

1 Assignment-free chirality detection in unknown samples via  
2 microwave three-wave mixing

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7 **Abstract**

8 Straightforward identification of chiral molecules in multi-component mixtures of unknown composi-  
9 tion is extremely challenging. Current spectrometric and chromatographic methods cannot unambigu-  
10 ously identify components while the state of the art spectroscopic methods are limited by the difficult and  
11 time-consuming task of spectral assignment. Here, we introduce a highly sensitive generalized version of  
12 microwave three-wave mixing that uses broad-spectrum fields to detect chiral molecules in enantiomeric  
13 excess without any prior chemical knowledge of the sample. This method does not require spectral as-  
14 signment as a necessary step to extract information out of a spectrum. We demonstrate our method by  
15 recording three-wave mixing spectra of multi-component samples that provide direct evidence of enan-  
16 tiomeric excess. Our method opens up new capabilities in ultrasensitive phase-coherent spectroscopic  
17 detection that can be applied for chiral detection in real-life mixtures, raw products of chemical reactions  
18 and difficult to assign novel exotic species.

## 19 Introduction

20 Many biomolecules, including DNA, proteins, and amino acids, are chiral, meaning they exist in two versions  
21 that are non-superimposable mirror images. Chirality is such a ubiquitous property in biology that more  
22 than 50 percent of active ingredients in pharmaceuticals are chiral [1]. Chiral molecules span other multi-  
23 billion dollar industries like the food industry, agriculture, and fragrances. In 2016, the first chiral molecule  
24 was detected in space[2], re-sparking conversations on the implications of molecular chirality for the origins  
25 of life. However, current established methods cannot determine enantiomeric excess, a signature of life, in  
26 complex raw samples like the ones collected from extraterrestrial environments.

27 Despite these broad applications, a general method for detecting and measuring enantiomeric excess  
28 remains elusive. While notable progress has been made towards detection of slight enantiomeric excess on  
29 the 0.4% level [3], detection of enantiomeric excess in unknown complex samples has proven challenging.  
30 Chromatography has long been the go-to method for enantiomeric analysis among synthetic chemists, how-  
31 ever, as detection is based on chemical interactions, it cannot be generalized to unknown samples. Mass  
32 spectrometry and Nuclear Magnetic resonance (NMR) rely on chiral derivitization reagents and can be sen-  
33 sitive to contaminants[4–6]. For unknown multi-component mixtures, polarimetry can be inconclusive, as  
34 the calculation of specific rotation requires knowledge of concentration and it is often referenced to neat  
35 samples[7–9].

36 Spectroscopic methods such as vibrational, photoelectron circular dichroism[10–14], and microwave spec-  
37 troscopy [15–23] can be mixture compatible and provide highly accurate information on species identity. So  
38 far, these methods have been limited by spectral assignment; prior to any chirality experiment, the spectrum  
39 of the molecule had first to be collected and fully assigned. Considerable efforts have been made to automate  
40 and simplify spectral assignment[24–27]; nonetheless, it is still a difficult and time-consuming task conducted  
41 mainly by trained spectroscopists.

42 In this work, we demonstrate a generalized assignment-free version of microwave three-wave mixing  
43 (M3WM)[28, 29] that can identify chiral species in enantiomeric excess in unknown complex samples. We  
44 achieve this by exploiting our high sensitivity and employing broadband excitation pulses to search experi-  
45 mentally for transitions in a three-level system, along with implementation of careful cancellation schemes  
46 to ensure that signals from species not in enantiomeric excess are subtracted. The resulting spectra, referred  
47 to here as “three-wave mixing spectra”, can provide direct proof on the existence of chiral species in enan-  
48 tiomeric excess and can be used for the study of previously hard-to-analyze samples: unassigned species and  
49 unknown complex mixtures.

### 50 Broadband three-wave mixing

51 Our broadband assignment-free three-wave mixing uses broadband microwave and RF excitation com-  
52 bined with careful cancellation schemes. The resulting three-wave mixing spectra include numerous tran-  
53 sitions from each chiral molecule that is present in enantiomeric excess. Each of those transitions stem  
54 from a M3WM excitation scheme, an example of which is shown in Figure 1(b). It is a three-level system  
55 of rotational energy levels that are connected via an *a*-type transition, a *b*-type transition, and a *c*-type  
56 transition, along each of three rotational axes. Two of these transitions are typically in the GHz frequency  
57 range, and the third transition is on the order of 100 MHz. Following the notation in [28], the stimulated  
58 microwave transition (with frequency  $\sim 10$  GHz) is referred to as the “drive” transition and the stimulated  
59 RF transition (with frequency  $\sim 100$  MHz) is referred to as the “twist” transition. The molecular ensemble  
60 emits radiation coherently at the “listen” frequency, which is detected and plotted as a spectrum. Previous  
61 M3WM experiments were limited to assigned, known species, required prior knowledge of the transitions,  
62 and reported enantiomeric excess based on a single excitation scheme like the one shown in Figure 1(b)[28–  
63 32].

