Tristearin as a Model Cuticle for High-Throughput Screening of Agricultural Adjuvant Systems

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ABSTRACT: The widely varied compositions and structures of plant cuticles create problems in the identification of suitable model systems for laboratory testing of adjuvants. We have compared the behavior of an extracted cuticle wax with tristearin, a well characterized crystalline triglyceride, which we propose as a model cuticle for ranking new adjuvant systems for their propensity to disrupt the cuticle barrier. The interaction of adjuvant products and their components with the extracted cuticle wax and tristearin was determined using differential scanning calorimetry and small angle X-ray scattering approaches. The interaction of the additive with tristearin caused a concentration-dependent change in the crystallite level, and correlated between the extracted wax and tristearin. Tristearin was subsequently used to compare the effectiveness of a range of adjuvant products and their major components. This approach has utility to quantify the effects of adjuvant components and enable more judicious selection of adjuvant candidates to progress to plant trials.

INTRODUCTION

Agrochemical adjuvants are commonly used in crop application of pesticides to promote increased bioavailability of the pesticide by improved foliar uptake. The effect of adjuvants on the foliar uptake of pesticides occurs by two main mechanisms, namely, control over the physiochemical properties of the applied product such as the droplet size and spreading and/or control over the interaction of pesticide with the target crop surface. Both uptake mechanisms are aided by the use of an adjuvant, generally consisting of an oil/surfactant mixture. The adjuvant oil is usually based on either hydrocarbon or vegetable oil sources with nonionic surfactants, by far the most commonly used surfactant type. Other adjuvant systems exist which contain a number of different materials, but these serve more specific purposes such as improved rainfastness, improved delivery efficiency, as well as reduced environmental toxicity.

Adjuvants act upon the cuticle of the leaf of the plant and improve the delivery of the active ingredient through softening of the epicuticular wax layer, the outer barrier layer of the leaf. The epicuticular wax layer is a complex matrix of waxes which consists of long chain (>C20) alcohols, esters, and acids. Plant species have different epicuticular wax profiles which creates complexity in the comparison of efficacy between the adjuvant systems and in determining the contributions of components used within adjuvants. The use of model compounds and mixtures to mimic the crystalline epicuticular matrix has been studied and show excellent insights into the structure and behavior of plant cuticles. The ability to develop a method which allows comparison of the major components of an adjuvant system with that of the actual product would be an extremely useful tool in the development of new and novel adjuvant systems.

We propose the use of tristearin (glyceryl tristearate) as a model cuticle due to multiple potential advantages. Firstly, tristearin is a naturally occurring triglyceride and is readily available in high purity. Secondly, the crystalline behavior of tristearin is well characterized and has three major crystalline forms with the two most prominent being the kinetically stable α-crystallite and the thermodynamically stable β-crystallite. Tristearin is well characterized and has three major crystalline forms with the two most prominent being the kinetically stable α-crystallite and the thermodynamically stable β-crystallite.
The interaction of additives/components/impurities with the α-form of tristearin causes a change in crystallization behavior of the α-crystallite, due to disruption of the packing, leading to a loss in the level of crystallite material present. Thus, the effectiveness of the disruption by the additive is indicative of the more aggressive adjuvancy of the adjuvant mixture or the isolated component. Several studies have used different long chain waxes as an in vitro cuticle model but not as a method for the high-throughput screening for the quantification and greater understanding of the efficacy of different adjuvant components.

The aim of the first part of this study was to determine whether tristearin is a suitable model cuticle in comparison with the extracted cuticle wax. Ficus macrophylla was selected as the nominal source for the cuticle wax as the tree is evergreen, readily available in suitable quantities required for extraction, and the cuticle of other members of the moraceae family have been readily studied. The second aspect of this study was the use of tristearin films to rank the likely trends in efficacy of different adjuvant types (fatty acid ester and mineral oil-based adjuvants) and their major components. The effect of the adjuvants on the tristearin and extracted wax substitutes was studied using differential scanning calorimetry (DSC) and small angle X-ray scattering (SAXS). The structures of tristearin and the major components of selected adjuvants and the components from which they are prepared are illustrated in Figure 1.

**Figure 1.** Chemical structures of significant components of systems studied: 1, tristearin; 2, generalized structure for mineral oil (where n is typically >20); 3, oleate ester, where R = methyl or ethyl; 4, typical example of a phospholipid present in lecithin.

