Analytical Methods

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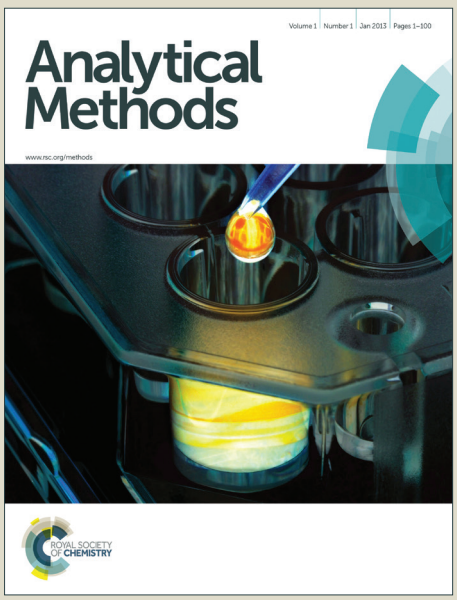

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Green detection of the olive fruit fly pest by the direct determination of its sexual pheromone

María del Carmen Alcudia-León, Soledad Cárdenas, Miguel Valcárcel and Rafael Lucena*

Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry. Marie Curie Building. Campus de Rabanales. University of Córdoba. 14071 Córdoba (Spain)

** Corresponding author: (tel/fax) +34-957-211-066; (e-mail) q62luror@uco.es*

Bactrocera oleae Gmelin, the olive fruit fly, is considered a serious pest in the cultivation of olive trees since the larvae feed on the fruit damaging the productivity and quality of the final products (olive fruit and olive oil). An extensive application of pesticides is usually employed to fight this pest producing secondary side-effects of environmental and safe concern. In this context, the development of green analytical methods focused on the detection of the pest may reduce these secondary problems. In this article, the combination of headspace and gas chromatography with mass spectrometric detection is proposed for the identification of olive fruit fly pest using one of its sexual pheromone component (1,7-dioxaspiro-[5,5]-undecane) as marker. The developed method is characterized by its simplicity, automation and robustness and follows the principles of green analytical chemistry. It provides, working under its optimal operation conditions, limit of detection of 26.55 µ/kg and precision, expressed as relative standard deviation, better than 4.7 % (calculated at 100 µg/kg). The relative recovery values (calculated at 100 µg/kg and 500 µg/kg) ranged between 93 % and 98 % for different olive cultivars which testifies for the applicability of the proposed method.

Keywords: Olive fruit fly, sexual pheromone, headspace, gas chromatography, mass spectrometry, green analytical chemistry

1. Introduction

Olive fruit fly (Bactrocera oleae Gmelin) infestation is a problem of major economical and social concern in the Mediterranean basin, that involves the 98% of the olive trees (Olea europea) cultivated in the world.[1,2] These insects lay their eggs on the fruits and once hatched, the larvae feed and grow into the fruit creating galleries throughout its mesocarp, destroying and consuming the pulp and allowing the development of secondary detrimental organisms like fungi. Finally, the larvae pupate in the fruit or on the ground after leaving the fruit.[2]

Olive fruit fly pest negatively affects to the quality of the final products, both olives and olive oil. It leads to drastic reduction in crop, premature fruit drop and reduction of the quality of the produced oil as it is strongly related to the physiological conditions of the fruit from which it is extracted. In general, the action of parasites prior to harvest or fungal activity during the period between harvest and oil extraction are the main external agents responsible for the breakdown of metabolic processes in the olive and subsequent deterioration of oil quality.[3-5] The decreased polyphenols concentration and thus the antioxidant power of the resulting oil, which is a quality parameter since it defines the stability of the product, is a critical example of this phenomenon. An increase of the oil acidity, peroxide index and UV-absorbance can be also highlighted.[6] These deleterious effects on the final product depend on different factors, such as the stage of the pest, the severity of the attack but also the olive variety being attacked.

