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Article

Low-Frequency Raman Scattering Spectroscopy as an Accessible Approach to Understand Drug Solubilization in Milk-Based Formulations during Digestion

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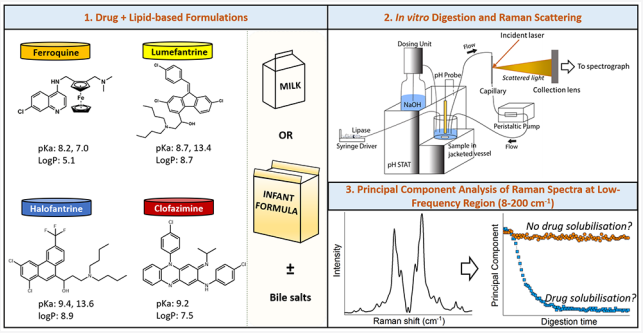
ABSTRACT: Techniques enabling *in situ* monitoring of drug solubilization and changes in the solid-state of the drug during the digestion of milk and milk-based formulations are valuable for predicting the effectiveness of such formulations in improving the oral bioavailability of poorly water-soluble drugs. We have recently reported the use of low-frequency Raman scattering spectroscopy (region of analysis <200 cm^{-1}) as an analytical approach to probe solubilization of drugs during digestion in milk using ferroquine (SSR97193) as the model compound. This study investigates the wider utilization of this technique to probe the solubilization behavior of other poorly water-soluble drugs (halofantrine, lumefantrine, and clofazimine) in not only milk but also infant formula in the absence or presence of bile salts during *in vitro* digestion. Multivariate analysis was used to interpret changes to the spectra related to the drug as a function of digestion time, through tracking changes in the principal component (PC) values characteristic to the drug signals. Characteristic low-frequency Raman bands for all of the drugs were evident after dispersing the solid drugs in suspension form in milk and infant formula. The drugs were generally solubilized during the digestion of the formulations as observed previously for ferroquine and correlated with behavior determined using small-angle X-ray scattering (SAXS). A greater extent of drug solubilization was also generally observed in the infant formula compared to milk. However, in the case of the drug clofazimine, the correlation between low-frequency Raman scattering and SAXS was not clear, which may arise due to background interference from clofazimine being an intense red dye, which highlights a potential limitation of this new approach. Overall, the *in situ* monitoring of drug solubilization in milk and milk-based formulations during digestion can be achieved using low-frequency Raman scattering spectroscopy, and the information obtained from studying this spectral region can provide better insights into drug solubilization compared to the mid-frequency Raman region.



KEYWORDS: *low-frequency Raman spectroscopy, drug, solubilization, in vitro digestion, X-ray scattering, milk, infant formula*

INTRODUCTION

The dissolution of poorly water-soluble lipophilic drugs in the gastrointestinal (GI) tract is often the limitation of absorption that leads to poor and variable oral bioavailability.[1] Lipid-based vehicles have received great interest as a formulation approach to overcome these challenges by maintaining the drug in a solubilized form during digestion.[2] These lipid-based formulations can range in composition from simple triglycerides to mixtures of glyceryl esters with fatty acids and surfactants or solvents. Full cream milk, having an average fat content of 3.8 w/v %, provides a natural source of triglycerides along with proteins and other micronutrients.[3] Ingestion of milk results in enzymatic lipolysis of triglycerides primarily in the intestinal region of the GI tract to form digestion products (diglycerides, monoglycerides, and fatty acids) that, due to the amphiphilic nature of the molecules, can self-assemble in aqueous solution to form a range of liquid crystalline structures.[4] These structures can potentially provide a favorable environment into which lipophilic drugs can partition, enabling their availability for absorption. Several studies have shown an enhanced oral bioavailability of poorly water-soluble drugs after coadministration with milk,[5,6] and an enhancement in drug solubilization during digestion was also observed in *in vitro* studies.[7−9] The investigation of drug solubilization during digestion is therefore critical to enable optimization of milk

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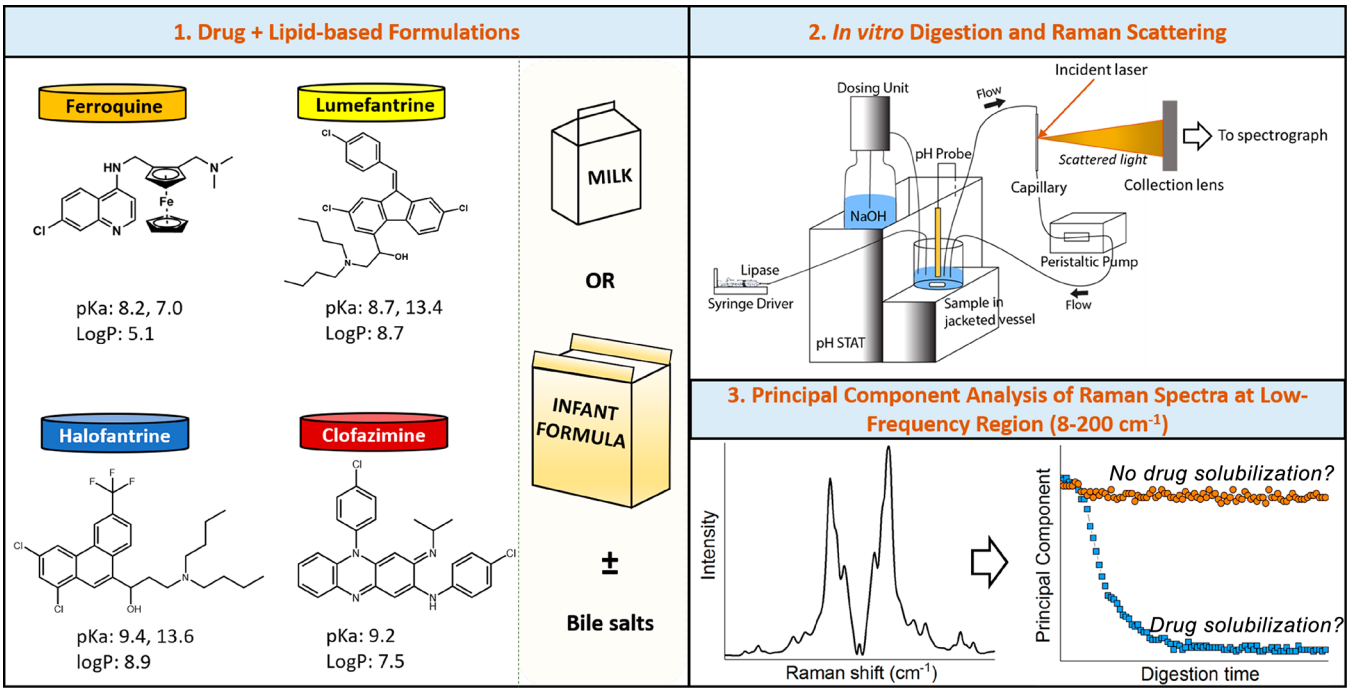

Figure 1. Schematic diagram for the design of the present study. The solid drug in powder form was added to milk or infant formula with or without added bile salts. The drug/formulation mixtures were digested under small intestinal conditions using a pH-stat digestion system coupled to a capillary for in-line low-frequency Raman spectroscopy. Principal component analysis of the Raman spectra in the low-frequency Raman shift region from 8 to 200 cm^{-1} was conducted to monitor changes related to the drug during digestion by extracting the drug signals from the background formulations. pK_a and logP values of the drugs were obtained from SciFinder (https://scifinder.cas.org/) and estimated using Advanced Chemistry Development (ACD/Laboratories) Software V11.02.

and milk-based formulations such as infant formula as a drug delivery vehicle.[7,8]

Methods typically used to study drug solubilization during digestion of lipid-based formulations include offline analytics, where drugs are separated by ultracentrifugation into a solid pellet and aqueous and lipid phases, and the drug concentration was determined by high-performance liquid chromatography (HPLC).[10,11] This requires a difficult separation of the lipid layer (where present) and the aqueous layer, followed by solvent extraction of the drug from all phases, which can potentially lead to poor drug recoveries and erroneous estimation to the amount of drug available for absorption. The use of digestion inhibitors to stop further lipolysis of the lipids may also potentially affect the partitioning of the drugs.[12] The development of analytical tools that enable *in situ* monitoring of drug solubilization during digestion coupled with solid-state analysis of the drug is therefore invaluable.