### MATERIALS AND METHODS

Tristearin (glycerol tristearate) (Figure 1) was purchased from Tokyo Chemical Industry (Tokyo, Japan). The adjuvant Hasten (alkyl ester adjuvant), whose major components are a mixture of canola oil methyl and ethyl esters 3 (Figure 1), was used as supplied by Victorian Chemicals (Coolaroo, VIC, Australia). Inbound (Mineral oil adjuvant 1 (MO1)) and Empower (Mineral oil adjuvant 2 (MO2)) are mineral oil-based adjuvants based on Mineral Oil SN70 and Mineral Oil 150 2 (Figure 1), respectively, and were used as supplied by Victorian Chemicals (Coolaroo, VIC, Australia). Liberate (lecithin adjuvant) is an adjuvant based on soy lecithin and alkyl methyl esters 3 (Figure 1) and was also used as supplied by Victorian Chemicals (Coolaroo, VIC, Australia).

Differential scanning calorimetry pans and lids were purchased from Perkin Elmer (Glen Waverley, VIC, Australia). Microscope coverslips were purchased from VWR International (Tingalpa, QLD, Australia). 1,1,2,2-Tetrachloroethane (>98% purity), silica gel (Kieselgel 40), methyl oleate (99% purity GC standard), and ethyl oleate (98% purity GC standard) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Milli-Q water (18.2 MΩ resistivity at 25 °C) was from a Milli-Q Academic water purification system from Millipore (Sydney, NSW, Australia).

### EXTRACTION OF LEAF CUTICLE WAX

Nontarnished leaves of multiple trees of *Ficus macrophylla* were freshly picked (1.0 kg) and placed in a large beaker (10 L). Chloroform (2 L) was added to the mixture and agitated to allow the solvent to coat all leaves. After 1 min, the solvent was decanted into a second beaker (5 L), to which magnesium sulfate was added. Filtration of the magnesium sulfate, followed by removal of the solvent by using a rotary evaporator (40 °C, 200 mbar) yielded a dark brown oil (2.84 g), which solidified upon cooling. The extract was taken up in chloroform (15 mL) and passed through silica gel (75 mL) using gradient elution with chloroform (4 × 100 mL) followed by 96:4 chloroform/methanol (2 × 100 mL). Removal of the solvent again by using a rotary evaporator (40 °C, 200 mbar) yielded a brown oil, which solidified upon cooling to form a creamy solid (1.9 g), in a yield of 0.2%, based upon the mass of leaves extracted.

### DIFFERENTIAL SCANNING CALORIMETRY (DSC)

All DSC samples were run on a DSC8500 from Perkin Elmer (Glen Waverley, VIC, Australia). For DSC measurements, two different sample preparation approaches were used to investigate the interaction of the adjuvant with tristearin — premixing the adjuvant/component with tristearin prior to casting the film in the DSC pan or the addition of the adjuvant system to the precast tristearin film.

**Preparation of Samples — Premixing Adjuvant/Component with Tristearin before Casting of the Film.** All premix samples were prepared by the same method. A typical example is described here for the premixed 2.5% ethyl oleate sample. Ethyl oleate (5.4 mg) and tristearin (180 mg) were weighed into a vial (4 mL) and to this 1,1,2,2-tetrachloroethane (1 mL) was added. The mixture was vortex mixed (30 s) and placed in an oven (37 °C) to allow complete dissolution. The vial was removed from the oven and an aliquot was immediately removed by an Eppendorf pipette (20 μL), to which the sample was carefully added to a DSC pan (aluminum, 50 μL) in a separate oven (85 °C). The solution was then left for 30 min at 85 °C to allow complete evaporation of the solvent. The DSC pan was removed from the oven and placed on a metal plate, at 20 °C, to aid the formation of the rapidly cooled tristearin film. A lid was sealed.
onto the sample pan with a Perkin Elmer universal crimper press. The DSC sample was held isothermally at 30 °C for 1 min, before heating at a ramp rate of 5 °C/min, to 75 °C. The peak area of the α-crystallite peak was recorded as the area under the graph from 45 °C to the point tangent to the baseline intersected the DSC trace.