The olive fruit fly pest has been controlled in the last decades by the use of organophosphate insecticides in the form of bait or cover sprays targeted against adults. Although these pesticides have been recently substituted by pyrethroids,[7] a safer alternative, it would be desirable to reduce their application since (i) they are environmental pollutants; (ii) they may contaminate the fruits and the produced olive oil

creating a problem of food safety concern; and (iii) they may kill other species such as beneficial arthropods.[2] Moreover, the appearance of insecticide resistance in Batrocera oleae has also been identified.[7-9]

Pest control methods include mass trapping, particle film technology and biological control.[2,10] None of them are useful for early dectection of the pest. Recently, a new alternative has been proposed by our research group for the rapid and green detection of pests based on the determination of their sexual pheromones.[11] In olive fruit fly, the main component of the sex pheromone, 1,7-dioxaspiro-[5,5]-undecane, was identified by Baker et al.[12] This pheromone can be directly related to the olive fruit fly although structural related compounds are pheromones of other insects. In contrast to the majority of Tephritidae, in which pheromones are usually produced by the males, it is produced by both male and female individuals.[13,14]

In this article, we proposed a green, fit-for purpose method for the determination of the pest pheromone as presence marker based on headspace-gas chromatography/mass spectrometry (HS-GC/MS). This approach can be considered within the green analytical chemistry (GAC) framework[15] for two reasons. On the one hand, its practical application should reduce the use of pesticides allowing their rational use, since pesticides are applied only when and where they are necessary. On the other hand, the analytical method fulfills almost all the twelve principles of GAC[16] regarding simplicity, automation, minimal consumption of organic solvents and derivatization reagents with direct sample analysis.

2. Experimental section

2.1 Reagents and samples

All reagents were of analytical grade or better. Sigma-Aldrich (Madrid, Spain) provided the major component of sex pheromone of olive fruit fly: 1,7-dioxaspiro-[5,5]-undecane. Stock standard solution of the analyte was prepared in n-hexane (Panreac, Barcelona, Spain) at a concentration of 1 g/L and stored in the dark at 4 ºC (the standard is stable under this conditions). Working solutions were prepared by the appropriate dilution of the stock in n-hexane.

For this study, four olive cultivars growing in Córdoba (Spain) were selected, namely: Picual, Cornicabra, Arbequina and Hojiblanca. 250 g of each cultivar were collected in January 2014 and October 2014 and they were immediately transported to the laboratory under refrigeration. Once in the laboratory, the unwashed samples were stored at -18 ºC until the analysis.

In the optimization and analytical validation of the proposed methodology, fortified olive fruit samples were employed. For this purpose, aliquots of 5 g of blank olive fruits were spiked with a standard of the analyte prepared in methanol and the standard was maintained in close contact with the sample matrix for 24 h at 4ºC before the analysis.

2.2 HS-GC-MS Instrument

Sample analyses were performed with an HP6890 gas chromatograph (Agilent, Palo Alto, CA, USA) equipped with an HP5973 mass spectrometric detector based on a quadrupole analyzer and an electron multiplier detector. An MPS2 32-space headspace autosampler (Gerstel, Mülhein and der Ruhr, Germany) was used as sample introduction interface. The autosampler includes a robotic arm and an oven for sample

heating/headspace generation. Samples were equilibrated at 80 ºC for 90 min with continuous stirring at 750 rpm. 2.5 mL of the homogenized headspace was finally injected via an automated injector fitted with a gas-tight HS-syringe maintained at 200 ºC and 80 ºC, respectively. System control was achieved with an HP1701CA MS ChemStation (Agilent Technologies).

The analyte was separated from the sample matrix components on a HP5MS fused silica capillary column (30 m x 0.25mm i.d.). The chromatographic temperature program was as follows: 40 ºC for 2 min, ramped to 120 ºC at 5 ºC/min and finally, increased to 250 ºC at 50 ºC/min and held for 2 min. The mass spectrometer detector operated in selected ion monitoring mode recording the m/z fragment-ions 98 and 101 in a single window. Electron impact ionization (70 eV) was used for analyte fragmentation. The injector, MS source and quadrupole temperatures were kept at 100ºC, 230 ºC and 150 ºC, respectively. The analytes were identified according to the retention time and the qualifying ions, while the peak area (sum of the intensities of m/z fragment-ions 98 and 101) was used for quantification.