## 64 Results

65 **M3WM spectra of chiral and non-chiral species.** Figure 2 highlights the difference between microwave  
66 spectra and three-wave mixing spectra. Figure 2(a) shows the microwave spectrum of a mixture of a chiral  
67 molecule ((R)-myrtenal) and a non-chiral molecule (benzyl alcohol), compared to spectra of the individ-  
68 ual components. In this frequency range, numerous rotational transitions from both species are present.  
69 Figure 2(b) shows the three-wave mixing spectrum of the same mixture, taken under similar conditions.  
70 Three-wave mixing spectra are non-zero only for chiral molecules in enantiomeric excess. Only transitions

71 from enantiopure R-myrtanal are observed, as transitions from the non-chiral benzyl alcohol do not survive  
72 subtraction. It is noticeable that the transitions in the three-wave mixing spectrum are significantly fewer  
73 in number than the lines in the microwave spectrum of myrtanal, which is expected as not all transitions  
74 can participate in a M3WM chirality detection scheme, like the one showed in Figure 1(b).

75 Both spectra were recorded at 7 K, from 13000-18250 MHz and a He buffer gas flow of 10 sccm. The  
76 M3WM spectrum is assembled from 485 individual spectral segments, with 22.5 MHz local oscillator steps  
77 between them and acquired with a 35 MHz broadband drive pulse and an RF pulse with a range of 60-105  
78 MHz for a total integration time of 3.5 h.

79 **M3WM spectrum of racemic samples.** M3WM spectra are designed to detect species in enantiomeric  
80 excess. Figure 3 shows the comparison between the M3WM spectrum of an enantiopure sample of (R)-1,2-  
81 propanediol, shown in blue, plotted against the M3WM spectrum of a racemic sample of 1,2-propanediol, in  
82 red. The M3WM signal of enantiopure (R)-1,2-propanediol shows three noticeable signals corresponding to  
83 the lowest and the third-lowest in energy (0.88 kJ/mol) conformer[33]. In contrast, these three-wave mixing  
84 signals are not present in the spectrum of the racemic sample, in red, which has been shifted by -15 (a.u) on  
85 the y-axis for clarity. The details of the methods used to eliminate non-chiral signals are described in detail  
86 below.

87 Both spectra were recorded in the range between 14500-15100 MHz with a He buffer gas flow of 10 sccm,  
88 at 7 K. Each spectrum is assembled from 72 individual spectral segments, with 22.5 MHz local oscillator  
89 steps between them and acquired with a 35 MHz wide drive pulse and a twist pulse range of 80-105 MHz  
90 for a total integration time of 1 h.

91 **M3WM spectrum of multi-component mixtures.** Three-wave mixing spectra can provide useful  
92 chirality information of multi-component mixtures without any prior chemical processing, separation, or  
93 spectral assignment. This capability is relevant to asymmetric synthesis and chemical analysis of complex  
94 real life samples. In Figure 4, we show the three-wave mixing spectrum for a mixture of terpenes. Terpenes  
95 are naturally occurring chiral building blocks that have been used for decades as starting materials for the  
96 synthesis of natural products and active ingredients in pharmaceuticals, due to their abundance and low  
97 cost[34–36]. All transitions in Figure 4 belong to enantiopure (-)- $\beta$ -pinene, (R)-fenchone, and (R)-carvone.  
98 The inset zooms into the transition around 16492 MHz which consists of two separate M3WM signals: a  
99  $\beta$ -pinene M3WM signal at 16491.7 MHz and a second one from fenchone at 16492.5 MHz. For such mixtures,  
100 even polarimetry measurements can be inconclusive, as the sum of the angles for multiple components can  
101 cancel each other out. Equal amounts of neat (-)- $\beta$ -pinene, (S)-carvone, and (R)-fenchone would have a total  
102 specific rotation  $[\alpha]_{20}^D$  of  $+7^\circ$ , the sum of each component, which carries significantly less chemical information  
103 than a spectrum. On the contrary, the three-wave mixing spectrum of such mixture, as seen in Figure 4,  
104 shows distinct transitions for each separate species. If microwave spectra of the species are available, even if  
105 unassigned, then no additional measurements are required to determine the exact identity of the species. For  
106 readily available chiral building blocks like the ones used here, species were easily and accurately identified.  
107 The spectrum of the mixture was recorded in the range between 16200-18000 MHz with a He buffer gas flow  
108 of 10 sccm, at 7 K. Each of the 150 spectral segments was recorded with a drive pulse of 35 MHz bandwidth.  
109 The total acquisition time was 2 h. Two separate twist ranges of 65-85MHz and 85-105MHz were used for  
110 increased RF power to assure transitions of less polar species are sufficiently driven.