X-ray scattering and Raman spectroscopy are the two most widely used techniques to identify the solid-state form of drugs and have also been used to characterize drug solubilization and precipitation during digestion of lipid-based formulations.[13,14] For example, precipitation of a model neutral drug, fenofibrate, during digestion of self-emulsifying drug delivery systems has been detected using small-angle X-ray scattering (SAXS) and mid-frequency Raman spectroscopy.[15,16] Recently, utilizing SAXS as the detection tool, simultaneous determination of digestion-mediated polymorphic transformation and solubilization of a weakly basic antimalarial drug has been reported during digestion of a milk system.[7] The basis of the two techniques is different as SAXS involves the scattering of X-rays by electrons from planes of atoms in preiodically-ordered molecules,[17] whereas Raman spectroscopy probes the vibrational transitions in and between molecules.[18] Changes in vibrational modes can, therefore, be associated with different intra- and intermolecular interactions.[19]

Differentiation between solid-state forms of drugs using Raman spectroscopy is generally performed by analyzing subtle spectral differences (peak shifts and relative intensities) in the fingerprint region (between 200 and 1800 cm^{-1}), where changes arise from differences in the intramolecular environment.[20] However, recent advancements in technologies (specifically volume Bragg gratings) have allowed the rapid collection of spectra to within 10 cm^{-1} of the laser line in dispersive systems with CCD detectors. This significantly improves the specificity of the instrumentation toward intermolecular interactions by accessing the low-frequency region of the Raman spectra in a rapid time frame.[21,22] This has allowed probing of the vibrational modes of the long-range crystalline lattice (phonon vibration) and hence has been increasingly used to investigate crystallization of an amorphous material and vice versa.[20,21]

We have recently explored the use of low-frequency Raman spectroscopy as a technique to monitor the solubilization behavior of a poorly water-soluble compound in milk during *in vitro* digestion using ferroquine as the model active pharmaceutical ingredient (API).[23] Analysis of the low-frequency Raman spectra showed complete disappearance of the spectral features attributable to the drug during digestion, indicative of drug solubilization, which was in agreement with SAXS measurements. The aim of this study is to evaluate further the potential use of low-frequency Raman spectroscopy to probe the solubilization of a wider range of drugs during digestion in a wider range of complex media (inclusive of

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Mol. Pharmaceutics 2020, 17, 885−899

infant formula and milk with the presence of bile salts). This is important for establishing the general applicability of the *in situ* low-frequency Raman approach as an accessible and sustainable technique that could be used in place of the synchrotron X-ray scattering approach by the pharmaceutical industry for screening formulations for drug solubilization during digestion. Ferroquine and a range of other poorly water-soluble model compounds (lumefantrine, halofantrine, and clofazimine) were studied during digestion across the milk and milk-like systems. Solubilization behavior of the drugs during digestion measured usinglow-frequency Raman spectroscopy was compared to results from synchrotron SAXS, and HPLC was used to quantify the distributions of the drugs in the separated phases after digestion. A schematic representation of the concept of the study is summarized in Figure 1.

MATERIALS AND METHODS

Materials. Ferroquine granules that contained 50 wt % ferroquine (SSR97193) were supplied by Sanofi (Paris, France). Lumefantrine was purchased from Haihang Industries (Shandong, China; min 99% purity). Halofantrine base was purchased from GlaxoSmithKline (King of Prussia, PA; >99% purity). Full cream bovine milk was purchased from local supermarkets in Melbourne (Australia) and Dunedin (New Zealand). Infant formula (brand not disclosed due to "commercial-in-confidence") was provided by Medicines for Malaria Venture (MMV). Full nutritional information on the milk and infant formula is summarized in Table S1. Clofazimine, sodium taurodeoxycholate hydrate (≥95%), Trizma maleate (reagent grade), and 4-bromophenylboronic acid (4-BPBA, >95%) were purchased from Sigma-Aldrich (St. Louis, Missouri). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Cayman Chemical (Michigan, USA). Calcium chloride dihydrate (>99%) and sodium hydroxide pellets (min 97%) were purchased from Ajax Finechem (Seven Hills, New South Wales, Australia). Sodium chloride (>99%) was purchased from Chem Supply (Gillman, South Australia, Australia). Sodium azide (≥99%) was purchased from Fluka (Sigma-Aldrich, St. Louis, Missouri). Dichloromethane and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). USP grade pancreatin extract was purchased from Southern Biologicals (Nunawading, Victoria, Australia). Unless otherwise stated, all chemicals were used as received without further purification, and water was sourced from Millipore Milli-Q water purification systems at the point of use.

Methods. *Sample Preparation and in Vitro Lipolysis.* Digestion of ferroquine (186 mg granules containing 93 mg ferroquine), halofantrine (40 mg), and clofazimine (50 mg) in milk and/or infant formula was performed on a pH-stat apparatus (Metrohm 902 STAT titration system, Figure 1) using methods described previously.[8,23,24] Briefly, ferroquine granules, halofantrine, clofazimine, or lumefantrine (60 mg) were added to 2.75 mL of water. To simulate a gastric step, 0.25 mL of 1 M HCl was added to the drugs, except for ferroquine, prior to the addition of 17.5 mL of milk or infant formula in digestion buffer. The amount of fat in milk and infant formula was kept constant at 0.67 g. The digestion buffer was 50 mM tris-maleate containing 5 mM $CaCl_2.2H_2O$, 6 mM NaN_3, and 150 mM NaCl, pH 6.5. Due to the hydrophobicity of lumefantrine, halofantrine, and clofazimine, a small amount of freeze-dried bile salts/phospholipid powder was added to the drug solution to promote wetting. Final concentrations of the bile salt and phospholipid in the drug/milk mixtures were 1.34 mM and 0.28 mM, respectively, and preparation of the powder is detailed in the Supporting Information. The drug/milk samples were transferred to a digestion vessel maintained at 37 °C using a temperature-controlled water bath, and the pH of the samples was adjusted to 6.500 ± 0.003 prior to digestion, which was initiated through a remote injection of 2.25 mL of pancreatic lipase solution (reconstituted from freeze-dried lipase prepared using methods described in the Supporting Information). The activity of the lipase was approximately 700 tributyrin unit (TBU) per mL of digest, which was determined based on prior digestion of 6 g of tributyrin per 20 mL of digestion buffer at pH 7.0. The pH of the sample was maintained at 6.5 during lipolysis using 2 M NaOH solution controlled using Tiamo version 2.5 software (Metrohm, Herisau, Switzerland).

Raman Spectroscopy. The experimental configuration for low-frequency Raman spectroscopy has been described previously.[25] In brief, a 785 nm laser source (Ondax, Inc. Monrovia, CA, USA) was filtered by two BragGrate bandpass filters (OptiGrate Corp. Oviedo, FL, USA) and focused (~500 μm sample spot) on the quartz capillary containing the digestion media at a 135° angle relative to the collecting lens. The backscattered light from the sample was collected and filtered through a set of volume Bragg gratings (Ondax, Inc. Monrovia, CA, USA) and focused into a LS 785 spectrograph (Princeton Instruments, Trenton, NJ, USA), which dispersed the scattered light onto a PIXIS 100 BR CCD (Princeton Instruments, Trenton, NJ, USA). Spectra were collected using WinSpec/32 software (Princeton Instruments, Trenton, NJ, USA) over a spectral window of −360 to 2030 cm^{-1} with 5−7 cm^{-1} resolution. A spectrum was collected every half a minute (0.5 s acquisition time, ×60 accumulations per frame) for a total of about 45 min, i.e., 90 frames.

Small-Angle X-ray Scattering. The digesting formulation was aspirated through a fixed quartz capillary mounted in the X-ray beam (13 keV, wavelength λ = 0.954 Å), using a peristaltic pump operating at approximately 10 mL/min. The sample-to-detector distance was approximately 0.6 m, covering $0.04 < q < 2.00$ $Å^{-1}$). The 2D SAXS patterns were recorded using a Pilatus 1 M detector with a 5 s acquisition time and a delay of 15 s between measurements, i.e., one measurement every 20 s. The raw data were reduced to 1D scattering functions $I(q)$ by radial integration using the custom in-house software ScatterBrain version 2.71.

HPLC. Quantification of ferroquine and clofazimine in the digested milk phases after ultracentrifugation has been described previously.[12,24] For lumefantrine and halofantrine, 200 μL of samples were collected at various time points during digestion of milk or infant formula and pipetted into polycarbonate centrifuge tubes followed by the addition of 4-BPBA (2 μL of 0.5 M 4-BPBA in methanol). Samples were ultracentrifuged for 40 min at 329 177*g*, 37 °C, using an Optima MAX-TL ultracentrifuge (Beckman Coulter, Indiana, USA) with a TLA-100 rotor. The resulting aqueous supernatant layer was removed using a 1 mL syringe and 25 gauge needle, and the tubes were cut into two separate sections: one containing the upper lipid layer and the other containing the bottom solid pellet. The tubes were placed in individual 1.5 mL centrifuge tubes and stored in the freezer prior to the extraction of the drugs. Methanol (200 μL) was added into each 1.5 mL centrifuge tube and vortexed. The ultracentrifuge tubes were removed from the 1.5 mL centrifuge tubes, and 800

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Mol. Pharmaceutics 2020, 17, 885−899

μL of dichloromethane (containing halofantrine as an internal standard for lumefantrine samples, and vice versa) was added to extract the lumefantrine or halofantrine from the lipid and pellet phases. The samples were centrifuged for 15 min at 14 462*g* to remove precipitated proteins, and the supernatant was diluted in 65 vol % methanol (mobile phase B) and 35 vol % 23 mM sodium dihydrogen phosphate with a 30 mM sodium hexanesulfonate ion pairing agent, pH 2.3 (mobile phase A). The HPLC system consisted of a Shimadzu CBM-20A system controller, LC-20AD solvent delivery module, SIL-20A autosampler, and a CTO-20A column oven set at 35 °C, coupled to an SPD-20A UV-detector (Shimadzu Corporation, Kyoto, Japan). A reverse-phase C_{18} column (4.6 × 75 mm^2, 3.5 μm; Waters Symmetry, MA, USA) was used for the separation, and the drugs were detected at 255 nm. The mobile phase gradient consisted of 65−90% B for 10 min, 90% B for 1 min, 90−65% B for 0.5 min, and 65% B for 3.5 min. The retention time for lumefantrine was 8.3 min and 7.4 min for halofantrine.

