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How Low Can It Go: ATR-FTIR Characterization of Compounds Isolated from Ginger at the Nanogram Level †

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Abstract: This proof-of-concept study demonstrated the potential of attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy for the structural characterization of natural products when only very small quantities of the target compound are available. Four known compounds (6-gingerol, 6-shogaol, 8-gingerol and 10-gingerol) were isolated from ginger (*Zingiber officinale*) rhizome using semi-preparative high performance liquid chromatography (HPLC). A portion of each fraction was evaporated on the ATR plate and spectra collected using a standard FTIR instrument. The minimum amount required to detect some spectral features appeared to be around 50 ng for the gingerols, and around 25 ng for 6-shogaol. Various peaks are assigned and interpreted to demonstrate the range of structural information that can be obtained. Evaporated ATR-FTIR spectroscopy could be an inexpensive and rapid method to aid structural elucidation of natural compounds, even when collected from a single semi-preparative HPLC run.

Keywords: detection limit; structural elucidation; natural products

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1. Introduction

Infrared (IR) spectroscopy has been an important part of the analytical chemist's toolkit since the 1930s [1]. It operates on the principle that dipole-active covalent bonds can absorb light from the infrared (700 nm–1 mm) region, which excites the bonds temporarily. If a full spectrum of IR light is used to illuminate a sample, IR-active bonds will absorb IR light at specific wavelengths, characteristic of the bond. By determining the wavelengths that are absorbed (either by measuring the reflectance or transmittance of the IR light), an analyst can ascertain the types and relative proportions of chemical bonds present in the sample. More detail on the principles behind IR spectroscopy can be found in several recent reviews [2–4]. Although IR spectroscopy has been somewhat displaced by more modern analytical techniques such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry, it can still be an important tool for structure elucidation. Its main benefits are that the instrument is much cheaper than other high-end methods and it is virtually free to run, both of which make it highly suited for rapid screening studies and/or educational purposes.

The most common type of IR spectroscopy used in analytical chemistry is Fourier transform infrared (FTIR) spectroscopy, which uses a Fourier transform algorithm to rapidly measure absorbance across the whole wavelength range. Many FTIR spectrometers use an attenuated total reflection (ATR) sample plate, which requires direct contact between the plate and the sample. Additionally, many ATR sample plates have rather small

sampling areas, which is generally considered a drawback as it makes representative sampling more difficult. However, the small sampling area may be beneficial for situations where only a limited quantity of sample is available for FTIR analysis.

This study aimed to demonstrate the potential of ATR-FTIR in facilitating the structural identification of compounds using minimal sample sizes. As a proof-of-concept, four known compounds were isolated from a ginger (*Zingiber officinale*) matrix, and their FTIR spectra were obtained and interpreted.

2. Materials and Methods

2.1. General Procedure

The general procedure of this work was as follows: Firstly, the polar constituents from a ginger sample were extracted using 90% methanol, following previously published protocols [5]. A ginger mass of approximately 3 g (dry weight) was used with around 25 mL total volume of 90% methanol.

Following this, the concentration of the target compounds (6-gingerol, 6-shogaol, 8-gingerol and 10-gingerol) were measured using a previously developed HPLC-DAD method [5], performed on an Agilent 1200 Series HPLC system.

Using the same method but with a larger injection volume (100 µL), 5 mL/min solvent flow rate and semi-preparative column (Agilent Eclipse XDB-C18; 150 × 9.4 mm; 5 µm pore size), the target compounds were fractionated from the methanol extract and collected. The volume of eluent containing the target compound from each fraction was recorded.

An accurately measured portion of the eluent was placed on the ATR plate of the FTIR spectrometer (Bruker Alpha II FTIR instrument) and allowed to evaporate. The FTIR spectra were collected between 4000–400 cm^{-1}, as the sum of 24 scans with a resolution of 4 cm^{-1}. Between different compounds and experiments, the ATR plate was thoroughly cleaned using Kimwipes® and liberal amounts of isopropyl alcohol.