## 111 Discussion

112 Three-wave mixing spectra can be recorded for any sample that contains molecules that are vaporizable and  
113 have non-zero electric dipole moments across all rotational axes. Microwave spectroscopy is mixture, solvent,  
114 isomer, and isotopologue compatible meaning that no chemical processing is necessary in most cases prior  
115 to analysis. Chiral information can be extracted on-the-spot as only transitions from species that are chiral  
116 and in enantiomeric excess survive cancellation. As shown in figures 2 and 3 signals from racemic samples  
117 or non-chiral molecules average to zero.

118 A promising application would be the direct chiral detection of the raw constituents of one pot asymmetric  
119 synthesis reactions, similar to ones by [37, 38]. Inside this flask, there are: reactants, solvents, products,  
120 by-products, and catalysts. Even though large polyatomic molecules like catalysts can't be easily seen, a  
121 comparison between the three-wave mixing spectrum before and after the reaction can identify any new

122 chiral products, in enantiomeric excess produced, as in figure 4. Even molecules very similar in structure like  
123 terpenes can be unambiguously identified with microwave spectroscopy. Since any separation or purification  
124 of the sample is unnecessary for analysis, our method can act as a tool for the general search of chiral catalysts.  
125 Unlike polarimetry, once the spectra are acquired the exact transitions can be used to unambiguously identify  
126 the species produced.

127 Microwave three-wave mixing works best for strongly polar molecules as the matrix elements for rotational  
128 transitions depend linearly on the magnitude of the dipole moments across the A, B, and C rotational axes[28].  
129 In this work, beta-pinene with dipole moments of  $|\mu_a| = 0.43$ ,  $|\mu_b| = 0.58$ ,  $|\mu_c| = 0.11$  Debye was the least  
130 polar molecule under study [39]. Even though enantiopure samples were used for all experiments, three-wave  
131 mixing signals scale linearly with enantiomeric excess (ee), thus signals from species of enantiopurity above 30  
132 percent should be sufficiently above noise level to be detected. This could be improved with straightforward  
133 electronics updates.

134 An important parameter of the experiment is the frequency range of the twist pulse. We know from experience  
135 that most molecules display transitions between 60-110 MHz so we chose to use this range for the “twist”  
136 pulse during all data acquisition. However, for a more complete analysis of unknown samples additional  
137 frequency ranges can be easily explored. We have encountered no chiral molecule without three-wave mixing  
138 transitions with a twist between 25-250 MHz, which is the range of our current RF amplifier: our method is  
139 thus expected to detect any common vaporizable small chiral molecule. Additionally, as molecules grow in  
140 size, their microwave spectra get more congested and they should typically exhibit richer M3WM spectra.

141 As mentioned above, the information about the existence of any chiral species in enantiomeric excess in a  
142 sample is straightforward but further analysis is needed for identifying each species of an unknown mixture.  
143 To determine the identity of the species one needs to search for the transitions in available spectral libraries  
144 like splatalogue[40], CDMS[41], or published experimental and calculated spectra. For more exotic species,  
145 it is possible to perform the experiment in reverse, going from broadband fields to resonant to identify all  
146 transitions of the three-level system. Then, double resonance experiments similar to the one performed by  
147 M.A.M Drumel et al.[42] can be conducted to determine the rotational constants and the structure of the  
148 unidentified species.

149 In summary, we have introduced a generalized version of M3WM that includes the capability of acquiring  
150 microwave three-wave mixing spectra in unassigned samples. M3WM spectra can provide direct evidence  
151 on enantiomeric excess on-the-spot without the need of prior spectral assignment via the combination of  
152 broadband excitation and careful signal cancellation. Our new method can be applied to particularly hard-  
153 to-analyze samples like unknown multi-component mixtures and hard-to-assign species and provides new  
154 methods for ultrasensitive phase-coherent spectroscopic detection.

## 155 Methods

156 **Experimental setup.** The main components of the buffer gas cell apparatus have been described in detail  
157 elsewhere[43]. Molecules flow continuously through a copper tube heated at 40 °C into the buffer gas cell  
158 held at 5-7 K. A schematic of the apparatus is shown in figure 1(a). Cold He buffer gas flows continuously  
159 into the cell at a typical flow rate of 10 standard cubic centimeters per minute (scm). Microwave horns  
160 are oriented with polarizations of  $\hat{x}$  and  $\hat{y}$  for excitation and detection, respectively. Two equally spaced  
161 copper electrodes are attached to the cell through 1” sapphire insulators to produce an electric field in  
162  $\hat{z}$  direction. As in traditional M3WM, the “drive” and “listen” microwave horns are placed at 90°. For  
163 additional polarization control while maintaining the cold environment inside the cell, sapphire windows (4  
164 inches diameter) were added on two sides of the cell and microwave absorber foam was placed on the outside,  
165 as shown in figure 1(a). We observed that covering the inside of the buffer gas cell with microwave absorber  
166 significantly increased the gas temperature.