Multivariate Analysis. Principal component analysis (PCA) was applied to the Raman spectra to qualitatively monitor drug dissolution during *in vitro* digestion in milk and infant formula using Orange software version 3.16.[26] This analysis was useful as a first-line approach to determine whether changes relating to drug signals with digestion time were detectable. Injection of lipase and the progress of digestion of milk and infant formula resulted in changes to the baseline of the spectra, and as such, the linear baseline correction of the low-frequency Raman data between −250 and 250 cm^{-1} was performed. After baseline correction, the spectral region between 8 and 200 cm^{-1} was then subjected to vector normalization prior to PCA of the ferroquine and lumefantrine samples. PCA was also performed on the mid-frequency region (300−1800 cm^{-1}) after rubber-band baseline correction and normalization. The emissive nature of clofazimine meant a modified preprocessing was required compared to nonemissive samples. In this instance, the spectral region was reduced to 1000−1800 cm^{-1}, and the data was preprocessed using a rubber-band baseline correction with vector normalization.

PCA was carried out on sample combinations shown in Table 1, where time-dependent spectral changes relating to the digestion of milk/infant formula blanks + drug could be distinguished from spectral changes associated with the digestion of milk/infant formula blanks. These also allowed for comparisons between the extent of drug solubilization in milk vs infant formula.

Table 1. Combinations of Samples Analyzed by PCA

samples			
ferroquine	halofantrine	lumefantrine	clofazimine
milk	milk	milk	milk
milk + ferroquine	milk + halofantrine	milk + lumefantrine	milk + clofazimine
infant formula		infant formula	infant formula
infant formula + ferroquine		infant formula + lumefantrine	infant formula + clofazimine
tris + ferroquine		tris + lumefantrine	tris + clofazimine

RESULTS

The Raman spectra of ferroquine, halofantrine, lumefantrine, and clofazimine powders are shown in Figure 2. Strong distinctive Raman scattering peaks that can be observed in the low-frequency region are indicative of highly ordered crystalline structures, while the intramolecular vibrations of the molecules are observed in the Raman spectral region between 300 and 1800 cm^{-1}. In the case of ferroquine, strong Raman bands exist at 1104 and 1363 cm^{-1}, attributed to ring breathing and C−H bending of the ferrocene group, respectively.[27] For halofantrine, the C—C stretching vibration of the phenanthrene ring gives rise to the prominent peak at 1354 cm^{-1}.[28] In the case of lumefantrine, the band at 1633 cm^{-1} can arise from the C═C stretching, and aromatic ring stretching is observed at 1588 cm^{-1}. Major Raman peaks of the drugs and their characteristic chemical functional groups (in mid-frequency region only) were summarized in Table S2. The broad underlying peak observed in the spectrum from the clofazimine powder sample is due to fluorescence (Figure 2h).

Characteristic peaks for ferroquine, halofantrine, lumefantrine, and clofazimine were still observed after dispersion in milk and infant formula at concentrations between about 2 and 4.5 mg/mL before digestion (Figure 3). Visual observations of the drug peaks at low- and mid-frequency regions after digestion in milk/infant formula revealed complete disappearance of peaks only in the case of ferroquine (Figure 3a,b) and halofantrine (Figure 3c,d). Solubilization of ferroquine in milk has been previously reported,[23] and the Raman spectra were replotted as in Figure S1 for comparison. Reduction in the intensities of the drug peaks in the low-frequency region after digestion in infant formula and milk can be attributed to the loss of crystallinity due to drug solubilization or the formation of amorphous solids. Similar behavior has been observed during the digestion of ferroquine in milk shown in our previous study.[23] In the mid-frequency region, major FQ peaks in the FQ+IF samples were not observed, although a small pertaining peak (at around 1104 cm^{-1}) that was slightly shifted toward higher wavenumbers could be seen. This may be attributed to changes in the nature of ferroquine interactions with surrounding molecules as triglycerides were predominantly present before lipase injection, and fatty acids/monoglycerides are the main compounds present after digestion.

Characteristic Raman peaks for lumefantrine and clofazimine were still present after digestion in milk and infant formula (Figure 3e−l). No significant decrease in the intensities of the lumefantrine peaks was seen after digestion, although slight broadening was observed in the infant formula sample in the mid-frequency region. The peaks in the clofazimine spectrum in the mid-frequency region decreased in intensity compared to lumefantrine after digestion, but the visual interpretation of the changes to the drug peaks in the low-frequency region is difficult.

Hence, to elucidate whether spectral changes of the drug peaks indeed occurred during digestion, principal component analysis (PCA) was performed on the Raman spectra on both the low- and mid-frequency regions to detect for subtle differences in the spectra that could not be otherwise interpreted by visual assessment alone. Analysis of the changes in Raman spectra for ferroquine in the milk and infant formula systems was also performed. It is important to note that the analysis of drug solubilization performed herein using PCA was not intended to be strictly quantitative, but the trends obtained can be compared qualitatively with those from SAXS measurements.

Ferroquine. Multivariate analysis of the ferroquine data from the low-frequency region was conducted and the plot of

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Mol. Pharmaceutics 2020, 17, 885−899

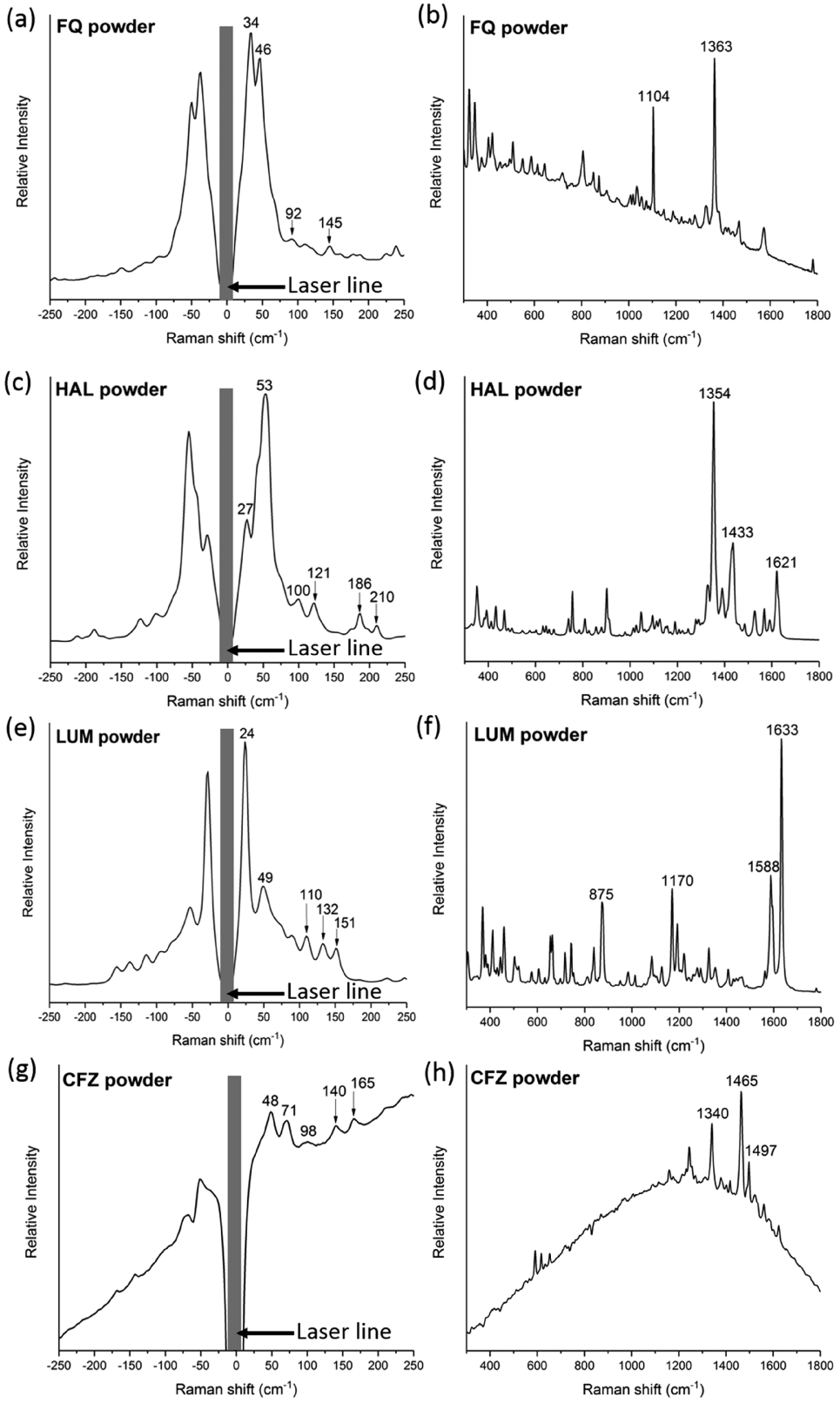

Figure 2. Raman spectra of ferroquine (FQ; panels a and b), halofantrine (HAL; panels c and d), lumefantrine (LUM; panels e and f), and clofazimine (CFZ; panels g and h) API powders at low- and mid-frequency regions.