2.2. Experiment 1

In Experiment 1, a sample of commercially dried ginger was used [6]. This dried ginger was directly extracted using 90% methanol, before HPLC analysis and semi-prep fractionation was performed as described in Section 2.1. Fractions were collected from a single run.

The fraction volume used for each FTIR analysis was 60 µL.

2.3. Experiment 2

In Experiment 2, a 50 g sample of fresh, commercial ginger (*Zingiber officinale*) was purchased from a local supermarket (Woolworths, North Rockhampton). It was oven dried at 60 °C until reaching a constant mass. The sample was then ground to a fine powder (<1 mm size) before extraction, HPLC analysis and semi-prep fractionation were performed described in Section 2.1. Fractions were obtained from three consecutive semi-prep runs and pooled for each compound. The fractions were then freeze-dried (−50 °C, <100 mT) and re-dissolved in 500 µL of methanol.

The fraction volume used for each FTIR analysis was 30 µL.

2.4. Data Analysis and Interpretation of FTIR Spectra

The FTIR spectra were exported in Opus format (*.0) and visualized and peak wavelengths determined in the Vektor Direktor software (Kax Group; Sydney, Australia). For plotting and interpretation, wavenumbers below 800 cm^{-1} were trimmed, as this region was consistently found to be dominated by noise, yielding no useful spectral information.

Graphs were drawn in GraphPad Prism 9.5.1.

The IR peaks were identified using relevant literature [7,8] and Brian Smith's columns in *Spectroscopy Online* [9].

3. Results and Discussion

3.1. First Experiment

3.1.1. Determination of Mass of 6-Gingerol and Related Compounds

The concentration of 6-gingerol in the 90% methanol extract was determined to be 0.647 mg mL^{-1}, using HPLC. The injection volume was 100 μL, so 0.646 mg mL^{-1} × 0.1 mL = 0.0646 mg = 64.6 μg was injected. A volume of 0.5 mL of eluent was collected across the 6-gingerol peak, therefore the concentration of 6-gingerol in the collected fraction solution would be 64.6 μg/0.5 mL = 129.3 μg mL^{-1}. Of this, 60 μL was used to gather the FTIR spectrum, which equates to 129.3 μg mL^{-1} × 0.06 mL = 7.76 μg.

The ATR platform contains a diamond crystal of approximately 1.5 × 1.5 mm (area = 2.25 mm^2) where the sample spectra is measured, while it is surrounded by an outer ring of approximately 11 mm diameter (area = 95.03 mm^2). As the solution to be analysed fills the outer ring, the IR spectra will only be collected from 2.25/95.03 × 100 = 2.37% of the total surface area. This assumes equal distribution of the analyte within the solvent, equal depth of the solvent within the entire outer ring, and that the solvent evaporates uniformly across the area bounded by the outer ring. If this is true, then the mass of 6-gingerol available for IR spectra collection would be 7.76 μg × 2.37% = 0.184 μg = 184 ng.

Using a similar process, the equivalent masses of 6-shogaol, 8-gingerol and 10-gingerol used for IR analysis were calculated and shown in Table 1.

Table 1. Equivalent amounts of 6-gingerol, 6-shogaol, 8-gingerol and 10-gingerol used for collection of the FTIR spectra in experiment 1.

Compound	Equivalent Mass Used (ng)
6-gingerol	184
6-shogaol	24
8-gingerol	40
10-gingerol	70

3.1.2. FTIR Spectra

As shown in Figure 1, the FTIR spectra of all samples except 8-gingerol showed several clear peaks.

3.2. Second Experiment

3.2.1. Determination of Mass of 6-Gingerol and Related Compounds

The second experiment used the oven-dried sample, produced from commercial fresh ginger. The 6-gingerol concentration in the 90% methanol extract of this sample was measured using HPLC and determined to be 1.402 mg mL^{-1}. Again, the injection volume was 100 μL, meaning that the equivalent mass of 6-gingerol per injection was 1.402 mg mL^{-1} × 0.1 mL = 0.1402 mg = 140.2 μg.