167 The sample input consists of three main parts: a copper tube, a diaphragm valve, and a nipple loosely  
168 packed with glasswool. Depositing the sample on glasswool results in even evaporation and significantly  
169 reduces signal fluctuations over time, leading to highly repeatable measurements.

170 **Elimination of non-chiral signals.** The most vital part of the experiment is to ensure that all signals  
171 stem from chiral species by successfully eliminating all non-chiral signals. We used three different methods  
172 to do so: a) polarization control as described above, b) fast subtractions, c) an updated microwave circuit  
173 design which rapidly and simultaneously changes the sign of the “drive” and “twist” pulses.

174 A key component of the success of non-linear microwave spectroscopy in a buffer gas cell is its high spectral  
175 acquisition velocity[43]. Each data point of the three-wave mixing spectrum consists of  $2.5 \times 10^6$  averages.  
176 The calm, controlled environment of the buffer gas cell enables careful subtractions between measurements of  
177 opposite twist phase every few hundreds of  $\mu\text{s}$  for each data point of the spectrum. This is important since we  
178 noticed that any “asymmetries” in the electronics or the data acquisition process can cause non-chiral signals  
179 to leak through. To solve this issue, we used a two-channel arbitrary waveform generator with very low time  
180 jitter (Siglent SDG6052X) to generate the “drive” and “twist” pulse. The timing window between each  
181 measurement and each experimental cycle was long enough ( $80 \mu\text{s}$ ) to prevent any signal cross-talk between  
182 measurements. A 9400 series Quantum Composer was also used to precisely control the timing between  
183 the two chirp pulses of each experimental cycle to ensure careful subtraction. It is not clear that a similar  
184 experiment could be conducted in an apparatus with pulsed valves where shot-to-shot variability is often  
185 significant. An updated microwave circuit design ensures high phase coherence between the twist and drive  
186 pulses by mixing the twist pulse with the beat note between the upconversion and downconversion steps.  
187 Figure 5 shows a comparison between the conventional circuit for microwave spectroscopy and the updated  
188 design. In the new design, two different local oscillators, LO1 and LO2, are used for the upconversion and  
189 the downconversion step and their beat frequency is mixed with the twist pulse. Specifically, mixer (M3)  
190 was added to the circuit taking LO1 and LO2 as inputs (the frequency difference between them was set to 2  
191 KHz). This beat note is AC-coupled and amplified, then fed into mixer (M4) where it is combined with the  
192 twist pulse. The offset local oscillators cause any 1D (non chiral) signals to alternate phase with the 2 KHz  
193 beat note between the two local oscillators, and thus average to zero. The phase of the twist also alternates  
194 phase with the 2 KHz beat note between the two local oscillators, and so the M3WM signal survives and  
195 averages to a non zero value. This signal is recorded alternatively with a generated twist phase of  $\phi = 0$  and  
196 a twist phase of  $\phi = \pi$ , and signals from these two configurations are further subtracted before the spectrum  
197 is assembled. This final step removes small ( $< 30 \text{ dB}$ ) bleedthrough of 1D signals resulting from imperfect  
198 mixing in the twist generation (M4).

199 **Data acquisition.** All spectra were collected with similar conditions to demonstrate the applicability  
200 of the method to a wide variety of species without selective optimization. A  $4\mu\text{s}$  long 35MHz broadband  
201 microwave pulse is used as the “drive” pulse followed by  $2\mu\text{s}$  long RF twist pulse with a frequency chirp of  
202 60-105 MHz. The “twist” pulse is overlapped with the drive pulse by  $1\mu\text{s}$ . The resultant coherent molecular  
203 signal (or “free induction decay” (FID)) following the double excitation is collected by a second orthogonally  
204 polarized horn and digitized to form the spectra measured such as in figure 2(b).