the first two principal components, which covered 95% of the sample variance, is shown in Figure 4a. Differences in clustering of the data were apparent when the drug was added to infant formula compared to milk or dispersion in tris buffer alone. Based on the loadings plot shown in Figure 4c, the positive first principal component (PC1) was correlated to milk and infant formula, whereas negative PC1 was correlated to ferroquine. PC2 separates the ferroquine signal from the

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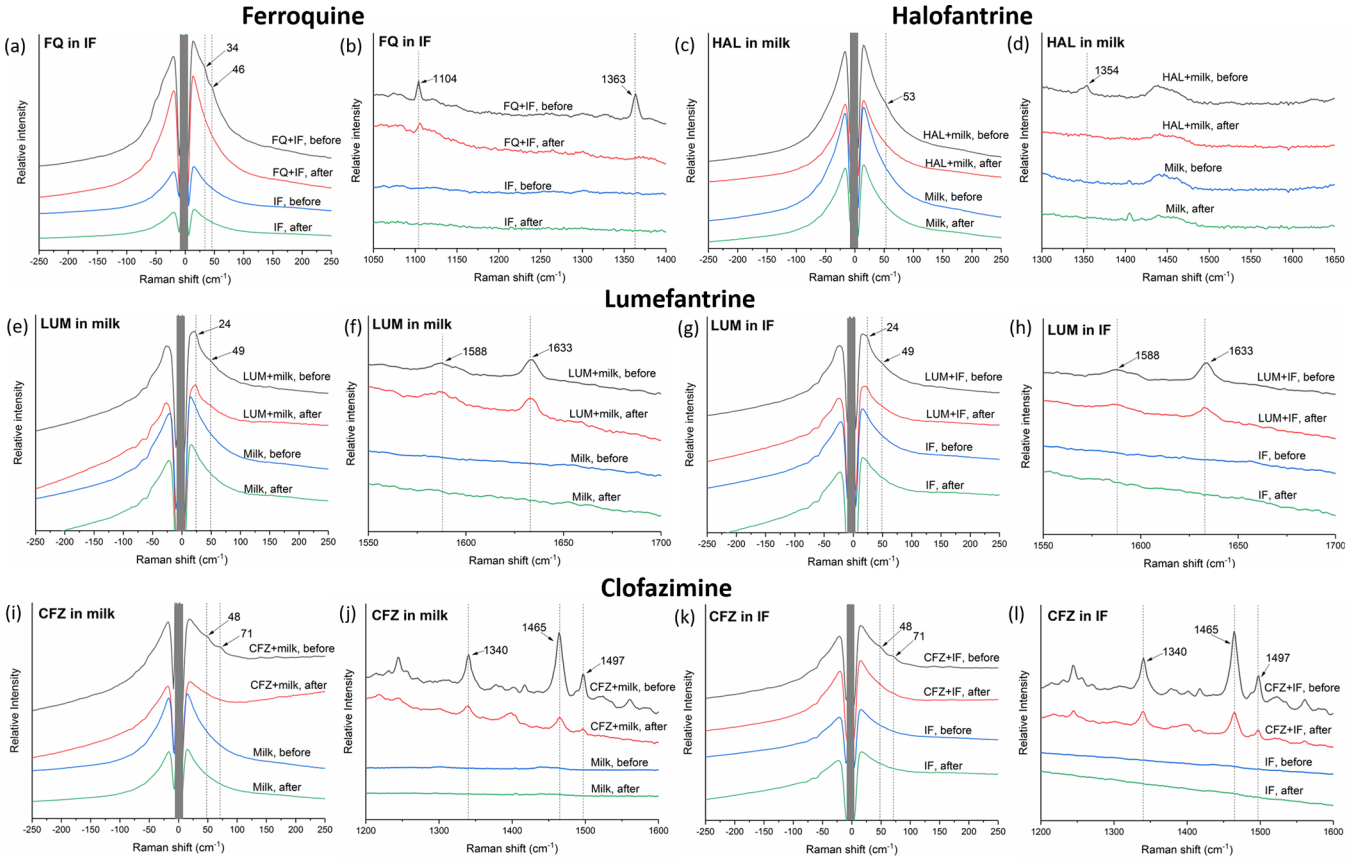

Figure 3. Raman spectra of ferroquine (FQ; a and b), halofantrine (HAL; c and d), lumefantrine (LUM; e–h), and clofazimine (CFZ; i–l) in milk and/or infant formula (IF) for the low- and mid-frequency (drug region-focused) spectral regions before and after digestion. Spectra for the blank milk and IF samples (without bile salt micelles for FQ and with bile salt micelles for LUM, HAL, and CFZ) were included for comparison purposes.

digested milk signal. During digestion, there was a clear change toward negative values of PC2 for ferroquine in milk and infant formula, while no changes toward negative PC2 occurred when dispersed in tris buffer as digestion progressed. To better visualize these changes, PC2 was plotted against dispersion (<0 min) and digestion (>0 min) times in Figure 4b. The value of PC2 for the spectrum of blank milk and infant formula was invariant during digestion (both displaying close-to-zero PC2 values), as was that for ferroquine in tris buffer (showing positive PC2 values), which was anticipated due to the lack of drug in the former two systems and the presence of suspended crystalline drug particles in the latter. In contrast, digestion of the ferroquine-containing milk and infant formula samples resulted in a clear drop in the PC2 from positive to negative values, which subsequently plateaued after about 15–20 min of digestion. These changes could be attributed to the disappearance of drug peaks (that were correlated to positive PC2) due to the loss of drug crystallinity. The lower initial PC2 value for the ferroquine suspension in infant formula compared to milk may indicate greater initial solubilization of ferroquine in the infant formula.

A similar trend in behavior for the ferroquine-containing systems was apparent when PCA was conducted on data obtained for the mid-frequency Raman spectral region (300–1800 cm^{-1}). The two main peaks in the mid-frequency spectrum for ferroquine seen in Figure 3b dominate the loading for PC2 (Figure 4f), which when tracked during digestion also showed no change in the absence of digestion and a strong decrease during digestion in the presence of milk or infant formula (Figure 4e).

Halofantrine. Multivariate analysis of the low-frequency Raman spectra collected during digestion of halofantrine in milk showed a drop in the PC1 values (35% variance, Figure 5a) that was mainly correlated to changes in the drug peaks (loadings plot in Figure 5b), although a slight contribution from the milk-only background could be seen. In contrast to the low-frequency Raman spectra, multivariate analysis of the mid-frequency Raman spectral region from 300 to 1800 cm^{-1} did not provide a clear separation between milk and halofantrine-containing milk samples due to interfering signals from the milk lipids. Comparisons between the loadings plot for PC1 from the mid-frequency range (12% variance) in Figure 5d and the Raman spectra for halofantrine in Figure 2d showed the presence of additional peaks that were not specific to the drug.

Lumefantrine. Multivariate analysis of lumefantrine in milk and infant formula during digestion using the low-frequency region is presented in Figure 6, where the first two principal components covered 98% of the sample variance. The first PC mainly differentiates samples containing lipids from no lipids, i.e., tris buffer only (Figure S2), regardless of the types of milk formulation, as a separate cluster was seen for lumefantrine in tris dispersion. Inspection of the subsequent PCs revealed that PC2 predominantly captured signals relating to lumefantrine of which peaks at 24, 110, 132, and 151 cm^{-1} were correlated with positive PC2. No significant decrease in the PC2 values was observed for lumefantrine after digestion in