2.3 Analytical procedure

The samples were ground and homogenized using a crusher (Moulinex Moulinette, 700W). An aliquot of 5 g was placed into a 10 mL headspace vial, which was then hermetically sealed with a silicone septum and placed into the autosampler. The robotic arm took each vial from the tray and transferred it into the oven where it was heated at 80 ºC for 90 min under mechanical stirring at 750 rpm to ensure the quantitative release of the analyte to the headspace of the vial. Then, 2.5 mL of the headspace was introduced by means of a syringe into the gas chromatograph/mass spectrometer, for separation and identification/quantification.

3. Results and discussion

3.1 Study of headspace conditions

In this extraction process, some variables have to be taken into account and conveniently selected with the aim of obtaining the highest sensitivity and selectivity levels. These variables have been studied by the "one variable at time" method using a blank olive fruits spiked with the target analyte at a concentration of 250 µg/kg. Table 1 summarizes the variables, the initial value, the interval studied and the optimum level for each one.

The extraction temperature was studied in the interval from 40 to 80 ºC, where the higher limit is fixed by security reasons since the high content of water in the olive fruit,[17] can produce a vial overpressure at higher temperatures. The results, which are shown in Fig. 1, indicated an increase of the analytical signal with the temperature in the studied interval. A value of 80 ºC was selected for this variable.

The addition of organic modifiers to the samples may improve the transference of the analytes from the sample to the headspace as it has been reported by other authors.[18] Three solvents, including n-hexane, acetonitrile and ethyl acetate were tested as modifiers at concentrations in the range 0.5 - 10 % (v/v). Acetonitrile and ethyl acetate produced a negative effect of the signal over the whole interval while a negligible effect for n-hexane was observed at concentrations lower than 5% with a decrease in the analytical signal at higher values. Attending to these results no solvent was added to the samples. Moreover, both the standard and working solution of the pheromone were

prepared in n-hexane in order to avoid the influence of the organic solvent in the analytical signal.

The effect of the headspace volume injected in the chromatograph was evaluated in the range from 0.5 to 2.5 mL. As the peak area of the analyte increased with the volume injected, 2.5 mL was selected for further studies.

Sample amount was studied in the interval 0.5-7 g. As it can be observed in Fig. 2, the signal increased with the sample amount up to 5 g remaining almost constant for higher amounts. Therefore, 5 g was selected as the best value.

Finally, the extraction time was studied to determine the equilibrium time. The results are shown in Fig. 3 and indicated that the peak area increased with time and a plateau was observed above 90 min. Thus, 90 min was selected.

3.2 Analytical figures of merit

The proposed procedure was analytically characterized for the determination of 1,7-dioxaspiro-[5,5]-undecane in olive fruit samples. A calibration graph was constructed by extracting blank olive samples spiked with the analyte in the range from 100 µg/kg to 5 mg/kg, obtaining a good linearity (R=0.9986).

The detection limit (LOD) and quantification limit (LOQ) were calculated using a signal-to-noise ratio (S/N) of 3 and 10 times, respectively. LOD was 26.5 µg/kg while LOQ resulted be 88.4 µg/kg. The precision of the proposed method, expressed as relative standard deviation, was calculated for nine independent analyses at a concentration level of 100 µg/kg and resulted to be 4.7 %.

In order to evaluate de applicability of the proposed method for the determination of the 1,7-dioxaspiro-[5,5]-undecane in olive fruits, different samples coming from four different cultivars (Picual, Cornicabra, Arbequina y Hojiblanca) were analyzed. In the

analysis of those olive fruits sampled in January 2014, the analyte was not detected (concentrations below the LOD) which was in-line with the farmers information concerning the absence of the pest during this sampling time. These samples were employed to develop a recovery study at two concentration levels, 100 and 500 µg/kg, each sample being analyzed in triplicate. The obtained results, which are listed in Table 2, showed recoveries in the range from 93 to 98%. These values demonstrate the potential of the proposed HS-GC/MS method for the determination of major component of the sexual pheromeone of the olive fruit fly.