Preparation of Samples — Surface Application of the Adjuvant to Premade Tristearin-Only Films. All surface-applied samples were prepared by the same method. A typical example is described for the 2.5% alkyl ester adjuvant system. The adjuvant-free tristearin (180 mg) was weighed into a vial (4 mL) and 1,1,2,2-tetrachloroethane (1 mL) added, and the crystalline film was prepared as described above. Then the alkyl ester adjuvant (10 mg) was added to Milli-Q water (4 mL) and roll-mixed for 30 min. An aliquot (40 µL) was added to the DSC pan which contained the crystalline tristearin film, and was then placed in an oven (40 °C) for 60 min to dry. The sample was removed from the oven and a lid was sealed with the sample pan with a Perkin Elmer universal crimper press.

The DSC sample was held isothermally at 30 °C for 1 min before heating at a ramp rate of 5 °C/min to 75 °C. The peak area of the α-crystallite peak was recorded as the area under the graph from 45 °C to the point tangent to the baseline intersected the DSC trace.

Small Angle X-ray Scattering (SAXS). Preparation of Samples — Premixing Adjuvant/Component with Tristearin. All premix samples were prepared by the same method. The glass cover slip (20 mm × 20 mm) was cleaned by a sequential wash process, in which the slide was immersed in chloroform, then ethanol, then water, followed by ethanol and finally chloroform before air drying. The glass slide was then placed in an oven (85 °C) on a metallic shelf for improved heat conduction. A typical example is for the 2.5% ethyl oleate premixed system. Ethyl oleate (6.0 mg) and tristearin (240 mg) were weighed into a vial (4 mL) and to this, chloroform (2 mL) was added. The mixture was vortex mixed (30 s) and left to stand (10 min) to allow complete dissolution. An aliquot was removed by an Eppendorf pipette (250 µL) and carefully added onto the glass slide to prevent loss of any of the solution. The slide was left for 5 min at 85 °C to allow complete evaporation of the solvent. The glass slide was removed from the oven and immediately placed on a metal plate at 20 °C to aid the formation of the tristearin film. The glass slide was stored in a plastic container to prevent dust contamination until SAXS measurements.

SAXS Acquisition and Analysis. The SAXS/WAXS beamline at the Australian Synchrotron, Clayton, Australia, was used to detect the crystalline structure of tristearin present in a sample and the change in the crystallite intensity with increased adjuvant or adjuvant component. Adjuvant/tristearin-coated glass slides were mounted on a steel 40 well plate (126 mm × 78 mm) with the center of each glass slide coinciding with the machined hole through the plate to allow the X-ray beam to pass through the sample. Twelve samples were mounted on each plate. SAXS data were collected for 1 s with a Pilatus 1 M camera (active area 169 × 179 mm² with a pixel size of 172 × 172 µm²), and a sample to detector distance of 1532 mm. The acquired synchrotron SAXS patterns were integrated from 2D scatter patterns to a one-dimensional intensity of scattering function (I(q)) vs the scattering vector (q) using the Scatterbrain software package, developed at the Australian Synchrotron. The length of the scattering vector is defined by the equation q = (4π/λ)sin(2θ/2), where 2θ is the scattering angle, and λ is the wavelength of the X-rays (0.661 Å). Deconvolution of the diffractograms to determine the levels of α and β-crystallities of tristearin was conducted using eXPFit, an add-in for Microsoft Office Excel, designed by Dr. Roger Nix of the University of London.

RESULTS AND DISCUSSION

Correlation of Thermal Behavior between Macrophylla Extract and Tristearin. Comparison of the thermal behavior of the extracted Ficus macrophylla cuticle wax and tristearin in the presence of alkyl ester adjuvant was undertaken by differential scanning calorimetry (DSC) to determine the ability of tristearin to act as a model cuticle in these measurements.

The extracted cuticle wax from Ficus macrophylla is a mixture of different lipids, and presents a broad melt profile with multiple melt maxima (Figure 2). The onset of the overall melt transition appeared at 53.27 ± 1.99 °C (n = 15) with the enthalpy of melt transition of 38.30 ± 7.81 J/g. In comparison to the extract, tristearin showed three clear thermal transitions. First is the melt transition for the α-crystallite peak, second, the subsequent crystallization of the β-form, and finally, the peak of the melt transition of the β-form. The α-crystallite form of tristearin had a melt temperature (onset) at 53.40 ± 0.37 °C, (n = 20) and the melt transition showed an enthalpy of 84.45 ± 4.89 J/g, both of which matched literature values for tristearin. The two wax systems therefore showed very similar onset melt temperatures, even with different melt transition profiles. The plant wax has a significantly lower enthalpy of transition which can be attributed to the plant cuticle wax extract being more amorphous due to the complex composition, and thus presenting a lower level of wax crystallites.

