Identification of Unknown Pesticides in Food Using Both LC/MSD TOF and Ion Trap MSn

Application

Food Safety

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Abstract

Traditionally, the screening of unknown pesticides in food was accomplished by gas chromatography/mass spectrometry (GC/MS) methods using conventional library searching routines. However, many of the new polar and thermally labile pesticides are more readily and easily analyzed using liquid chromatography/mass spectrometry (LC/MS) methods, even though no searchable libraries currently exist (with the exception of some limited user libraries). There is, therefore, a need for LC/MS methods to detect true pesticide unknowns.

This application develops an identification scheme for unknown pesticides using a combination of liquid chromatography/mass selective detector (LC/MSD) with time-of-flight (TOF) and ion trap (MSn) options, and searching for the empirical formula using the accurate mass and the ChemINDEX or Merck Index® databases. The approach is different from the conventional library searching of ions. The procedure here consists of multiple steps.

First is the initial detection of a possible unknown pesticide in actual market-place vegetable extracts (tomato or another salad vegetable) using either the LC/MSD TOF or the LC/MSD ion trap.

Second is the application of the "Dual Approach." This consists of accurate mass identification of an empirical formula or molecular mass (usually containing an A+2 isotope signature, such as from S or Cl), followed by MS/MS ion trap confirmation of the chlorinated and/or sulfur-containing pesticides and their major fragment ions and chlorine or sulfur signatures.

Third is the database analysis of the empirical formula, which is carried out with either the Merck Index CD, containing 10,000 compounds, or the ChemINDEX CD, containing 77,000 compounds, and a laptop computer.

In the examples given, the unknowns are identified as the organophosphate insecticide, malathion, in cucumbers; and the insect growth regulator, buprofezin in tomato extracts.

The fourth and final step involves confirmation with authentic standards.

Introduction

The identification and quantitation of insecticides in vegetables is of great importance to individuals and health organizations around the world. The European Union (EU), has set new Directives for pesticides at low levels in vegetables in order to
meet these health concerns. For example, new laws such as the European Directive 91/414/EEC [1], or the Food Quality Protection Act (FQPA) [2] in the US have increased the standards for human health, workers, and environmental protection. They also require re-registration for older pesticides.

Furthermore, the review programs have withdrawn authorizations for many of the crop protection products currently on the market, 177 compounds in the US and 320 in Europe. Moreover, it was announced in Europe that a total of 110 products will be withdrawn in the near future [1]. The revision of the review program in Europe dictates that over 450 existing active substances will be taken off the market by 2008 with about 400 pesticides remaining in use.

Next, the quality standards within the new regulations include the re-assessment of the maximum residue limits (MRLs) for vegetables, and EU directives are setting different MRLs for each pesticide within each food group. Typically, the MRLs are lower than the previous ones. Furthermore, the new Directive leads to different MRLs for each EU country, which are still being decided. The EU Directive states that individual country MRLs will be maintained in the new program. Finally, banned compounds have the lowest MRLs, which is set now at 0.01 mg/kg (ppm). With the planned program to remove so many compounds from the market, it is important, even necessary, that screening for unknown pesticides may be done by LC/MS on vegetable extracts. Because it is not always possible to know which banned substances may be used, it is of vital importance to environmental food monitoring that there be a system to give fast and accurate screening of unknown substances in food and food products.

Thus, there is an important need for research studies and methods development on the analysis of unknown pesticides in food by new LC/MS methods, such as the combination of accurate mass using LC/MSD TOF, and MS/MS using LC/MSD ion trap. Our study in this report is one of the first of its kind to examine the new Agilent LC/MSD TOF combined with LC/MSD ion trap, and the use of commercial databases, such as the Merck Index and the ChemINDEX database to identify unknown pesticides in food.

Several advantages of the combination of LC/MSD TOF and ion trap are that accurate mass and empirical formulas may be combined with the MS/MS spectra or even MS^n for spectral information. The identification of unknowns using the LC/MSD TOF and ion trap consists of six steps, which are outlined below.

