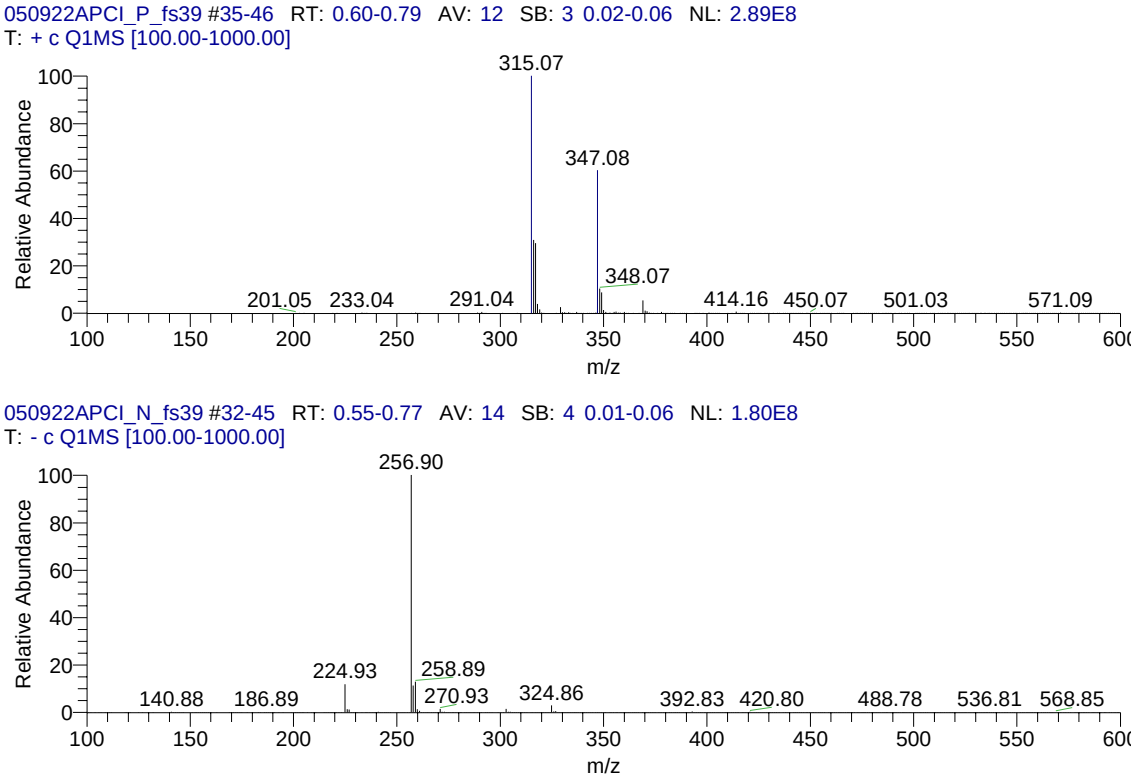


Figure 102 Tribufos ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

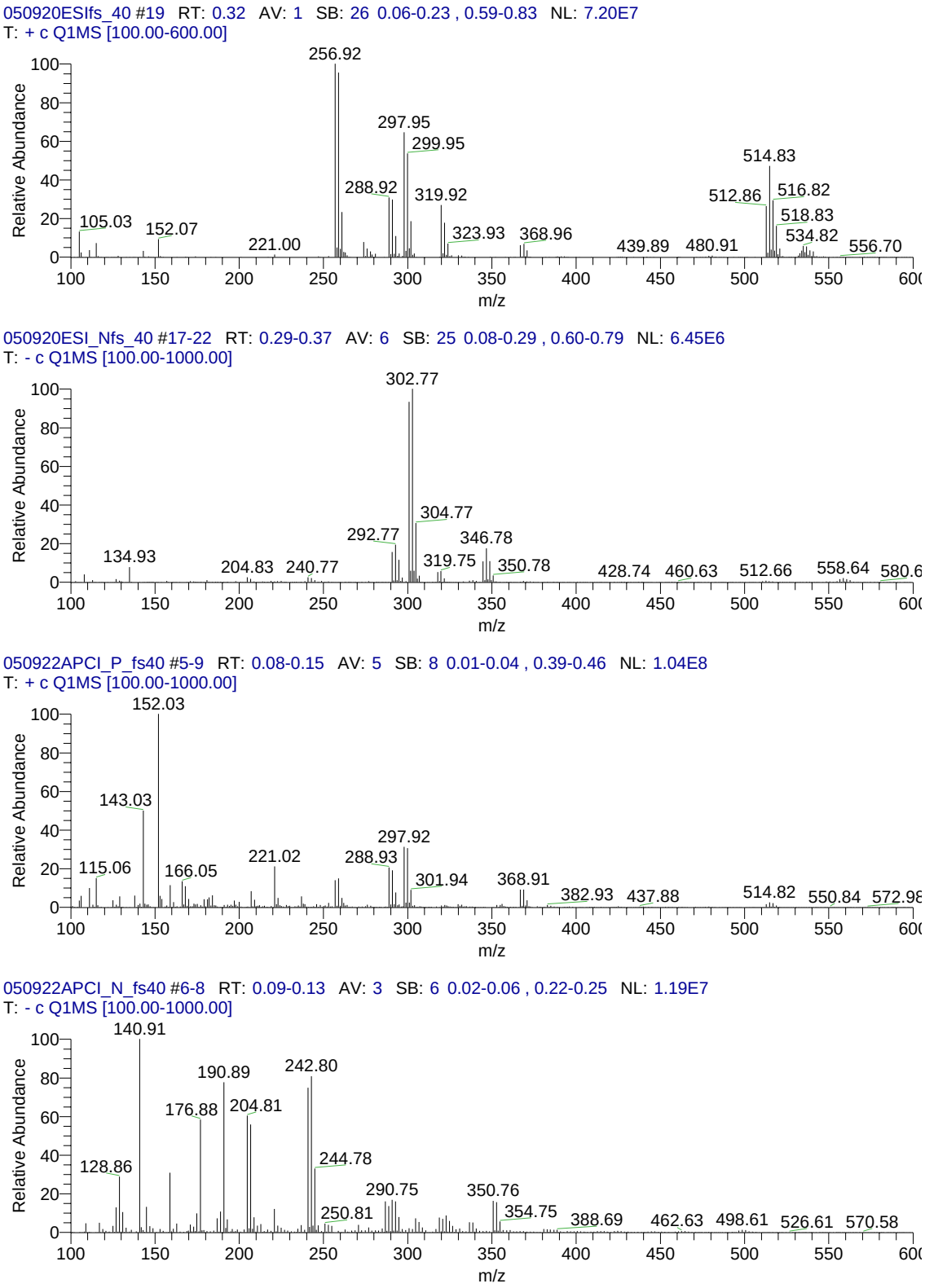


Figure 103 Trichlorfon ESI positive, ESI negative, APCI positive, APCI negative full scan spectra

CHAPTER 4: SAMPLE CLEANUP AND METHOD APPLICATION

When analyzing biological samples, almost always the first step is cleanup, followed by sample concentration. The preferred first step is solid phase extraction (SPE) because it is relatively inexpensive, easy to automatise, and a quick method.

It was clear from previous [35] work, that SPE unlikely to be successful because the analytes of interests encompass a very wide range of physical properties. Particularly the highly hydrophilic analytes, like acephate and methamidophos are very difficult to retain together with hydrophobic OP pesticides.

Also based on experiences in [35] the better cleanup method is lyophilization or freeze drying. When working with urine as matrix, freeze drying yielded very good result. It is not as easy to automatise as SPE, but the actual manual preparation is limited, and small amounts of solvent can remove most of the analytes. Reasons against lyophilization are that it requires specialized equipment, and it is a long process, creating a bottleneck in the cleanup procedure.

Should lyophilization fail, there are number of options, essentially variants of liquid-liquid extraction (LLE):

- Manual liquid-liquid extraction. It can yield good information on feasibility for a certain solvent, but uses a lot of it and practically impossible to automatise.
- Cartridge based LLE, such as Varian's ChemElut. It is a form of LLE, but because the hydrophilic carrier increases surface area, it can have good results with less solvent.

- Accelerated solvent extraction (ASE) is a LLE carried out under pressure and potentially elevated temperatures. While it uses more solvent than the ChemElut system does, it is an automated process

Liquid-Liquid Extraction on Solid Backing

Since ChemElut cartridges perform similarly to LLE in a flask, it is a good starting point for investigating LLE feasibility. ChemElut cartridges are made by Varian, Inc., and they serve as an alternative to the conventional liquid-liquid extraction. These cartridges are ultrapure polypropylene tubes filled with Hydromatrix. Hydromatrix is a purified and of controlled particle size diatomaceous earth.

When performing liquid-liquid extraction with a ChemElut cartridge, the aqueous sample first added to the tube. The Hydromatrix adsorbs the water on the surface of the particles and generates a very large potential contact surface wit the extraction solvent. After an approximately 3-5 minutes waiting, the eluent is added and led gravity-flow through the system. The stationary Hydromatrix does not let the aqueous sample to escape from the cartridge. The organic solvent surrounds the immobilized sample and the extraction process takes place. ChemElut cartridges are a good alternative to manual liquid/liquid extraction. In the experiments testing the ChemElut cartridges, 1ml serum samples were used spiked with 500 ng/mL OP pesticides. Recoveries were calculated according to the method described in Appendix 4B.

The results of the experiments are summarized in Table 20 and Table 21. First (Table 20), serum samples were tested on 3mL ChemElut cartridges with ethyl ether, ethyl acetate, dichloromethane, 1-chlorobutane, toluene and hexane as extraction solvent

candidates. In the second round of experiments (Table 21), ethyl acetate, dichloromethane and hexane were tested by themselves and in combination. In the first screening 5 mL solvent was used. In the second group the amount of solvent was increased to 2 x 5mL. From the tested neat and combination solvents, ethyl acetate was the most promising throughout the range of the analytes.

Analyte	Solvent					
	Diethyl Ether	Ethyl Acetate	Dichloro methane	1-Chloro butane	Toluene	n-Hexane
MMP	2.3%	17.8%	4.2%	1.0%	0.9%	0.1%
Acephate	0.7%	8.7%	1.8%	0.1%	0.1%	0.0%
Oxydemethon Methyl	3.6%	9.9%	2.9%	1.0%	0.9%	0.6%
Dimethoate	3.5%	9.5%	2.3%	1.3%	0.8%	0.3%
Trichlorfon	4.3%	13.9%	4.2%	3.7%	10.0%	2.7%
Dichlorvos	8.7%	2.4%	3.1%	5.3%	3.1%	0.2%
Methidathion	28.6%	12.4%	20.2%	10.9%	8.7%	8.5%
Propetamphos	0.0%	0.0%	33.2%	14.3%	19.0%	9.1%
Ethoprop	27.3%	23.4%	17.2%	8.4%	9.6%	3.8%
Azinphos Me	0.0%	0.0%	28.7%	16.8%	14.1%	41.3%
Pirimphos Me	0.1%	14.6%	9.0%	7.3%	6.8%	13.5%
Tetrachlorvinphos	11.7%	21.9%	0.0%	6.0%	7.1%	5.8%
Fonofos	0.0%	0.0%	12.4%	7.7%	8.2%	6.2%
Bensulide	0.0%	0.0%	0.0%	25.1%	26.8%	4.1%
Coumaphos	0.0%	6.4%	0.0%	37.4%	33.9%	15.3%
Chlorpyrifos	0.0%	0.0%	3.5%	8.0%	7.6%	0.4%
Ethion	0.0%	0.0%	0.0%	39.0%	12.1%	11.6%
Tribufos	22.4%	26.8%	0.0%	33.0%	34.4%	29.8%
Temephos	0.0%	14.0%	0.0%	36.2%	31.4%	46.0%

Table 19 Recoveries for lyophilization extraction

Analyte	Solvent					
	Diethyl Ether	Ethyl Acetate	Dichloro methane	1-Chloro butane	Toluene	n-Hexane
MMP	16.3%	42.7%	35.7%	0.6%	0.6%	0.2%
Acephate	3.5%	23.8%	17.3%	0.0%	0.0%	0.0%
Oxydemethon Methyl	4.3%	33.3%	87.5%	0.2%	1.1%	0.3%
Dimethoate	81.2%	84.8%	87.7%	74.6%	78.4%	0.1%
Trichlorfon	80.9%	38.8%	40.4%	71.3%	93.9%	3.8%
Dichlorvos	75.4%	16.2%	9.0%	10.3%	25.3%	3.6%
Methidathion	23.7%	23.6%	19.1%	25.7%	27.6%	27.8%
Propetamphos	12.7%	13.1%	10.9%	16.7%	14.2%	18.5%
Ethoprop	38.0%	30.6%	22.4%	30.9%	37.6%	26.6%
Azinphos Me	22.8%	18.0%	16.8%	16.6%	17.1%	11.5%
Pirimphos Me	13.0%	13.5%	10.0%	14.9%	15.9%	21.5%
Tetrachlorvinphos	16.7%	14.5%	16.6%	18.8%	19.8%	10.3%
Fonofos	14.3%	11.1%	12.9%	20.5%	20.3%	26.9%
Bensulide	11.8%	10.9%	11.5%	20.1%	21.6%	0.0%
Coumaphos	13.3%	12.0%	5.8%	16.2%	14.2%	16.4%
Chlorpyrifos	8.8%	9.4%	4.8%	10.5%	8.7%	19.2%
Ethion	8.0%	8.6%	3.1%	6.5%	6.3%	18.2%
Tribufos	9.4%	10.5%	4.8%	10.7%	9.5%	17.2%
Temephos	0.5%	5.9%	1.5%	2.4%	2.2%	10.6%

Table 20 Recoveries for extraction on ChemElut Cartridges

Analyte	Solvent						
	Ethyl Acetate	Dichloro methane	n-Hexane	Blend of Ethyil Acetate Dichloromethan e n-Hexane	1 Ethyl Acetate 2 Dichloro-methane	1 Ethyl Acetate 2 n-Hexane	1 Dichloro methane 2 n-hexane
MMP	45.7%	24.2%	0.3%	12.4%	38.3%	20.7%	0.2%
Acephate	44.3%	36.1%	0.2%	19.7%	45.4%	19.6%	0.1%
Oxydemethon Methyl	43.5%	71.8%	0.5%	45.6%	60.6%	26.0%	0.1%
Dimethoate	75.6%	74.7%	9.2%	68.4%	63.9%	54.5%	0.1%
Trichlorfon	52.7%	22.6%	8.3%	23.2%	41.9%	33.0%	0.2%
Dichlorvos	36.1%	1.2%	0.4%	0.7%	17.0%	5.7%	0.3%
Methidathion	38.1%	18.8%	46.9%	56.5%	31.4%	45.9%	35.9%
Propetamphos	18.0%	9.5%	36.1%	34.2%	14.5%	32.3%	22.0%
Ethoprop	40.7%	15.1%	28.5%	37.6%	32.2%	39.0%	9.6%
Azinphos Me	31.3%	13.4%	31.5%	52.7%	25.2%	36.1%	20.9%
Pirimphos Me	16.9%	5.7%	41.0%	33.4%	14.1%	31.8%	32.2%
Tetrachlorvinphos	22.0%	9.0%	25.9%	45.6%	18.2%	30.8%	15.6%
Fonofos	18.6%	6.2%	38.2%	31.6%	15.6%	25.1%	17.9%
bensulide	14.2%	6.9%	5.4%	38.3%	12.7%	30.4%	0.1%
Coumaphos	15.6%	4.2%	33.4%	35.1%	14.7%	29.0%	24.8%
Chlorpyrifos	13.4%	3.3%	33.7%	17.4%	12.5%	21.5%	23.4%
Ethion	12.8%	3.8%	30.6%	11.5%	11.7%	18.9%	27.5%
Tribufos	16.7%	8.0%	29.2%	20.9%	14.6%	25.9%	26.6%
Temephos	10.3%	2.2%	17.5%	6.2%	9.2%	12.3%	17.1%

Table 21 ChemElut LLE experiment with combination of solvents

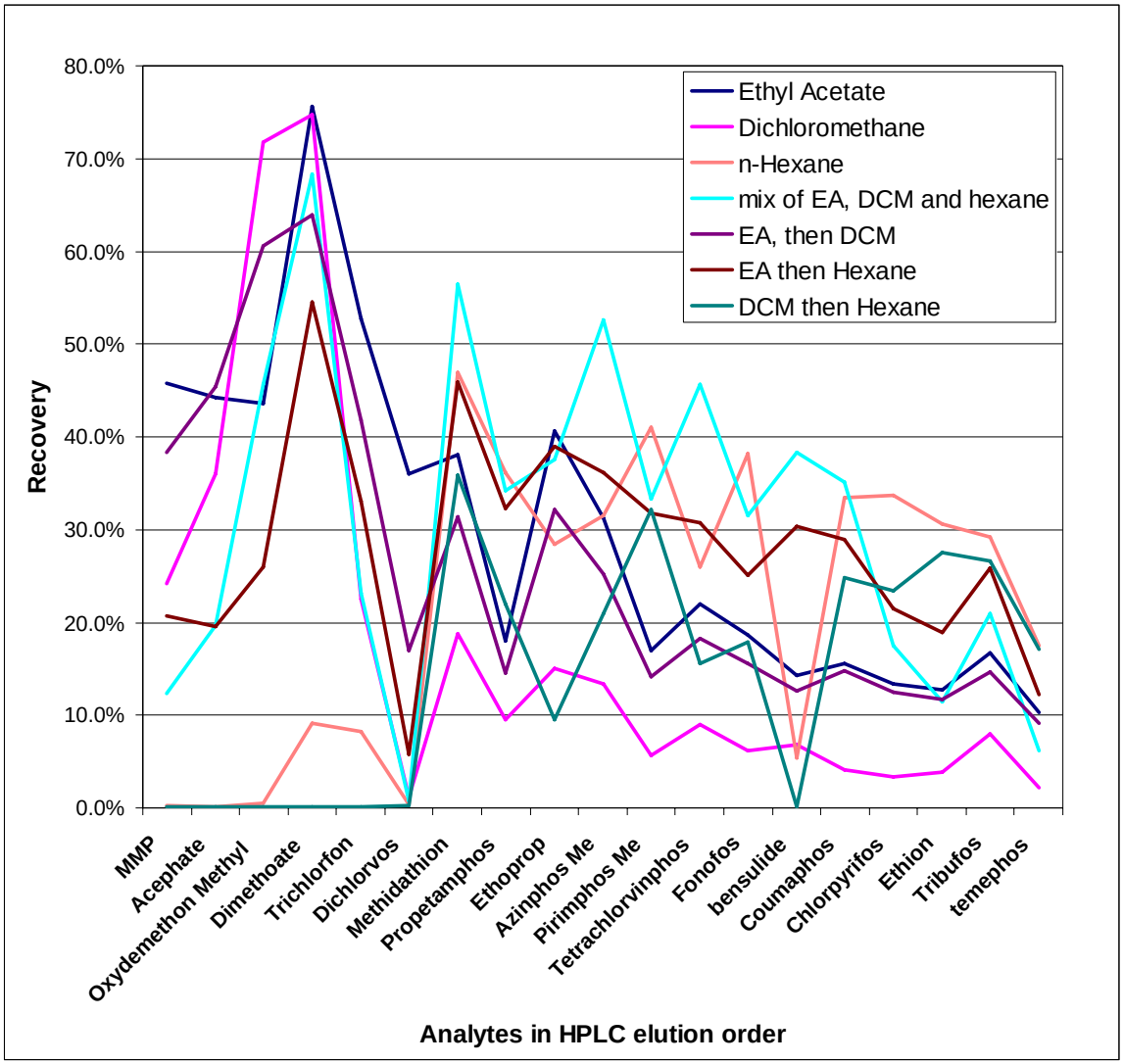


Figure 104 ChemElut LLE experiment with combination of solvents

Accelerated Solvent Extraction (ASE)

Accelerated solvent extraction is a variant of solid backed LLE. The most common carrier is Hydromatrix, a purified form diatomaceous earth marketed by Varian Inc. Just like in the case of LLE cartridges, the particles of the solid backing provide a large surface area and increase the contact area between the matrix and the eluting solvent. ASE extraction is carried out in pressurizable stainless steel cartridges. The application of pressure further increases relative surface by pressing the matrix inside the solid particles. Applying pressure allows for the use of elevated temperatures, where the pressure prevents the solvent from boiling. Using hot solvents for extraction can accelerate the process because the lowered viscosities and increased diffusion.