Comparison of the effect of adjuvant on the two wax systems was undertaken by the preparation of a series of samples in triplicate, which contained increasing levels of the premixed adjuvant. The effect of the addition of alkyl ester adjuvant, added to both tristearin and cuticle wax at levels of 0–4 and 0–15% w/w as an example of the results from the DSC studies, are shown in Figure 3.

As the level of additive in the cuticle extract was increased, a systematic decrease was observed in both the melt onset peak, from 54 to 48 °C and in the peak area (Figure 3A). The change in the peak area is obscured visually due to a shift in the baseline, but the trend, when converted to change in area as

Figure 2. Representative DSC traces of tristearin (bottom) and cuticle wax extracted from Ficus macrophylla (top).
shown in Figure 4 is clear. Both of these effects are related to the cuticle wax being a mixture of different materials, all of which contribute to the melt transition. Some of these components will have a higher or lower sensitivity to the presence of the additive. A similar pattern was observed for all additives studied which involve the extracted *Ficus macrophylla* cuticle wax. Although this complex composition reduced the resolution of the transitions compared to tristearin, there are sufficient systematic changes in the thermal transition using this extract to provide direct comparison with the systems exposed to tristearin.

As the level of alkyl ester adjuvant was increased in the tristearin film, the formation of the \( \alpha \)-crystallite peak was suppressed and a marked change in the \( \alpha \)-crystallite peak shape occurred. At low levels of additive, only small changes in the onset melt temperature and peak area occurred. Above 0.5\% w/w alkyl ester adjuvant levels, the onset melt temperature and peak area deceased substantially. The \( \alpha \)-crystallite peak completely disappeared upon the addition of 4\% w/w of the alkyl ester adjuvant. As the enthalpy of the \( \alpha \)-crystallite decreased, the onset temperature of the melt transition also decreased and the peak shape broadened due to increased disruption of the crystalline matrix of tristearin.

The most significant difference between the two wax systems was that even when high loadings of the adjuvant were added to the cuticle wax, there was a proportion of the melt transition that was not influenced by the presence of adjuvant. This was not the case with tristearin, when even at low levels of additive, there was a complete suppression of the melt transition under study. The changes in the peak area for all the additives studied are more clearly observed when the area of the melt transitions is plotted against the additive concentration (Figure 4). The peak area of the melt transition relates to the level of crystallites formed when the film is crystallized and is dependent upon both the nature and the level of the additive present. This gives an accurate and reproducible comparison for the study of the effect of additives on the wax matrix system.

**Effectiveness of Different Adjuvants on Extracted Cuticle Wax.** The addition of alkyl ester adjuvants to the extracted cuticle wax induced a reduction in the crystallite peak area of 12\% at only 2.5\% w/w adjuvant, which reduced to 55\% at 7.5\% w/w alkyl ester adjuvant. At levels of addition of alkyl ester adjuvant above 7.5\% w/w a plateau occurred, indicating a limit of the effect of the adjuvant on the cuticle wax extract. The MO1 adjuvant (Figure 4B) behaved in a slightly different manner from that of the alkyl ester adjuvant, with a greater interaction at lower additive levels. There was a 19\% reduction in the peak area at 2.5\% w/w additive level but a reduced interaction at higher levels. Slightly greater than 10\% w/w additive was required to achieve a 51\% loss in the peak area. At this point, a limit was reached, where the adjuvant system was effective at suppression of the cuticle wax crystallites. The MO2 adjuvant required greater additive levels of 15\% w/w to achieve the maximum suppression of the cuticle wax (Figure 4C), but unlike the previously discussed adjuvants, the level at which the suppression of the wax crystallites occurred was significantly lower, at 72\% loss of melt transition. This difference may be due to the fact that the MO2 adjuvant has a much lower surfactant content than the alkyl ester adjuvant or the MO1 adjuvant. The phospholipid mixture lecithin (Figure 4D) adjuvant was by far the least effective adjuvant system studied, in which a 2.5\% w/w loading caused only an 8\% suppression of the peak area and required over 15\% w/w lecithin adjuvant to achieve the maximum extent of interaction with the wax.

