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A Chemical Approach to Obtaining α-copaene from Clove Oil and Its Application in the Control of the Medfly

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Abstract: The *Ceratitis capitata* (Wiedemann) fruit fly pest has been widely ecologically controlled by means of using attractant substances. This study supports the idea that α-copaene, a naturally occurring substance found in numerous plants, might be used as a semiochemical to control this pest. The possibility of obtaining this natural compound in abundant quantities may reveal its potential use in integrated pest management. The main goal of this study was to demonstrate, on a small scale, how the extraction of clove oil by fractional distillation and other laboratory-assisted techniques can facilitate the obtaining of abundant amounts of α-copaene for its use in the control of the medfly. As a result, the male attraction of α-copaene isolated from clove oil was confirmed to be 5–6 times higher than commercial trimedlure. In its field projection, five distilled fractions with an α-copaene content of less than 10% were shown to have from a quarter to half of the attractive power exerted by trimedlure on males. It can be concluded that the use of selected distilled fractions of α-copaene can be enough to obtain large quantities of this compound to be applied successfully in ecological programs to lure medflies.

Keywords: *Ceratitis capitata*; clove oil; α-copaene; trimedlure; attractant activity; semiochemical

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1. Introduction

In line with the European Green Deal 2050, the EU biodiversity strategy for 2030 highlights the necessity of recovering damaged ecosystems through lowering the usage and toxicity of pesticides [1,2]. Even though per-hectare pesticide use in Europe has been consistent since 1990, worldwide pesticide consumption roughly doubled between 1990 and 2010, then stabilized over 2010 to 2019 at a level of about six million tons of pesticides used per year [3]. These levels of pesticide consumption (e.g., mainly cover-sprayed or as toxic baits) reinforce the use of attractants in integrated pest management (IPM) for fruit fly control (e.g., attracticide technique, mass-trapping, or sterile insect technique (SIT)) [4,5]. However, other ecological strategies such as biological methods were also implemented (e.g., the use of parasitoids, predators, biological insecticides, or bacterial symbionts) [6–10]. As a result, the use of lures in fruit flies has become one of the primary ecological strategies for pest control [11,12]. *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) continues to have a major economic impact, since its larvae have more than 300 species of fruits and vegetables as hosts worldwide [13,14]. Despite the great efforts that have been made controlling this fly with attractants, today the complete natural components and the way of action of most natural pheromones are still not well known [4,11]. The composition of the *C. capitata* pheromone has been described as a blend of different compounds, a lot of them minor or unknown and perhaps essential in the power of attraction [15,16]. Ecological control and monitoring of *C. capitata* have traditionally been performed by using baits, including a pheromone-related compound and/or food attractants combined with

an insecticide (i.e., chemical or biological origin) in different trap types [17–23]. Female selective catching has been based mainly on food-related compounds [24–27]. Traps baited with trimedlure, a male attractant related to an antenna odorant-binding protein, has been used in the Mediterranean area during more than 40 years; hence, it was considered the best and most practical attractant for *C. capitata* [28,29]. A great effort has been made to increase the efficiency and longevity of this synthetic parapheromone (i.e., trimedlure) in the field [30–32]. Despite this, although effective in most cases, it has not always been enough to control the pest, or the costs were too high for it to be applied [5,33].

In the search for alternatives, it has been observed that exposure to volatiles from essential oils (EOs) boost medfly attractiveness or mating success when employed as semiochemicals, e.g., in SIT programs [34,35]. For example, ginger root oil, α–copaene-containing oils, and orange and other citrus oils enhance male performance in *C. capitata* [36–39]. In fact, the oily liquid hydrocarbon α-copaene has been demonstrated to have an attractive power between 2 and 5 times higher than trimedlure on medfly [40]. The roots and seed-extracted EO from *Angelica archangelica* L. was the first evidence of the attractant efficacy of α-copaene on *C. capitata* [41]. More than 800 pounds of this oil were used in approximately 50,000 traps to control the effectiveness of the fly eradication process in Florida in 1956 [42]. Both tricyclic sesquiterpenes, α-copaene and α-ylangene, which were isolated from this oil after fractionation, were recognized as their active compounds [41,43]. Despite these findings, α-copaene's commercial scalability is still unestablished today. The main reasons are because (*1*) although the presence of α-copaene has been described as a component in many plants' EOs [44], its relative content is usually very low [45]; and (2) the length of the currently used synthesis pathway increases its complexity and costs. So, it would be useful to find a way of obtaining α-copaene in abundant quantities for its field scaling. Avoiding the complex synthesis of this compound, among the known plants containing α-copaene in greater proportion is the clove tree, *Syzygium aromaticum* (L.) Merr. & L.M. Perry (Syn. *Eugenia caryophyllus*), a maritime tropical tree of the Myrtaceae family whose flower buds are used as a spice and is highly rich in oil and used worldwide in cooking and perfumery [46]. The world production of clove oil oscillates around 2000 t, of which Indonesia produces about half [47]. To support its safety, this EO is included in the list of compounds approved by the European Commission for its use in organic production [48]. In fact, among its properties, clove oil has a significant antimicrobial activity and important antioxidant activity, and it has repellent activity for some storage pests [46,49,50].