The solvent of choice for OP pesticides was ethyl acetate. This selection was made based on the results using ChemElut cartridges (Table 20, Table 21). Once the extraction solvent was selected, extraction temperature, static time and pressure was varied and optimized. The results are tabulated in Table 22 and charted in Figure 105 and Figure 106. Increasing temperatures, static(extraction) time and pressure has a positive effect on most compounds, but has a dramatic decrease of recovery on trichlorfon, dichlorvos and tetrachlorvinphos. Fro that reason the parameters selected were 5 minutes static time, at room temperature (20C) and 1500 PSI nitrogen pressure.

Compound	Temperature (Pressure = 1500 PSI, 5 min Static time)				Temp = 20°C	
	20°C	40°C	75°C	100°C	10 min static time	2500 PSI
MMP	79.9%	81.9%	84.9%	77.4%	79.2%	71.9%
Acephate	49.5%	59.4%	88.4%	84.6%	51.5%	44.3%
Oxydem. Me	60.4%	81.6%	94.4%	81.7%	72.3%	73.6%
Dimethoate	98.5%	92.9%	95.5%	83.8%	93.5%	87.6%
Trichlorfon	81.2%	64.9%	31.7%	13.9%	59.9%	27.8%
Dichlorvos	91.2%	34.5%	1.4%	0.1%	34.7%	0.3%
Methidathion	84.5%	84.3%	89.1%	76.1%	88.8%	79.8%
Propetamphos	88.7%	91.4%	99.2%	94.3%	85.4%	89.4%
Ethoprop	81.4%	83.6%	94.9%	93.3%	87.1%	86.7%
Azinphos Me	87.5%	88.3%	90.6%	72.4%	36.2%	18.1%
Pirimphos Me	67.0%	71.6%	66.9%	45.1%	71.5%	59.7%
Tetrachlorvinphos	69.0%	54.7%	27.4%	3.2%	47.0%	18.0%
Fonofos	81.3%	89.8%	102.1%	97.8%	79.4%	82.4%
Bensulide	66.6%	81.4%	102.2%	91.6%	81.1%	83.2%
Coumaphos	74.0%	82.1%	102.2%	99.0%	69.3%	66.1%
Chlorpyrifos	62.9%	76.8%	93.1%	84.7%	70.3%	70.3%
Ethion	62.4%	76.8%	100.9%	97.8%	67.5%	70.3%
Tribufos	64.4%	75.8%	93.0%	90.7%	79.3%	83.3%
Temephos	56.2%	73.8%	103.2%	98.7%	58.8%	59.9%

Table 22 Effects on recoveries of varying ASE extraction parameters

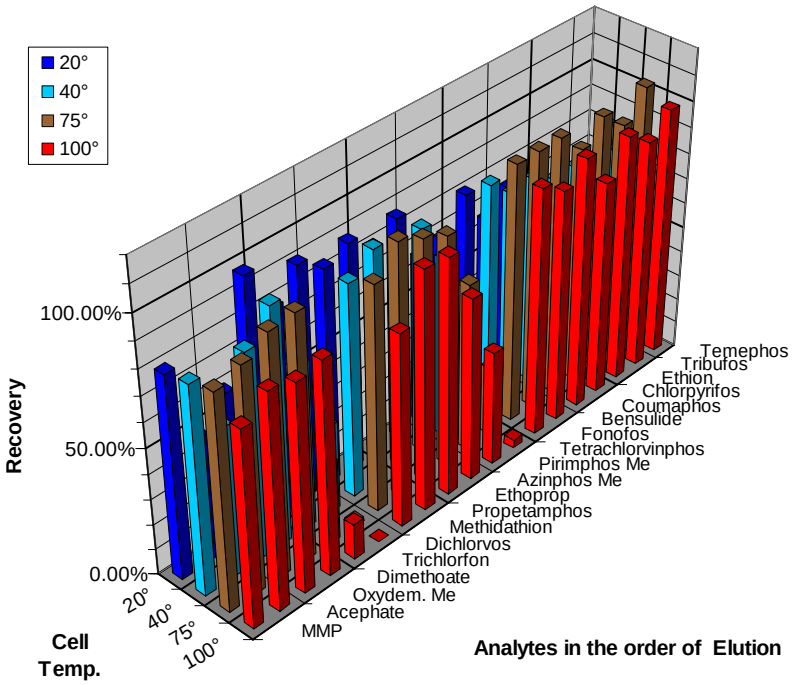


Figure 105 ASE temperature effect on recoveries

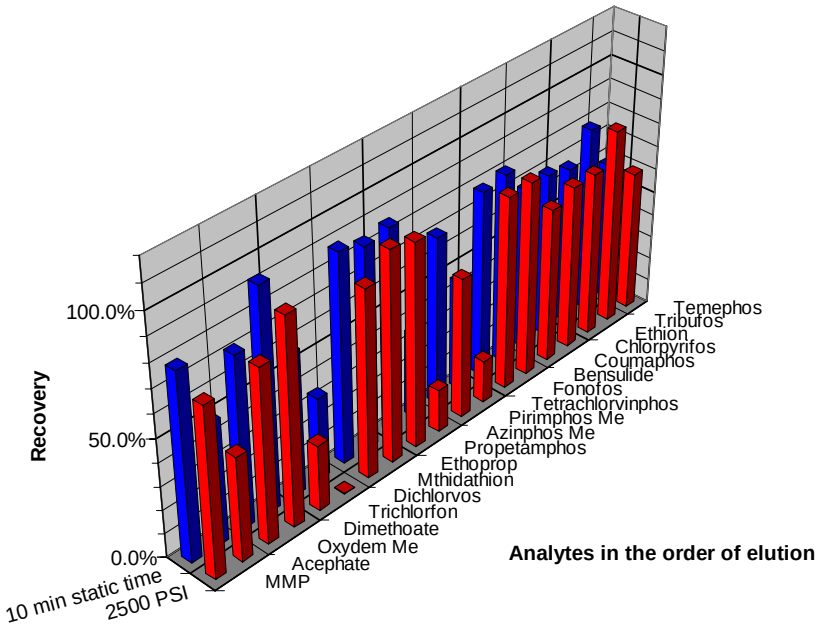


Figure 106 ASE dwell time and pressure effect on recoveries

Second step cleanup: normal phase chromatography on SPE cartridges.

After ASE extraction with ethyl acetate, the samples are clean enough to – after the lipid rich portion of the serum. If these samples are injected without further cleanup, the lipids will first concentrate in the column, causing retention time shifts and peak shape changes. Next the lipids migrating into the MS, contaminate the ion source and will cause rapid signal degradation. For this reason a second step of the cleanup process was developed. The approach to further purify the ASE extract was to transfer it to a sorbent filled cartridge and wash it with a polar solvent. The elution process was to be fractionated and tested for the analytes as well for contaminants.

The process was carried out as follows:

The instrument used was Rapid Trace (originally from Zymark corporation, now Caliper Life Sciences Hopkinton, MA) 3mL SPE cartridges were filled with 200mg sorbents each. The ASE extract (0.35 mL) was then transferred to the Rapid Trace. The automated system placed the extract on top of pre-wetted sorbent. Then the cartridge was washed with 8mL of ACN. 1 mL fractions were collected and analyzed for all the LC-group compounds. Half of the fractions were also dried down (1hr 105°C) to check solids content.

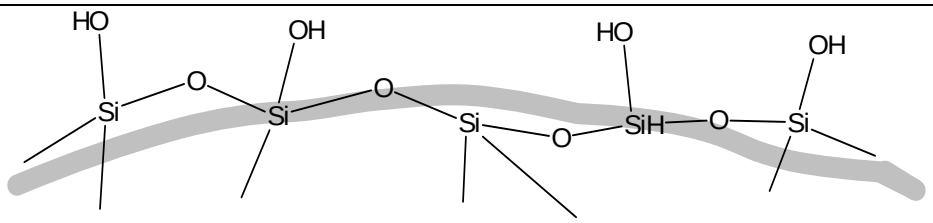
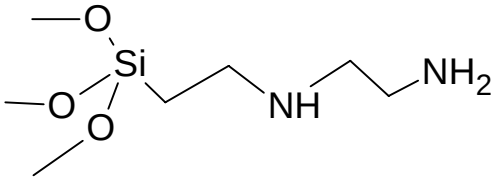
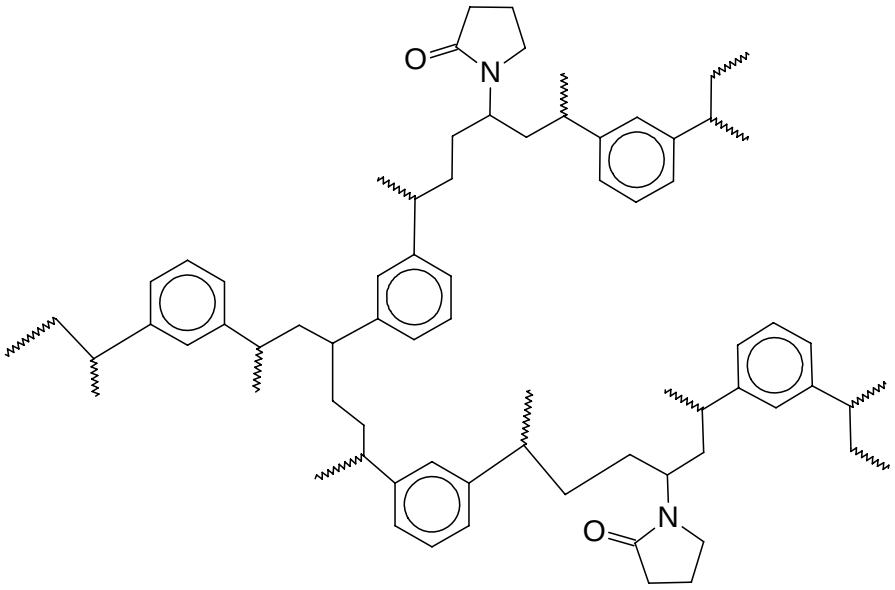
Silica Surface	
Supelco PSA Structure	
Varian HLB Sorbent	

Figure 107 Structure of various sorbents

The following sorbents were tested:

- Precipitated silica (Varian, Inc. Palo Alto, CA) (Figure 107)
- Alumina (Varian, Inc. Palo Alto, CA)
- LRA or Lipid removal agent, a synthetic calcium silicate from (Advanced Minerals, Santa Barbara, CA) [36]
- PSA or primary, secondary amine. It is a surface treated silica from Supelco, primarily used for foodstuff analysis (Figure 107)
- Varian HLB (Figure 107)

The cumulative solids were plotted on Figure 108.

Next, the cumulative amount of analytes were plotted for each sorbent and the cumulative solids/contaminants curve superimposed on each. Figure 109

All the conventional sorbents (silica, alumina) showed varying affinity to the different OP pesticides. LRA did not show a different retention for OP pesticides and contaminants.

The choice came down between PSA and Varian HLB. PSA worked excellent, except for its strong retention of acephate. Because acephate is one of our important compounds, the choice became the Varian HLB bulk sorbent.

In the extraction method, the first 1.5mL is saved, concentrated and used for HPLC and GC both. As it seen on Figure 109, this cut allows close to 100% of the analytes and less than 10% of the contaminants.

There is a possibility of carryover of trace water. Ethyl acetate can contain up to 7% water at room temperature. And the repeated solvent exchanges may not remove all of it. Trace of water can damage GC columns over time. To remove residual water, a

combination of 200 mg bulk HLB and the same amount of anhydrous sodium sulfate was tried. Dichlorvos. Tetrachlorvinphos and phosmet showed decreased recoveries (Table 23, Table 24) so this step is not applied in the final method.

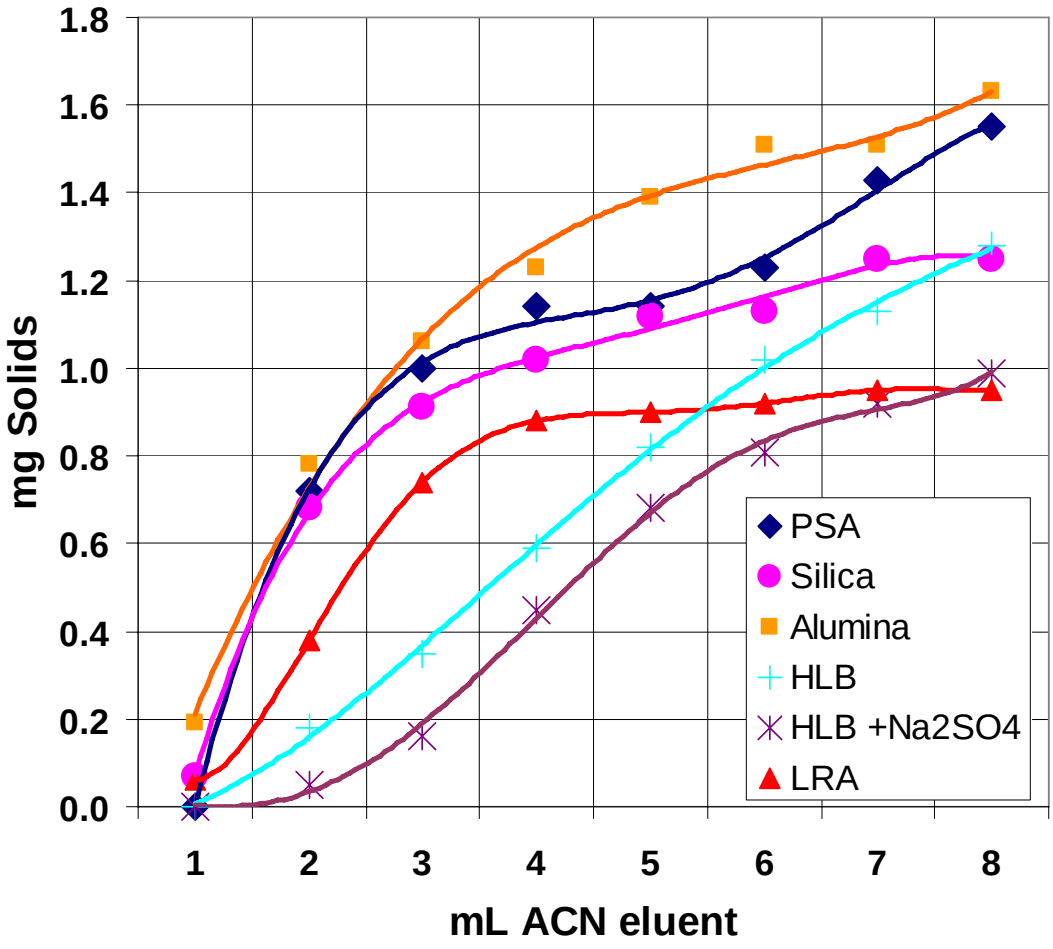


Figure 108 Comparison of various sorbents for removing contaminants from serum extract