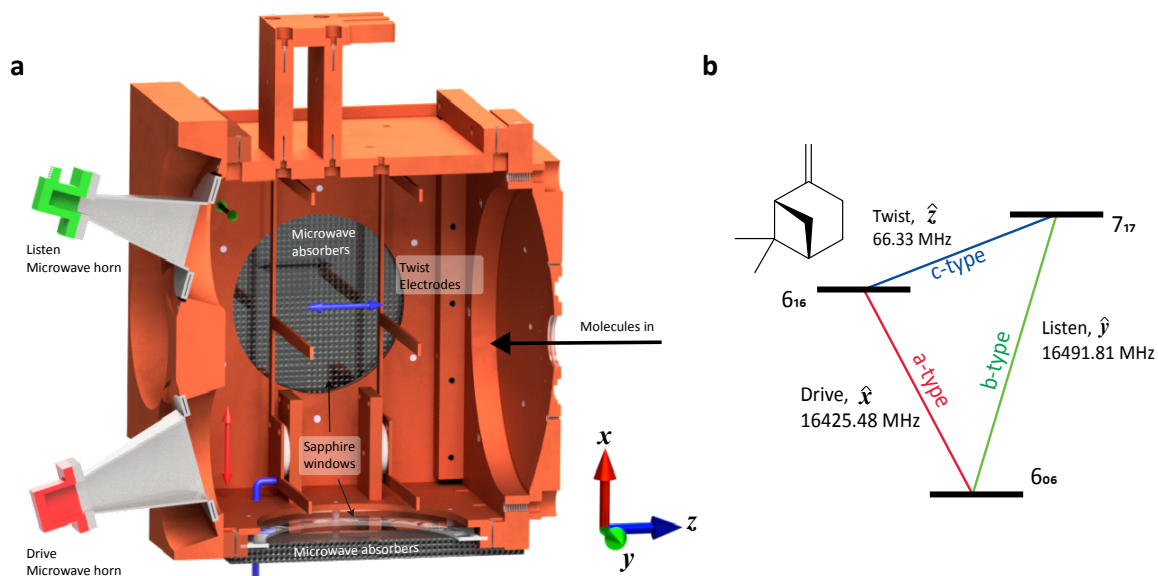


Figure 1: **Three-wave mixing spectra in a buffer gas cell** (a) Cut-away of the buffer gas cell used for acquiring three-wave mixing spectra. Arrows colored in red, blue, and green indicate the polarization of the drive, twist, and listen pulses. The back and bottom side of the cell have sapphire windows covered on the outside with microwave absorbers. (b) A typical M3WM excitation scheme for  $\beta$ -pinene. The three transitions are polarized perpendicular to one another and form a triangle that consists of an a-type, a b-type, and c-type transition.