1. Analyze the vegetable extract with ion trap in full-scan mode looking for important unknown peaks or alternatively, using in-source CID on LC/MS TOF.
2. Next check for A+2 isotope patterns to see if Cl, Br, or S is present.
3. Search Merck Index or ChemINDEX for possible unknowns using molecular mass (nominal mass weight), A+2 isopes, and molecular formula.
4. Proceed to ion trap MS/MS with proposed ideas for structure and do MS^2 or MS^3. Identify ion fragments, accurate mass fragments, and possible structures.
5. Combine with LC/MSD TOF data of accurate fragment ions and protonated molecule to make tentative identification.
6. Obtain and analyze appropriate standard for final confirmation.

Given are two detailed examples of this process using store-purchased salad vegetables, cucumbers and tomatoes, both of which contained "unknown white powders" that were subsequently identified by the above process for various "unknown pesticides."

**Experimental Methods**

**Standard Vegetable Extraction [3]**

1. Tomato, lettuce, pepper, and cucumber, in 2-kilogram portions, were obtained from the market place and homogenized by chopping.
2. Then 15 g of the homogenized vegetable was accurately weighed and placed into a 200-mL PTFE centrifuge tube.
3. Ethyl acetate (45 mL) and 6.5 M NaOH (1 mL) were added and blended in a high-speed blender (Polytron) for 30 s at 21,000 rpm [4]. After this time, 13 g of anhydrous Na_2SO_4 was added and the extraction repeated for 30 s.
4. The combined extracts were then filtered through a thin layer of 20 g of anhydrous Na_2SO_4. The solid was washed with 50 mL of ethyl acetate and the combined extracts were evaporated to dryness on a vacuum rotary evaporator using a water bath at 45 ±5 °C.
5. The dried residue was dissolved by sonication in 15 mL of methanol. The extracts, which contained 1 g of sample per mL, were filtered through 0.2-µm PTFE filters (Millex® FG, Millipore) prior to LC/MS analysis.

**Rapid Vegetable Extraction**

1. Select tomato or cucumber containing white powder from a commercial market place.
2. Carefully wash the vegetable skin three times with methanol to remove the white powder, recovering a total of 2 to 5 mL of wash, (depending on the size of the vegetable) into a 150-mL Pyrex® beaker.
3. After mixing, transfer the methanol wash to a 5-mL syringe and filter through a Millex-FH PTFE filter and aliquot 0.3 mL.
4. Dilute with 0.6 mL of deionized water.
5. Analyze by either LC/MSD TOF or LC/MSD ion trap.

**LC/MSD TOF Methods**

Instrument: Agilent model LC/MSD TOF with electrospray source

LC pumps were Agilent 1100 binary pumps, injection volume 50 µL with standard Agilent ALS

Column: ZORBAX Eclipse® XDB-C8, 4.6 mm × 150 mm × 5 µm, part number 993967-906

Mobile phases: A: ACN, B: 0.1% formic acid in water

Gradient: 15% A to 100% A over 30 minutes, at 0.6 mL/min

Positive ESI, capillary: 4000 V

Nebulizer 40 psig, drying gas 9 L/min, gas temp 350 °C

Skimmer 1: 20 V, Skimmer 2: 10 V, Capillary exit offset: 50 V, Capillary exit: 70 V

Octopole 3 V, Oct RF 100 V, Octopole ∆: 2 V, Lens 1: -3 V, Trap drive: 50, Lens 2: -50 V

Target: 50,000, max. accumulation time: 200 ms, scan m/z 50 to 800, averages: 5

Rolling average: on, MS/MS m/z 2.0 isolation width, amplitude: 1.2 V, fragmentation cutoff m/z 83

**LC/MSD Ion Trap Methods**

Chromatographic conditions identical to those using LC/MSD TOF for direct comparison of peaks.