In this experiment, three injections were performed, with the 6-gingerol peak collected for each run (i.e., 140.2 μg/injection × 3 injections = 420.6 μg collected in total). The total eluent volume collected for this peak was 7.0 mL; however, this was freeze-dried and re-dissolved in 0.5 mL of methanol, making the equivalent concentration of 6-gingerol in the re-dissolved solution: 420.6 μg/0.5 mL = 841.2 μg mL^{-1}. This resulting solution (30 μL) was placed on the ATR plate (841.2 μg mL^{-1} × 0.03 mL = 25.2 μg), which corresponds to 25.2 μg × 2.37% = 0.598 μg = 598 ng available for collection of the IR spectra. This was approximately 3 times more than in experiment 1.

Again, following the same procedure, the equivalent masses for the other three compounds were calculated and displayed in Table 2. In general, the masses were higher than in Experiment 1, with the exception of 10-gingerol.

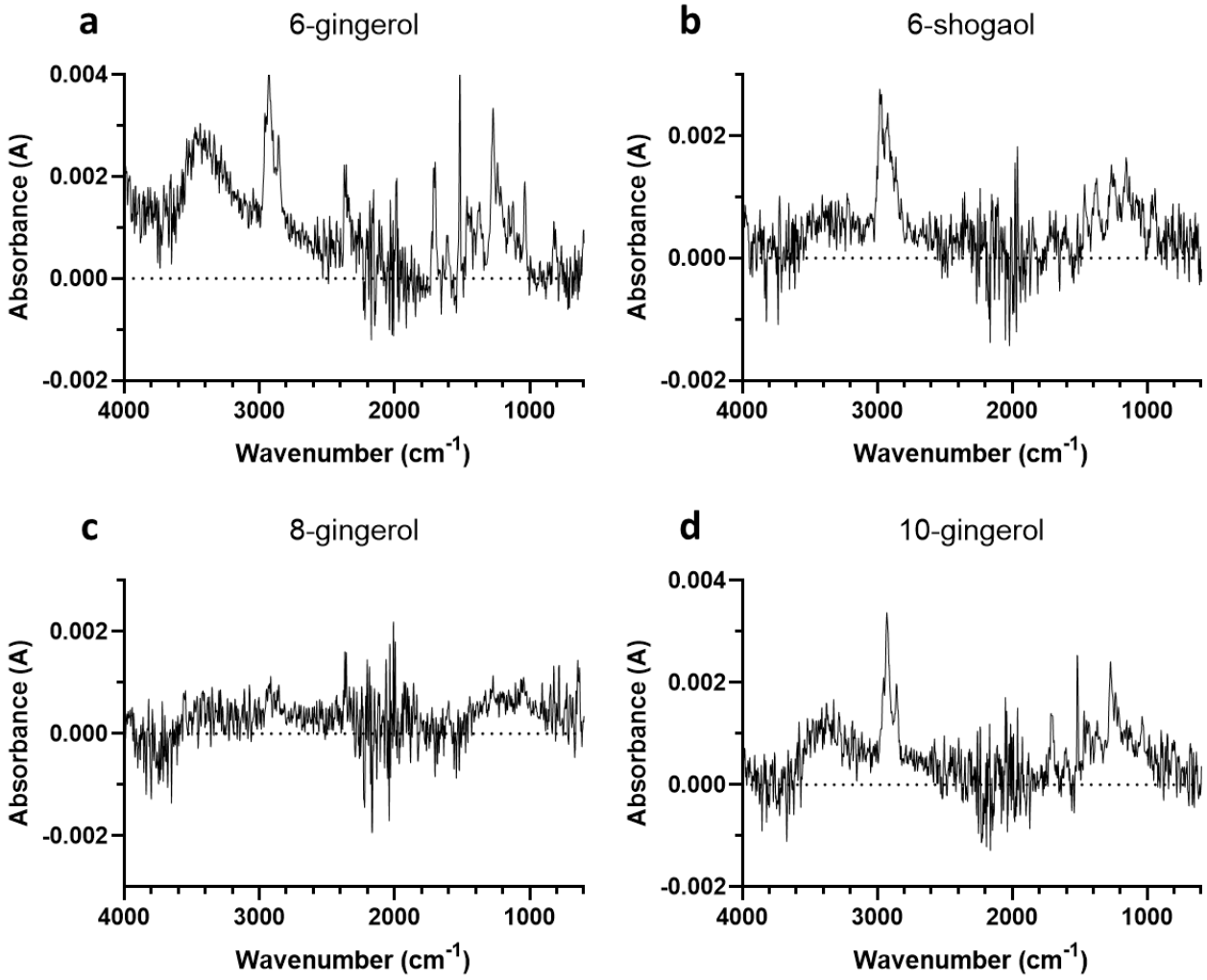

Figure 1. FTIR spectra of the evaporated fractions from experiment 1. Masses provided are approximate only. (**a**) 184 ng of 6-gingerol, (**b**) 24 ng of 6-shogaol, (**c**) 40 ng of 8-gingerol, (**d**) 70 ng of 10-gingerol.

Table 2. Equivalent amounts of 6-gingerol, 6-shogaol, 8-gingerol and 10-gingerol used for collection of the FTIR spectra in experiment 2.

Compound	Equivalent Mass Used (ng)
6-gingerol	598
6-shogaol	76
8-gingerol	90
10-gingerol	53

3.2.2. FTIR Spectra

Figure 2 shows the FTIR spectra for the four compounds isolated in Experiment 2. All of the spectra showed discernable peaks—including 8-gingerol, which had previously not shown any clear peaks in Experiment 1 (Figure 1c).