Based on this background, the goal of this work was to prove that clove oil is an abundant source that can be used successfully to extract α-copaene at commercial levels for use as a semiochemical on *C. capitata*. In this sense, the following working phases were planned: (*a*) An initial laboratory de-eugenolyzed clove essential oil (DCO, henceforth) fractional distillation and the study of α-copaene content in the obtained fractions; (*b*) A fractional distillation of an industrial fraction obtained as secondary by-product of β-caryophyllene industrial production and study of their α-copaene fractions content; (*c*) Obtention of α-copaene by open silica gel column chromatography and high performance liquid chromatography (HPLC), from the rich fractions of crude industrial products; (*d*) Study of the enantiomeric identity of α-copaene from DCO by enantioselective gas chromatography; and, (*e*) Study of the attraction of *C. capitata* males to the source oils and some selected fractions obtained.

2. Materials and Methods

2.1. Insects

Flies from a *C. Capitata* colony held continuously in the insectarium of the Centre for Agricultural Chemical Ecology (CEQA, Valencia) were used in the biological assays. Conditions of rearing were 27 ± 2 °C, 50–60% relative humidity, and a photoperiod of 16:8 (L:D). Larvae were fed with a mixture of wheat bran/sucrose/yeast autolysate/nipasol/nipagin/water/hydrochloric acid of 35% (20:5:1:0.5:0.5:10:0.1) (*w*/*w*).

Experiments were performed with emerged flies of 6–8 days old, held in the same insectarium conditions, and fed only with sucrose, yeast autolysate (4:1, *w*/*w*), and water.

2.2. Biological Assays

In this study, *C. capitata* males' attraction was tested with two products derived from clove oil and provided by the enterprise Acedesa (Murcia, Spain): (*1*) De-eugenolyzed clove essential oil (DCO) (Clove terpenes, Acedesa), which is used to obtain β-caryophyllene, a product widely used in perfumery; and (*2*) an Industrial Fraction (IF) (Crude caryophyllene No. 5060, Acedesa), which is a by-product in the industrial distillation process of the DCO corresponding to the first distillation fractions. Activity of both products, distillation fractions from DCO and IF, and α-copaene obtained from DCO were compared with trimedlure (TML) (98% purity) as a control that was provided by Agrisense (Pontypridd, UK).

Attractant activity of *C. capitata* was determined by introducing 100 males of 1-day old in a methacrylate cage of dimensions 30 × 30 × 40 cm^3 (wide × long × high) with the upper face open and covered with a cloth and keeping them in the conditions of the insectary. Assays were carried out in the morning (a.m.) with flies 6–8 days-old. The appropriate amount of each sample dissolved in 100 µL of n-hexane (HPLC grade) was put on dental cotton (height = 5 mm, Ø = 10 mm) and placed in a petri dish (50 mm Ø) in the center area of the base of the box. During the test time, after removing the food from the cage, the number of flies perched inside the petri dish was recorded every 3 min for 15 min. The attraction trials began with the lowest concentration, so as not to saturate the environment, and a minimum of 15 min was waited between each trial.

To compare the differences in the *C. capitata* male-attractive power between α-copaene obtained from DCO and TML, the dose used was 0.5 µg, and a dose of 5 µg was used to compare male attraction between TML, DCO, IF, and their respective distilled fractions. Each dose was tested with flies of three different generations, and, for each generation, each dose was tested 5 times in cages with one hundred males per $cage^{-1}$.

2.3. Obtention of Vacuum Distillation Fractions and Pure α-copaene

A vacuum-sealed, silver-plated column with dimensions of 40 cm long × 3 cm Ø i. and packed with Dixon rings was used to obtain the distilled fractions. An angled Bertrand collector–separator was used to maintain the vacuum during fraction collection. The vacuum was maintained at 5 mm Hg throughout the whole distillation. The chemical composition of distilled fractions was studied by gas chromatography (GC). A Fisons 8000 GC equipped with a Hewlett Packard HP-1 (crosslinked methyl silicone) column (25 m × 0.32 mm; film thickness = 0.52 µm) was used. Compounds were identified by their retention times and characteristic fragmentation patterns in mass spectra obtained by gas chromatography–mass spectrometry (GC–MS) and literary references [51]. The mass spectra were obtained by the electronic impact technique in a Varian Saturn II spectrometer, coupled with GC.

Extraction of α-copaene from DCO and IF fractions was performed by two methods: (*1*) *Open column.* The α-copaene separation was carried out with silica gel (63–200 µm) in proportion to 50 g g^{-1} of DCO and using hexane as eluent. Column monitoring was made by analytical thin-layer chromatography (silica gel 60 F254 chromatographs), using as revealing I_2; (*2*) *Preparative HPLC.* A Varian chromatograph was used, with a model 9065 pump and a model 9065 PolyChrom UV detector. The column used was LiChrosorb Si 60 of 7 µm (25 × 2.5 cm^2).

Purity confirmation of the isolated compounds was carried out in analytical HPLC [column Spherisorb, 5 µm (25 × 0.7 cm^2)] by studying them with eluents of different polarities. In parallel, a UV detector–photo diode array was used to record chromatograms at different wavelengths. UV spectra were obtained at three different times corresponding to the rising, apex, and falling of each chromatographic peak. Thus, a compound was considered pure when a single peak was recorded at the different wavelengths and the UV spectrum overlapped with the internal standard.