Those samples collected in October 2014 presented the analyte at different concentrations as it can be observed in Table 3. The obtained values were in accordance with those data published by the Andalusian Goberment (Andalucia, Spain) for this period.[19] These official data, which were obtained by visual inspection of the fruit, are schematically presented in the map of Fig. 4, where the incidence of the pest is shown using a color legend. The olives analysed were sampled in a zone high pest incidence and it was studied at four different locations (indicated as A, B, C, D). The detection of the pheromone is indicative of the pest presence that has caused a great economical problem in 2014 in the olive oil sector according to the news.[20]

As an example, Fig. 5 shows a typical chromatogram obtained for the analysis of a olive sample containing the analyte at 185 µg/kg.

4. Concluding remarks

This article presents a analytical methodology for the determination of 1,7-dioxaspiro-[5,5]-undecane, the sexual pheromone of Bactrocera oleae Gmelin, in olive fruit. Bactrocera oleae Gmelin is the main olive tree pest and it has a severe effect on the

productivity and quality of the olive-related products.[21] In fact, this effect has been especially severe in 2014 according to the published news.

The proposed method can also be considered as a green methodology in a double facet. On the one hand, it permits the early detection of the pest avoiding the massive use of pesticides that can be focused only on infected area and for the detection of contaminated fruit avoiding the manufacture of lower quality products. On the other hand, the method is safe for both the analyst and the environment as no organic solvents are required except for the preparation of the analytical standards required for method calibration. The sample treatment is minimized as only a homogenization of the fruits is required.

The proposed methodology, based on the robust combination of HS-GC/MS, is simple, rapid and completely automated and therefore it can be employed in routine analysis. It allows the determination of the pheromone at lower concentration, 88.4 µg/kg with precision better than 4.7 %.

ACKNOWLEDGMENT

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FIGURE CAPTION

Figure 1. Effect of the extraction temperature on the extraction of the 1,7-dioxaspiro-[5,5]-undecane. Each level has been evaluated in triplicate.

Figure 2. Effect of the sample amount on the extraction of the 1,7-dioxaspiro-[5,5]-undecane. Each level has been evaluated in triplicate.

Figure 3. Effect of the extraction time on the extraction of the 1,7-dioxaspiro-[5,5]-undecane. Each level has been evaluated in triplicate.

Figure 4. Official data about the incidence of the olive fruit fly pest incidence in Andalucía in October 2014. The location of the sampled locations (A, B, C and D) are also presented.

Figure 5. Chromatogram of a Hojiblanca olive fruit variety containing 1,7-dioxaspiro-[5,5]-undecane, marked with an asterisk, at a concentration of 185 μg/kg. The mass spectrum of the target analyte is shown in the figure detail. The retention time of the pheromone peak is 14.59 min.

Table 1. List of the variables involved HS-GC/MS determination of 1,7-Dioxaspiro-[5,5]-undecane.

Variable	Initial value	Interval studied	Selected value
Extraction temperature (ºC)		40 - 80	80
Organic modifier (0-10 % v/w)	0%	N-hexane (0,5 -10 %) Acetonitrile (0,5 -10 %) Ethyl acetate (0,5 – 10 %)	0%
Injected volume (mL)	1	0.5 - 2.5	2.5
Sample volume (g)	2.5	0.5 - 7	5
Extraction time (min)	30	2 - 140	90