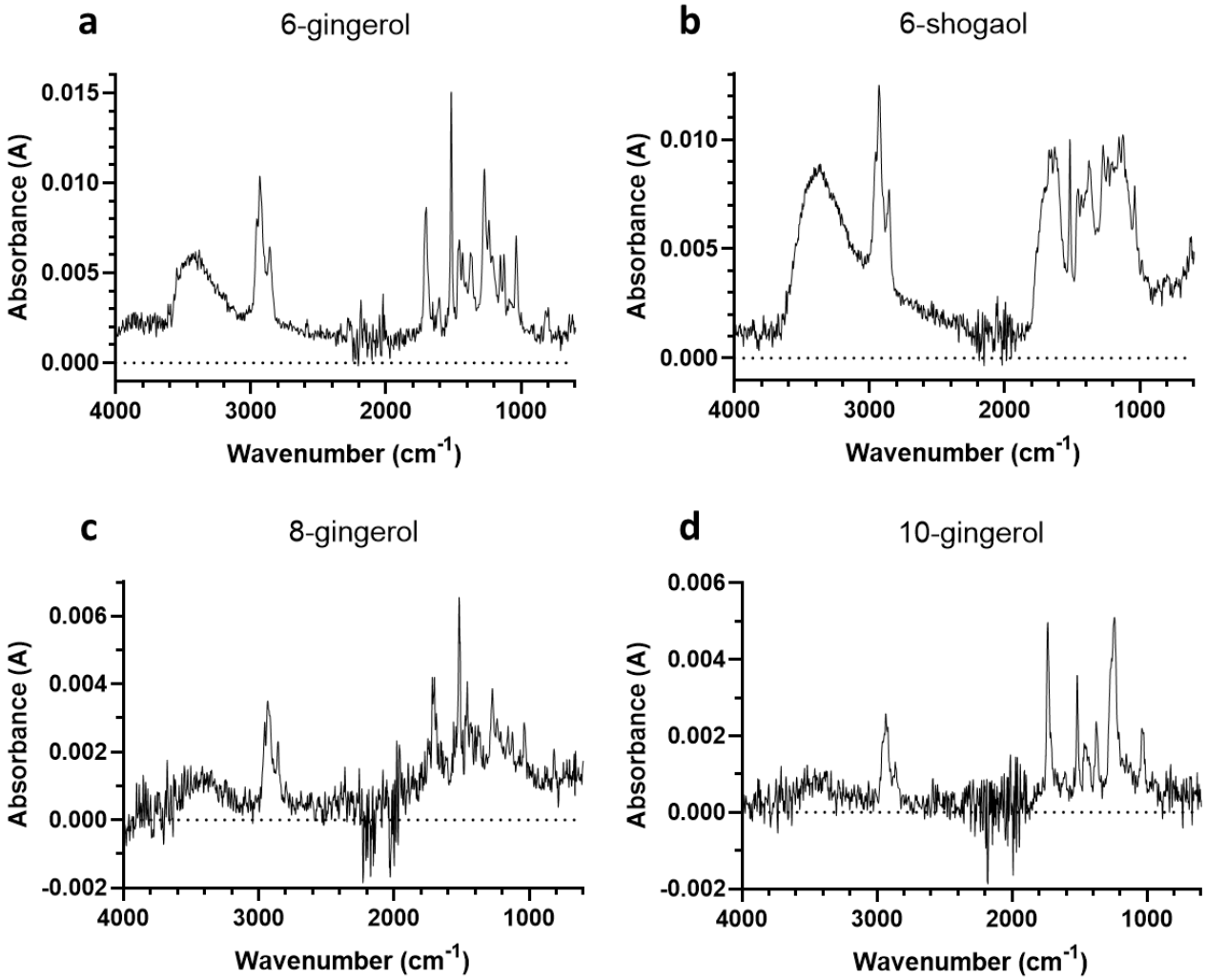

Figure 2. FTIR spectra of the evaporated fractions from experiment 2. Masses provided are approximate only. (**a**) 598 ng of 6-gingerol, (**b**) 76 ng of 6-shogaol, (**c**) 90 ng of 8-gingerol, (**d**) 53 ng of 10-gingerol.

3.3. Assignment of FTIR Spectra

Each spectrum was examined independently, and the discernable peaks were recorded in Table 3. However, the region between 2600–1800 cm^{-1} contained a large amount of noise attributed to the FTIR instrument characteristics. As this region does not contain any relevant information about bonds pertinent to these samples, it was disregarded in the spectral analysis.

Table 3. Peak locations for the FTIR spectra from Experiment 1 and 2, and their responsible bonds. Note that some of the peaks were not assigned.

Assigned Bond	**6-Gingerol**		**6-Shogaol**		**8-Gingerol**		**10-Gingerol**	
Equiv. mass (ng)	184	598	24	76	40	90	70	53
Experiment	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1[#]	Expt 2	Expt 1	Expt 2
O-H stretch (alcohol, intermolecular bonded)	3439 br	3381 br		3364 b		~3146 br w	~3377 br w	
CH_3 asymmetric stretch	2958 sh	2954 sh	2977	2952 sh		2954 sh	2952 sh w	2958 sh w
CH_2 asymmetric stretch	2927	2934	2923 w	2929	2915 w	2932	2927	2936