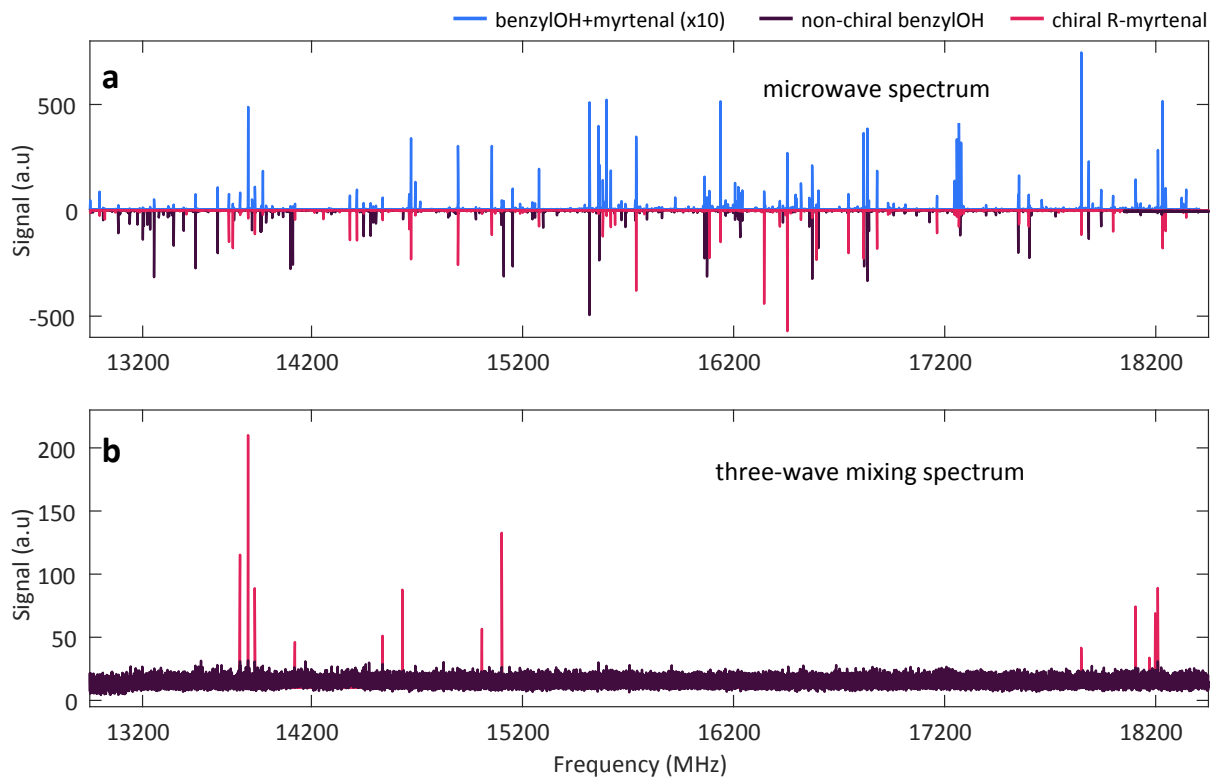


Figure 2: **Comparison between microwave spectra and three-wave mixing spectra.** (a) Top in light blue, microwave spectrum of the mixture of (R)-myrtenal (chiral) and benzyl alcohol (non-chiral). Amplitudes are multiplied by a factor of 10 for better visibility. Inverted, in dark purple and pink, the microwave spectra of the individual species, benzyl alcohol and myrtenal respectively. (b) Three-wave mixing spectrum of the mixture of (R)-myrtenal and benzyl alcohol. Each transition belongs to a chiral species in enantiomeric excess. In pink, only transitions from enantiopure (R)-myrtenal survive cancellation.

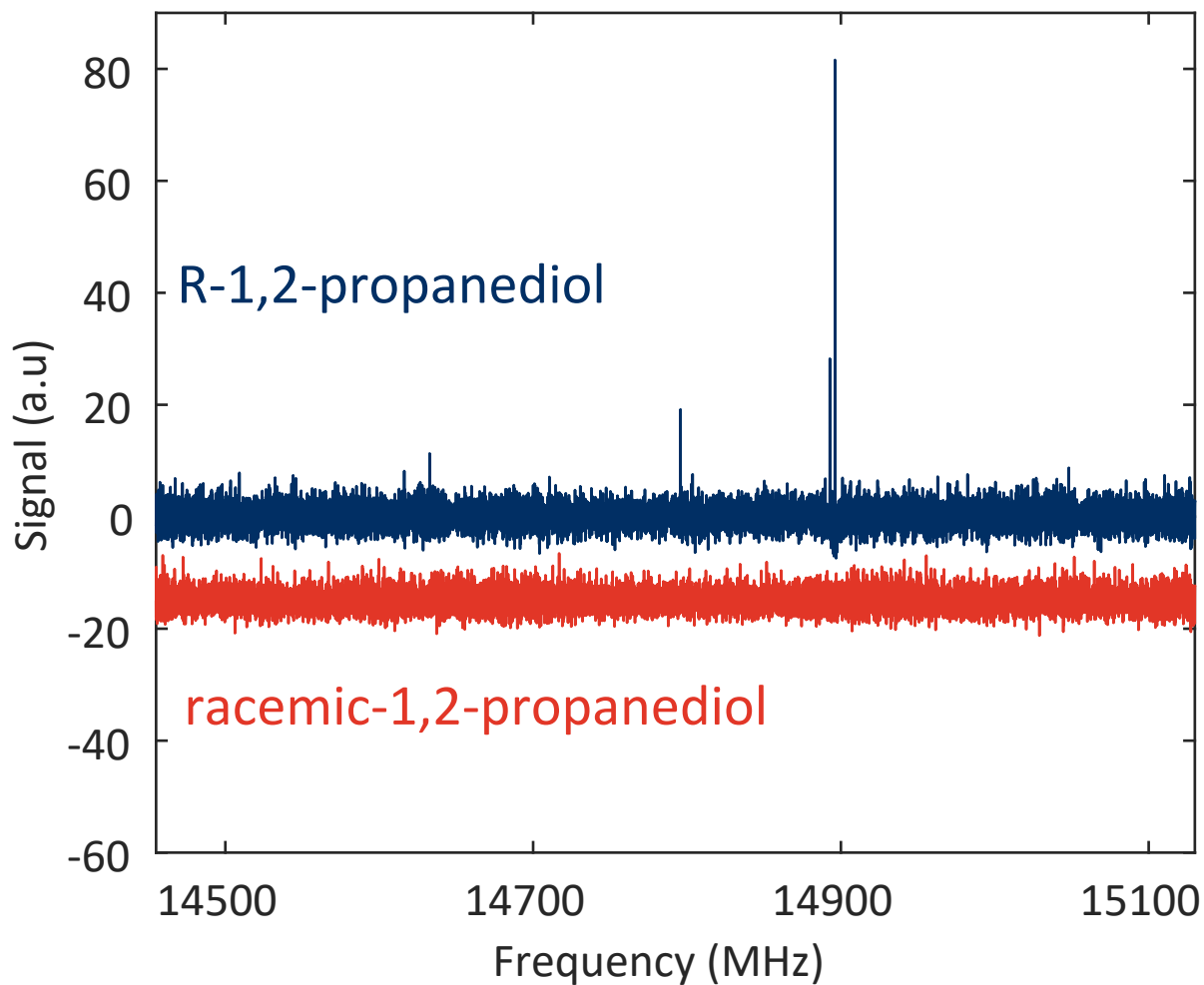


Figure 3: **Three-wave mixing spectra of enantiopure vs racemic samples.** (blue) Three-wave mixing spectrum of enantiopure (R)-1,2-propanediol. The three noticeable three-wave mixing signals belong to the two lowest energy conformers of 1,2-propanediol. (red) Three-wave mixing spectrum of racemic 1,2-propanediol. An offset of -15 (a.u) on the y-axis has been added to the racemic spectrum for clarity to show that no signal survives subtraction as expected.