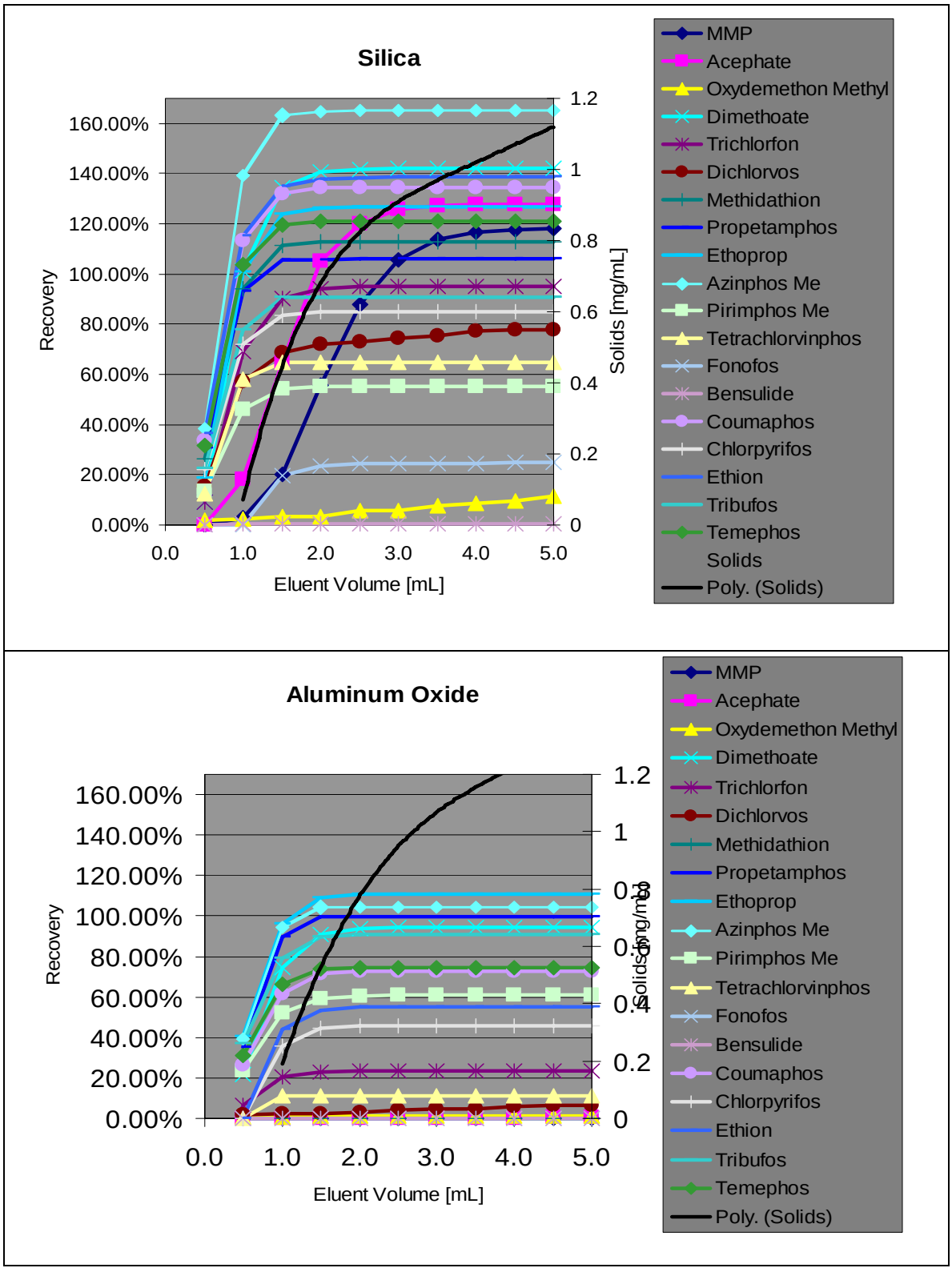


Figure 109 Elution of OP pesticides over different sorbents

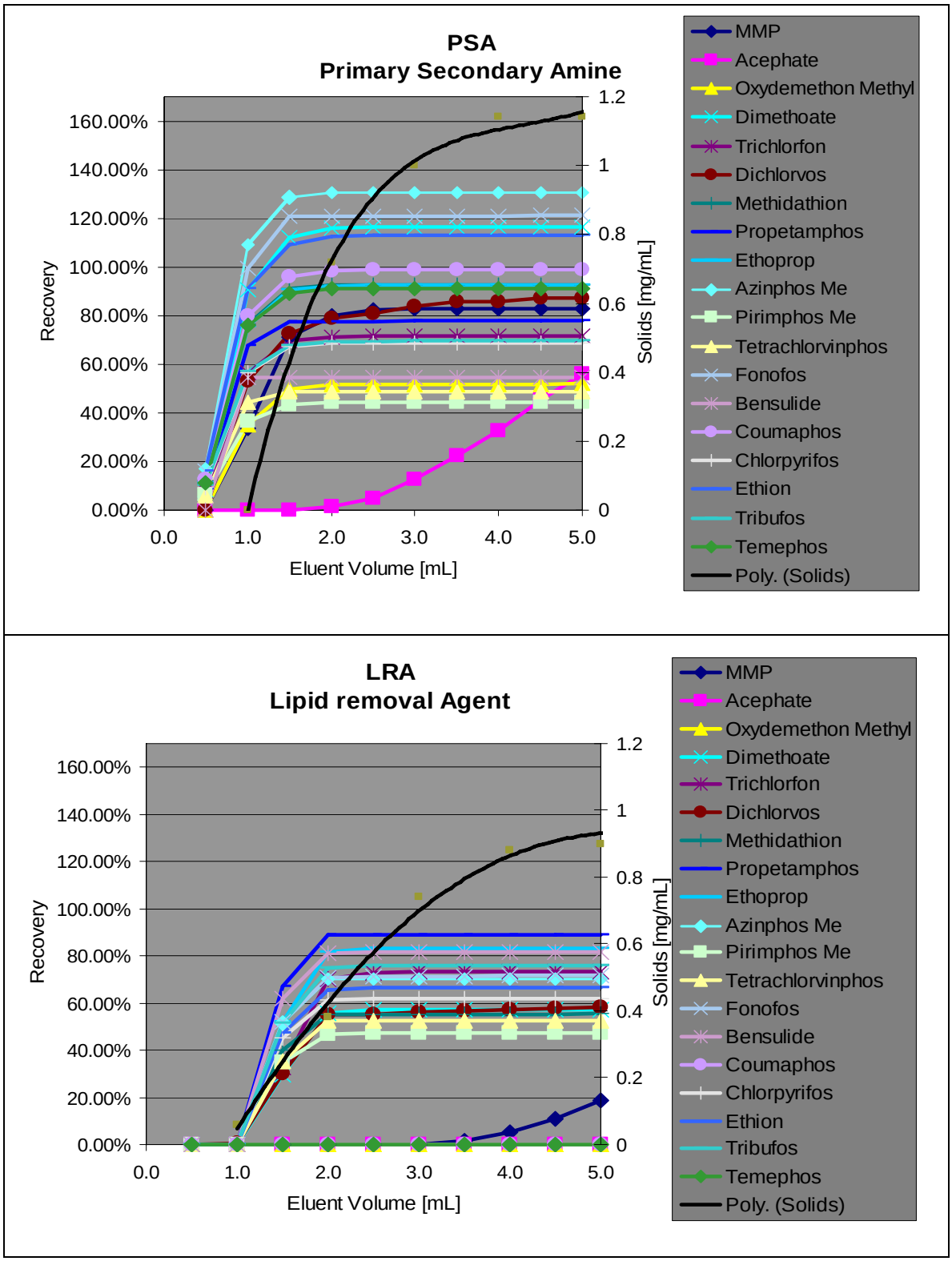


Figure 109, continued: Elution of OP pesticides over different sorbents

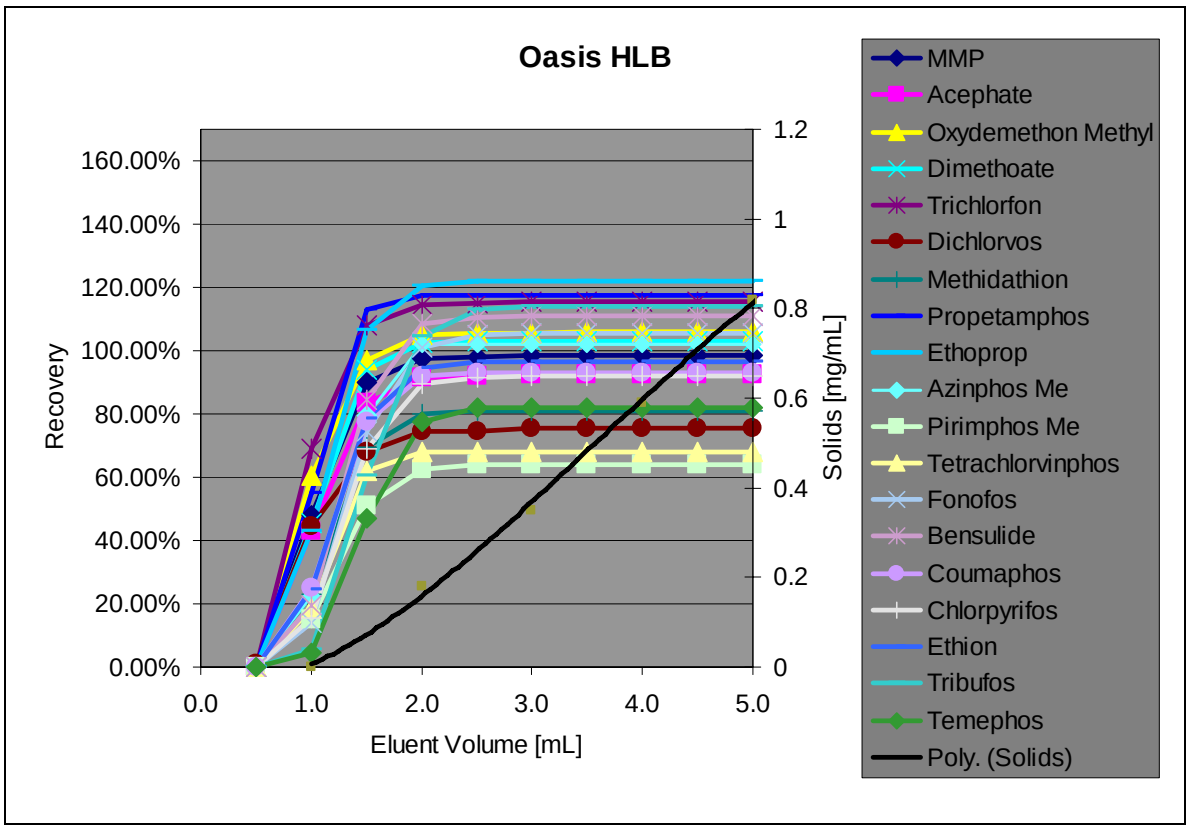


Figure 109, continued: Elution of OP pesticides over different sorbents

	Average Recoveries		Standard Deviations	
	200 mg HLB	200 mg HLB + 200 mg Na_2SO_4	200 mg HLB	200 mg HLB + 200 mg Na_2SO_4
Mevinphos	69.2%	36.8%	7.0%	17.2%
Chlorethoxyfos	55.8%	67.9%	4.4%	9.0%
Phorate	59.7%	75.9%	3.3%	7.7%
Cadusafos	82.7%	81.9%	6.3%	12.7%
Dicrotophos	82.2%	73.7%	12.0%	12.1%
Sulfotepp	62.4%	50.1%	2.4%	10.7%
Diazinon	71.6%	91.5%	16.8%	58.3%
Terbufos	57.1%	69.5%	13.2%	38.0%
Fonofos	72.2%	94.1%	16.7%	62.1%
Phostebupirim	62.5%	73.3%	9.3%	20.1%
Chlorpirifos Me	50.3%	46.9%	6.0%	1.7%
Me parathion	45.9%	64.6%	14.1%	3.4%
Malathion	64.9%	37.4%	12.1%	9.5%
Fenthion	55.2%	58.1%	9.7%	4.4%
Et Parathion	47.0%	70.6%	21.3%	26.2%
Fenamiphos	56.7%	69.5%	13.7%	3.0%
Profenofos	31.7%	12.6%	11.5%	5.9%
Phosmet	30.1%	2.2%	21.1%	0.8%
Phosalone	62.6%	74.7%	13.0%	9.4%

Table 23 Two step cleanup: recoveries of GC group OP pesticides.
Concentrations are 500ng/mL

	Average Recoveries		Standard Deviations	
	200 mg HLB	200 mg HLB + 200 mg Na_2SO_4	200 mg HLB	200 mg HLB + 200 mg Na_2SO_4
MMP	65.9%	66.0%	1.0%	2.3%
Acephate	43.9%	50.7%	2.5%	14.7%
Oxydem Me	65.5%	63.7%	8.7%	9.0%
Dimethoate	80.2%	80.9%	9.8%	5.6%
Trichlorfon	53.6%	27.5%	11.2%	12.3%
Dichlorvos	47.2%	18.3%	40.1%	1.5%
Methidathion	77.9%	74.6%	8.0%	5.7%
Ethoprop	75.6%	82.3%	2.7%	11.2%
Propetamphos	69.9%	77.4%	1.7%	14.2%
Azinphos_Me	69.3%	66.2%	8.5%	5.9%
PirimphosMe	60.6%	51.4%	9.2%	5.1%
Tetrachlorvinphos	48.4%	14.2%	14.7%	11.2%
Fonofos	68.1%	74.8%	0.4%	7.2%
Bensulide	63.9%	73.3%	4.1%	8.3%
Coumaphos	64.8%	72.6%	6.2%	15.1%
Chlorpyrifos	62.2%	69.1%	4.7%	10.8%
Ethion	58.9%	73.0%	2.5%	13.5%
Tribufos	63.9%	76.9%	0.6%	11.5%
Temephos	52.1%	65.3%	3.9%	16.1%

Table 24 Two step cleanup: recoveries of LC group OP pesticides
Concentrations are 500ng/mL

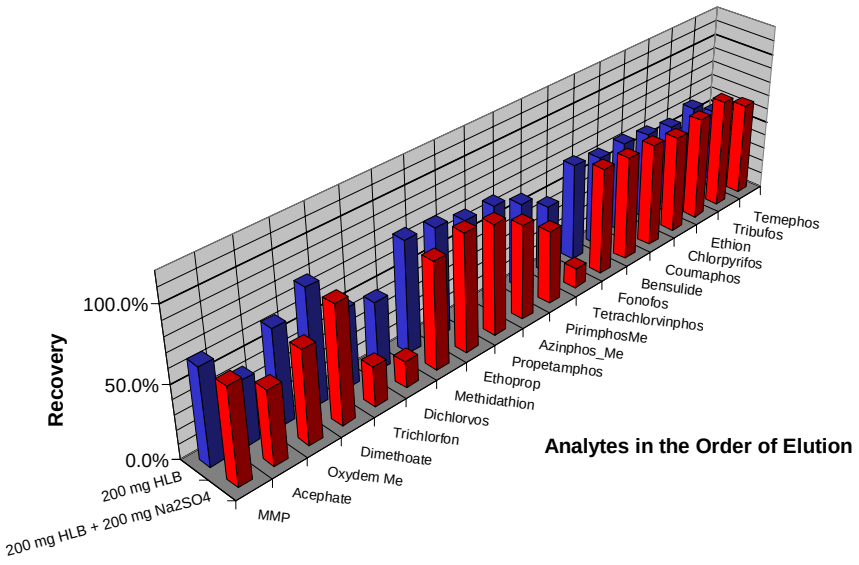


Figure 110 Two step cleanup: recoveries of LC group OP pesticides

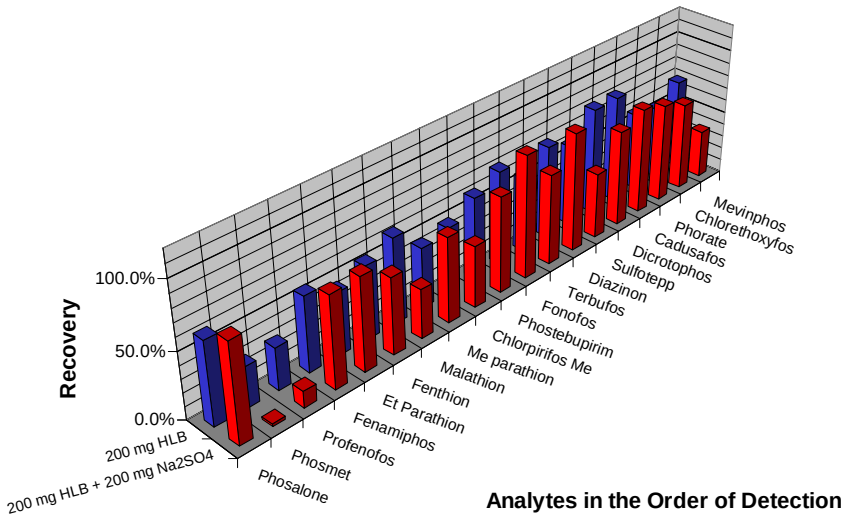


Figure 111 Two step cleanup: recoveries of GC group OP pesticides

Serum Extraction

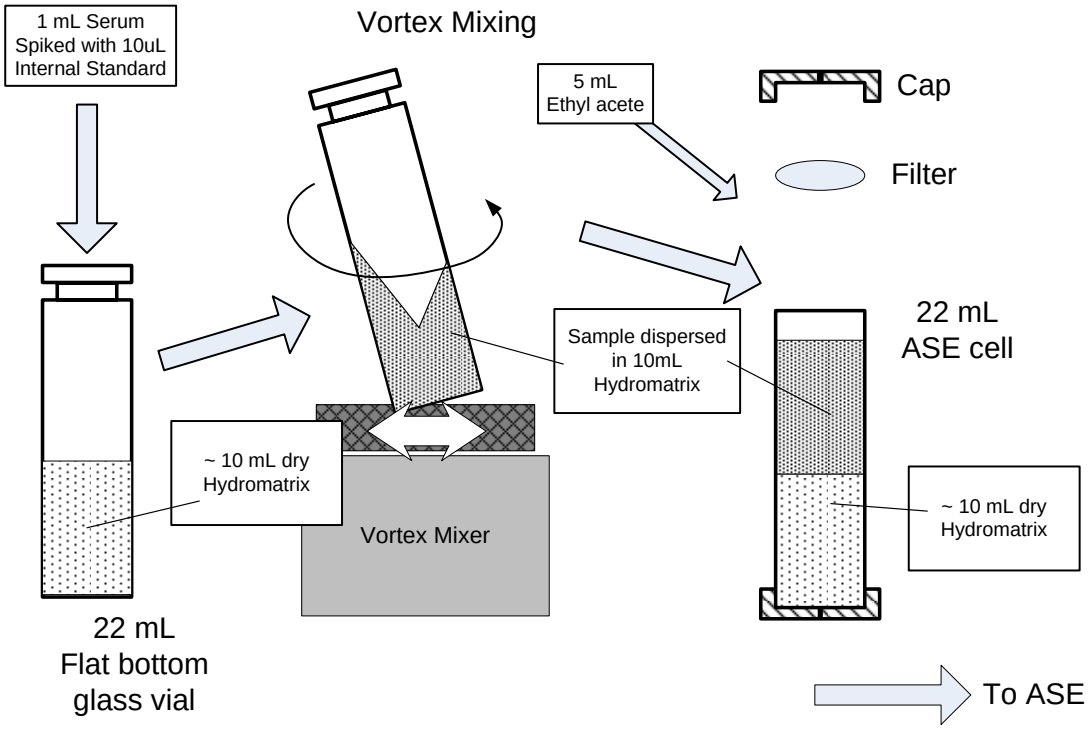


Figure 112 Sample dispersion process prior ASE extraction

Frozen unknown and blank serum samples were first thawed out. 1 mL of each was aliquoted into 15 mL conical bottom centrifuge tubes. All samples were spiked with 10µL internal standard solution. Each centrifuge tubes were mixed with vortex mixer for ten seconds. Next, the standard series were spiked with 10µL standard solutions. The centrifuge tubes were vortex mixed once more for ten seconds.