CH_2 symmetric stretch	2857	2859	2861 sh w	2855		2855	2857	2864 w

-O-CH_3 symmetric stretch		2845 sh w	2845 sh w	2826 sh w			2845 sh w	
						2798 w		
						1715 sh		1734
C=O stretch, aliphatic ketone	1703	1701				1699	~1705 br	1713 sh w
C=C stretch, disubstituted (trans)				~1664				
C=C stretch, conjugated alkene				1627				
	1608 w	1604 w			1598 w	~1604 w	~1600 w	~1608 w
						1559 sh		
								1540 w
C=C stretch, benzene ring	1515	1515	1513 w	1517		1517	1517	1517
						1488 w		
C-CH_3 asymmetric bend	1462 w	1458	1462 w	1458		1458	1466 w	1463 w
-CH_2- scissors?							1449 w	1449 w
	1431 w	1433 w				1431 w	~1433 w	
						1398	1404 w	
O-H in-plane bend (phenol)?	1369 w	1373	1373 w	1377		1375	1369 w	1375
C-O stretch, aromatic ether	1270	1270	1264 w	1272	1270 w	1272	1270	1270 sh w
C-O-H stretch (phenol)	1235 w	1235 w		1235 w		1235 w	1235 w	1241
	1214 w	1210 sh w				1210 w	1212 w	1208 sh w
Saturated C-C-C stretch, ketone?	1154 w	1152	1154 w	1152		1155 w	1148 w	1152 w
C-OH stretch, 2° alcohol?	1121 w	1126		1124		1124 w		
C-O stretch, saturated ether	1033	1035		1037		1035	1037 w	1037
C=C bend, disubstituted (trans)			~963 w					
1,2,4-trisubstituted benzene		818				814		

br = broad peak, sh = shoulder, w = weak peak. # peaks very hard to distinguish.