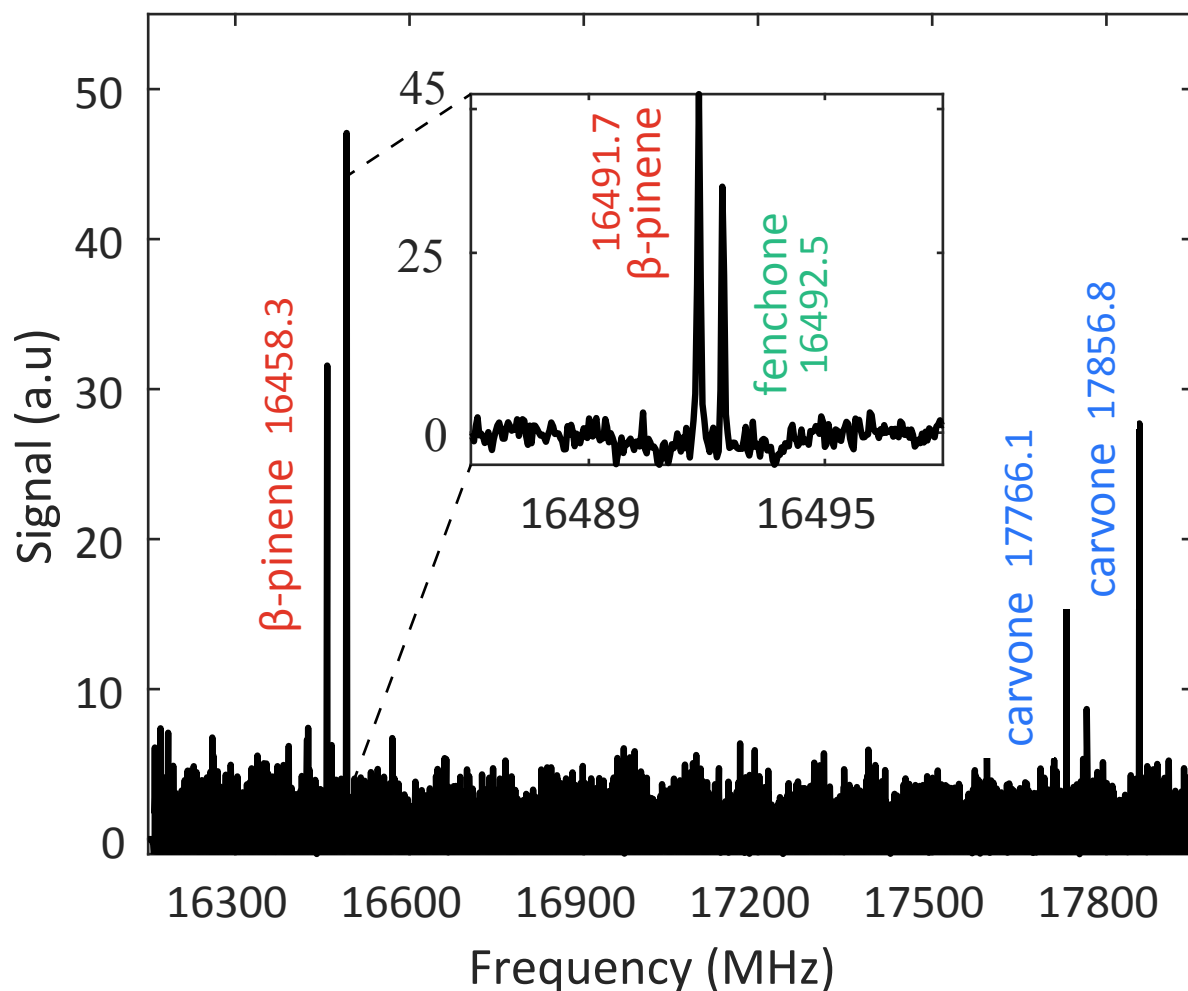


Figure 4: **Three-wave mixing spectra for a mixture of chiral terpenes.** Enantiopure (-)- $\beta$ -pinene, (R)-fenchone, and (R)-carvone were identified within the mixture with a narrow scan between 16300-18000 MHz and two twist ranges from 65-85 MHz and 85-105 MHz. The inset zooms into the transition around 16492 MHz which consists of two M3WM signals: a  $\beta$ -pinene M3WM signal at 16491.7 MHz and one from fenchone at 16492.5 MHz.