In equal number as the centrifuge tubes, ASE cartridges were prepared as follows:

Cartridge body and cap were hand tightened, and then one fiberglass based filter disc was placed inside. 10 mL of Hydromatrix absorbent was placed in the cartridges.

The same number of 20 mL flat bottomed vials was also filled with 10 mL Hydromatrix. Each serum sample was then pipetted on top of the corresponding glass vial, where the Hydromatrix promptly absorbed it. Each vial then was vortex mixed at high speed to disperse the serum in the Hydromatrix. The absorbent/serum mix was then transferred in a previously "half filled" cartridge.

A second filter disk was placed on top of the cartridge; the top cap was put in place and hand tightened. The cartridges were placed on the ASE rack, the receptacle vials were put in place and labeled. The extraction solvent reservoir was filled with fresh ethyl acetate and 1500 PSI nitrogen pressure applied.

The extraction process consisted of two extraction cycles of ten minutes duration at 1500 PSI and room temperature. Machine purge (automatic flush between samples) was turned on to prevent cross contamination.

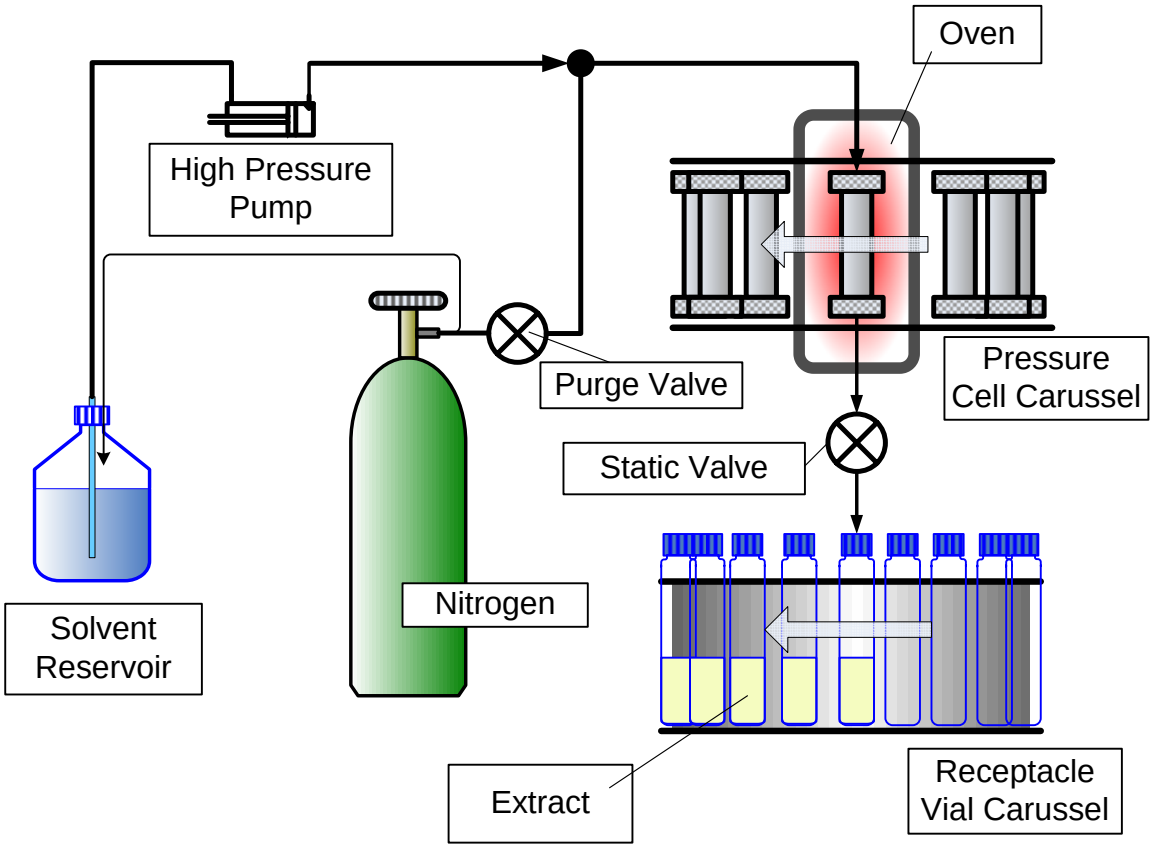


Figure 113 Accelerated solvent extractor

After the extraction the receptacle vials were collected, and the ethyl acetate extract was transferred into 50 ML Turbovap II tubes (with 0.5 mL tips). The sensors of the Turbovap II evaporator were customized to signal endpoint at 350µL instead the regular 0.5 mL. Applied nitrogen pressure was 13 PSI, the water bath temperature was adjusted to 35°C.

The concentration took place in thee steps:

First the ethyl acetate was blown off until the sensor stopped the process, or approximately .35 mL. Next 5 mL acetonitrile was added to each tube and the process repeated. Finally an other 5 mL of acetonitrile was added to each tube, but this time the

evaporator was programmed, to stop the drying process at 6 minutes after sensors detect the meniscus. This program resulted in approximately 100 μL final extract volume in acetonitrile.

The extract samples were filled in HPLC vials, and refrigerated until the next step: HPLC-MS-MS.

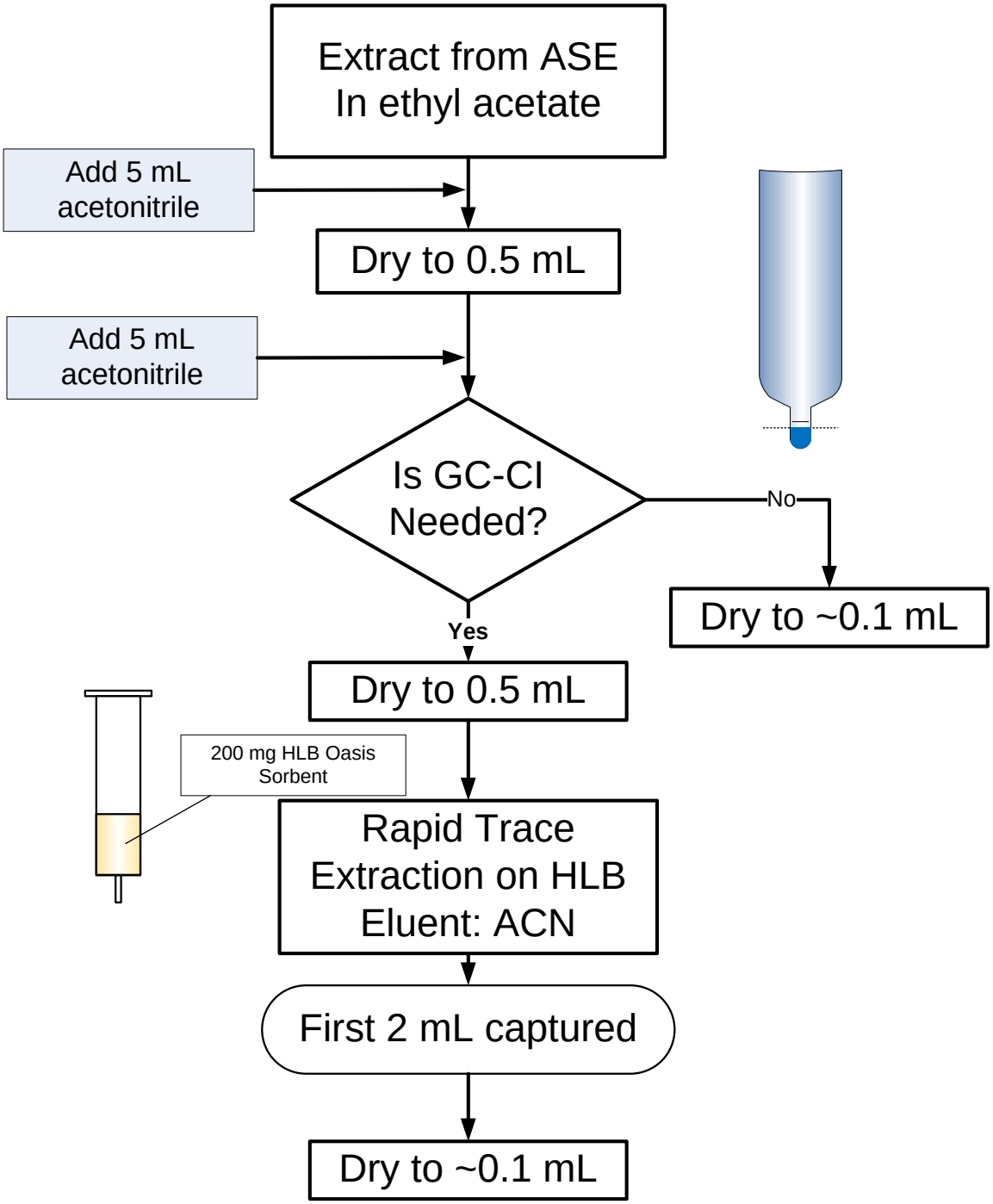


Figure 114 Comprehensive cleanup diagram

Determination of Instrument Detection Limits LC-MS-MS and GC-MS-MS groups

LODs were determined by using a series of neat standard solutions. The starting concentrations were 1.00, 2.50, 5.00, 10.00, 25.00, 50.00, 100, 250 and 500 ng/mL, identical concentrations for each analyte. These standards were previously prepared in acetonitrile.

The internal standard stock solution was of the concentration of 2.5 µg/mL in acetonitrile. This solution was diluted twenty times with acetonitrile. The resulting diluted internal standard solution had the concentration of 125 ng/mL for each isotope labeled standard.

To make the standard series for the "LC analyte group", 10 µL of the native standard solution was pipetted into a LC vial, 10 µL of diluted internal standard solution added, and then followed with 20 µL of acetonitrile. This process was repeated for each concentration levels. The resulting series had concentrations of 0.250, 0.625, 1.25, 2.50, 6.25, 12.5, 25.0, 62.5, 125 ng/mL in serum €for native compounds and 31.3 ng/mL for isotope labeled standards. The standard series samples were injected at 1µL each, using the HPLC-MS-MS method described previously.

For the "GC analyte group" the same standard series was used as a starting point, but with a different preparation: 20µL of the original standard series, then 20 µL diluted internal standard was pipetted into a 15 mL conical bottom centrifuge tube. The tube then was placed in a Turbovap LV evaporator. Temperature was 40°C, nitrogen pressure was set to 15 PSI. The drying process was carefully monitored and stopped just before complete dryness. 80 µL toluene was added to the centrifuge tube, mixed thoroughly on a vortex mixer, then transferred in a GC vial and capped.

The 1 μL of the prepared samples were injected in the GC-MS-MS system.

For the LC and GC experiment both, three injections were made.

The LC-MS-MS experiment was only conducted in positive APCI mode because – as it was shown earlier – not all OP pesticides ionize in ESI mode.

Since not all OP pesticides ionize in negative mode, positive ionization was selected.

LOD-s were determined as follows:

For each compound a linear calibration plot was established by using linear regression and 1/concentration weigthing. The vertical axis of the calibration curve was the area ratio (area of analyte peak divided by the internal standard peak area). The horizontal axis was the concentration. Once the calibration curve was defined, for each concentration and each analyte a "calculated concentration" was determined, based on the slope and intercept of the calibration curve and the response factor for the particular measurement. Next, for each concentration of the standard series the standard deviation of the "calculated concentrations" was determined and plotted over the actual concentrations. Limit of detection was determined by establishing a "standard deviation – concentration" relationship trend line and extrapolating it to zero concentration. LOD is defined as three times the expected value of the standard deviations of the calculated concentrations at zero concentration.

Effect of drying the samples.

During the cleanup process the samples are dried in the presence of acetonitrile to remove water and ethyl acetate. To determine the effect of the drying process, and to determine if complete dryness causes sample loss, the following experiment was carried out:

A stock solution was prepared which contained all the OP analytes at 500 ng/mL: concentration, but no internal standard. 0.5 mL of this solution was added to Turbovap II tubes started to dry the samples at 40°C and 12 PSI nitrogen pressure. Drying was stopped at 10 and 20 minutes, the samples reconstituted to 0.5 mL and internal standard added. The experiment was done in multiples of 3.

A second set of experiments was also completed with one difference: at the beginning approximately 5 µL dodecane was added, the experiment was similarly stopped at 10 and 20 minutes. Everything was carried identically to the first set of experiment.

The reconstituted samples were analyzed with the HPLC-MS-MS system; response factors were averaged for identical samples then compared

	Start	10 min	20 min	start	10 min, 2µL dodecane	20 min, 2µL dodecane
MMP	100.00%	45.20%	21.11%	100.00%	61.92%	41.76%
Acephate	100.00%	86.57%	66.91%	100.00%	94.46%	79.76%
Oxydemethon Methyl	100.00%	92.93%	101.83%	100.00%	92.46%	98.10%
Dimethoate	100.00%	85.47%	76.35%	100.00%	87.99%	84.31%
Trichlorfon	100.00%	28.00%	15.10%	100.00%	52.35%	22.05%
Dichlorvos	100.00%	6.67%	3.37%	100.00%	22.47%	3.09%
Methidathion	100.00%	94.91%	91.72%	100.00%	99.42%	98.68%
Propetamphos	100.00%	86.07%	51.42%	100.00%	97.18%	76.52%
Ethoprop	100.00%	35.29%	11.90%	100.00%	76.42%	19.36%
Azinphos Me	100.00%	99.95%	108.24%	100.00%	97.25%	102.03%
Pirimphos Me	100.00%	77.67%	54.49%	100.00%	84.65%	70.65%
Tetrachlorvinphos	100.00%	120.74%	125.45%	100.00%	121.15%	146.78%
Fonofos	100.00%	38.89%	5.64%	100.00%	76.56%	20.09%
Bensulide	100.00%	104.90%	111.46%	100.00%	100.20%	107.56%
Coumaphos	100.00%	103.83%	108.55%	100.00%	101.26%	105.96%
Chlorpyrifos	100.00%	74.77%	44.84%	100.00%	87.31%	66.36%
Ethion	100.00%	99.13%	95.45%	100.00%	98.24%	103.38%
Tribufos	100.00%	99.19%	84.62%	100.00%	97.97%	92.87%
Temephos	100.00%	110.98%	118.84%	100.00%	111.10%	111.43%

Table 25 Effect of drying on recoveries of LC compound group, with and without addition of dodecane "catching solvent"

From Table 25 and figures

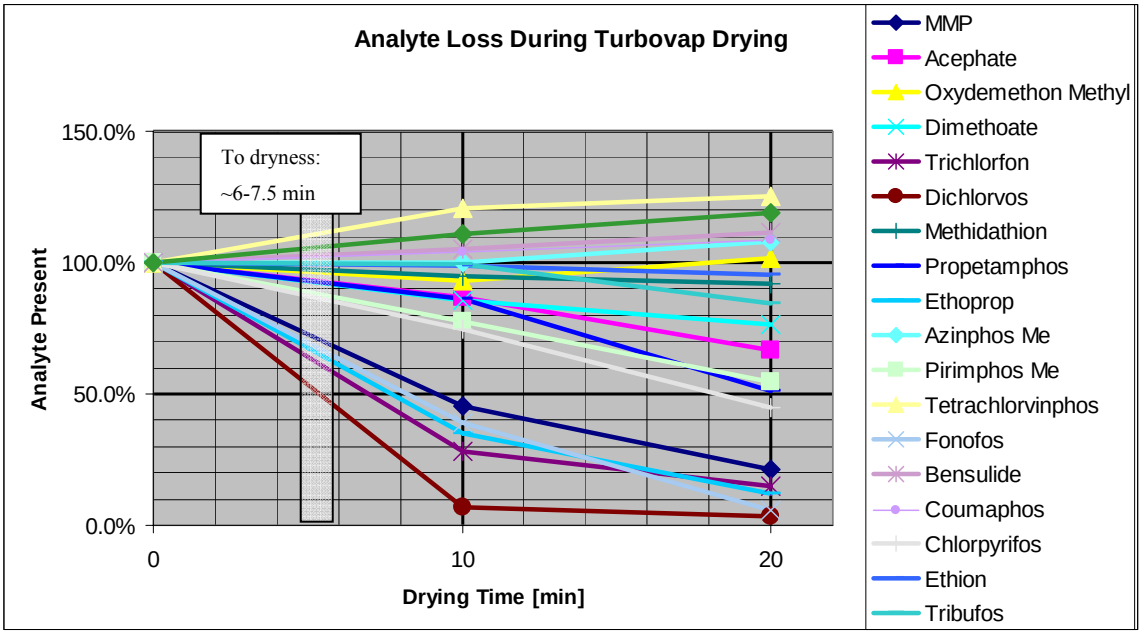


Figure 115 Recoveries of LC compound group as the function of drying time

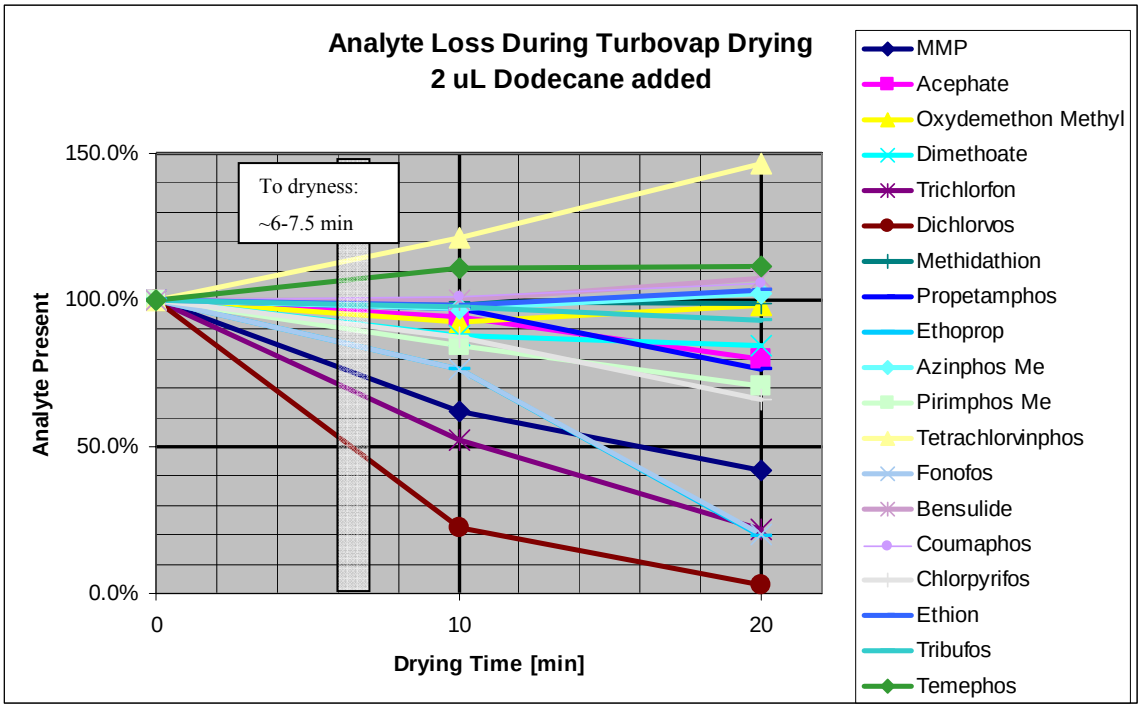


Figure 116 Recoveries of LC compound group as the function of drying time, 2µL dodecane added