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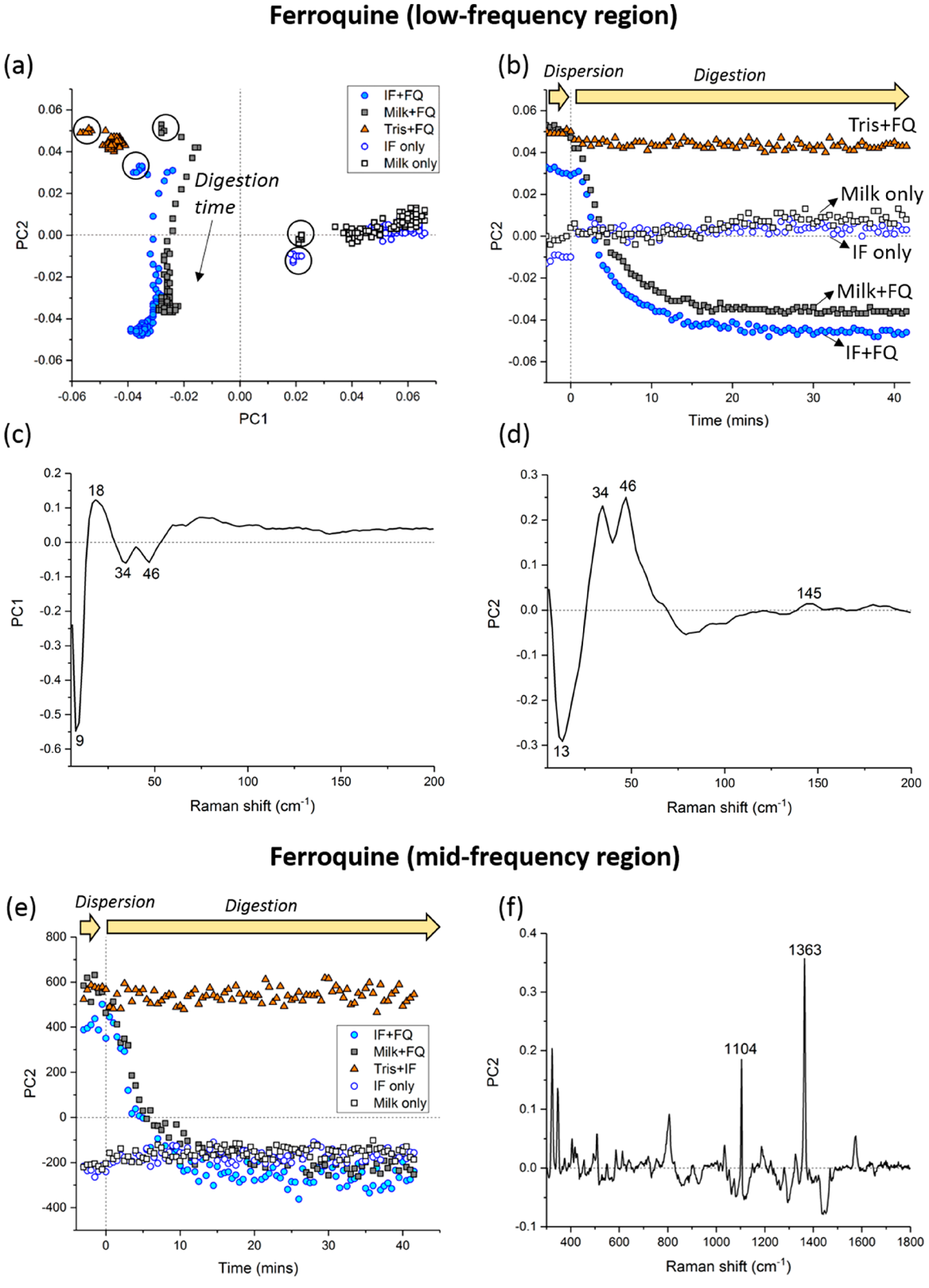

Figure 4. PCA of ferroquine in milk and infant formula during dispersion and digestion. (a) Two dimensional PCA scores plot for milk, infant formula (IF), and ferroquine (FQ) in milk, IF, and tris buffer generated from analysis of the low-frequency Raman shift region from 8 to 200 cm^{-1}. Circled regions point to clusters before lipase injection. (b) The plot for the second principal component (PC2) against dispersion and digestion time and (c, d) the corresponding loadings plot for PC1 and PC2. (e) PC2 vs dispersion and digestion time from the mid-frequency region 300−1800 cm^{-1} with (f) the loading. Lipase was injected to initiate digestion at 0 min.

tris and milk that could be associated with poor solubilization of lumefantrine in these formulations, and a slight drop in the PC2 values was observed in infant formula. To support these observations, multivariate analysis of the mid-frequency Raman

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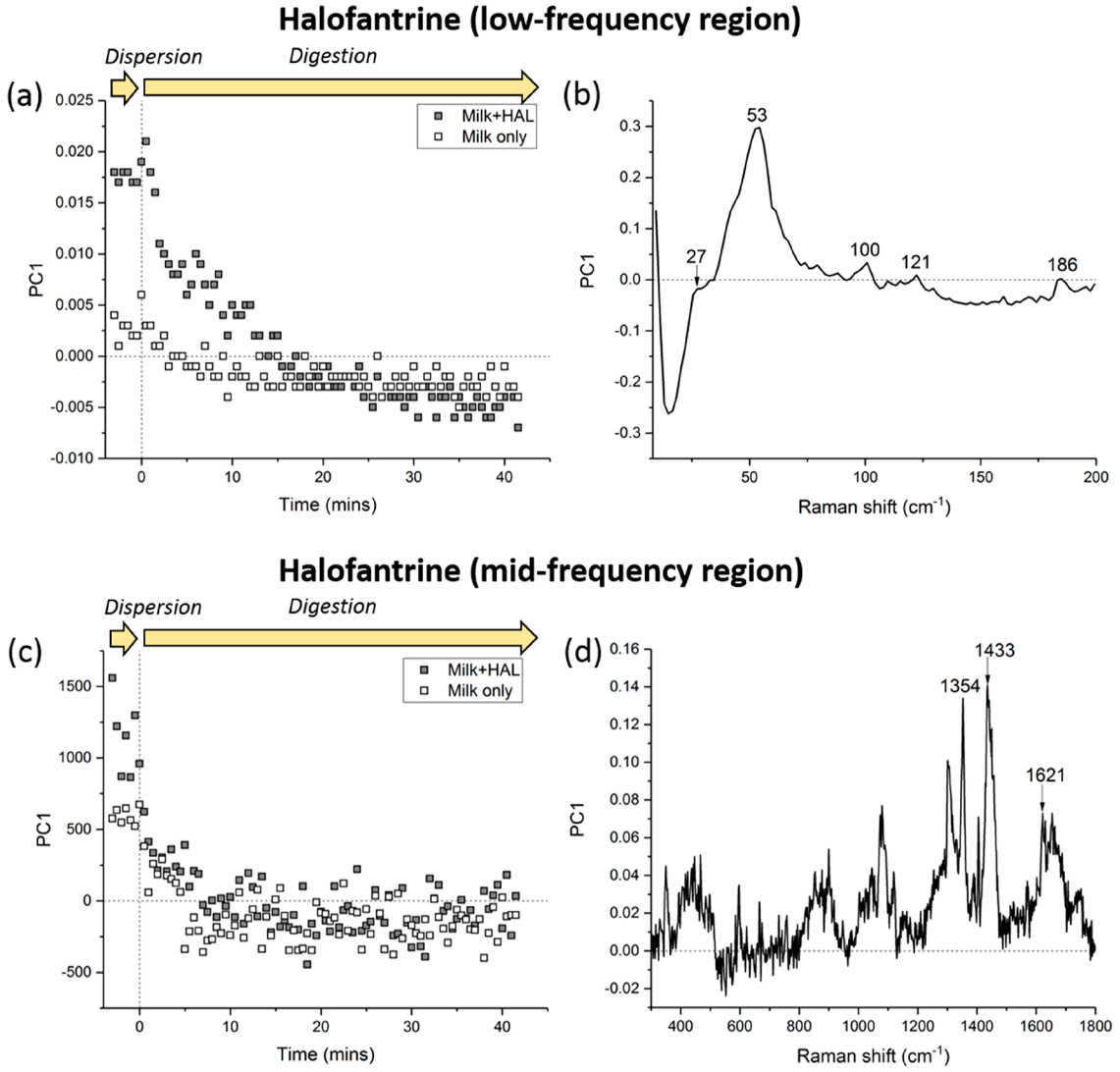

Figure 5. (a) Changes in PC1 values obtained from multivariate analysis of the low-frequency Raman spectra from 8 to 200 cm^{-1} during dispersion and digestion of milk and milk-containing halofantrine and (b) the corresponding loading for PC1. (c) Plot of PC1 against dispersion and digestion times from the multivariate analysis of the Raman spectra in the mid-frequency region from 300 to 1800 cm^{-1} and (d) the corresponding loading for PC1. Positions of the major Raman spectral shifts characteristic to halofantrine were annotated.

spectral region from 300 to 1800 cm^{-1} was performed and no changes in PC2 values (Figure 6c) were observed during digestion in milk when compared to digestion in tris buffer. Despite the similar trends between the analyzed data in the low- and mid-frequency regions, the spread of the PC2 values was much larger for the mid-frequency region, providing more confidence in the conclusion that lumefantrine is not significantly solubilized in digesting milk or infant formula.