**Effectiveness of Different Adjuvants on Tristearin.** The profiles for adjuvant effectiveness against tristearin in Figure 4E–H are visually similar to those for the extracted wax appearing as sigmoidal type dose–response curves, except that they demonstrated complete elimination of \( \alpha \)-crystallites with the sufficient addition of adjuvant, making the enthalpy reach baseline levels. It is apparent visually that the alkyl ester induced the greatest change at lower adjuvant concentrations than the two MO adjuvants, which in turn were much more effective than the lecithin system.

**Comparison of Trend in Effectiveness of Adjuvants between Waxes Using Half-Maximal Effective Concentration.** The trend in effectiveness of the different adjuvants against the crystallites in the extracted cuticle wax is more easily compared quantitatively when plotted as the half-maximal effective concentration (EC\(_{50}\)). This index can then
be used as a measure of the additive potency towards the crystalline material and is analogous to the measurement of drug potency. The significant advantage of the use of the EC₅₀ methodology is the direct comparison of effectiveness between different adjuvants obtained for either wax system, and direct visual comparison of the trends between them. The EC₅₀ was calculated with the GraphPad Prism 7 graphical software, with the use of an asymmetric sigmoidal analysis function, on each of the triplicate series for both wax systems, for each of the adjuvants tested. An average value of the EC₅₀
was calculated from the individual values obtained and shown in Figure 5. There is a ranking of effectiveness apparent from the EC$_{50}$ values for the cuticle extract.

![Figure 5. Comparison of the additive effect on different wax systems by EC$_{50}$ methodology.](image)

As can be observed, the trends of the EC$_{50}$ values of additives for both the extracted cuticle wax and tristearin, the proposed cuticle wax mimic, are very similar. The alkyl ester-based adjuvant had the lowest EC$_{50}$ value for both the extracted cuticle wax and tristearin. The mineral oil-based adjuvants both show the same trends, that is, the MO1 adjuvant was overall more effective than MO2 at the suppression of the crystallites in both systems. Finally, the least effective adjuvant, for both systems, with the highest EC$_{50}$ value, was the lecithin-based adjuvant. There was a greater error associated with the measurements based on the extracted cuticle wax data compared to that of the proposed cuticle model wax, tristearin. This is most likely due to the extracted cuticle wax being a mixture of different compounds, potentially including nonwax components of the leaf, which leads to a weaker, more inconsistent crystallization compared to that of the cuticle wax extracted from Ficus macrophylla leaves which have a melting point within the range studied (35–80 °C) but have no interaction with adjuvant products studied. However, the correlation of effectiveness with the adjuvant type against the two wax systems indicates that the extracted solutes that do interact with the adjuvant are representative of cuticle waxes.

Confirmation of Structural Changes in Tristearin To Corroborate DSC as a High-Throughput Screening Method for Adjuvants. The thermal behavior of tristearin is well established from previous DSC-based studies allowing the determination of changes in α-crystallite formation.$^{11a,c}$ However, DSC is still a nonspecific technique, where any heat transfer will induce a signal. In comparison, diffraction measurements provide confirmatory direct and definitive proof of changes in phase transitions through the direct measurement of the structure. Small angle X-ray scattering, when coupled with a synchrotron X-ray source, provides high quality rapid diffraction data to correlate with the DSC data. Premixed samples were prepared similarly to those of the DSC samples but on glass microscope coverslips and the diffraction was measured using SAXS.

A representative example of the SAXS patterns obtained from transmission experiments on the films are shown in Figure 6, where ethyl oleate, a component of adjuvant systems, was the additive premixed with tristearin.