Construction of calibration curves

First a series of stock solutions was prepared for each analyte. The stock solutions were 1000 µg/mL concentration in acetonitrile. By blending the forty stock solutions at equal volume, a 25 µg/mL blended stock was made. Using the blended stock as a starting solution, the following standard concentrations were developed by gradual dilution:

1.00, 2.50, 5.00, 10.0, 25.0, 50.0, 100, 250, 500, 1000 ng/mL

10µL of above standards was added to 1 mL serum resulting in the following concentration series:

0.010, 0.025, 0.050, 0.100, 0.250, 0.500, 1.00 ng/mL analyte in serum

Calibration curves were constructed following the isotope dilution method. For the forty compounds of interest we obtained seventeen isotopically labeled variants for using them as internal standards. A mixture of internal standards was dissolved in acetonitrile at a concentration of approximately 0.25 ng/mL. This level was selected because all the reference compounds are well detectable at this concentration. Increasing the amount of reference compounds may causes problems because they contain small amounts of the non labeled variant. This can introduce an "offset amount of" analyte, not coming from the matrix.

For each analyte a corresponding internal standard was selected. After the analysis was completed the date files were collected and evaluated using Thermo Finnigan's Xcalibur software. For all calibration curves the linear model was chosen with 1/x weighing. 1/x weighing puts more emphasis to the lower concentration points. This is more desirable for trace analysis.

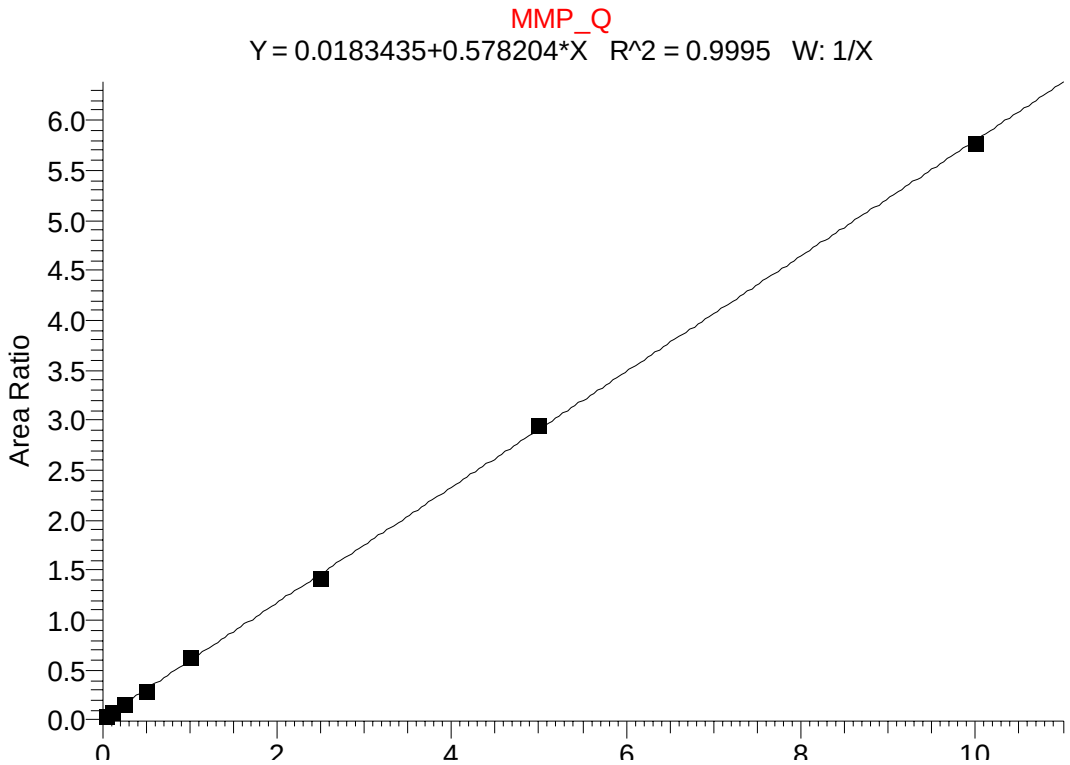


Figure 117 Methamidophos Calibration Plot

Name	Type of Label	Location of the Label
Acephate	D6	Deuterated dimethyl
Bensulide	D14	Deuterated diisopropyl
Chlorpyrifos	D10	Deuterated diethyl
Chlorpyrifos methyl	D6	Deuterated dimethyl
Coumaphos	D10	Deuterated diethyl
Dimethoate	D6	Deuterated dimethyl
Disulfoton	D10	Deuterated diethyl
Ethyl parathion	D10	Deuterated diethyl
Fenthion	D6	Deuterated dimethyl
Fonofos**	^{13}C6	
Malathion	D10	Deuterated diethyl
Methamidophos	D6	Deuterated dimethyl
Oxydemeton methyl	D6	Deuterated dimethyl
Phorate	^{13}C4	
Phosmet	D6	Deuterated dimethyl
Sulfotepp	D20	Deuterated tetraethyl
Terbufos	^{13}C4	

Table 26 Available isotope labeled internal standards

LOD-s for the LC group

Instrument LOD-s were determined by injecting a series of neat standards dissolved in acetonitrile, thus not performing the actual cleanup. Final LOD-s were calculated from triplicates as described above:

OP name	LOD [pg]:
MMP	0.29
Acephate	0.26
Oxydemethon Methyl	1.19
Dimethoate	0.27
Trichlorfon	1.13
Dichlorvos	8.99
Methidathion	0.16
Propetamphos	1.82
Ethoprop	0.97
Azinphos Me	0.52
Pirimphos Me	0.18
Tetrachlorvinphos	3.32
Fonofos	0.44
Bensulide	0.75
Coumaphos	1.38
Chlorpyrifos	0.95
Ethion	0.25
Tribufos	0.92
Temephos	6.07

Table 27 LC group compound detection limits in pure form

Precision and accuracy of the method

Precision of the method is described by the standard deviation of measurements at a certain concentration. Accuracy is the quality that compares the measured values with the expected concentrations. Both, accuracy and precision are displayed as percentages: the

mean of measured concentration/expected concentration ratio and the relative standard deviation, respectively.

We used two concentrations, 0.25 ng/mL and 5 ng/mL to show accuracy and precision of the method

	Standard Deviation		Accuracy	
OP concentration in serum Analyte	0.25 [ng/mL]	5 [ng/mL]	0.25 [ng/mL]	5 [ng/mL]
MMP	5.5%	1.8%	97%	100%
Acephate	16%	5.2%	94%	98%
Oxydemethon Me	0.2%	10%	124%	95%
Dimethoate	10%	6.4%	93%	99%
Trichlorfon	8.3%	8.3%	81%	99%
Dichlorvos	14%	8.1%	154%	97%
Propetamphos	8.7%	6.4%	102%	97%
Methidathion	20%	4.9%	93%	94%
Ethoprop	9.8%	5.3%	103%	97%
Azinphos Methyl	20%	4.4%	98%	97%
Pirimphos Methyl	4.7%	3.2%	102%	96%
Tetrachlorvinphos	14%	3.5%	93%	96%
Fonofos	9.4%	4.9%	100%	99%
Bensulide	12%	4.6%	83%	95%
Coumaphos	6.2%	4.6%	99%	98%
Chlorpyrifos	6.8%	4.6%	95%	99%
Ethion	11%	3.7%	102%	97%
Tribufos	17%	4.4%	96%	97%
Temephos	21%	7.1%	89%	102%

Table 28 Accuracy of the LC group assay, 1 mL serum, n=5

	Standard Deviation		Accuracy	
OP concentration in serum	0.25 [ng/mL]	5 [ng/mL]	0.25 [ng/mL]	5 [ng/mL]
Analyte				
Mevinphos	9.1%	7.9%	94%	107%
Chlorethoxyfos	3.1%	5.3%	88%	100%
Dicrotophos	19%	1.4%	93%	119%
Sulfotepp	5.9%	5.3%	76%	105%
Cadusafos	6.7%	5.1%	90%	109%
Phorate	5.1%	17%	99%	100%
Diazinon	0.7%	1.6%	78%	110%
Fonofos	19%	3.4%	97%	102%
Phostebupirim	19%	6.8%	103%	99%
Chlorpyrofos Me	13%	2.8%	91%	99%
Me Parathion	20%	8.7%	110%	101%
Malathion	5.0%	13%	138%	101%
Fenthion	18%	7.8%	85%	106%
Et Parathion	21%	6.2%	95%	102%
Fenamiphos	10%	19%	81%	118%
Phosalone	5.2%	3.7%	76%	108%

	Standard Deviation		Accuracy	
OP concentration in serum	0.25 [ng/mL]	5 [ng/mL]	0.25 [ng/mL]	5 [ng/mL]
Analyte				
Mevinphos	18%	2.4%	118%	97%
Chlorethoxyfos	10%	11%	75%	104%
Dicrotophos	19%	8.6%	107%	101%
Sulfotepp	5.1%	2.2%	105%	103%
Cadusafos	15%	3.0%	102%	101%
Phorate	2.8%	1.2%	95%	99%
Diazinon	23%	4.0%	96%	112%
Fonofos	5.1%	4.1%	93%	98%
Phostebupirim	5.6%	2.2%	106%	97%
Chlorpyrofos Me	44%	8.0%	109%	103%
Me Parathion	43%	7.2%	167%	100%
Malathion	45%	17.6%	99%	107%
Fenthion	46%	3.8%	126%	100%
Et Parathion	2.3%	22%	98%	116%

To to test the precision of the method, serum samples of three concentrations were prepared: .250 ng/mL, 1 ng/mL and 5 ng/mL. These samples were prepared, spiked and

extracted individually, then the concentrations were determined and plotted. At this point was it determined that was it determined that length of extraction time plays an important role when extracting larger number of samples on ASE. The ASE is a carousel system, where the assembled cells with serum inside are waiting for their turn to be extracted. While the cells wait at room temperature, enzymatic and hydrolytic decomposition is taking place. This theory was tested by keeping spiked serum at room temperature for up to 24 hrs and testing recoveries at different times. As it shows in

Figure 118, about half of the compounds lose more than 50% of their original concentration. If, - as in the original method – we use two five minutes static cycles when all the other time adds up a 24 cartridge carousel takes over six hours to process. This time delay while working with full carousels, can and do effect detection limits and the precision of the measurement. The main problem is, that not all compounds have their own isotope labeled equivalent as internal standard. Different compounds hydrolyze at a different rate and this will effect response factors and calculated concentrations.

The first attempt to solve the decomposition problem was to add 100µL 50 mM EDTA solution. The idea was that EDTA will chelate the metal ions in the A-esterases and denature these enzymes. Comparing Figure 118 and Figure 119 it is clear that the EDTA did not have the desired effect.

The second approach was to fine-tune the first part of the sample preparation. First, the ASE pressurized dwell time was reduced from 2x5 to 2x2 minutes, but using the same amount of ethyl acetate. This alone reduced the total run time to 3 hrs.

Second, the sample dispersion method was altered. Instead of adding the serum directly to the filled cartridge, it was separately pre-dispersed in 10mL of Hydromatrix. Lastly, 5

mL of ethyl acetate was added to the cartridge before closing, to denature any enzyme and to begin the extraction process while waiting in the line. The results were tabulated in Table 29. At the end of the table, averages were calculated for the range of OP pesticides covered (HPLC group).

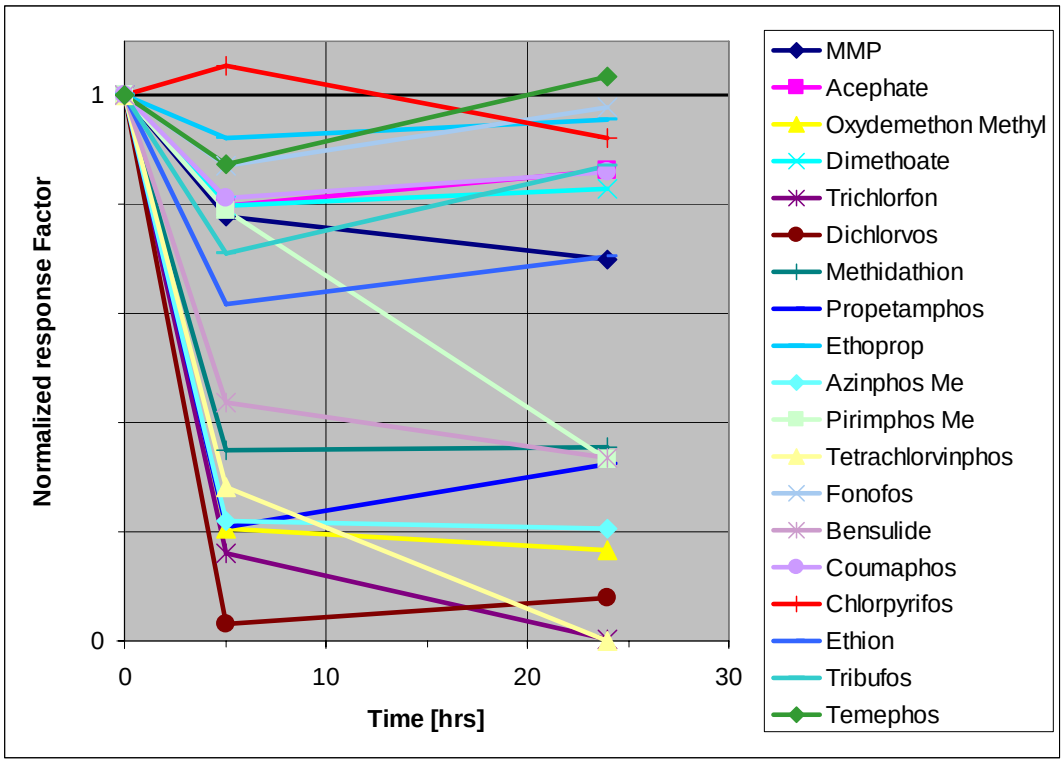


Figure 118 Decomposition of OP pesticides in serum

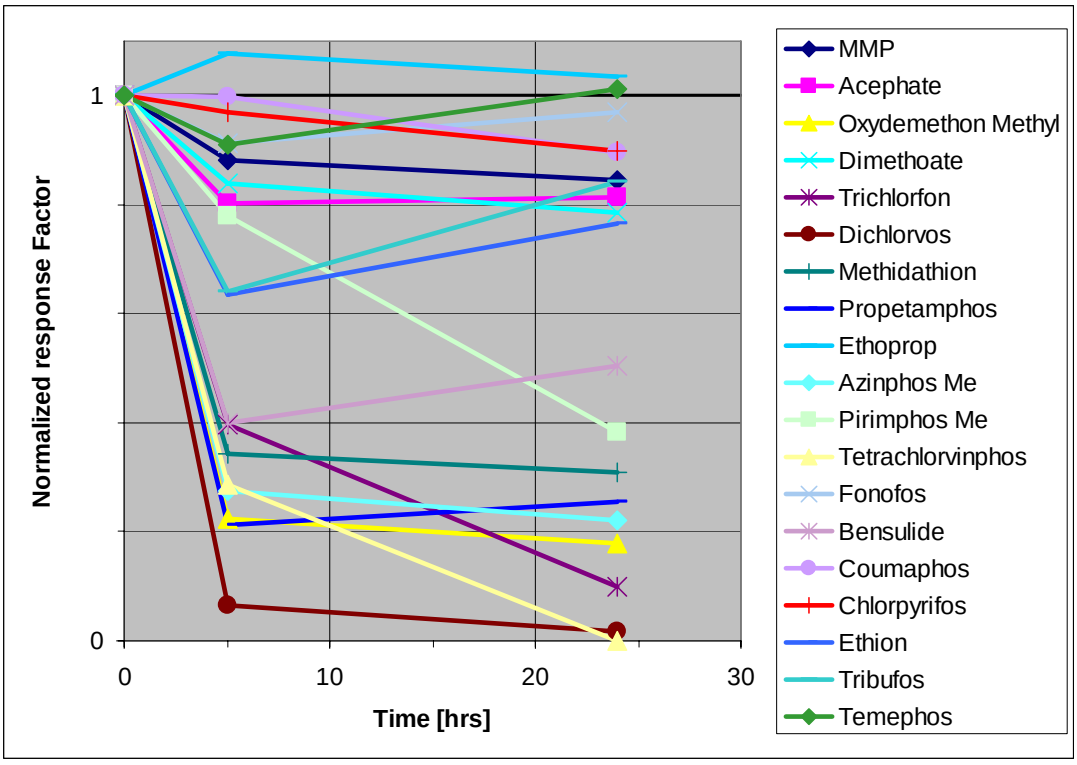


Figure 119 Decomposition of OP pesticides in serum, 100µL 50 mmolar EDTA solution is added