The peak assigned to O-H stretch was observed at a wavelength less than 3550 cm^{-1} in all samples where it was detected, providing confirmation that this O-H bond was able to participate in intermolecular hydrogen bonding [7]. In addition, the absence of water in the analyzed samples was confirmed by the lack of any significant peak located at ~1630 cm^{-1}, which typically arises from the O-H scissoring of water molecules. Consequently, this peak could be attributed to one or more alcohol (-OH) groups.

In all spectra, the ratio of the methyl/methylene (~2960/2930 cm^{-1}) peaks was much less than 1, indicating that the CH_2/CH_3 ratio of all compounds were ≥3. This agrees with the true CH_2/CH_3 ratios of the compounds, which ranges from 3 for 6-shogaol, 3.5 for 6-gingerol, 4.5 for 8-gingerol and 5.5 for 10-gingerol.

For nearly all spectra, there were no significant peaks between 3200-3000 cm^{-1} which could be attributed to alkene/aromatic C-H stretch; indicating that larger portion of the compound was not conjugated or aromatic.

Evidence of C=O stretch from an aliphatic ketone was found at around 1703 cm^{-1} in most of the FTIR spectra.

Only the 6-shogaol spectrum contained peaks at 1664 and 1627 cm^{-1}. The first peak (1664 cm^{-1}) fell outside the range for vinyl, vinylidene, or *cis* alkene bonds but was just bordering the range for a *trans*-substituted alkene bond [8].

The most intense peak in most of the FTIR spectra was found at ~1515 cm^{-1}, which was attributed to C=C stretch of a benzene group. However, it was moderately shifted from the theoretical value of 1480 cm^{-1} for benzene, indicating the presence of some substituent group(s) on the benzene moiety. This finding agreed with the known structures of the four compounds, which include a 1,2,4-trisubstituted benzene ring (Figure 3).

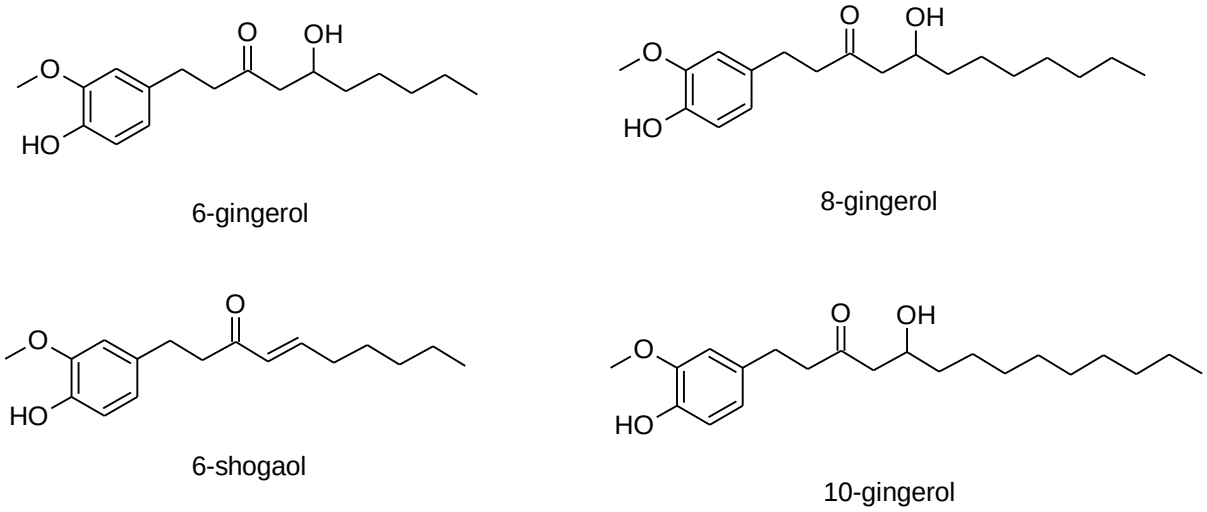

Figure 3. The structures of 6-gingerol, 6-shogaol, 8-gingerol and 10-gingerol.