Clofazimine. In the final case study, solubilization of clofazimine in milk, infant formula, and tris buffer during digestion were characterized by analyzing the Raman spectra using PCA. The three principal components covered 99% of the total variance in the samples, with the third PC being positively correlated to crystallized clofazimine (see the loadings plot for PC3 in Figure 7b). No clear separation between drug and background samples could be observed in PC1 and PC2 that mainly segregate signals relating to milk from IF and tris buffer (Figure S3). It can be observed that a decrease in the PC3 values for clofazimine occurred during digestion of milk and infant formula but stayed relatively constant for clofazimine in tris buffer, indicating drug solubilization in the digesting milk lipid formulations. The magnitude of the PC3 values after digestion may indicate similar solubilization of clofazimine in infant formula compared to milk when considering the contribution from milk and infant formula alone (Figure 7a).

For the mid-frequency region, using the same preprocessing as the other nonemissive drugs, separation of the drug from the background milk/IF signals was not successful; and although preprocessing of the data with no vector normalization showed drug/background separation with a decreasing PC1 indicative of drug dissolution, a significant emissive background was observed (Figure 7c,d). Therefore, PCA was repeated using a reduced spectral region (1000−1800 cm^{-1} with baseline correction and vector normalization), which allowed for the more effective removal of the emissive signal. Herein, the first PC separates milk and IF blanks from clofazimine (Figure 7e). Unlike the low-frequency Raman spectral analysis, a decrease in PC1 values that were correlated to clofazimine (loading plot in Figure 7f) could be observed during the digestion of IF but

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Mol. Pharmaceutics 2020, 17, 885−899

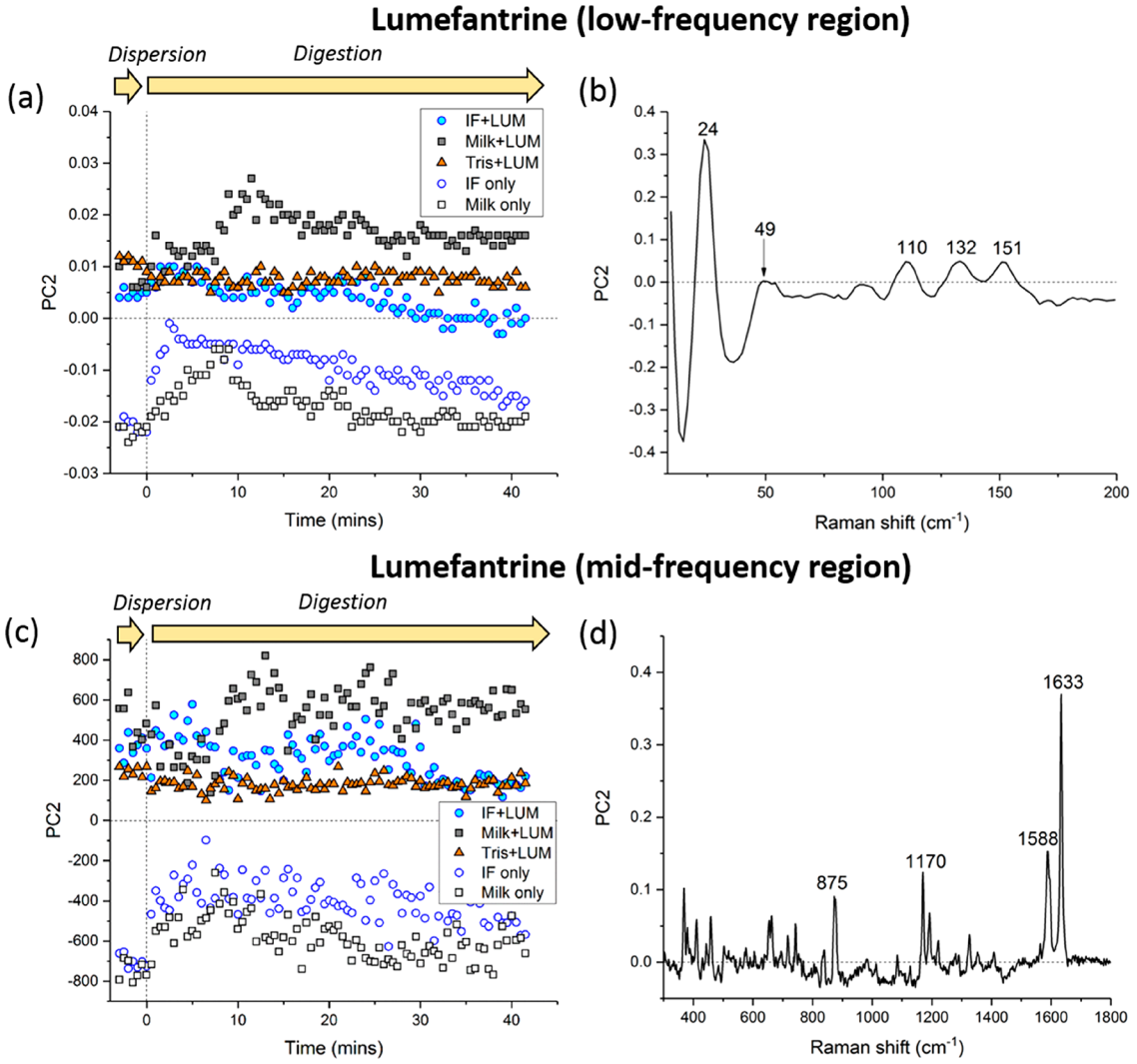

Figure 6. Plot of PC2 against dispersion and digestion times for milk, infant formula, and lumefantrine (LUM) in milk, LUM in infant formula (IF), and LUM in tris buffer generated from analysis of the (a) low-frequency region from 8 to 200 cm^{-1} and (c) the id-frequency region from 300 to 1800 cm^{-1}. (b) The corresponding loading plots for PC2 were shown in panels b and d, respectively. Major peaks belonging to lumefantrine are annotated. All samples contained bile salt micelles, and lipase was injected to initiate digestion at time 0 min.

not milk. This suggested a greater extent of drug solubilization during digestion in IF compared to milk, which was in line with previous SAXS studies.[24]

Meanwhile, the second PC mainly extracts information that may be related to clofazimine/fatty acid salt complexes (Figure S3e,f). The increase in PC2 values with the digestion of clofazimine in milk and IF but not tris was consistent with visual observations of the Raman spectra before and after digestion (shown in Figure 3j,l), where the appearance of a broad peak at around 1400 cm^{-1} was seen. Although this peak has been previously reported to be present in the Raman spectra of the hydrochloride salt of clofazimine but not the free base,[29] it may be possible that the formation of this broad peak was due to the acid/base interactions between ionized clofazimine and fatty acids liberated from the lipolysis of triglycerides rather than the characteristic peak of the clofazimine-HCl salt since the pH of milk + drug samples before and after digestion were constant at pH 6.5. These observations highlight key differences between low- and mid-frequency Raman associated with the vibrational modes probed, where the low-frequency modes are associated with vibrations across a crystal lattice (intermolecular) which requires long-range order, while in the mid-frequency, the vibrations are intramolecular in nature and thus do not require long-range order.

Comparison between Raman Scattering (Low-Frequency Region), SAXS, and HPLC. The drop in the relevant PC values for the low-frequency Raman scattering relevant to the drug crystallinity have been shown to be linked to digestion[23] and would, therefore, be considered to be analogous to the reduction in the area under the diffraction peaks observed previously during digestion using *in situ* SAXS measurements.[7,8,12,24] These two *in situ* techniques have advantages over analytical separation of the digestion phases and drug assay by, e.g., HPLC, as mentioned in the introduction; however, some analogy can be made between the dependence of the amount of drug in the pellet phase determined by HPLC over time with X-ray or Raman scattering due to the presence of nonsolubilized drug during digestion. The amount of drug partitioned in the pellet phase (HPLC) before and after digestion in milk and infant formula is presented in Figure 8. In general, Raman scattering and

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Mol. Pharmaceutics 2020, 17, 885−899