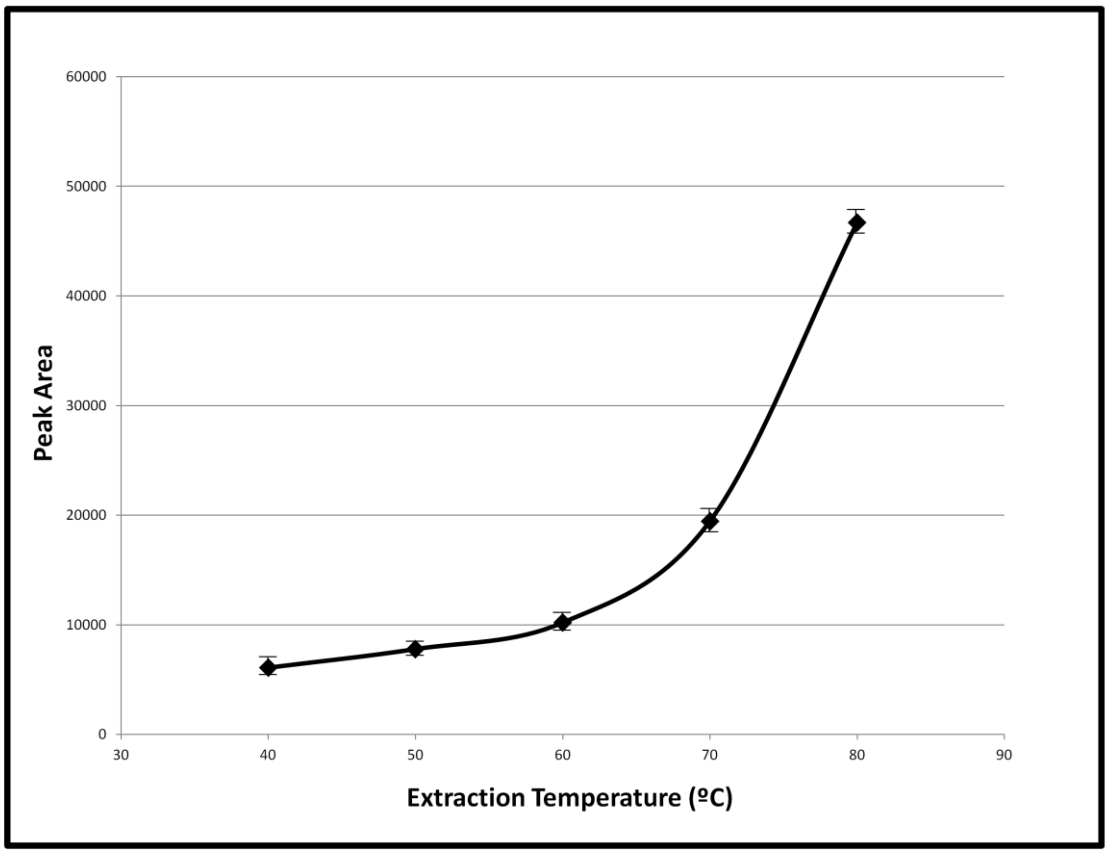
Table 2. Recovery study performed on blank olive samples spiked with 1,7-dioxaspiro-[5,5]-undecane.

Sample	Recovery ± SD	
	100 µg/kg	**500 µg/kg**
Picual	96 ± 4	98 ± 5
Cornicabra	95 ± 4	93 ± 4
Arberquina	93 ± 4	97 ± 5
Hojiblanca	94 ± 4	96 ± 4

SD, standard desviation (n=3)

Table 3. Analysis of different olive fruits sampled in October 2014 in different locations (A, B, C and D) that are described in Figure 4.

Analyte	**Picual (A)** µg/kg ± SD (n=3)	**Cornicabra (B)** µg/kg ± SD (n=3)	**Arbequina (C)** µg/kg ± SD (n=3)	**Hojiblanca (D)** µg/kg ± SD (n=3)
1,7-dioxaspiro-[5,5]-undecane	221 ± 10	352 ± 16	279 ± 13	185 ± 9