Infrared spectroscopy (IR) spectra were obtained as a liquid film on a Nicolet 710 FT-IR spectrophotometer, spanning the 4000–600 cm^{-1} region. V_{max} is indicated for the main absorption bands. One-dimensional nuclear magnetic resonance (NMR) (^{1}H, ^{13}C, and DEPT) and the correlation spectroscopy (COSY) spectra were recorded on a Varian Gemini 300 MHz spectrometer, in $CDCl_3$ as a solvent.

The enantiomeric identity of the α-copaene isolated from DCO was determined by enantio-selective gas chromatography. For this, a 30 m long × 0.25 mm Ø i. trifluoroacetyl gamma-cyclodextrin (G-TA) column was used. The chromatograph conditions were an initial temperature of 80 °C for 60 min, rising 1 °C min^{-1} up to 170 °C, and using He as carrier gas at a pressure of 35 kPa. The optical rotation values were determined in a PerkinElmer polarimeter using light with a wavelength corresponding to line D of the sodium emission spectrum, using $CHCl_3$ as a solvent. The concentrations were expressed in g 100 mL^{-1}.

2.4. Data Analysis

The male attraction of the studied compounds (i.e., DCO and IF, their respective distilled fractions, α-copaene and trimedlure) at different times was compared using a one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) multiple comparison test. The presumptions of normality and homoscedasticity were checked with the Shapiro–Wilk and Bartlett test, respectively. Data were log-transformed prior to ANOVA to satisfy conditions of equal variance. Non-transformed means and standard error (SE) are presented, and a p value less than 0.05 was considered statistically significant. The statistical Statgraphics software program (version XVIII, Statpoint Technologies, Inc., Warrenton, VA, USA) was used to perform the analysis.

3. Results

3.1. Obtaining Distillation Fractions Rich in α-copaene

3.1.1. De-Eugenolyzed Clove Essential Oil

Data corresponding to the composition of a total of seven oil distillation fractions obtained are shown in Table 1. In the results, DCO2 (2nd fraction) presented the highest concentration of α-copaene (6.06%), although DCO1 (5.79%) and DCO3 (3.48%) were also selected for attraction assays. All together, DCO1 to DCO3 fractions had a yield of 30.7% of DCO with an average content of 3.8% of α-copaene. Fractions from DCO4 to DCO6 represented 53.4% of DCO with a content in α-copaene of 1.5%. These less-enriched fractions could be used in redistillation to obtain new, more concentrated, fractions in α-copaene. The most abundant sesquiterpenes found in DCO composition were β-caryophyllene (87.63%) followed by α-humulene (8.46%) and α-copaene (2.12%).

Table 1. Distilled fraction composition of de-eugenolyzed clove essential oil (DCO).

Fraction	Vol (mL)	%	T[a] (°C)	α-cubebene (%)	α-copaene (%)	β-caryophyllene (%)	α-humulene (%)	δ-cadinene (%)
Total	500			0.76	2.12	87.63	8.46	1.03
DCO1	5.3	1.06	40–106	5.15	5.79	62.32	2.21	ND
DCO2	14.1	2.82	106–114	4.81	6.06	82.91	3.03	ND
DCO3	133.9	26.78	114–122	1.30	3.48	88.51	5.02	0.05
DCO4	82.7	16.54	122	0.54	2.3	89.21	6.58	0.28
DCO5	153	30.6	122	0.18	1.28	87.88	8.66	0.51
DCO6	31.5	6.3	122	ND	0.52	85	13	1.03
DCO7	79.5	15.9	–	ND	ND	52.19	16.63	8.24

ND: not detected.

3.1.2. IF Industrial Fraction

This cheaper commercial fraction contained 4.62% of α-copaene. Twenty distilled fractions were obtained but after their analysis with GC were reunited in three fractions

(IF1, IF2, and IF3) according to its content in α-copaene (Table 2). Two fractions (IF1, IF2) very rich in α-copaene were obtained; between them they contained 23% of the total α-copaene of the IF and had a richness in α-copaene of 8.04%. IF3 was the most abundant and contained only 4.1% of α-copaene.

Table 2. Distilled fraction composition of the industrial oil (IF).

Fraction	Vol (mL)	%	T[a] (°C)	α-cubebene (%)	α-copaene (%)	β-caryophyllene (%)	α-humulene (%)
Total	500			2.46	4.62	86.27	6.02
IF1	16	3.2	68–109	10.02	9.75	65.63	1.78
IF2	50	17.6	109	5.12	7.50	82.00	2.63
IF3	396	79.2	–	1.93	4.10	86.68	6.23

3.2. Isolation of α-copaene

The separation of α-copaene and β-caryophyllene was carried out with very good yields by open column and preparative HPLC.

3.2.1. Open Column

1. DCO. A practically pure fraction in α-copaene was obtained eluting with hexane the initial DCO. This fraction had a richness of 99% (GC) and a yield of 85% of the $DCO_{\alpha\text{-copaene}}$, corresponding to 1.8% of the total oil. In Figure 1, the analytical HPLC chromatogram is shown. This $DCO_{\alpha\text{-copaene}}$ was used in attraction tests. In the following fractions, α-cubebene, β-caryophyllene, and α-humulene were separated. The β-caryophyllene fraction had a richness of 98% and contained 85% of the total $DCO_{\beta\text{-caryophyllene}}$.