The diffractogram of tristearin alone shows a clear diffraction peak at $q = 0.124$ Å$^{-1}$, which corresponds to the α-form of tristearin (see Figure S1 in the Supporting Information for full tristearin diffractogram).$^{11b,18}$ The addition of ethyl oleate as an additive induced a slight broadening of the diffraction pattern, but no change in the level of α-crystallite of tristearin occurred below 1% ethyl oleate. Above 1%, the appearance of a small peak at $q = 0.140$ Å, which corresponds to the β-crystallite of tristearin, was apparent.$^{11b,18}$ With the increased addition of ethyl oleate additive, the α-crystallite peak decreased further in intensity, replaced by the β-crystallite. At 3% (w/w) ethyl oleate and above, no α-crystallite remained and only the β-crystallite of tristearin was present.
Reduction in the level of \( \alpha \)-crystallite of tristearin upon increased amount of ethyl oleate was calculated for both DSC and SAXS data sets (Figure 7A), expressed as the EC\(_{50}\) value (Figure 7B). Determination of the SAXS data were run in triplicate and, where required, deconvolution of the peaks was conducted using the eXPFIT macro, designed for the use with Microsoft Excel.19

The SAXS data set is similar to that of the DSC data set for the use of ethyl oleate as an additive, in that they both show sigmoidal responses to the inclusion of the additive. The EC\(_{50}\) value of the effect of ethyl oleate on tristearin was calculated to be 1.48% by DSC and 1.84% by SAXS. The overall slightly higher EC\(_{50}\) values obtained from SAXS measurements is ascribed to the lower temperature at which SAXS measurements were conducted (room temperature). In contrast, the DSC measurement of the intensity of the \( \alpha \)-peak essentially begins at 45°C, at which point the system will be more fluid. So, although both techniques are probing the level of disruption of the tristearin films, they are conducted under very different conditions. The close concordance between the two techniques provides further confidence in DSC as a strong representative ranking approach for comparing effectiveness across adjuvants or components of adjuvant systems. Additional supporting examples of these correlations are provided in Figure S2 in the Supporting Information.

**Comparison of Major Components with Complete Adjuvant Products.** Using the methodology described in the earlier sections, calculation of EC\(_{50}\) values for the complete adjuvant products, compared to the major component from which they are composed can be made (Figure 8). The raw data providing the EC\(_{50}\) values are illustrated in Figures S3–S5 in the Supporting Information.

![Figure 8](image-url)

The lecithin adjuvant product, which contained a mixture of soy lecithin and alkyl oleate esters as the main components, showed behavior that fell between that of the two components, indicating that methyl oleate is required to boost the poor performance of lecithin, but is not synergistic in its combination. In contrast, the mineral and vegetable oil components (ethyl and methyl oleate) were more effective than the corresponding complete adjuvant product. This suggests that the oil component of the adjuvant product is more able to disrupt the model cuticle film than the complete adjuvant system. It is acknowledged that the commercial products may also contain minor amounts of particularly...
surface-active components that may provide a large effect on cuticle disruption; the methods demonstrated here provide a means to more rapidly isolate and compare their individual contributions to the overall product formulations.

Comparison of the Effectiveness of Adjuvant Systems on Surface Application. The studies shown above were performed by premixing the extracted cuticle wax or tristearin with the system of interest. Although demonstrated to give useful trending information, the use of the model cuticle for the study of adjuvants and their components are of greater values if they correlate with real world application conditions. The conventional application of adjuvant products is through spray deposition onto the leaf surface of the crop with subsequent drying of the deposited droplets on the leaf surface through environmental exposure. To that end, the application of the adjuvant products to a film of tristearin with subsequent drying was also undertaken to allow comparison of the premix and surface-applied methodologies. The study of the aqueous application of the individual components was not possible as homogeneous systems could not be formed due to immiscibility of the oils with water, consequently the aqueous surface application studies were limited to the complete adjuvant products.

The application of aqueous emulsions of the adjuvant products were undertaken with care to ensure that the complete film of tristearin was covered by a thin layer of emulsion. The samples were dried at 40 °C to ensure that no polymorphic change to the tristearin film occurred due to the drying process. The samples were run in triplicate by DSC with the change in peak area of the α-crystallite form of tristearin measured and expressed as the average EC50 value. As the data was asymmetric, this was calculated with the GraphPad Prism 6 graphical software using asymmetric sigmoidal analysis. Comparison of the effectiveness of the adjuvant product emulsions when premixed with tristearin (data repeated from Figure 7), to that when applied to precast tristearin films is shown in Figure 9.