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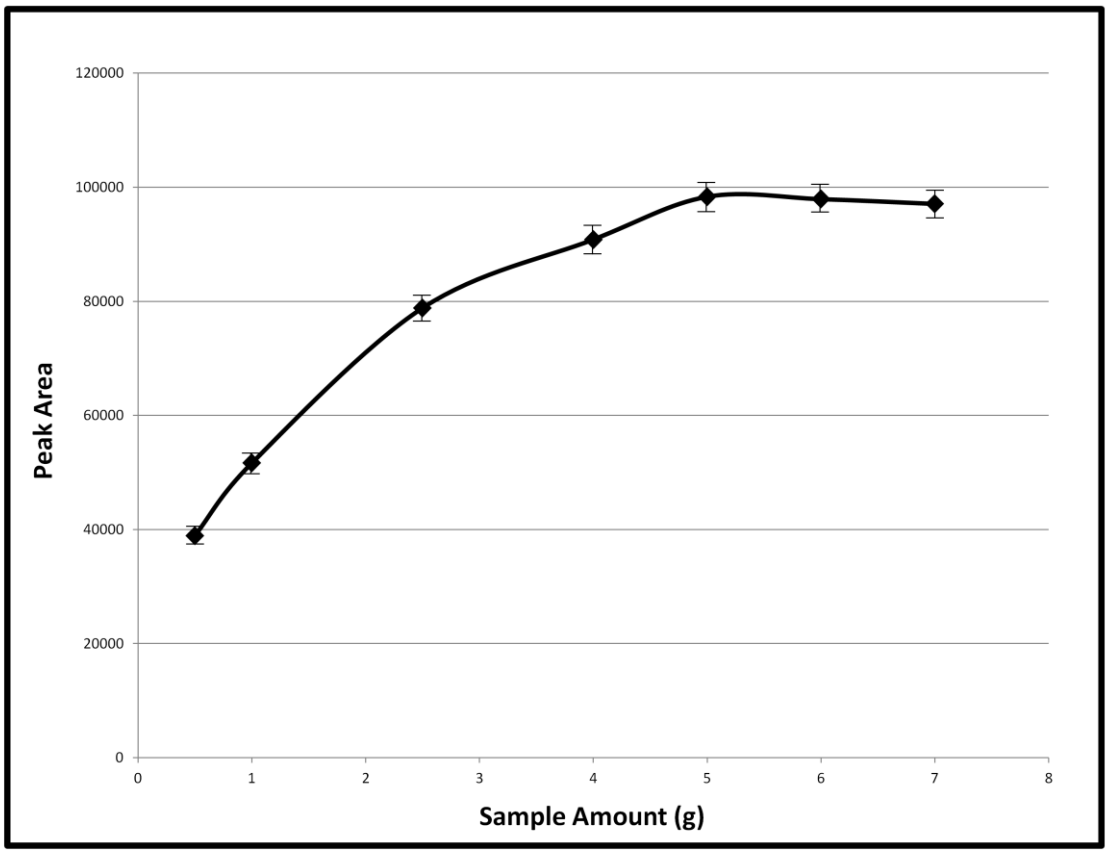

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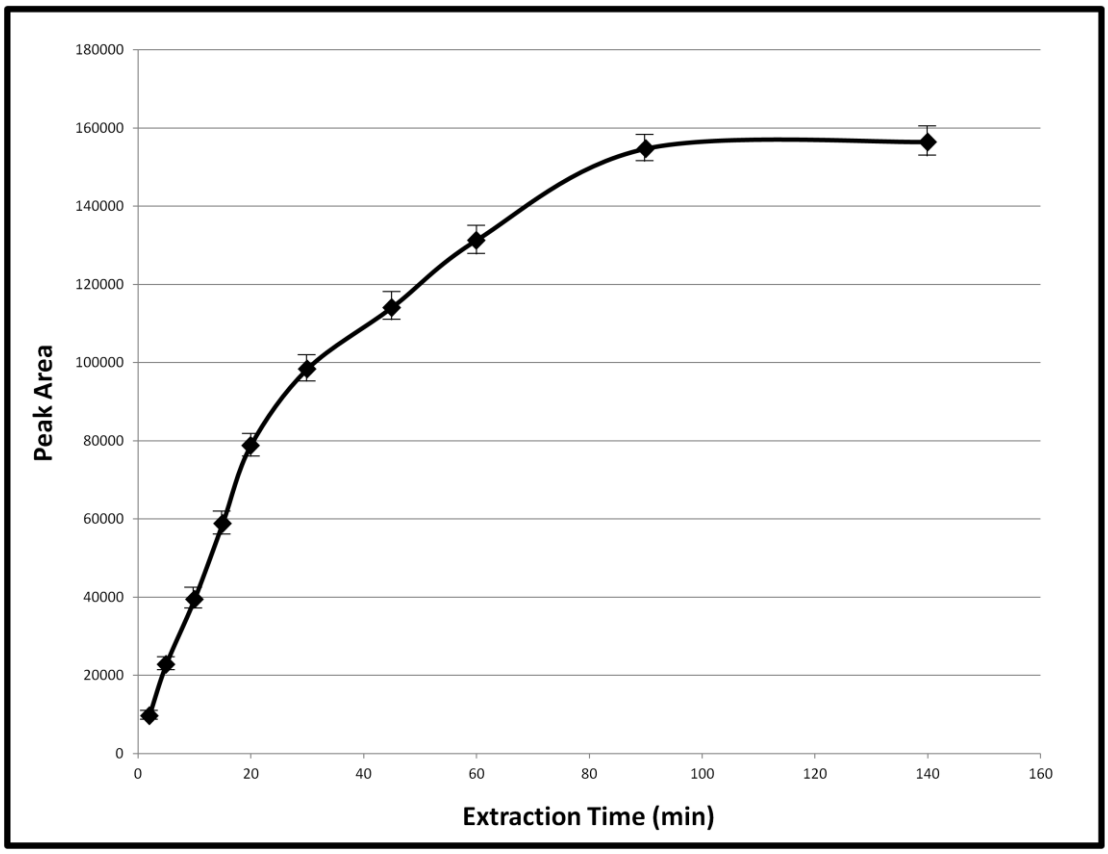