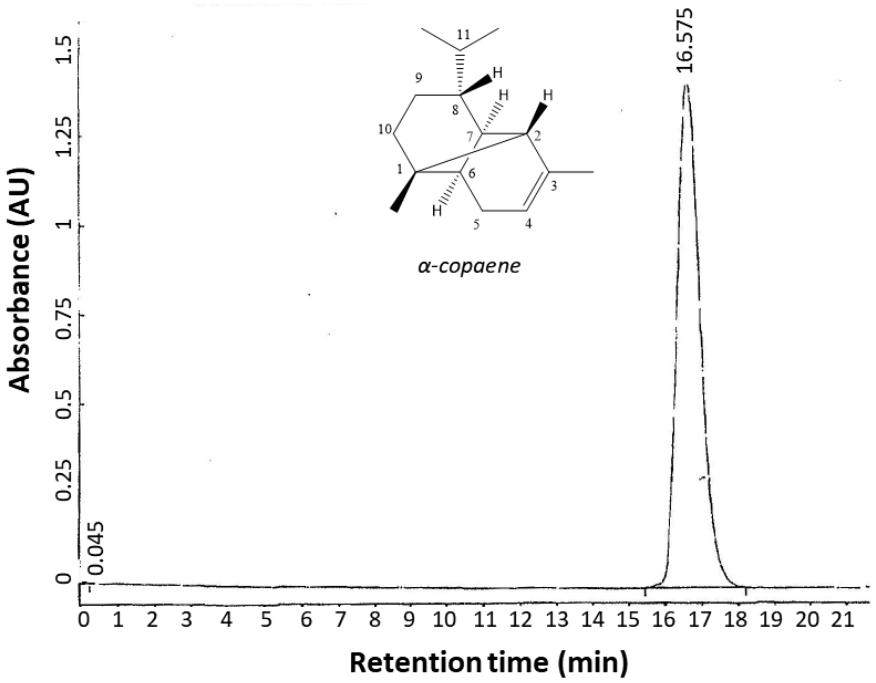


Figure 1. Analytical HPLC chromatogram of α-copaene. Column Spherisorb, 5 μm (25 × 0.7 cm^2); Hexane, 1.5 mL min^{-1}. Detector UV Varian 9065 PolyChrom 229 nm.

Characterization of α-copaene was performed by IR spectra, ^1H NMR, ^{13}C NMR, and MS according to the following parameters: $[\alpha]^{25}_D = -2.36°$ (*c* 0.89, $CHCl_3$); IR: ν_{max} 3029, 1665 y 786 cm^{-1}; ^1H NMR: δ_H 5.2 (sa, 1H, C=CH), 2.2–2.0 (m, 3H, H-2 + H-5), 1.7 (sa, 3H, $C=CCH_3$), 0.8 [dd, *J* = 7, 5 Hz, 6H, $CH(CH_3)_2$] y 0.7 (s, 3H, CCH_3) ppm; ^{13}C NMR: δ_c 143.9

(C_6), 116.0 (C_5), 54.2 (C_4), 44.7 (C_1), 44.3 (C_9), 39.4 (C_2), 36.9 (C_{10}), 36.2 (C_{11}), 32.2 (C_7), 30.0 (C_3), 23.1 (C_8), 21.8 (CH=CCH3), 19.9, 19.6 y 19.3 (3xCH3) ppm; MS: m/z 204 (M^+,20), 161 (96), 133 (11), 119 (100), 105 (93), 93 (48), 77 (20) y 55 (21).

Also, a practically pure fraction in α-copaene was obtained with the same solvent from DCO1- to DCO4-gathered fractions. This fraction had a richness in α-copaene of 99% (GC) and a yield of 89%, corresponding to 2.9% of the total of the four fractions collected. DCO1- to DCO4-gathered fractions had a β-caryophyllene yield of 90%, containing 97% of the total $DCO_{\beta\text{-caryophyllene}}$.

2. IF industrial fraction. A practically pure fraction in α-copaene was obtained with a richness of 99% (GC) and a 90% yield of the $IF_{\alpha\text{-copaene}}$, corresponding to 3.7% of the total IF. The β-caryophyllene fraction contained 98% of total $IF_{\beta\text{-caryophyllene}}$, with a 92% yield.

3.2.2. Preparative HPLC

Isolation of α-copaene was performed by HPLC from the DCO1 + DCO2 + DCO3 and IF fractions. The chromatogram corresponding to the DCO1 + DCO2 + DCO3 fraction is shown in Figure 2 (a similar chromatogram was obtained with the IF fraction). Both fractions were remarkably rich in β-caryophyllene. In both cases, the α-copaene fraction obtained was practically pure (99.5%, GC).