There are similar trends observed between the premixed approach and surface-applied approach. First, the alkyl ester adjuvant product had the lowest EC50 of the products using both application methods. Secondly, the mineral oil-based products showed the same pattern of effectiveness, in that the MO1 adjuvant showed the lowest EC50 of the mineral oil-based adjuvants and MO2 adjuvant the least effective.

Notably the scale of the difference between the performance of the two mineral oil adjuvant products was significantly greater using the surface-applied methodology, even though the same pattern of effectiveness was observed. For the premix approach, all the components are evenly distributed through the tristearin, providing a means of maximum interaction. The effectiveness of adjuvants using the surface-applied methodology might be expected to be much more dependent on the surfactant component, as the penetration of the tristearin layer has to occur before disruption of the tristearin crystallites can occur, mimicking the real-life process of foliar interaction of adjuvants and pesticides. The mineral oil adjuvant MO2 has a much lower surfactant content than MO1, and as such may have compromised ability to wet the tristearin film as a prerequisite to penetration.

The lecithin-based adjuvant performed surprisingly well in the surface-applied format, as the surface activity of the phospholipid component likely acts similarly to surfactants in providing a wetting function and enabling penetration of the methyl oleate into the film to exert its effect on the crystallites. In the premix format, lecithin cannot serve this function, thus, it appears that the nature of the oil component needs to be considered in deciding the approach to use these high-throughput approaches for ranking adjuvant effectiveness as a route to more judicious choice of formulations for plant trials.

In conclusion, the use of tristearin as a model cuticle system for the high-throughput screening of performance-relevant differences between adjuvants and adjuvant components was demonstrated. Both differential scanning calorimetry and small angle X-ray scattering provided concordant ranking of effectiveness, indicating that DSC as a laboratory-based approach is suitable for this purpose. The studies do not inform other important factors dictating overall field performance of adjuvant systems including the droplet size, spray cone dimensions, potential for drift, and soil wetting behavior. However, this methodology assesses the important adjuvant behavior at the major barrier at the plant level, and thereby enables valuable insight into the factors for selection of likely new adjuvant candidates to progress into more expensive plant-based screening methods.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02656.

XRD profiles of tristearin polymorphs; dose–response curves for increasing levels of adjuvants on tristearin and cuticle extracts determined from the peak area, enthalpy of transition, measured using SAXS and DSC (PDF)

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Notes
The authors declare the following competing financial interest(s): At the time of the studies Andrew Killick and
Peter Jones were employees of Victorian Chemicals, who manufacture some of the adjuvants in this study. Graham Webster was an employee of Monash University but is now an employee of Victorian Chemicals.

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**REFERENCES**

(1) (a) Hazen, J. L. Adjuvants - terminology, classification, and chemistry. *Weed Technol.* 2000, 14, 773−784. (b) Tu, M.; Hurd, C.; Randall, J. M. *Weed Control Methods Handbook: Tools and Techniques for Use in Natural Areas;* The Nature Conservancy: Arlington, VA, 2001.

(2) (a) Katagi, T. Surfactant effects on environmental behavior of pesticides. *Reviews of Environmental Contamination and Toxicology;* Whitacre, D. M., Ed.; Springer: New York, NY, 2008; pp 71−177. (b) Green, J. M.; Beestman, G. B. Recently patented and commercialized formulation and adjuvant technology. *Crop Prot.* 2007, 26, 320−327. (c) Rodham, D. K. Colloid and interface science in formulation research for crop protection products. *Curr. Opin. Colloid Interface Sci.* 2000, 5, 280−287. (d) Thacker, J. R. M.; Young, R. D. F. The effects of six adjuvants on the rainfastness of chlorpyrifos formulated as an emulsifiable concentrate.

(3) (a) Kirkwood, R. C. Recent developments in our understanding of the plant cuticle as a barrier to the foliar uptake of pesticides. *Pestic. Sci.* 1999, 55, 198−200. (b) Stock, D.; Geoff, B. Physicochemical properties of adjuvants: values and applications. *Weed Technol.* 2000, 14, 798−806. (c) Wyss, G. S.; Charudattan, R.; Rosskopf, E. N.; Littell, R. C. Effects of selected pesticides and adjuvants on germination and vegetative growth of *Phomopsis Amaranthicola,* a biocontrol agent for *Amaranthus spp.* *Weed Res.* 2004, 44, 469−482. (d) Castro, M. J. L.; Oyeda, C.; Cirelli, A. F. Advances in surfactants for agrochemicals. *Environ. Chem. Lett.* 2012, 14, 85−95.