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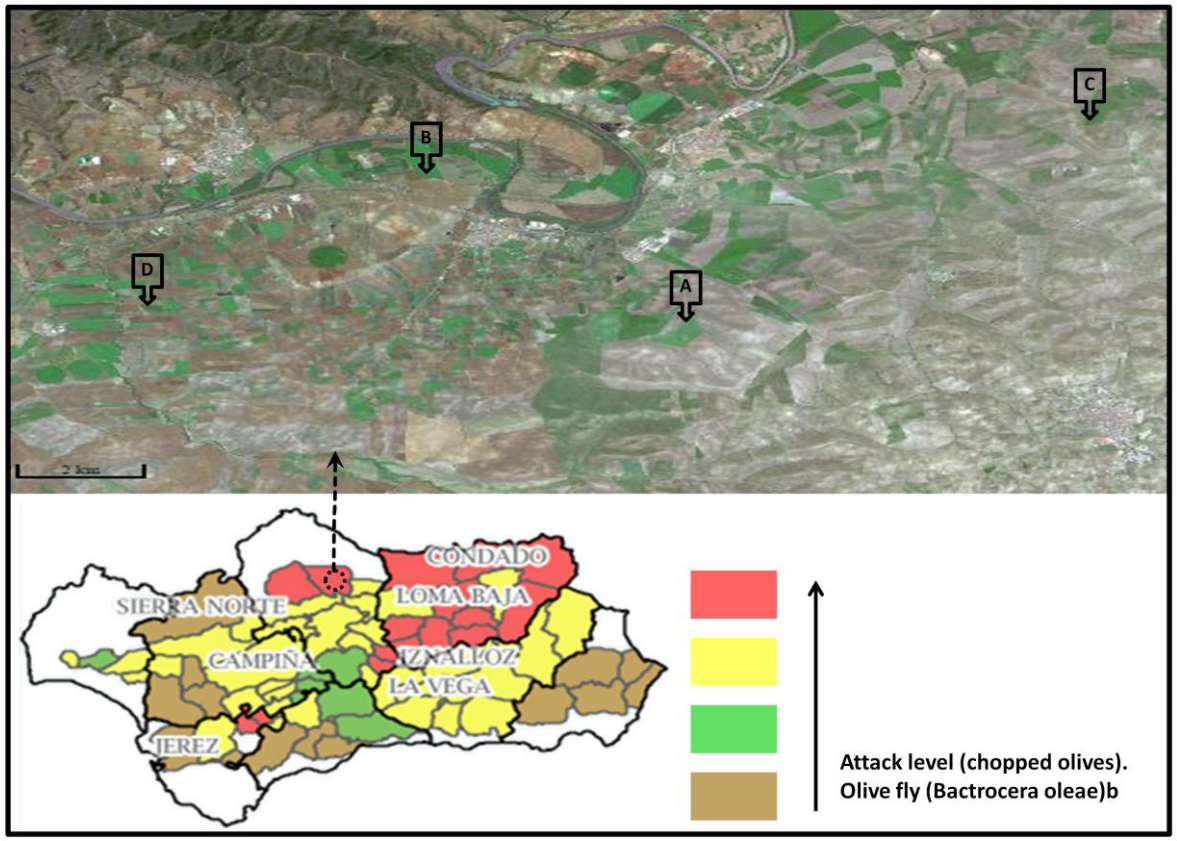