Instrument: LC/MSD Ion Trap Classic

LC pumps were Agilent 1100 binary pumps, injection 50 µL with standard Agilent ALS

Column: ZORBAX Eclipse XDB-C8, 4.6 mm × 150 mm × 5 µm, part number 993967-906

Mobile phases: A: ACN, B: 0.1% formic acid in water

Gradient: 15% A to 100% A over 30 minutes, flow rate: 0.6 mL/min

Positive ESI, Capillary 3000 V

Nebulizer 40 psig, drying gas 9 L/min, gas temp 350 °C

Skimmer 1: 20 V, Skimmer 2: 10 V, Capillary exit offset: 50 V, Capillary exit: 70 V

Octopole 3 V, Oct RF 100 V, Octopole ∆: 2 V, Lens 1: -3 V, Trap drive: 50, Lens 2: -50 V

Target: 50,000, max. accumulation time: 200 ms, scan m/z 50 to 800, averages: 5

Rolling average: on, MS/MS m/z 2.0 isolation width, amplitude: 1.2 V, fragmentation cutoff m/z 83

Reference A Sprayer 2 set to constant flow rate during the run
Results and Discussion

Cucumber Extract

Figure 1 shows the ion trap total ion chromatogram (TIC) for the rapid extract of the white powder present on a store-purchased cucumber. The largest peak in the chromatogram, deemed the Star*TIC, has a spectrum shown in Figure 1 (bottom panel) with ions at \( m/z \) 331 and 353. The spacing of \( m/z \) 22 indicates a sodium adduct. Because the analysis was carried out in positive ion, the \([M+H]^+\) is the \( m/z \) 331, and the \([M+Na]^+\) is the \( m/z \) 353. Furthermore, note that the \( m/z \) 331 ion also contains an A+2 ion at \( m/z \) 333, which is \( \sim 8\% \) of the \( m/z \) 331 ion. The two important A+2 ions to consider in unknown ID are chlorine and sulfur (occasionally bromine). Sulfur-34 is present in natural abundance of 4.8%; thus, the \( \sim 8\% \) peak is most likely due to two sulfur-34 atoms present in the unknown at \( m/z \) 331.

The next step is the search of the Merck Index for the molecular weight and sulfur atom content. Figure 3 shows an example search sheet on a laptop computer. Because the ion trap gives the protonated molecule (\( m/z \) 331) the search is done by subtracting a proton to give the molecular weight of 330. The molecular weight is then searched across one mass unit to 331. The molecular formula has only the S2, which is a requirement now of the database search.

Figure 2 shows the LC/MS/MS of \( m/z \) 331. The ions present include: \( m/z \) 285, 240, 127, and 99. These ions will be used later for structural identification after a database search on the Merck Index finds a possible structure. These ions will then be compared with that structure.

Figures 4a and 4b show the two results of the Merck Index database search. The compounds found were penicillin O and malathion, with molecular weights of 330.43 and 330.36, respectively.
Results of library search for MWt = 330-331
Molecular Formula = S2*

**Figure 4a. First result for database search for MW 330-331.**

**Figure 4b. Second result for database search for MW 330-331.**
The fragmentation ions shown in Figure 5 fit the ions from the MS/MS experiment of the \( m/z \) 331 (Figure 2). Therefore, malathion is a good possibility of an identification from the Database search of the Merck Index.

![Fragmentation possibilities for malathion.](image)

The next step is obtaining the accurate mass with the LC/MSD TOF. Figure 6a shows the TIC and the nearly identical retention times between the two instruments for the unknown Star*TIC because of using the same HPLC column. Figure 6b shows the accurate mass for the \( m/z \) 331.0435 and only one formula match of \( \text{C}_{10}\text{H}_{19}\text{O}_{6}\text{PS}_{2} \).

![Figure 6a. LC/MS TOF analysis of a cucumber extract.](image)

When this formula is entered into the Merck Index database the only match is malathion (Figure 7).