Relative standard deviations of calculated concentrations	no vortex, no ethyl acetate			**5 mL ethyl acetate** but no vortex mixing			**5 mL ethyl acetate, vortex mixing**		
	Concentration [ng/mL]								
Analyte	0.25	1	5	0.25	1	5	0.25	1	5
MMP	6.9%	3.2%	3.8%	4.5%	6.1%	2.5%	7.4%	8.6%	1.6%
Acephate	30.4%	9.9%	3.8%	25.2%	14.7%	4.2%	10.2%	11.0%	2.9%
Oxydem Me	20.8%	44.3%	26.1%	26.9%	30.3%	11.7%	N/F	16.8%	11.3%
Dimethoate	11.2%	9.2%	8.1%	13.3%	6.1%	8.7%	11.0%	11.8%	4.0%
Trichlorfon	100.0%	108.3%	75.5%	19.8%	12.7%	12.2%	9.0%	11.9%	4.7%
Dichlorvos	N/F	N/F	N/F	N/F	19.2%	38.9%	15.0%	26.3%	24.3%
Methidathion	25.0%	10.3%	5.9%	28.3%	7.4%	12.2%	12.0%	10.1%	7.3%
Ethoprop	18.9%	9.4%	5.7%	24.3%	11.5%	13.8%	18.5%	7.2%	7.1%
Propetamphos	38.3%	37.9%	12.0%	60.2%	31.2%	26.6%	13.0%	11.1%	10.1%
Azinphos Me	69.3%	20.7%	9.2%	21.1%	18.9%	9.8%	26.4%	5.5%	10.7%
Pirimphos Me	37.2%	32.8%	21.3%	16.8%	10.4%	32.5%	5.7%	9.9%	6.5%
Tetrachlorvinphos	160.4%	123.0%	95.6%	49.0%	18.4%	19.7%	22.2%	10.5%	8.4%
Fonofos	23.6%	8.1%	4.2%	7.0%	9.5%	6.4%	18.2%	6.2%	5.3%
Bensulide	56.2%	15.8%	18.8%	47.9%	28.8%	18.1%	15.6%	13.0%	9.2%
Coumaphos	19.2%	14.5%	10.9%	15.7%	5.1%	12.7%	8.5%	9.6%	6.4%
Chlorpyrifos	23.2%	20.8%	12.1%	31.1%	3.5%	8.7%	22.2%	15.1%	10.7%
Ethion	15.3%	7.6%	7.1%	8.8%	6.3%	20.3%	5.5%	9.8%	5.1%
Tribufos	34.7%	12.5%	13.7%	23.2%	25.8%	41.9%	15.4%	14.2%	4.2%
Temephos	39.5%	39.3%	27.5%	53.7%	36.9%	24.5%	26.6%	13.2%	4.1%
Average	*40.6%*	*29.3%*	*20.1%*	*26.5%*	*15.9%*	*17.1%*	***14.6%***	***11.7%***	***7.6%***

Table 29 Relative standard deviations of three concentration of analytes - HPLC group

Detection limits for the cleaned up serum samples are sown in Table 30

	LOD [ppt]
MMP	33
Acephate	22
Oxydemethon Methyl	29
Dimethoate	69
Trichlorfon	
Dichlorvos	
Methidathion	5.2
Propetamphos	38
Ethoprop	85
Azinphos Me	41
Pirimphos Me	22
Tetrachlorvinphos	
Fonofos	245
Bensulide	1.0
Coumaphos	17
Chlorpyrifos	61
Ethion	7.5
Tribufos	27
Temephos	16

Table 30 Limits of detection of the LC group compounds, serum samples

The problem with azinphos methyl and the power of tandem mass spectroscopy

Analyzing real samples from the field, several clear "hits" were detected for azinphos methyl. The peaks were clear, well defined, but the concentrations determined by using the "quantitation ion" (318->132) were substantially different from those indicated by the conformation ion. (318->77). QC results were within the expected range for both transitions.

Further investigation has shown that phosmet co-elutes (not fully resolved) with azinphos methyl and has the same molecular mass, 317. This usually isn't a problem using tandem MS, but in this case one of the fragments, 77 is the same. Phosmet is analyzed in the GC-MS-MS part of the method, so this coincidence only became apparent while comparing ion ratios of the transitions 318->132 and 318-77 in standards, QC-s and actual samples.

This problem was solved by changing confirmation ion from 318-77 to 318-> 104.

Analyte	Parent Ion m/Z	Fragment m/Z	Optimum Collision Energy [V]
Azinphos Methyl	**318**	**77**	51
		132	21
		104	26
		125	24
Phosmet	**318**	160	33
		133	49
		77	52
		105	56

Table 31 Major transitions and optimum collision energies for azinphos methyl and phosmet

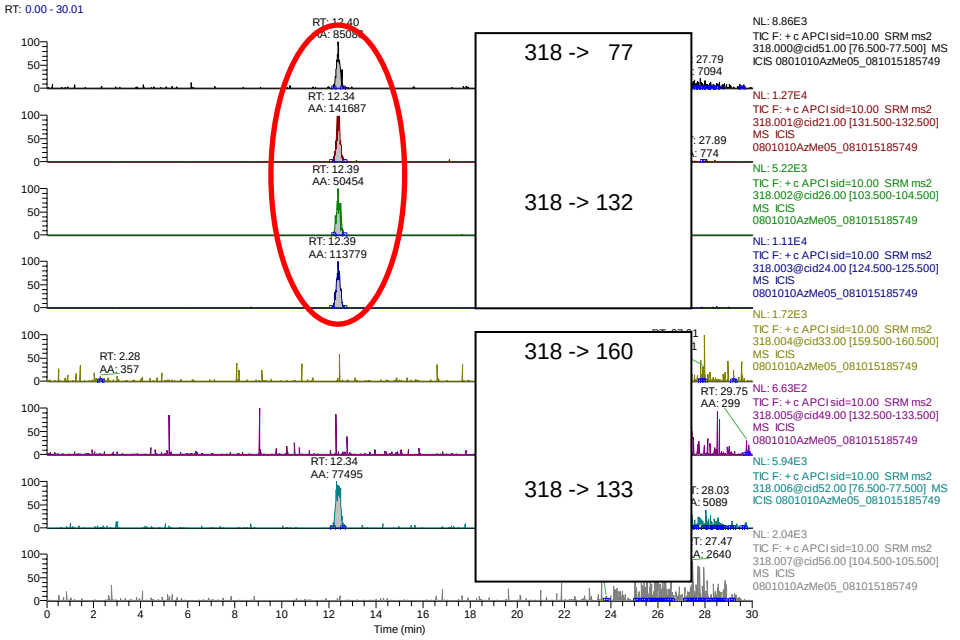


Figure 120 Azinphos methyl chromatography, monitoring all four major transitions for azinphos methyl and phoseth

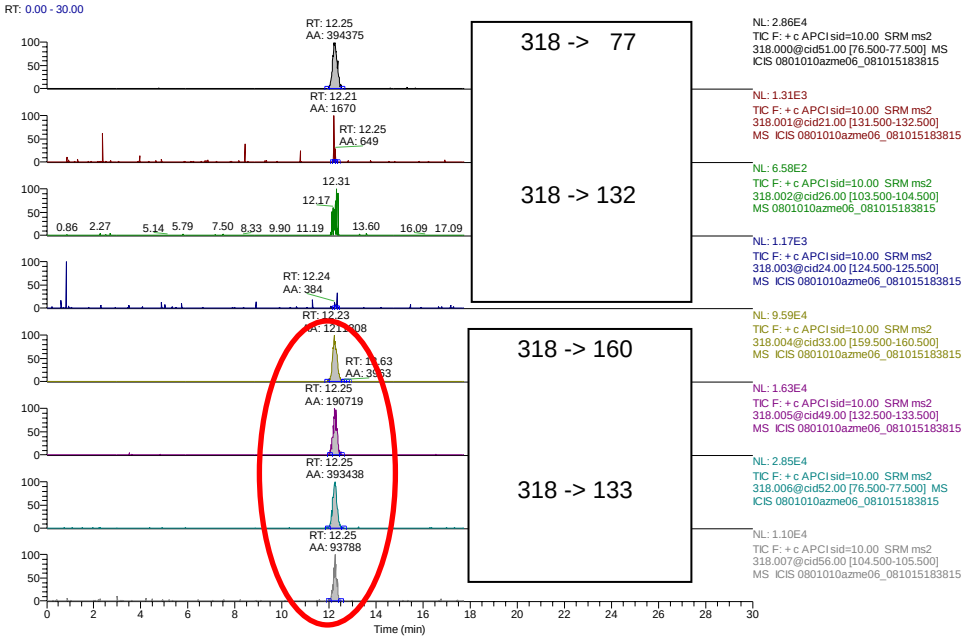


Figure 121 Phosmet chromatography, monitoring all four major transitions for azinphos methyl and phosmet

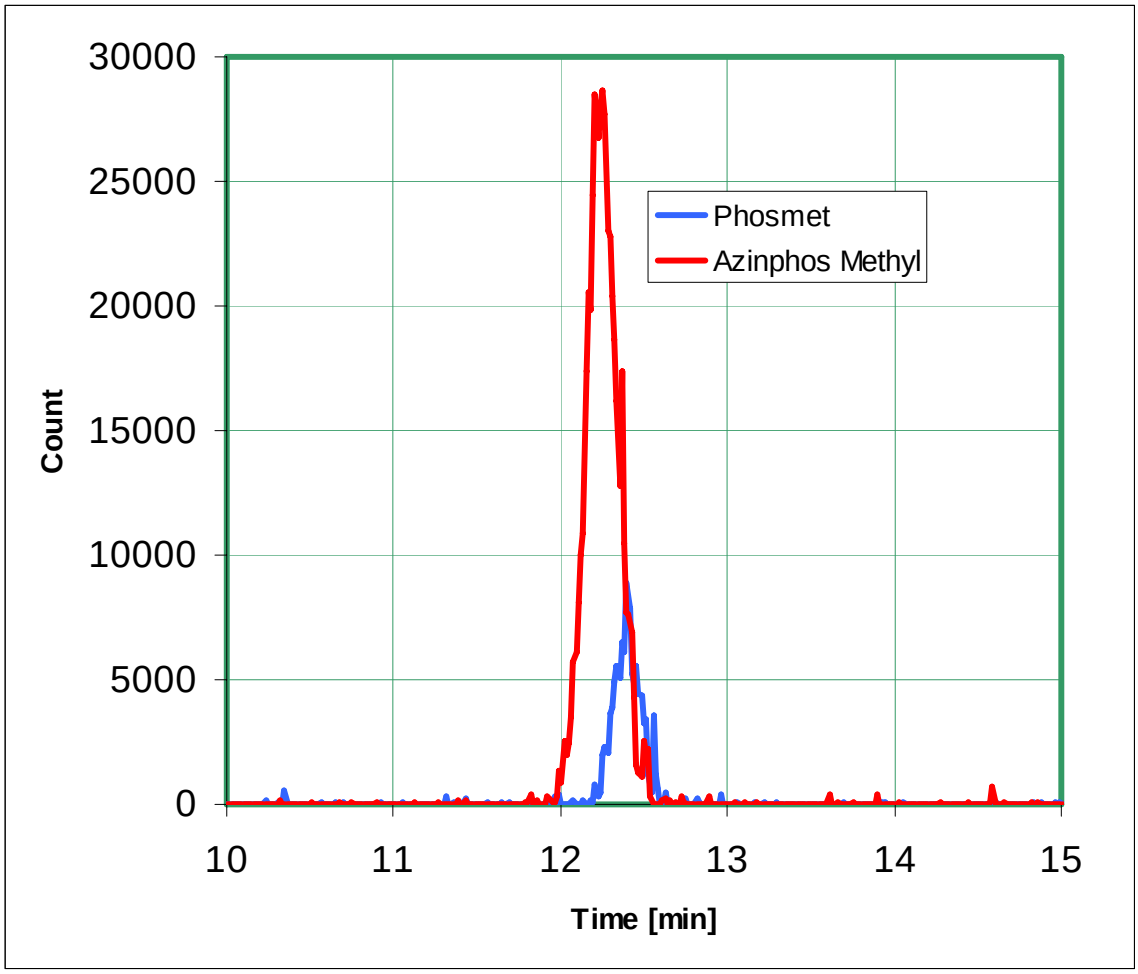


Figure 122 Transition 318 -> 77 signal from azinphos methyl and phosmet

Chapter 4 Discussion

The firs step of the method is the cleanup and it was to be developed last. Detection methods have to be established first, so the effectiveness of different approaches to the cleanup may be evaluated.

The purpose of the cleanup procedure is twofold: First to remove those contaminants from the sample that interfere with the detection or analytical separation. In serum these contaminants are mainly salts, proteins and lipids.[37] .

It is also important to concentrate the sample. The available serum sample is usually 1-5 mL. LC and GC methods only inject a few micro liters of sample. The solution containing the analytes, have to be concentrated.

The most important cleanup consideration in our work is that it has to be universal. The serum samples are usually part of studies, supplies are limited and most often they came from an anonymous individual. The cleanup and analytical methods are destructive. Once a sample is processed that amount of serum is used up, it can not be replenished.

Considering availability of instrumentation and materials, the usual approach to cleanup is as follows:

- Solid phase extraction (SPE)
- Lyophilization followed by solid-liquid extraction
- Various forms of liquid-liquid extraction (LLE)

Solid phase extraction

Solid phase extraction is the usual first and preferred approach. It is specific, uses less solvent than the varieties of LLE methods. It is also highly automatable. Methods developed for individual tubes are easily transferred to 96 well plates and automated liquid handlers. The problem with SPE in this application is the range of compounds and consequently the range of their properties. For example: some are extremely water soluble (methamidophos (MMP), acephate) others are very lipophilic. Some are ionizable in solution, most are not. This problem was confronted in a previous work [35], where SPE had to be abandoned. (The matrix was urine.) The reason was that the highly hydrophilic compounds could not be retained sufficiently on any sorbent and washed. Acephate and MMP always eluted with wash step. Eventually freeze drying and liquid – solid extraction with dichloromethane had to be applied.

Lyophilization

Lyophilization or freeze drying was tried as described in the experimental section, but if did not bring the same results as in urine matrix. The most likely explanation is that when serum dries, it forms a hard crust leaving limited area for the solvent to interact with. Urine on the other hand, - when freeze dried – turns into a crumbling powdery material which has a very large surface are.

Liquid- Liquid extraction

The method was developed with the intention to run many samples in the future, so LLE in a separatory funnel was never considered. LLE was first tested using ChemElut LLE cartridges. Using these cartridges shoved promise, but we did not have the

equipment to automate. For this reason and because of the need for better control the LLE portion of the method was transferred to ASE, where timing, temperature and pressure is controlled. As described previously, ethyl acetate solvent and 1500 PSI (~10,000 kPa) at room temperature was chosen as operating parameters. While the many of the more lipophilic (late eluting in HPLC) compounds improve their recovery at higher pressures and temperatures, three analytes: trichlorfon, dichlorvos and tetrachlorvinphos show abnormally low recoveries at alleviated pressure temperature or dwell time. The commonality of these three compounds is that they have chlorine near the ester bond, possibly weakening it and predisposing it to thermal breakdown. Interestingly, these three compounds are also among those five compounds in the GC-MS-MS section that were shown unpredictable retention behavior.

Different structural properties of OP pesticides were examined and correlated with ASE extraction data. Only the LC group was used because – as it was pointed out earlier in this chapter- after ASE extraction the samples are not clean enough for GC-MS-MS. It was found that the strongest correlation is between the number of nitrogen atoms in the molecule and the recovery percentage. Interestingly, the Correlation factor between $\log(K_{ow})$ and recovery is only 0.23, while the same between the number of nitrogen atoms and the recovery is -0.83.

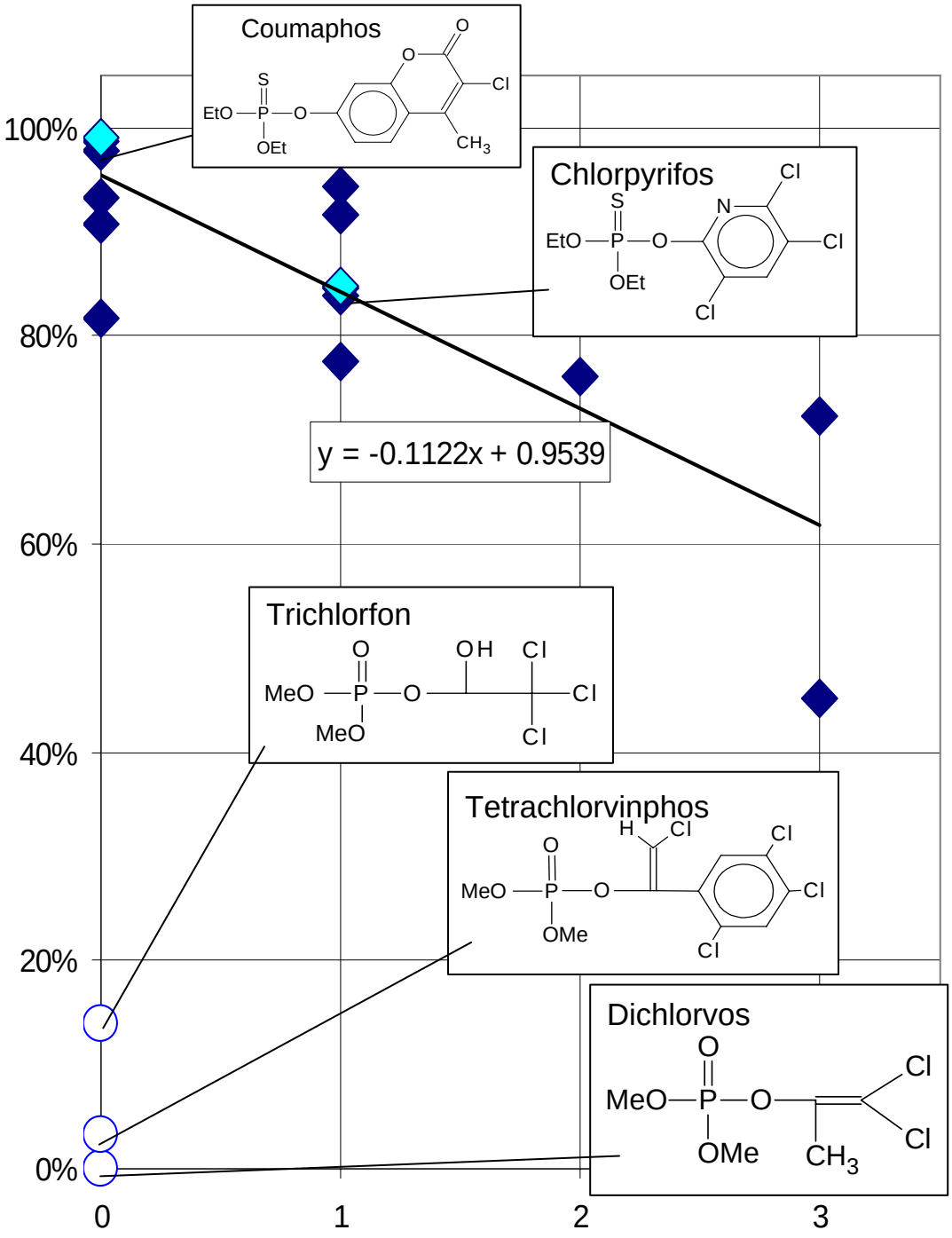


Figure 123 Dependence of ASE recovery on the number of nitrogen atoms in the OP molecule. Emphasis on the halogenated species.