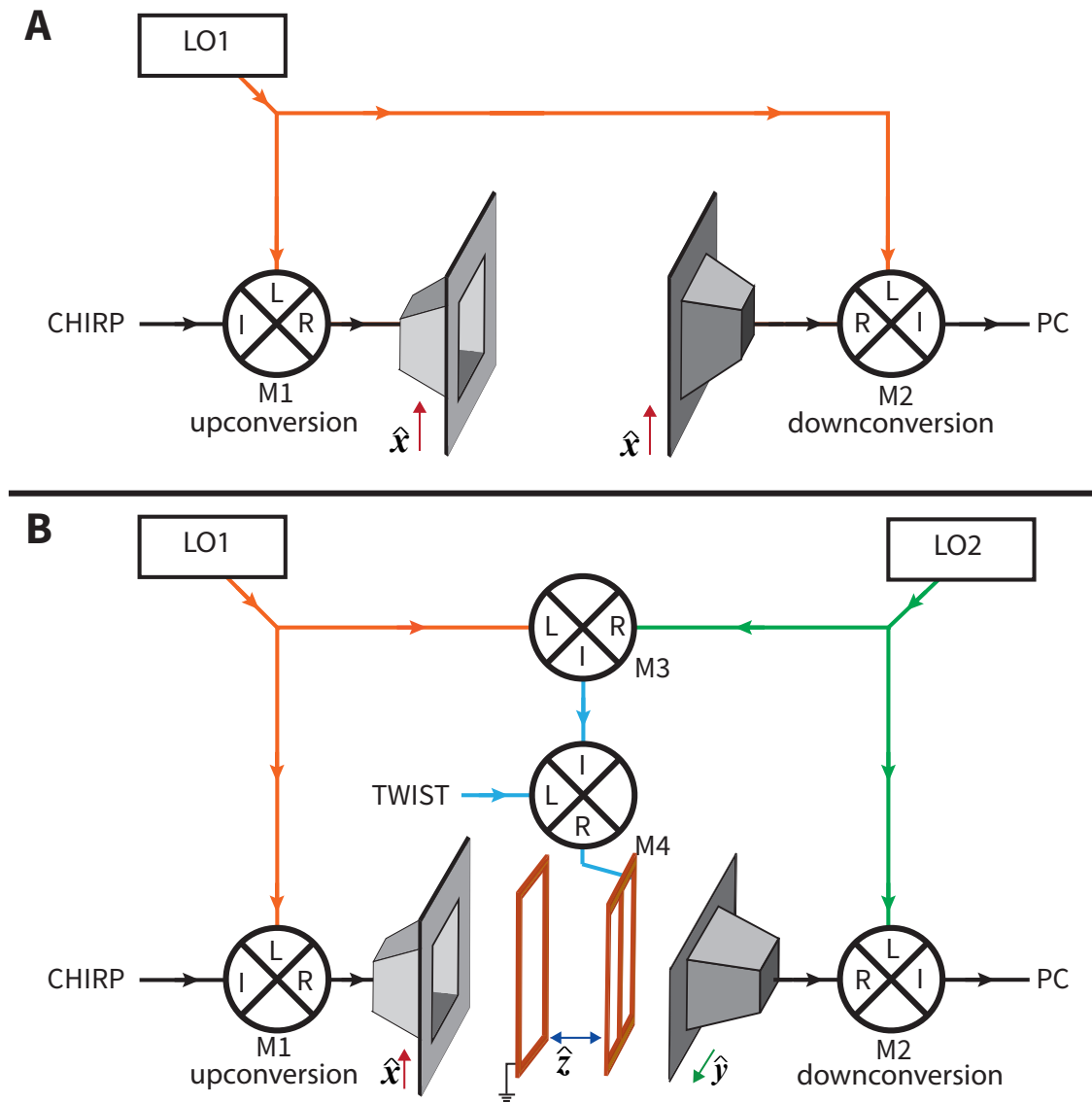


Figure 5: **Comparison of microwave circuit design.** (a) Schematic of the standard microwave circuit for microwave spectroscopy. (b) Schematic of the new circuit design for acquiring three-wave mixing spectra. The twist electrodes have been added for clarity. The upconversion and downconversion step are done with different local oscillators LO1 and LO2. Their beat note, 2 KHz in this work, is mixed with the twist to eliminate all non-M3WM signals.

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## 306 **Author Contributions**

307 G.K. realized the experimental setup, conducted the experiments and data analysis and wrote the manuscript  
308 with input from all authors. I.W. assisted in experimental design and data analysis, took and analyzed the  
309 data, and made figures used in the manuscript .L.S. designed microwave circuitry, assisted in experimental  
310 design, copy edited manuscript. D.P. assisted with experimental design, data analysis, and writing.

## 311 **Competing interests**

312 The authors declare no competing interests.

## 313 **Additional information**

314 Supplementary information is available for this paper.