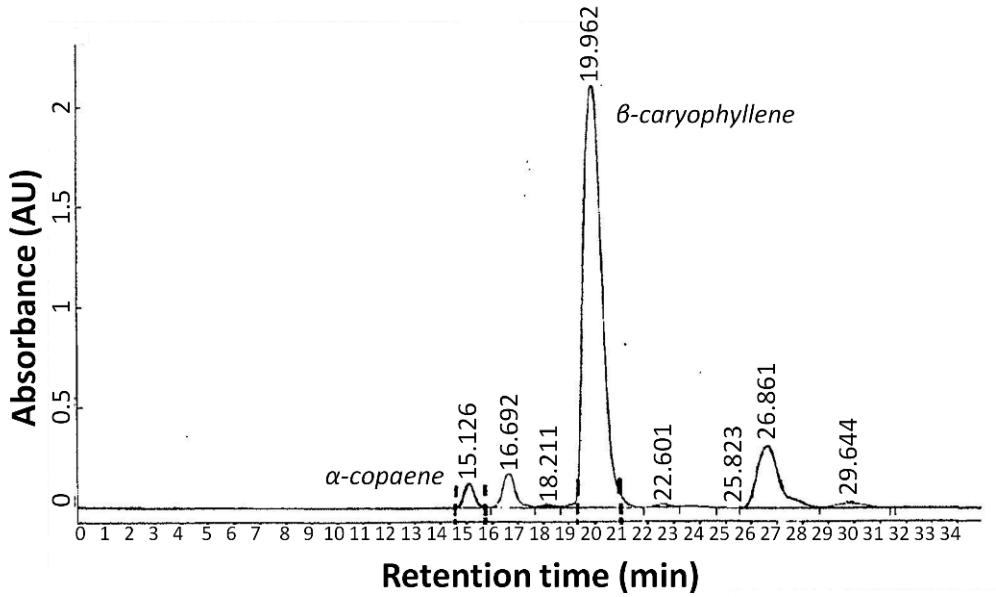
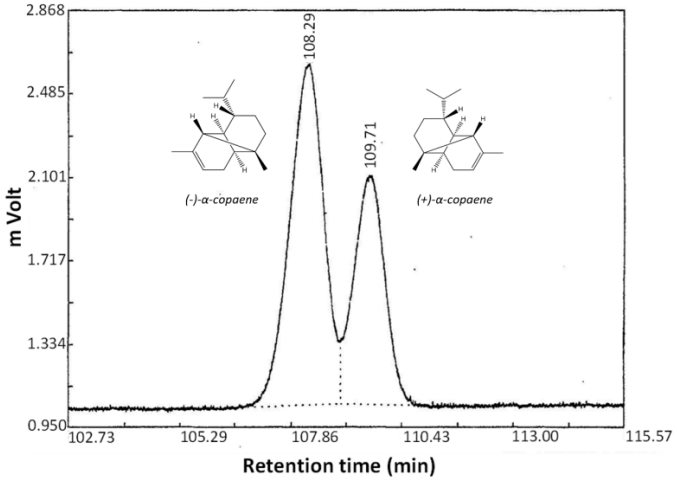


Figure 2. HPLC chromatogram of DCO1 + DCO2 + DCO3. Column LiChrosorb Si 60 of 7 μm (25 × 2.5 cm^2); Hexane, 5.0 mL min^{-1}. Detector UV Varian 9065 PolyChrom 224 nm.

3.2.3. Chiroptical Characterization of the α-copaene from the DCO

Chiroptical parameters obtained from the extracted $DCO_{\alpha\text{-copaene}}$ were $[\alpha]^{25}_D = -2.12°$ (c 0.89, $CHCl_3$), corresponding to a 67.6% portion of the enantiomer (−) and 32.4% of the enantiomer (+), taking as $[\alpha]_D$ value for the (+)-α-copaene +6.0°; considering the literature for the (+)-α-copaene: $[\alpha]_D$ = + 6.6°, c 0.8 [52]; $[\alpha]_D$ = + 5.29°, c 2.13 [53]; $[\alpha]_D$ = + 6.4°, c 1.20 [54]. These results were confirmed by gas–liquid chromatography with a quiral column as shown in Figure 3. Commercial (−) enantiomer (98% purity) was injected, its retention time being 108.29 min. Subsequently, α-copaene isolated from the DCO was injected, obtaining two peaks. The integration of their areas provided the following data: 62.37% of the (−) enantiomer and 37.63% of the (+) enantiomer, data in agreement with the value of the rotational power obtained.



Compound	Retention time (min)	Area (mV/Sec x 10)	Area (%)
(-)-α-copaene	108.29	873119	62.37
(+)-α-copaene	109.71	526791	37.63

Figure 3. Enantiomeric gas chromatography separation of α-copaene. Column G-TA 30 cm × 0.25 mm; 80 °C (60′), 1 °C min^{-1} until 170 °C; He 35 kPa.

3.3. Attraction Assays

The optimal test doses were previously determined to compare the differences in the *C. capitata* male-attractive power between α-copaene and trimedlure. As a result, an optimal dose for laboratory tests of 0.5 µg was obtained, since, in the tests carried out with 1 µg, the fly count could not be done reliably, because there were many flies attracted (>50%). In Figure 4 is shown the results of attracting males of *C. capitata* when applying 0.5 µg of both attractants (i.e., α-copaene and trimedlure) with a purity greater than 99% (α-copaene obtained from DCO by open column). It was observed that after 15 min the α-copaene was between 5 and 6 times significantly more active than the TML.