The peak at ~1369 cm^{-1} was assigned as O-H bend (phenol) rather than C-CH_3 symmetric bend, due to its broadness. However, there may also be some contribution from C-CH_3 symmetric bend in this region.

The presence of a mixed ether group (i.e., one of the ether carbons is part of a saturated methyl group, while the other ether carbon is part of an aromatic ring) was evident from two very strong peaks between 1300 and 1000 cm^{-1}—specifically at ~1270 and ~1035 cm^{-1} [10]. This was most clearly seen in the 6-gingerol spectra, although it could also be seen to a lesser extent in the 8-gingerol and 10-gingerol spectra. These peaks were also found in the 6-shogaol spectra (Figure 2b) but were more obscured by other broad peaks in this region.

After identifying the mixed ether group, the spectra were re-examined, and a minor shoulder at ~2840–2830 cm^{-1} identified in several spectra. This falls in the region characteristic of methoxy C-H stretch [11]. Consequently, when considered in conjunction with the mixed ether group (above), this demonstrated the presence of a methoxy group attached to a phenol group.

C-O-H stretch from a phenol group was observed at 1235 cm^{-1}, while the peak around 1154 cm^{-1} was attributed to a saturated C-C-C stretch, possibly due to a ketone. Another C-O-H stretch was seen at 1124 cm^{-1}, which was assigned to a 2° alcohol.

In the 6-shogaol sample from Experiment 1, there was a minor peak at around 963 cm^{-1}, which falls in the range of a disubstituted (trans) C=C bend. However, this peak was quite weak and should be interpreted with caution.

Finally, a peak at around 816 cm^{-1} observed in two samples was attributed to a 1,2,4-trisubstituted benzene.

3.4. Interpretation of FTIR Spectra

This section provides a summary of the structural information that was readily obtained from the FTIR spectra, as detailed in Section 3.3 and Table 3.

The following features were common to all four compounds:

- One or more O-H groups (less visible in 6-shogaol and 8-gingerol)
- One or more CH_3 groups

- One or more CH_2 groups
- A CH_2/CH_3 ratio of ≥3, indicating the presence of at least 3 CH_2 groups
- A methoxy group, as indicated by the presence of an aromatic ether and saturated ether group
- A methoxy (-O-CH_3) group based on the CH_3 absorbance at ~2845 cm^{-1} (note this was not observed in 8-gingerol, possibly due to the small sample mass. However, the main methoxy feature above was seen in 8-gingerol)
- One or more benzene rings
- Tentative: a phenol group
- Tentative: a ketone with a saturated C-C(=O)-C structure
- A secondary alcohol

The following features were found in the gingerol compounds, but not in 6-shogaol:

- An aliphatic (i.e., non-conjugated) ketone
- A 1,2,4-trisubstituted benzene (note that this was not seen in 10-gingerol)

Additionally, the second compound (6-shogaol) showed these spectral patterns:

- A disubstituted, *trans* alkene
- A conjugated alkene

As depicted in Figure 3, these features match extremely well with the known structures of the target compounds, enabling the identification of all functional groups in all the compounds (and their relative positions in several cases). The only slightly unusual finding was that the secondary alcohol peak was also seen in the 6-shogaol spectra; this may be due to some contribution from the phenol peak, as 6-shogaol does not have any other secondary alcohol groups.

3.5. Synthesis of the Derived Information

Indeed, FTIR spectroscopy is not well suited for complete structural elucidation on its own. However, when combined with other analytical techniques (in particular, mass spectroscopy), it can be highly valuable.