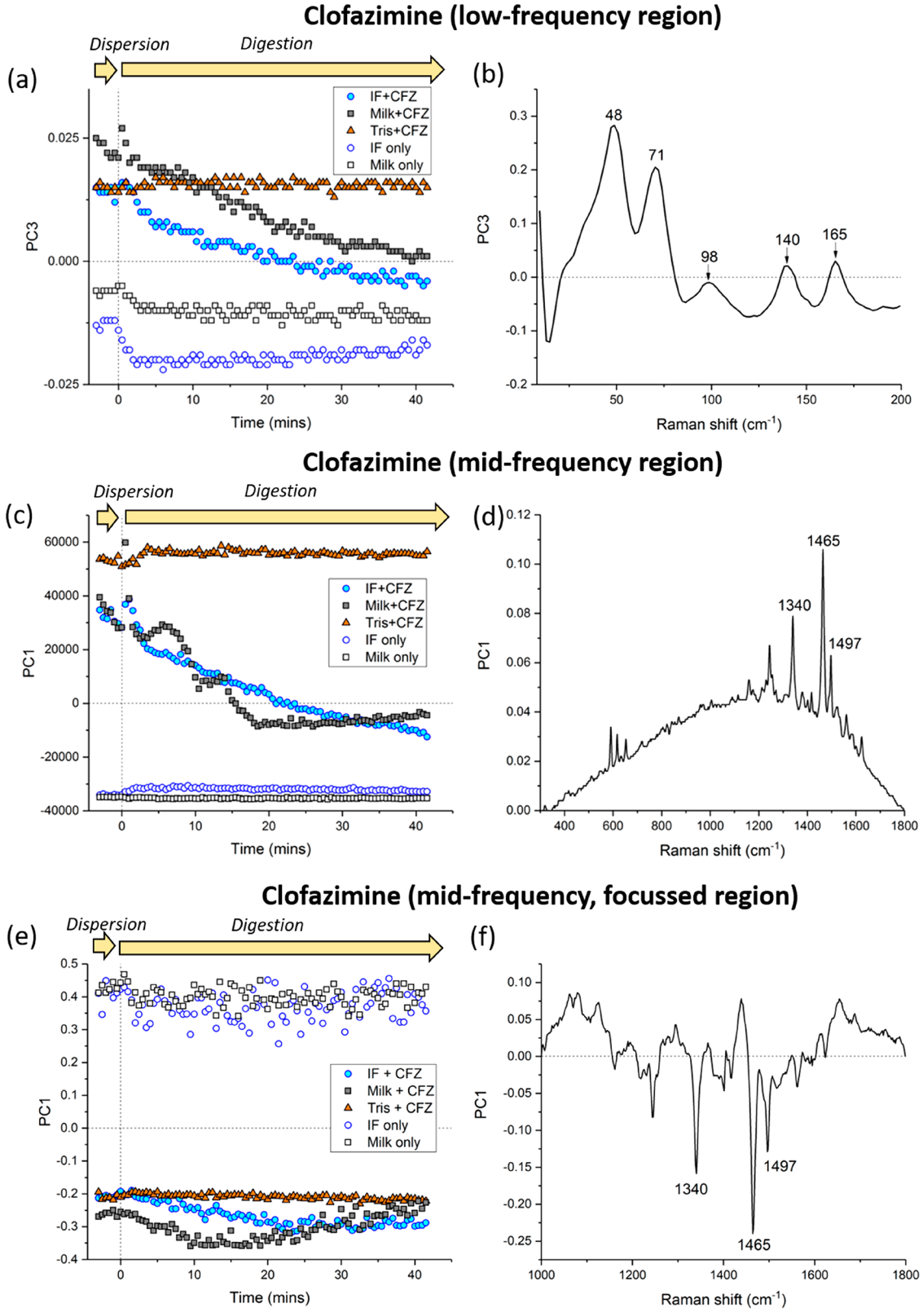

Figure 7. (a) Changes in PC3 against dispersion and digestion time for milk and infant formula, and for clofazimine (CFZ) in milk, infant formula (IF), and tris buffer from analysis of the low-frequency region from 8 to 200 cm^{-1} and (b) the loading for the PC3 data. (c) PC1 vs time in mid-frequency region (300−1800 cm^{-1}) and (d) the corresponding loading plot. (e) PC1 vs time for the focused mid-frequency range Raman spectra from 1000−1800 cm^{-1} and (f) the corresponding loadings plot. Major peaks for clofazimine are annotated. All samples contained bile salt micelles, and lipase was injected to initiate digestion at time 0 min.

SAXS exhibited similar solubilization trends for ferroquine-, halofantrine-, and lumefantrine-containing milk and infant

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Mol. Pharmaceutics 2020, 17, 885−899

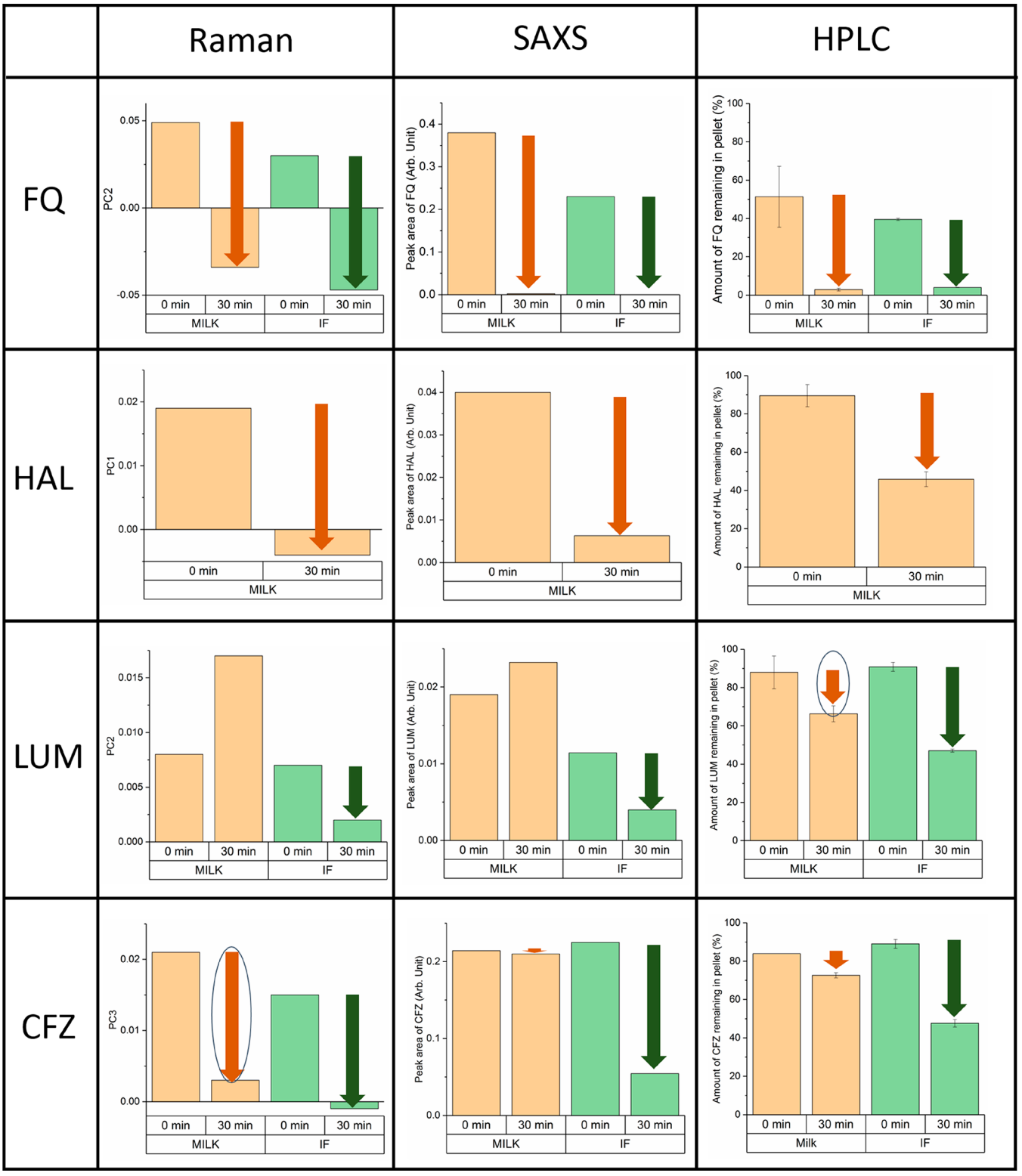

Figure 8. Comparisons between the drop in PC values, area under the diffraction peak, and the amount of drug partitioned in the pellet phase from low-frequency Raman scattering spectroscopy, SAXS, and HPLC, respectively, before and after 30 min digestion in milk and infant formula. The arrows point to the direction of change, where a decrease in the *y*-axis values is indicative of drug solubilization. Circled arrows highlight inconsistencies in the trend in drug solubilization compared to the other techniques.

formula before and after digestion, with the exception of clofazimine as highlighted in the figure (circled arrows in Figure 8).

Diffraction peaks for ferroquine disappeared during digestion and quantification of the pellet phase after ultra-centrifugation of the digested milk and infant formula phases showed the presence of <5% undissolved drug. The solubilization of halofantrine during digestion in milk was also evident from the near-complete disappearance of the diffraction peak characteristic of the drug crystals. In the case

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Mol. Pharmaceutics 2020, 17, 885−899

of lumefantrine, although no apparent solubilization was observed during digestion in milk based on the PC2 values in Raman scattering and the area under the diffraction peak at 1.63 cm^{-1} in SAXS, the amount of lumefantrine partitioned into the pellet phase after ultracentrifugation of the digested milk phases was quantified (using HPLC) to be about 67 ± 7%, with ~33% drug present in the lipid + supernatant layers after 30 min digestion. Interestingly, a separate investigation into the lipid layer isolated after ultracentrifugation using a polarized optical microscope revealed the presence of birefringent particles that may arise from the crystalline drug (Figure S4), since microscopic images of the lipid layer for the milk-only sample (without lumefantrine) showed an absence of birefringent particles. Potential localization of crystalline drugs into the lipid layer after ultracentrifugation could therefore cause an overestimation to the amount of solubilized lumefantrine measured usingHPLC, highlighting a potential limitation of separation-dependent methods.