Novelty and significance of the method

Currently there are three different approaches to analyze OP exposure. These methods can be grouped as indirect and direct (parent compound) methods.

- Urine Analysis of OP metabolites [13]

Most large studies use urinary analysis of OP metabolites. This method gives a window to OP exposure of few days. It is also a cumulative indicator of OP exposure.

Advantage of the urinary assay is that urine samples are easy and non-invasive to obtain and clean up is relatively easier than that of blood products. Some OP pesticides like chlorpyrifos, coumaphos, diazinon, parathion, malathion have specific metabolites detectable in urinary assay.

While urinary metabolite assay is the most common analysis of OP exposure, it has two shortcomings. Many OP pesticides do not have easily detectable specific metabolites, so the source of non specific metabolites has to be estimated from external data, such as usage statistics. The second and more general shortcoming is, that – as shown later on the chlorpyrifos example – the source of metabolites may be environmental degradation prior exposure. The degradation products are not cholinesterase inhibitors, not known to be toxic. This results in the underestimation of the health outcome – exposure correlation.

- Butyryl Cholinesterase adduct assay [38, 39]

Butyryl cholinesterase is a scavenger of anti-cholinesterase compounds and it is abundantly available in serum. The OP compounds of interest form adducts at the active

sites, lose their specific group. These adducts may be detected for a few months after exposure.

Comprehensive OP assay for OP adducts is currently under implementation at CDC. It will provide an alternative to the urinary assay.

It also will inherit one of the shortcomings of the above method: non-specificity. On the other hand, environmental degradation products do not form serum cholinesterase adducts.

2 Parent compound methods

Direct analysis of the parent compounds in serum

This is the method developed in this dissertation.

As shown in the graph, after exposure the first opportunity for detection is in the blood. While the window of detection is short, hours to few days depending the type of OP, sensitivity and metabolic conditions, direct analysis has significant advantages.

- It is a specific method. The compounds of interests are directly analyzed, exposure is determined, not deducted from secondary markers. (metabolites)
- It is a non-cumulative method, like a snapshot of state of OP exposure of a group in question.
- It relies on a regulated fluid: blood, so subsequent concentration correction is not necessary.
- It eliminates the otherwise always present question of the source of the secondary biomarker: is it exposure to OP or exposure to environmental degradants.

A practical significance of the method described in the thesis is that it is a sensitive method, with sub ng/mL limits of detection, useful for exposure studies, that covers all 39 EPA approved OP compounds.

Assay	Detected Compound	Specificity	Cumulative? Window of exp.
Urinary Metabolites	Nonspecific metabolites: Alkyl phosphate and thiophosphate metabolates Few specific metabolates	Only for the OP pesticides that have detectable specific metabolites	Yes, days
Butyryl Cholinesterase Adducts	Digested protein segment with OP remaining grouop still attached	Non specific	Yes, months
Serum OP (Dissertation method)	Original, non metabolized OP pesticides	Highly specific	No, Hours to days

Table 32 Summary of OP assays for human exposure

The Chlorpyrifos example.

Chlorpyrifos is a broad-spectrum, chlorinated organophosphate insecticide, acaricide and nematicide. It was first introduced in 1965, to control insects on food and animal feed crops. It is one of the most commonly used insecticide. It is still used for food crops, golf courses turf, non-structural wood treatment for termite control.

Once ingested, chlorpyrifos metabolizes according the mechanism illustrated in Figure 124.

In water and soil chlorpyrifos also degrades in a similar pattern. (

Figure 125) Both degradation pathway includes the oxon formation step. Environmental decomposition does not include the glucoronization step. Non specific metabolites are deithylphosphate and diethyl thiophosphate, while specific metabolites are 3,5,6 trichloropiridinol and 3,5,6 trichloropiridinol glucoronide.

While specific metabolites may provide a way to specify the OP in question, the source of the specific metabolite may be environmental degradation, prior ingestion and not metabolism of the original OP. This alternate pathway clouds the speciation. It also makes it difficult to assign health effects to OP exposure, since the metabolic pathway and the environmental degradation partially overlap.

The same is true for the non specific metabolites. Urinary assay of non specific metabolites is used to track cumulative exposure to OP pesticides. To interpret the results, one needs to have knowledge of the composition and types of the pesticides the population is exposed to. But even with this information it is not certain if the non specific metabolites detected in the urine were results of actual OP exposure or prior environmental degradation.[40]

Clarification of the OP exposure may be approached in several ways.

One is the above mentioned determination of the composition of likely exposure from agricultural usage and environmental data.

Second approach is – which was developed in this thesis – is to use the presence of unmetabolized parent compounds in blood as biomarker of OP exposure.

Why is the method unique?

The method developed is unique for two reasons:

1. The range of compounds it detects.

This method covers 35 of the 39 EPA registered OP pesticides. Before, the highest number was 21. [23].

Serum methods covering a limited number of OP pesticides existed before, but they concentrated on poisoning cases where the possible toxicants are already known and the concentrations are relatively high. These forensic cases don't need high sensitivity or selectivity.

2. Limits of detection

Detection limits are 1-2 orders of magnitude lower than previous methods, covering a larger number of OP pesticides.

The reasons for this increased limits of detection is three fold:

- The ASE / ethyl acetate extraction provides recovery through the range of OP pesticides.
- For the "GC group compounds" The secondary, normal phase cleanup (with HLB sorbent) eliminated much of the lipid contaminants, allowing for

chemical ionization for serum sample extracts. This purer extract also helped lowering the chemical background.

- Tandem mass spectrometry was used throughout the method. Tandem MS adds an other layer of selectivity and increases LOD

This lower detection limit, combined with the wide range of detectable compounds makes the method developed in this dissertation a uniquely applicable assay for the "snapshot detection" of OP pesticide exposure.

The cleanup and sample concentration part of the method may be used to detect of low levels of similar, ester type analytes. (ref to Angela's poster)

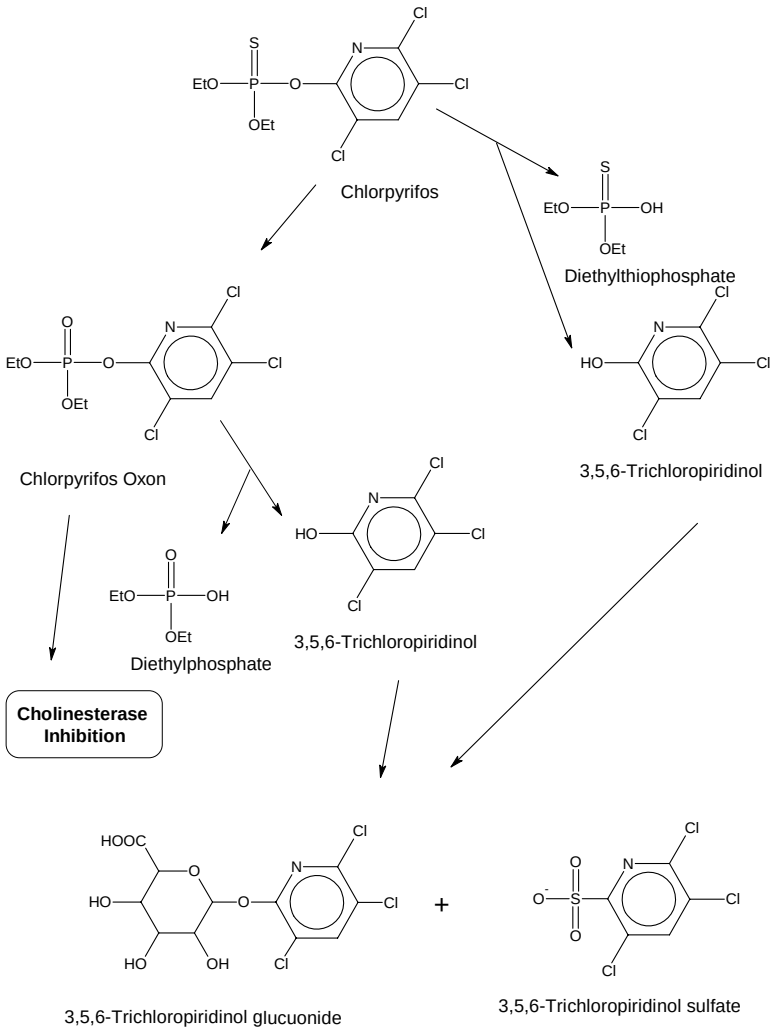


Figure 124 Metabolism of Chlorpyrifos

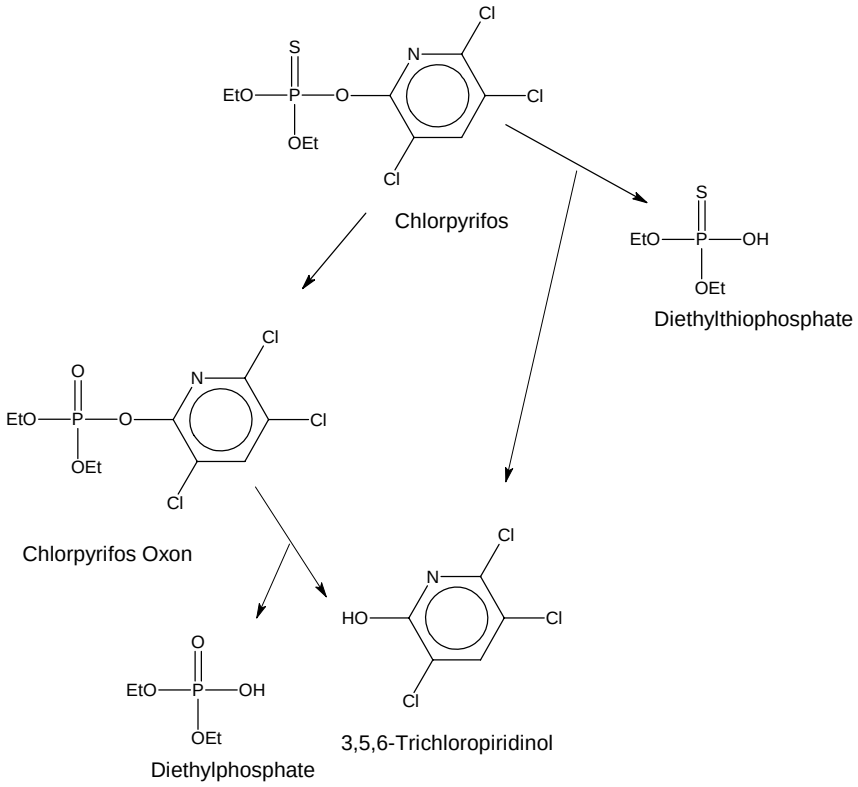
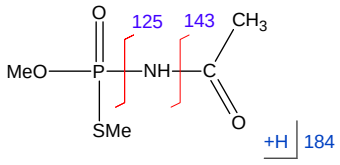
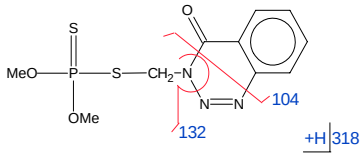
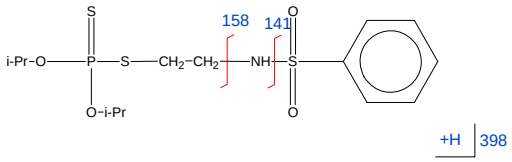
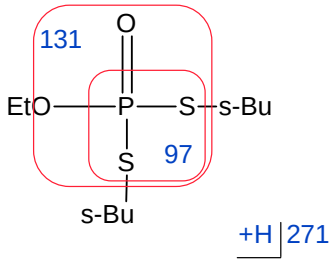
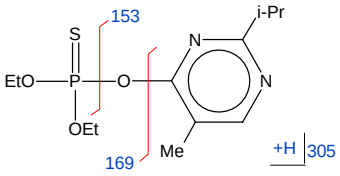
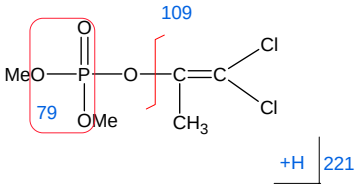
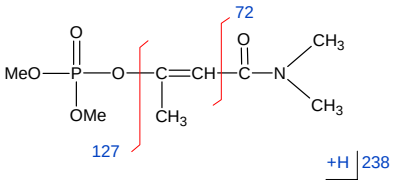
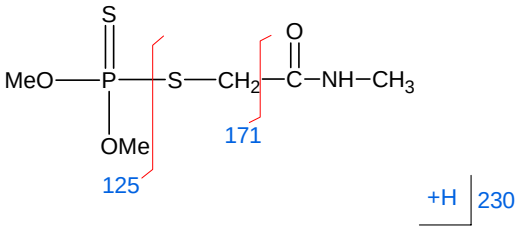
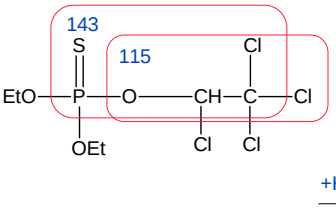


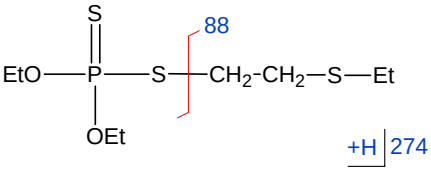
Figure 125 Environmental degradation of chlorpyrifos

CHAPTER 4 APPENDIX

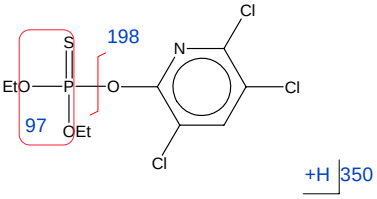
Appendix 4A: Suggested fragmentation of OP pesticides

Pesticide name	Chemical Structure
Acephate	
Azinphos-methyl	
Bensulide	
Cadusafos	
Diazinon	
Dichlorvos (DDVP)	
Dicrotophos	
Dimethoate	