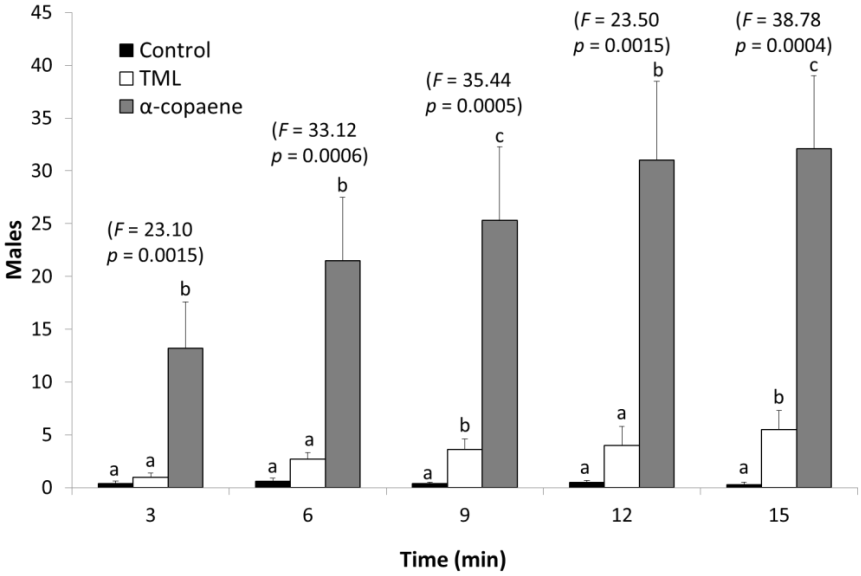


Figure 4. *Ceratitis capitata* male comparative attraction between trimedlure and α-copaene. Concentration of attractants was 0.5 µg. Values represent means ± SE (n = 3) with different generations. Each repetition (n) corresponds to the average of 5 cages with one hundred males $cage^{-1}$. Different letters for each time indicate statistically significant differences (One-way ANOVA, $p < 0.05$). All reported F values have (2, 6) degrees of freedom.

Figure 5 shows the attraction results using as attractants DCO, three fractions of its distillation (i.e., DCO1, DCO2, and DCO3; which have 2.73, 2.85, and 1.64-fold more α-copaene content than the source oil, respectively; see Table 1), the IF oil and the first two fractions obtained from its distillation (i.e., IF1 and IF2 with an α-copaene content of 2.11 and 1.62-fold more α-copaene content than the starting fraction, respectively; see Table 2), trimedlure, and the control without any attractant included. Among the results, the IF1 and IF2 subfractions were the most active on medfly attraction to the target area after TML. IF1 differed significantly from all previous ones from 9 min. The attractive power of IF1 and IF2 was approximately half that of TML. The DCO1, DCO2, DCO3, and IF fractions had approximately a quarter of the attractive power of TML. This lesser attraction is justified because DCO and IF oils, and their respective fractions, contained a lower proportion of α-copaene.

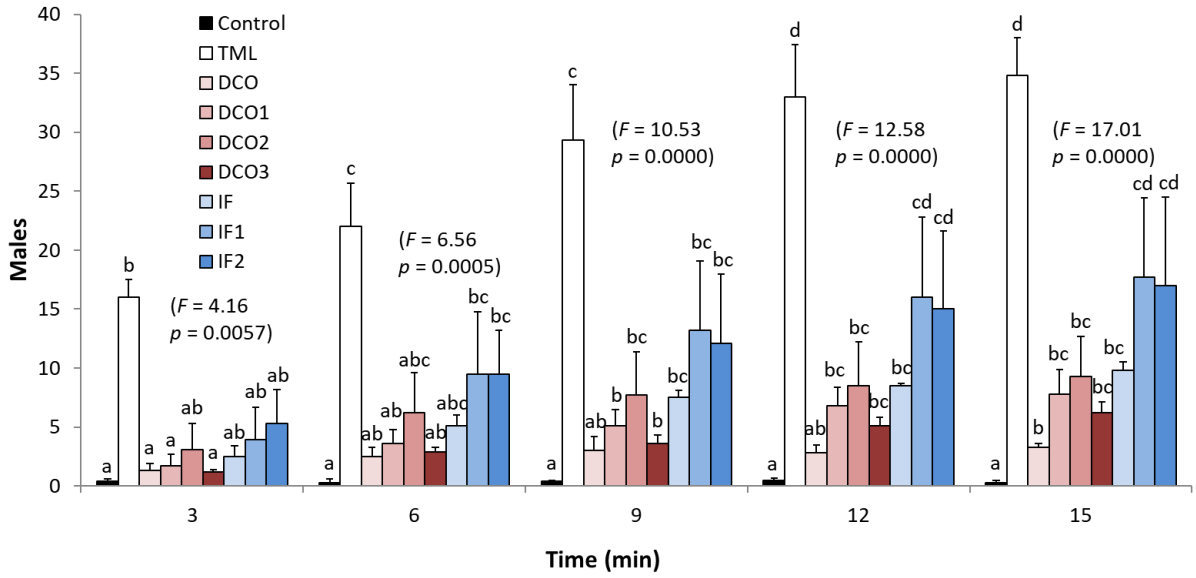


Figure 5. *Ceratitis capitata* male comparative attraction between trimedlure (TML), de-eugenolyzed clove essential oil (DCO), industrial fraction (IF), and their respective distilled fractions. Dose of the attractants was 5 µg. Content of α-copaene (µg) in the samples was (0.103) DCO, (0.29) DCO1, (0.303) DCO2, (0.19) DCO3, (0.173) IF, (0.487) IF1, and (0.375) IF2. Values represent means ± SE (n = 3) with different generations. Each repetition (n) corresponds to the average of 5 cages with one hundred males cage^{-1}. Different letters for each time indicate statistically significant differences (One-way ANOVA, $p < 0.05$). All reported F values have (8, 18) degrees of freedom.