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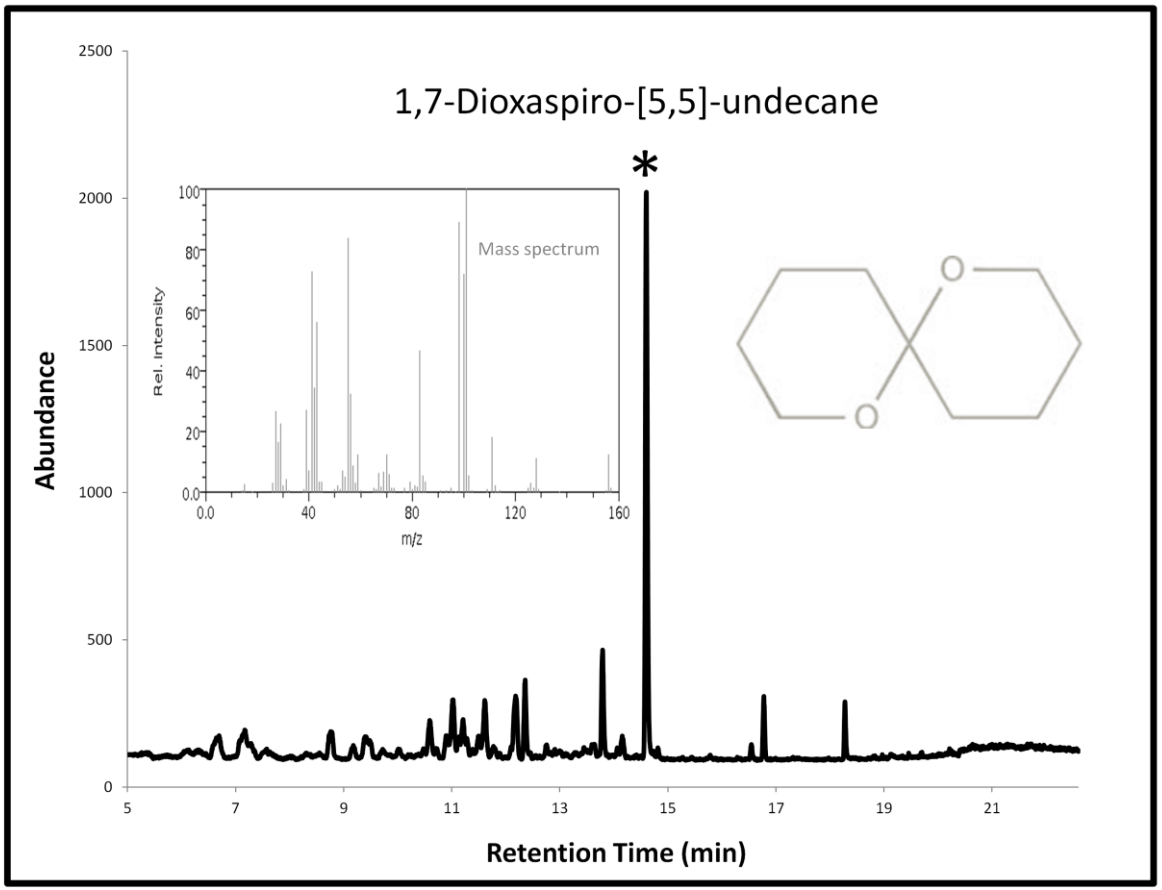

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