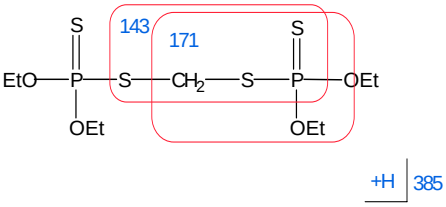




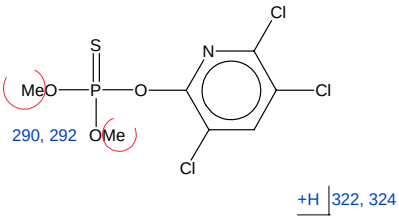
Chlorethoxyphos



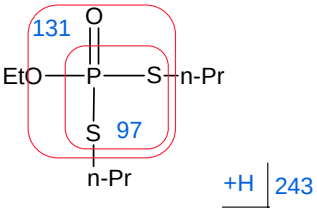
Disulfoton



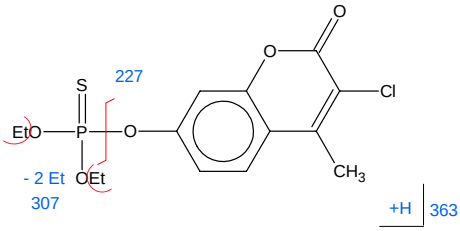
Chlorpyrifos



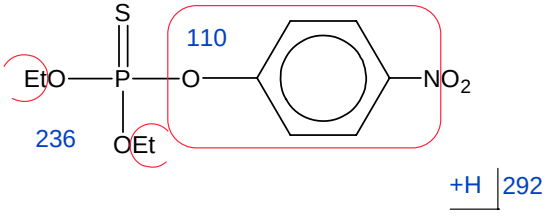
Ethion



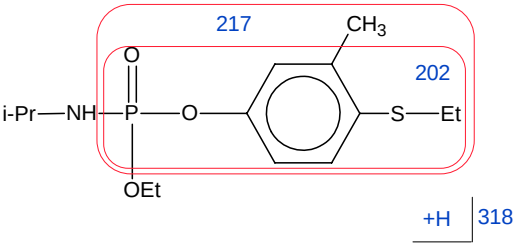
Chlorpyrifos methyl



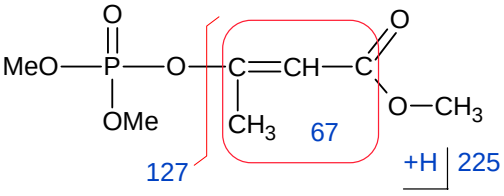
Ethoprop



Coumaphos

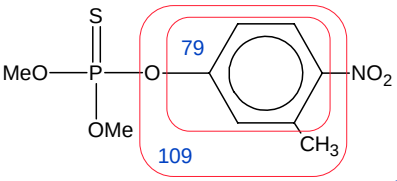


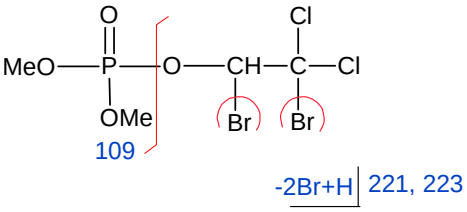
Ethyl Parathion



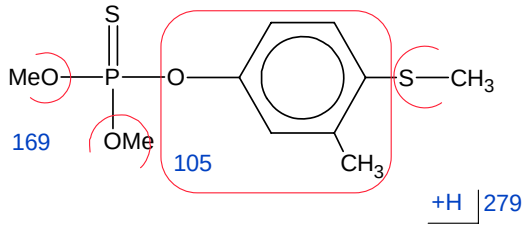
Fenamiphos

Mevinphos

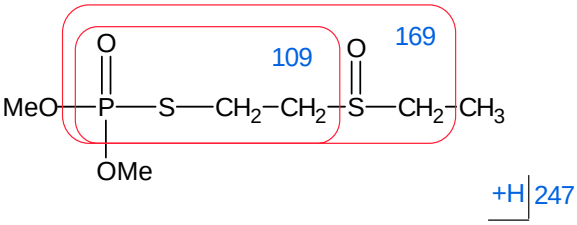




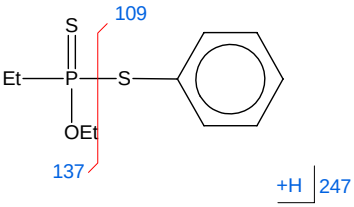
Fenitrothion



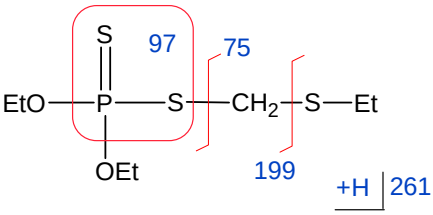
Naled



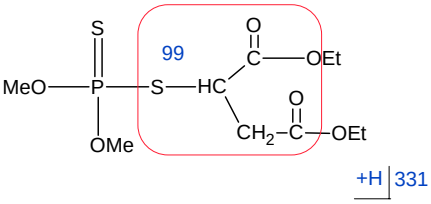
Fenthion



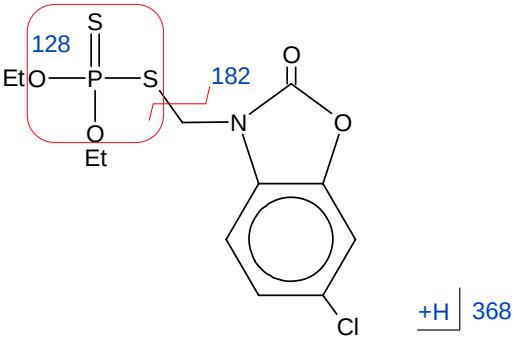
Oxydemeton methyl



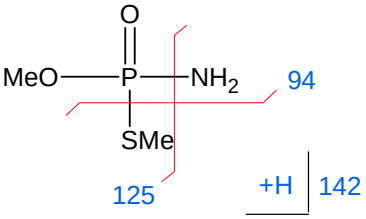
Fonofos



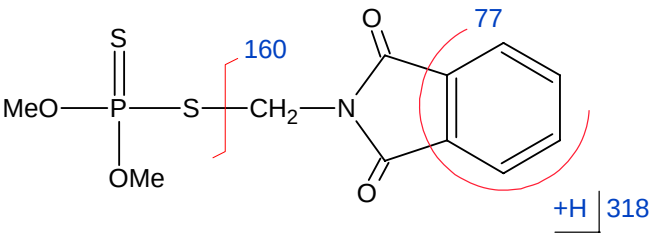
Phorate



Malathion

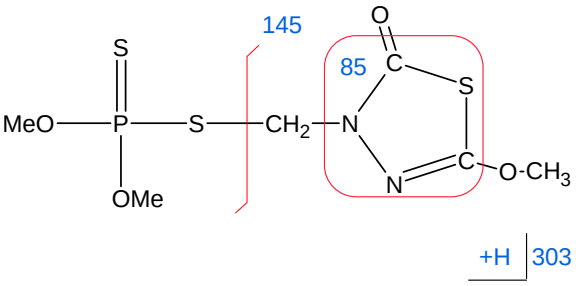


Phosalone

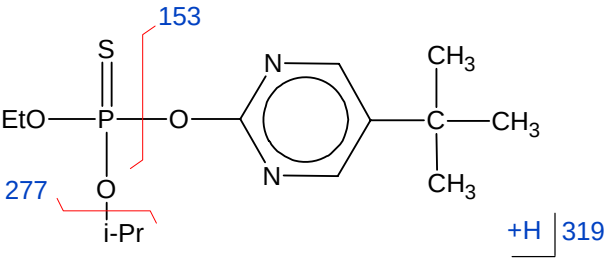


Methamidophos

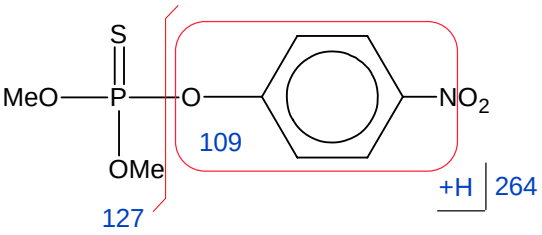
Phosmet



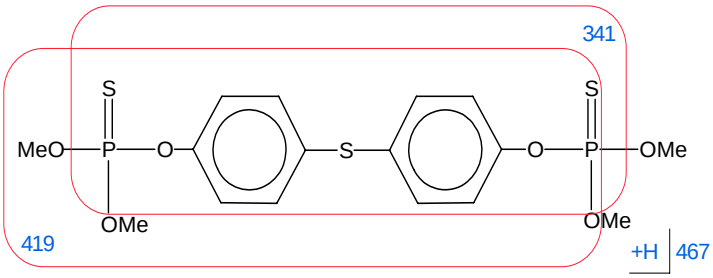
Phostebupirim (tebupirimfos)



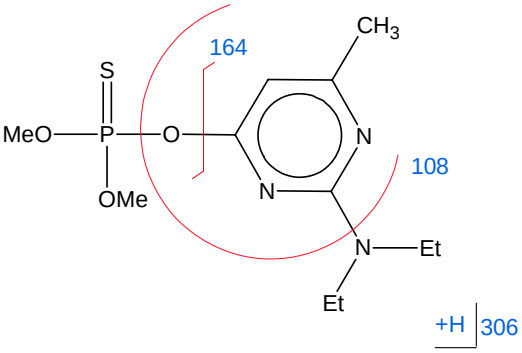
Methidathion



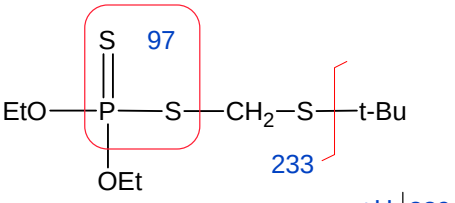
Temephos



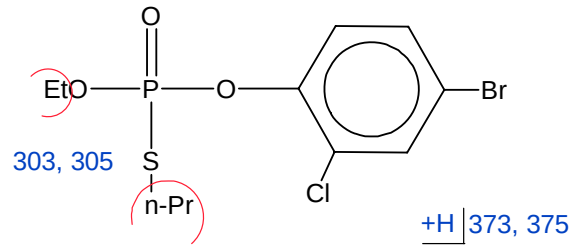
Methyl parathion



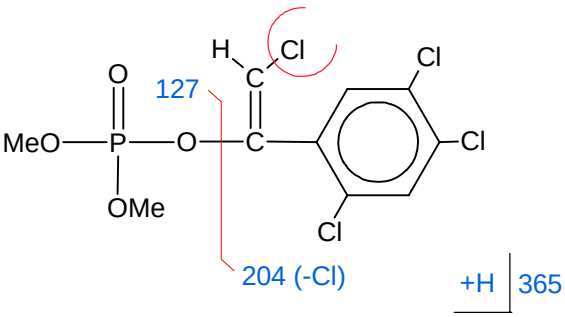
Terbufos6



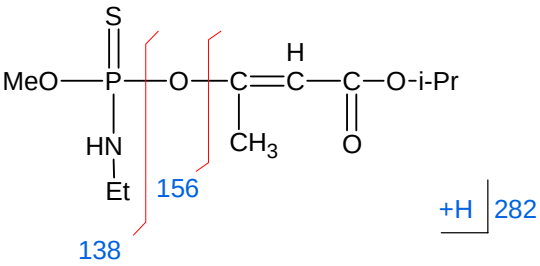
Pirimiphos methyl



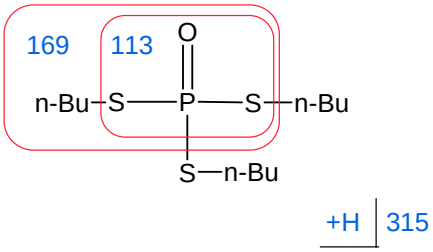
Tetrachlorvinphos



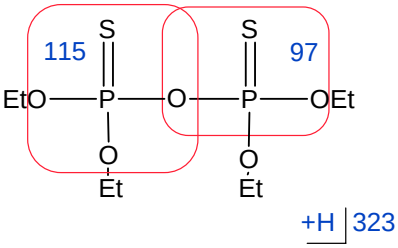
Profenofos



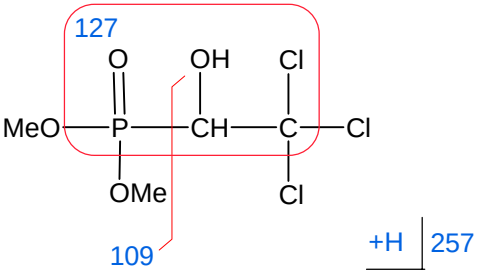
Propetamphos



Tribufos (DEF)



Sulfotepp



Trichlorfon

Appendix 4B: Determination of recovery using internal standard

Recoveries are the fractions of the analytes extracted after a clean up step, or after the entire cleanup process. To determine recoveries one need two samples of the matrix, A and B. Sample A is spiked with a predetermined amount of analyte and homogenized. Sample B is not spiked. Next both of the samples are put through the clean up process. After completing the cleanup, the same amount of analyte added to B as it was added sample A before. Next internal standard is added to both A and B and homogenized.

Both extracted and concentrated samples A and B is analyzed for all compounds of interest and response factor is determined for all in A and B.

The recovery for each analyte is calculated as follows:

$$rec = \frac{f_A}{f_B}$$

Equation **1** Calculation of recoveries fA and fB are the response factors for the particular analyte in samples A and B

Finally recovery calculations are done on multiple sample pairs, average recoveries and standard deviations are calculated. The experimental process is illustrated on

Figure 126. This approach of determining recoveries eliminates the matrix effect, because the cleanup sample (A) and the control (B) both contain extracted matrix.

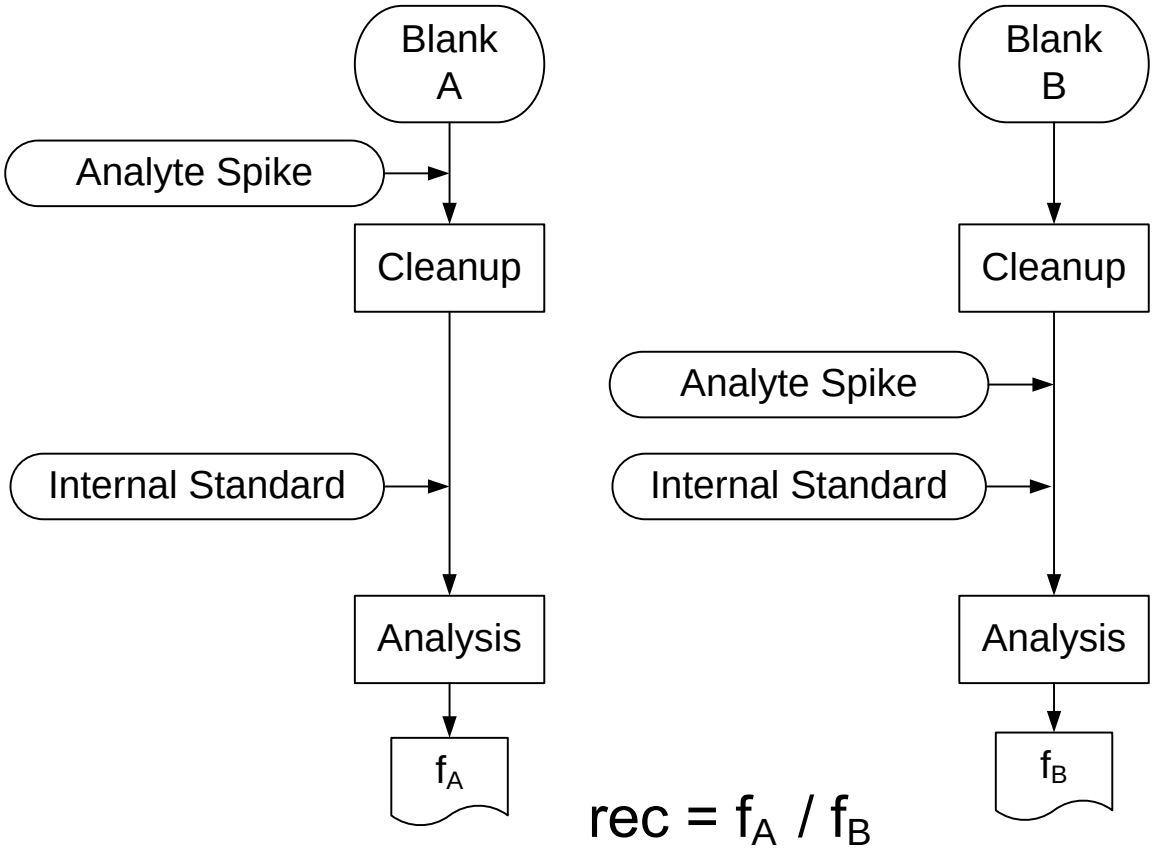


Figure 126 Flow chart of determining recoveries of a given cleanup process

Appendix 4C: Determination of limit of detection (LOD)

Limits of detections were calculated as described by Taylor [41] and illustrated on Figure 127. Multiple calibration series were plotted and for each known concentration ("actual concentration" on Figure 127) the response factor was determined. From the response factor, "calculated concentrations" were computed, using the calibration curve. As shown in the second part of Figure 127, the standard deviations of the calculated concentrations ware plotted against the actual concentrations. By fitting a line to the standard deviation series of data points, the intercept was determined. The intercept is the standard deviation of the calculated concentration at zero concentration, or S_0. Three times S_0 is defined as the limit of detection.

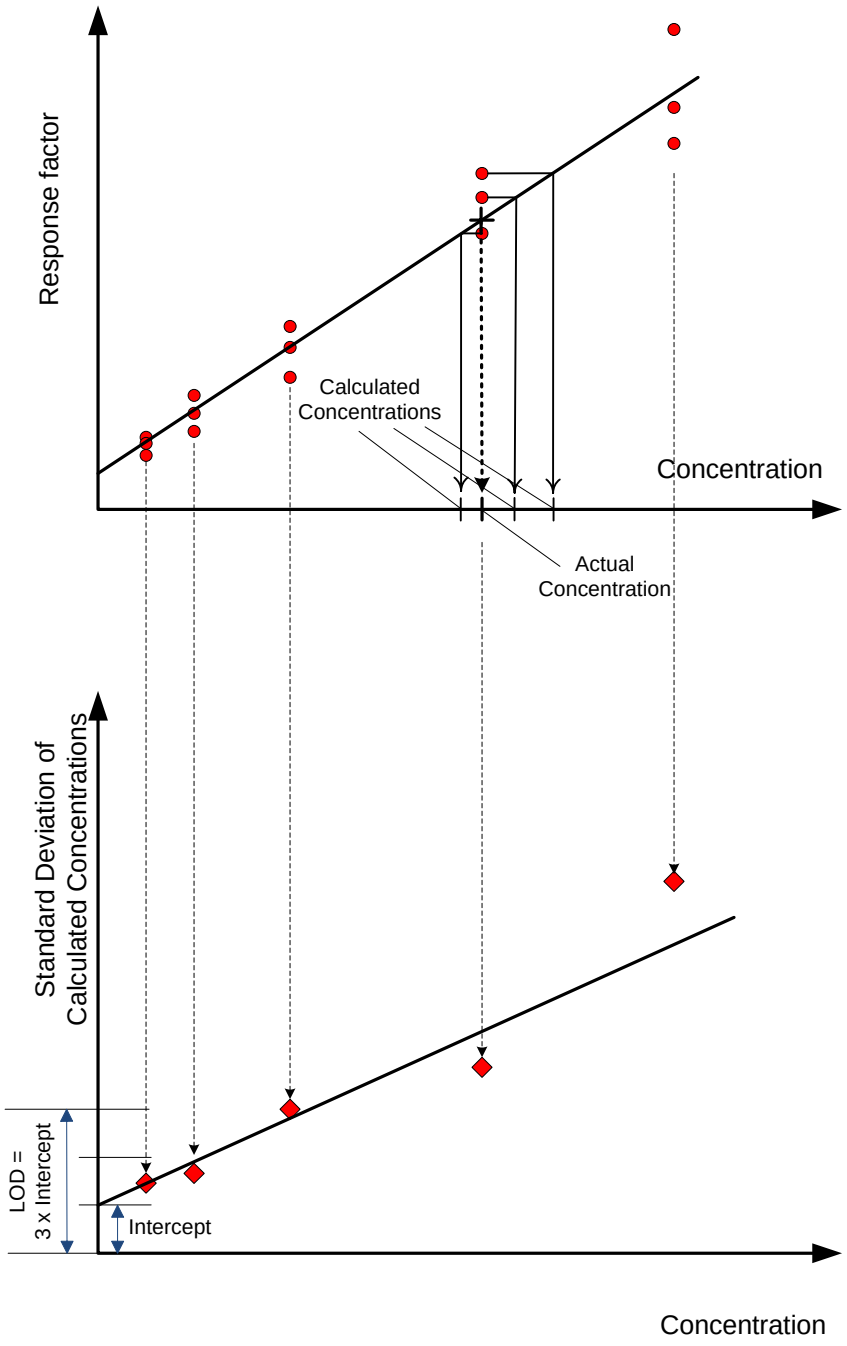


Figure 127 Determination of limits of detection

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