(5) (a) Pang, Y.-D.; Larson, I.; Barnes, T. J.; Prestidge, C. A.; Boyd, B. J. Adsorption of nonlamellar nanostructured liquid-crystalline particles to biorelevant surfaces for improved delivery of bioactive compounds. *ACS Appl. Mater. Interfaces* 2011, 3, 1771−1780. (b) Nadiminti, P. P.; Rookes, J. E.; Dong, Y. D.; Sayer, C.; Boyd, B. J.; Cahill, D. M. Nanostructured liquid crystalline particle assisted delivery of 2,4-dichlorophenoxyacetic acid to weeds, crops and model plants. *Crop Prot.* 2016, 32, 17−29.

(7) Nadiminti, P. P.; Dong, Y. D.; Sayer, C.; Hay, P.; Rookes, J. E.; Boyd, B. J.; Cahill, D. M. Nanostructured liquid crystalline particles as an alternative delivery vehicle for plant agrochemicals. *ACS Appl. Mater. Interfaces* 2013, 5, 1818−1826.

(8) (a) Singh, M.; Siyu, T.; Shiv, D. S. Adjuvants enhance weed control efficacy of foliar applied diuron. *Weed Technol.* 2002, 16, 74−78. (b) Schöherr, J.; Baur, P.; Uhlig, B. A. Rates of cuticular penetration of 1-naphthylacetic acid (NAA) as affected by adjuvants, temperature, humidity and water quality. *Plant Growth Regul.* 2000, 31, 61−74.

(9) (a) Koch, K.; Enskakt, H.-J. The hydrophobic coatings of plant surfaces: epicuticular wax crystals and their morphologies, crystallinity and molecular self-assembly. *Micron* 2008, 39, 759−772. (b) Enskakt, H.-J.; Boese, M.; Mader, W.; Barthlott, W.; Koch, K. Crystallinity of plant epicuticular waxes: electron and X-ray diffraction studies. *Chem. Phys. Lipids* 2006, 144, 45−59. (c) Macková, J.; Vašková, M.; Macek, P.; Hronková, M.; Schreiber, L.; Šantrůček, J. Plant response to drought stress stimulated by ABA application: Changes in chemical composition of cuticular waxes. *Environ. Exp. Bot.* 2013, 86, 70−75.

(10) (a) Fagerström, A.; Kocherbitov, V.; Westbye, P.; Bergström, K.; Arnebrant, T.; Engblom, J. Surfactant softening of plant leaf cuticle model wax − A differential scanning calorimetry (DSC) and quartz crystal microbalance with dissipation (QCM-D) study. *J. Colloid Interface Sci.* 2014, 426, 22−30. (b) Fagerström, A.; Kocherbitov, V.; Westbye, P.; Bergström, K.; Mamontova, V.; Engblom, J. Characterization of a plant leaf cuticle model wax, phase behaviour of model wax−water systems. *Thermochim. Acta* 2013, 571, 42−52.

(11) (a) Matovic, M.; van Miltenburg, J. C.; Los, J.; Gandolfo, F. G.; Flöter, E. Thermal properties of tristearin by adiabatic and differential scanning calorimetry. *J. Chem. Eng. Data* 2005, 50, 1624−1630. (b) Kellens, M.; Meeussen, W.; Gehrke, R.; Reynaers, H. Synchrotron radiation investigations of the polymorphic transitions of saturated monounsaturated triglycerides. Part 1: tripalmitin and tristearin. *Chem. Phys. Lipids* 1991, 58, 131−144. (c) Oh, J. H.; McCurdy, A. R.; Clark, S.; Swanson, B. G. Characterization and thermal stability of polymorphic forms of synthesized tristearin. *J. Food Sci.* 2002, 67, 2911−2917.

(12) Mullin, J. W. Crystallization. In *Crystallization,* 4th ed.; Butterworth-Heinemann: Oxford, 2001.

(13) Nadiminti, P. P.; Rookes, J. E.; Boyd, B. J.; Cahill, D. M. Confocal laser scanning microscopy elucidation of the microcrystallinity of the leaf wax cuticle. *ACS Omega* 2016, DOI: 10.1021/acsomega.8b02656.