Assuming some information could be obtained about the relative mass of the compound (i.e., precluding the presence of two or more benzene ring), the following process could be hypothesized for assembling the FTIR structural data (using 6-gingerol as an example):

- Begin with the benzene ring
- At the 1, 2 and 4 positions, add:
  - A phenol group
  - A methoxy group (alternatively, the benzene group could have two alkane chains, and the methoxy group could be located on one of them. Placing the methoxy group on the benzene ring would require some familiarity with other similar natural structures such as vanillin; or more detailed structural information using a different analytical technique)
  - Possibly an alkane chain (of unknown length, but at least 6 carbons long if this is the only alkane chain)
- Add a secondary alcohol group at the second carbon or further down the alkane chain
- Add a ketone group at the third carbon or further down the alkane chain
- Assuming that only one alkane chain was attached to the benzene group, at least 3 CH_2 groups would be required on this chain (i.e., excluding the C-OH and C=O carbons) to satisfy the ≥3CH_2/CH_3 ratio. Consequently, the alkane chain would have to be at least 5 carbons in length. Additionally, no alkene groups would be included in the chain, as the FTIR spectra did not show any alkene bonds aside from the benzene ring.

As can be seen from this simple process, this would produce a structure somewhat reminiscent of the known structures of 6-gingerol, 8-gingerol and 10-gingerol (Figure 4). Importantly, all of the functional groups are there, although their relative positions are not well defined.

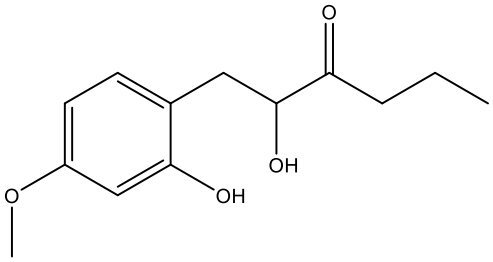

Figure 4. One potential theoretical structure, based almost solely off FTIR spectral data.

When considering the structure of 6-shogaol, it might be assumed that the secondary alcohol is retained (based strictly on the FTIR spectra). However, the new alkene bond would have to be added either 1 carbon away from the benzene ring, or 1 carbon away from the ketone group, to ensure that it satisfied the conjugated alkene criteria.

4. Discussion

Overall, the FTIR spectra provided a surprising amount of structural information, even when only tens of nanograms were available for analysis, rather than hundreds of nanograms. This highlights the potential importance of this technique for aiding the structural identification of natural products isolated in small quantities. Although FTIR spectroscopy is a non-destructive analytical technique, it would be challenging, if not practically impossible, to recover the analyte after its use in evaporated FTIR.

Distinguishing the FTIR spectra of 6-shogaol from the spectra of the gingerols was clear-cut due to the absence of a distinct peak around 1700 cm^{-1}, broad absorption peaks between 1680–1600 and 1280–1090 cm^{-1}, a much weaker peak at ~1517 cm^{-1}, and a slightly weaker peak at ~1270 cm^{-1}. This facilitated easy differentiation between these two compound classes. If longer shogaols (e.g., 8-shogaol, 10-shogaol, 12-shogaol), reported in ginger [6], were similarly isolated using semi-prep HPLC, it is anticipated that their FTIR spectra would readily identify them as shogaols.

The minimum amount required to detect certain spectral features appeared to be around 50 ng for gingerols (Figure 2d) and around 25 ng for 6-shogaol (Figure 1b). Consequently, FTIR spectroscopy holds promise as an inexpensive and rapid method for aiding the structural elucidation of natural compounds, even when isolated in small quantities.

Author Contributions: Conceptualization, J.B.J.; methodology, J.B.J.; software, J.B.J.; validation, J.B.J. and R.J.B.; formal analysis, J.B.J.; investigation, J.B.J., R.J.B. and J.S.M.; resources, J.B.J. and M.N.; data curation, J.B.J.; writing—original draft preparation, J.B.J.; writing—review and editing, J.B.J., R.J.B., J.S.M. and M.N.; visualization, J.B.J.; supervision, M.N.; project administration, J.B.J.; funding acquisition, M.N. All authors have read and agreed to the published version of the manuscript.

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