Thus, the accurate mass gives the same formula match that is consistent with the identification by ion trap. Furthermore, Figure 6b also shows the accurate mass ions for the possible fragmentation of the unknown at masses of \( m/z \) 353.0256, 285.0017, 127.0389, and 99.0081. These accurate mass ions are the same ones measured with the ion trap in MS/MS mode of the \( m/z \) 331 at nominal mass (with the exception of the \( m/z \) 353 adduct). The masses of these ions match the formulas shown in Figure 5 including the sodium adduct of the \( m/z \) 331. Thus, the probability of this being the correct identification is quite high.

The final step is the identification by standard matching. This was done by both ion trap MS/MS and by TOF; both gave this compound as the correct identification. Malathion is a banned substance for cucumbers so this identification represents an important finding. Based on the identification point scheme for unknowns, this compound receives 2.0 points for the protonated molecule, 2.0 points for the \( m/z \) 285 ion, and 1.5 points for the MS/MS transition from \( m/z \) 331 to 285 for a total of 5.5 points. Four identification points are required for banned substances in food (see reference by Stolker et al. [5] in book by Ferrer and Thurman [6]). The complementary nature of the two instruments is also shown with the next unknown example.
Figure 6b. LC/MS TOF spectrum of peak at 22.5 min (m/z 331).

Figure 7. Database search for C₁₀H₁₉O₆PS₂.
**Tomato Extract**

In this next example, a similar protocol is followed except that this time we use the LC/MSD TOF first to obtain an in-source CID spectrum. Figure 8a shows the Star*TIC for the rapid extraction of the tomato white powder.

![Figure 8a. LC/MS TOF analysis of a tomato extract.](image)

**Figure 8b.** LC/MS TOF spectrum of peak at 23.9 min (m/z 306).

The two formulae were both then searched in the Merck Index and only one formula gave a database hit. That compound was C_{16}H_{23}N_{3}OS, the insect growth regulator buprofezin. Buprofezin is used extensively on white flies according to the Merck Index (Figure 9). Thus, this compound was a good candidate for a positive identification.

![MERCK](image)

**Figure 9.** Database search for C_{16}H_{23}NO_{2}PS and C_{16}H_{23}N_{3}OS.
Note in the spectrum the presence of the ion at m/z 201.1059 (Figure 10). LC/MSD ion trap MS/MS of the m/z 306 ion gave the m/z 201 and the further MS\(^3\) yielded the m/z 116 ion.

Furthermore, the expected mass for the 201.1056 fragment ion matched the value from the LC/MSD TOF (201.1059) quite closely (0.0003 u), which gave a high certainty for identification. After obtaining the buprofezin standard, the final data show a perfect match, which further shows the ability of the LC/MSD TOF and LC/MSD ion trap to identify unknowns. Buprofezin is allowed for tomatoes; therefore, it is not a banned substance. The quantitative tolerance for buprofezin may then be measured by the standard extraction and analysis for vegetable acceptance to European Union standards [1].

**Conclusions**

- LC/MSD TOF and LC/MSD Trap are complementary and powerful tools for identification of pesticides in vegetables and represent a new approach for environmental food chemistry using LC/MS.
- The combination of these two tools, the twin mass spectrometer(s), with a pesticide database, Merck Index or ChemINDEX, works often for identification of unknown pesticides.
- The use of identification of fragment ions with MS\(^2\) and MS\(^3\) is also powerful when combined with accurate mass of LC/MSD TOF CID spectra.
- Combining TOF and Trap for unknown ID works [4, 7]! Future work should include chromatographic data to help in the identification of unknowns and to provide a simple pesticide library generated through calculation of empirical formula and isotope ratios.

**Figure 10.** LC/MS TOF spectrum and LC/MS/MS Ion Trap spectrum for m/z 306.

**Figure 11.** Reasonable structures of fragment ions consistent with the structure of buprofezin.
References

1. EU Food Directives, 2002, 91/414/EEC
2. Food Quality Protection Act, 1998 (FQPA)

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