4. Discussion

Most ecological methods applied currently to control fruit flies require the use of attractants [19,29,55,56]. The use of powerful and targeted attractants has become almost essential to control *C. capitata*. In the Mediterranean area, attractants are used basically in: *1.* monitoring to quantify the growth of the pest population and choose the most effective period for actions (i.e., widespread application, included SIT); *2.* mass capture procedures, placing a high density of traps with the objective of catching and killing as many adults (i.e., males and females) as possible; generally combining an effector (i.e., insecticides, insect growth regulators, sterilants, or pathogenic organisms) with a lure (i.e., food attractants like putrescine, trimethylamine, and ammonium acetate, combined with a semiochemical); and, *3.* lure and kill (i.e., attracticide), usually using a protein hydrolysate-insecticide in combination with a semiochemical (optional) and applied by pulverization or using attractant devices [57]. In practice, mass trapping is difficult (i.e., mainly because a high density of traps requires high costs and has low attraction for females, and due to the saturation and efficiency of traps), which makes the attracticidal technique the most widely used on a small scale in combination with other methods (i.e., chemical or biological). SIT is the most promising and the one that has given the best results, although it requires high costs for its implementation. The standard

parapheromone for monitoring and trapping male medflies continues still to be trimedlure (tert-butyl 4-chloro-2-methylcyclohexane-1-carboxylate). Although effective, it is short-lived; hence, the release rate has directly been related to increasing the temperature [32,58]. There are still significant efforts being made to extend the life of trimedlure dispensers in the field, since typical maximum efficacy does not exceed six weeks [59–61]. This forces in most cases making replacements of the dispensers with a significant increase in costs, especially under warm climate conditions (i.e., 28–43 °C) [62]. Also, medfly females are not attracted to either TML or α-copaene [52,63].

As an alternative, it is widely known that many EOs possess compounds that act with semiochemical activity (e.g., pheromone or allelochemical) capable of modifying the behavior of many species of insects [64–66]. In this sense, this study used clove oil as a source of α-copaene and verified its attractant efficacy on *C. capitata*. A background of difficulties with the chemical synthesis of α-copaene was revealed by some research. In fact, racemic α-copaene was synthesized for the first time by a 17-stage route based on a reaction of ketone-base-catalyzed cyclization [67–69]. Subsequently, other complex workarounds were devised, for instance, the conversion of a carvacrol-based monoterpenoid or the intramolecular cycloaddition of a dienyl-substituted 3-oxidopyrylium [70,71]. Recently, some biotechnological approaches have been successfully implemented, such as the over-expression of a potato α-copaene synthase gene, resulting in enhanced levels (i.e., up to 15-fold higher than controls) or the α-copaene synthesis from glucose by *Escherichia coli* transformation with a gene that heterologously expressed *Piper nigrum* α-copaene synthase [72,73]. These novel bioengineered approaches are promising, but their feasibility should be verified in pest control. Moreover, the extraction of α-copaene directly from plants, although present in many species, has the difficulty that its content is usually low [44,52].

Trying to solve these issues, in this work, a chemical approach has been performed using fractional distillation, a method that is extensively used in industry (e.g., biorefineries, desalination, or separation of contaminants, among many others) and whose base is the separation of components by their boiling points (i.e., using heat) [74–78]. In fact, some variants like hydrodistillation (HD) and steam distillation (SD) have been one of the most widely used techniques in the extraction of EOs; and successfully applied to clove oil extraction [46,79–82]. However, some other innovative techniques have also been applied to extract EOs from clove oil, such as microwave-assisted hydrodistillation, microwave-assisted steam distillation (i.e., MDAs), hydrodistillation assisted by ohmic heating (HDA), or supercritical fluid extraction assisted by cold pressing (SFEA). These modern techniques saved extraction time compared to conventional methods (i.e., cold pressing, HD, or SD), although not differing in major components' composition (i.e., eugenol, β-cariophyllene, α-humulene, and eugenyl acetate in clove oil), and yields varied with conditions (i.e., temperature, time, and power); however, no results on α-copaene were provided in this research [46,83,84]. Related to this, it was possible to extract low percentages of copaene (although it is not indicated which, α or β stereoisomer) with SD (3.41%) and superheated-SD (0.55%) techniques [85]. The yield and quality of extracted EOs from plants depends not only on the extraction method itself but many other factors, like plant organ used (e.g., aerial parts, inflorescence, or roots), agronomic practices (e.g., fertilization, irrigation, date of harvest), or cultivation location (e.g., microclimate, soil composition) [46]. As a handicap, the distillation method, although easy to operate and highly reproducible, requires a long extraction time and large volumes of solvent and energy [46]. At the industrial level, distillation necessitates a thoroughly rigorous process control and systems optimization to be effective and prevent contamination [86]. In this research, based on this background, the fractional distillation process has made it possible to concentrate clove oil into fractions with different contents in α-copaene, despite the difficulties of using plants as a source of this compound. In fact, the major component of clove oil is eugenol (ca. 50–70%), followed by eugenyl acetate, β-caryophyllene, and α-humulene (these three components together ca. 10–40%), with the α-copaene content not exceeding 10% (i.e., within minority compo-

nents) [46,87]. Clove oil has been tested in the field as a source of attractant pheromones to *C. capitata*. Moustafa et al. [88] used white Jackson traps with a piece of cotton saturated with diluted clove oil concentrations (12.5, 25, 50, 75, and 100%) in paraffin oil. Clove oil at a concentration of 50% produced the highest attraction for males compared with the other different concentrations of clove oil or trimedlure, but the components responsible for the attractiveness of the clove oil were not specified. Also, clove oil was demonstrated to be effective in the attraction of other related tephritids [89]. However, contrarily, clove oil has also an important property as a biopesticide [90–92]. As a result, in this study, a variety of fractions were obtained, which, in the case of the distillation of the DCO, turned out to have a content in α-copaene between 1.64 to 2.85-fold more concentrated than the original oil (i.e., corresponding to the first three fractions); and, in the case of fractions corresponding to the distillation of IF between 1.62 to 2.11-fold (i.e., corresponding to the first two fractions). Other techniques applied in this research to obtain α-copaene in a simpler way from clove oil were the use of an open column and preparative HPLC; both allowed obtaining practically pure fractions of α-copaene, although their implementation at the industrial level is currently unsustainable.

Regarding chirality, the fractions obtained turned out to be a mixture of both enantiomers, corresponding 67.6% to (−)-α-copaene and 32.4% to (+)-α-copaene (i.e., its left-handed and right-handed forms, respectively). The (+)-α-copaene has been described as the most effective in the attraction of medfly males, together with (−)-ceralure-B1, a non-natural iodinated cyclohexane ester, with similar effectiveness [93,94]. In fact, the first isolated form extracted from commercial angelica seed oil was also proved to be dextrorotatory and between two and five times more attractive to *C. capitata* than trimedlure [40]. However, the most common form seems to be the left-handed and therefore the most abundant, having been possible to obtain α-copaene extraction with good yields (e.g., from copaiba root oil) [54]. In addition, (−)-α-copaene demonstrated in field assays to have an attractant power like the right-handed enantiomer in the laboratory (i.e., 5–8 times higher) with a persistence of 1 mo. at an individual dose of 100 µL, with the possibility of increasing it to reach low populations [95]. In fact, by increasing electro-antennographic signaling activity on the medfly, exposure to volatiles from EOs (i.e., terpenes, terpenoids, and aromatic and aliphatic components) improved the success of male mating [34,38,46,96]; this behavior has been also observed, for example, in other tephritids (e.g., *Bactrocera oleae*) and some species of beetles [97–99].

Although the results of this research are hopeful, more studies are required to implement its usage at an industrial level and confirm their economic viability. According to the recent studies explained above, it is possible to extract clove oil using other techniques (e.g., MDAs, HDA, or SFEA) or biological methods (e.g., genetic engineering). However, most studies performed until now were small-scale. Further research is required to scale these methods at an industrial level and apply them in pest control to confirm α-copaene attractant efficacy under different field conditions. In any case, this study supports the use of clove oil as a source of α-copaene with a viable chemical approach and confirms the findings of previous research about its effectiveness in the attraction of *C. capitata*.

5. Conclusions

Male attraction induced by clove oil and some distilled fractions on *C. capitata* was studied to determine its possible field scaling. Four fractions richer in α-copaene (5.79%, 6.06%, 3.48%, and 2.3%) than the starting clove oil (2.12%) were obtained. The first three fractions had a quarter of the attractive power of TML, constituting an industrially interesting by-product for use as attractants for *C. capitata*. The clove oil industrial fraction (i.e., IF) contained 4.62% α-copaene. The first two fractions of IF-distillation were very rich in α-copaene, with 9.75% and 7.5%, respectively. The attractive power of these subfractions was approximately half that obtained with TML, also constituting an interesting industrial by-product as an attractant for this pest. In addition, the separation of α-copaene and β-caryophyllene was carried out with very good yields by open column and by preparative

HPLC. In this way, it was possible to isolate α-copaene by open column from the DCO, DCO1 to DCO4 together, and IF with yields of 85%, 89%, and 90%, and with a richness of 99%. Yields were not higher with preparative HPLC. The pure α-copaene isolated from DCO (i.e., 62.37% of the (−) enantiomer plus 37.63% of the (+) enantiomer) was between five and six times more active than TML in attracting *C. capitata* males. Further assays should be done to study the additive, synergistic, or antagonistic interaction between enantiomers. Consequently, considering the results obtained, the application of industrially distilled clove oil as male-medfly attractant is relevant and could be useful in integrated pest control programs to control this pest.

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