The area under the diffraction peak in SAXS and the HPLC data for clofazimine stayed relatively constant after digestion in milk, indicating only a low extent of drug solubilization. However, a significant drop in PC3 values (characteristic to clofazimine) for the low-frequency Raman data was observed (Figure 8, bottom panels), which may arise from the background interference from the fluorescence of the intense-red-colored clofazimine[13] and changes in turbidity of the milk following digestion. In IF, the extent of solubilization indicated by SAXS and HPLC was greater but still not complete as suggested by the PC3 values.

DISCUSSION

The development of *in vitro* techniques that enable *in situ* characterization of drug solubilization in simulated gastrointestinal environments during digestion of formulations to predict *in vivo* performance is invaluable. Our group has recently developed new synchrotron small-angle X-ray scattering (SAXS) approaches to probe drug solubilization during *in vitro* digestion.[15,30] SAXS is a powerful technique, enabling real-time monitoring of drug solubilization, as well as polymorphic[7] and pseudopolymorphic (e.g., hydrates) transformations,[31,32] while also allowing elucidation of evolution of lipid colloidal structures during a single digestion. However, the diffraction technique measures only the overall amount of crystalline drug present, does not allow differentiation between phases into which dissolved drug may partition and is not sufficiently sensitive to discriminate digestion-induced formation of amorphous solids.

The feasibility of low-frequency Raman spectroscopy as a technique to monitor solubilization of ferroquine during *in vitro* digestion in milk was recently shown,[23] with the current study aimed at expanding these studies into other poorly water-soluble drugs and milk-based formulations (i.e., infant formula) with the incorporation of bile salts. Raman spectroscopy is a light scattering technique that has been widely used in the pharmaceutical industry to predict the amount of active pharmaceutical ingredients in solid-state formulations, typically tablets and capsules.[33,34] In Raman scattering, a small proportion of light is scattered at a different wavelength to the incident laser, and the energy differences correspond to the vibrational energy transitions associated with changes in polarizability (distortion of electron clouds) of the molecule.[18] Strong Raman scattering signals can, therefore, be observed in drugs that contain aromatic groups; due to the low level of Raman scattering of water, this technique can also be used to identify solid drugs in aqueous suspensions.[18] Low-frequency Raman scattering has found particular recent interest because of the ability to use it to discriminate between different polymorphic forms[21,22,35] and solid-states of pharmaceuticals.[20,25,36,37]

Ferroquine, halofantrine, lumefantrine, and clofazimine are basic lipophilic drug molecules that exhibit strong Raman scattering signals in both low- and mid-frequency regions. The former can be derived from lattice vibrations of the crystals that may be sensitive to intermolecular interactions and solid-state forms, while the latter is from intramolecular vibrations of the various functional groups of molecules.[20,21] It is therefore anticipated that solubilization of the drugs will result in spectral changes in both regions, particularly in the low-frequency region as a crystalline drug is lost to the solution, providing a new means to track drug solubilization over time. Additionally, the hypothesis that there will be concordance between the Raman approach and SAXS for the same process was tested across different drugs and lipid systems.

The solubilization patterns of ferroquine, halofantrine, lumefantrine, and clofazimine during digestion showed some interesting differences. In the case of ferroquine and halofantrine, the decrease in intensity of the low-frequency Raman peaks could be visually observed after digestion in milk and/or infant formula. These observations were confirmed in more detail using multivariate analysis of the Raman spectra and correlated well with the kinetics of reduction in the intensity of the crystalline drug peaks in SAXS, further supporting the use of low-frequency Raman spectroscopy as an analytical approach to probe drug solubilization.

However, it was also determined that the ability to utilize low-frequency Raman scattering to probe drug solubilization is dependent on the drug being investigated. When low-frequency Raman spectroscopy and SAXS were used to study the solubilization of lumefantrine, poor drug solubilization was observed during digestion, particularly in milk, but a significant amount of drug was present in the lipid phase when analyzed using HPLC. This would typically be seen as a good result as it may indicate that the drug has dissolved at least into the lipid and is therefore available for absorption. However, analysis of the lipid phase with polarized light microscopy reveals the presence of a crystalline drug, and this can provide a misleading picture of drug distribution and solubilization since more drug appears to be solubilized than is actually the case. Other potential issues with separation-based approaches to drug distribution during digestion include variations in density of the drug/lipid layers, necessity for a digestion inhibitor that is usually introduced in a solvent, and the extended time and force required for separation of the layers following ultracentrifugation, which could potentially lead to uncertainties in absolute drug quantification in the offline HPLC analytics.

The low level of solubilization of lumefantrine during digestion of milk compared to the other drugs was somewhat surprising as the solubility of lumefantrine in oleic acid (which constitute close to 30% of the total fatty acids in milk)[38] was found to be greater than 10-fold that of the corresponding triglyceride in the drug solubility studies (see Table S3). The production of fatty acids from triglycerides is a crucial aspect for solubilization of basic lipophilic drugs, and the high solubility in the fatty acids would generally indicate that the drug should be well solubilized during digestion of the triglycerides in milk. The reason why this is not the case for

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Mol. Pharmaceutics 2020, 17, 885−899

lumefantrine specifically is not yet clear, whether there is a particularly poor wetting of the drug particles or some other interfacial factor is still to be confirmed.

Lumefantrine appeared to be more readily solubilized in infant formula compared to milk during solubilization, and this could arise from differences in the lipid compositions, with the infant formula likely to have more medium-chain triglycerides compared to milk.[24] Digestion of infant formula would therefore generate more medium-chain fatty acids, in which lumefantrine was particularly soluble. Table S3 clearly shows that lumefantrine was more soluble in the triglycerides and fatty acids of the medium-chain lipids compared to long-chain lipids on a weight-to-weight basis using tricaprylin and triolein as the representative medium- and long-chain lipids. The role of water-soluble components, stabilizers, and other excipients in infant formula that may impact solubilization of lumefantrine was unknown and warranted further investigation, but most infant formulas contain bovine whey protein, a constituent that was also present in milk.

While low-frequency Raman spectroscopy presents an accessible methodology for the *in situ* screening of formulations for drug solubilization during digestion, we show that this may be problematic for colored and fluorescent compounds. Clofazimine is a red phenazine dye, whose color in solution is also pH-sensitive.[39] The scattering from clofazimine could, therefore, be influenced by not only the intrinsic properties of the drug but also the changes in the turbidity of the milk formulations as digestion progressed. In the case of having a highly colored or fluorescent compound, the use of *in situ* SAXS may be necessary as it is usually not difficult to identify peaks in the high q range (equivalent to wide-angle diffraction), which are not impacted by background peaks from the digesting lipid formulations, making data interpretation much easier. Although the use of Raman spectroscopy also requires spectral preprocessing and multivariate analysis to extract features for monitoring drug solubilization, for appropriate drug types the technique can be used as an accessible alternative approach to SAXS without the uncertainty introduced by separation methodologies. Future studies toward more deeply understanding the differences between the two techniques and anticipating when they are likely to arise, for example by studying additional highly emissive compounds, will be an important further step toward a more universal use of this approach as a development tool for poorly water-soluble drugs in lipid formulations during digestion.

CONCLUSIONS

In this work, the solubilization of four basic model drugs (ferroquine, halofantrine, lumefantrine, and clofazimine) in milk-based formulations was studied in real-time using low-frequency Raman spectroscopy, and the behaviors were compared to synchrotron SAXS and phase separation approaches. The disappearance of the distinct phonon modes in the low-frequency Raman region could be observed for ferroquine and halofantrine during digestion in milk and/or infant formula, and no changes were evident in tris buffer, where no fat was present, clearly linking the changes in the Raman spectrum to drug solubilization. Signals arising from the digestion of lipids in milk and infant formula could also be detected using low-frequency Raman spectroscopy, which may contribute to interference during data processing. In contrast to ferroquine and halofantrine, lumefantrine was not well solubilized during digestion, particularly in milk, and separation of phases for analytical determination of drug solubilization was confounded by drug crystals distributing to the lipid phase. The approach of separating phases and assaying for the drug rather than performing an *in situ* measurement was therefore shown to provide a misleading result for lumefantrine. The SAXS and Raman techniques were not in agreement in the apparent solubilization of clofazimine; however, this inconsistency highlighted the potential limitation of the low-frequency Raman technique when applied to highly colored compounds with strong fluorescence. With the aforementioned limitation, *in situ* low-frequency Raman scattering offers a new accessible option for screening the solubilization of poorly water-soluble drugs during digestion.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.9b01149.

> Preparation of freeze-dried lipase; preparation of bile salt mixed micelles; nutritional information for milk and infant formula; characteristic Raman peaks at low- and mid-frequency regions for ferroquine, halofantrine, lumefantrine, and clofazimine powders; solubility of lumefantrine in triglycerides and fatty acids; low and mid-frequency Raman spectral region of ferroquine in milk before and after digestion; plot of PC1 vs dispersion and digestion time for lumefantrine in low-frequency region; PC1 and PC2 vs dispersion and digestion time for clofazimine in low-frequency region; PC2 vs dispersion and digestion time for clofazimine in mid-frequency focused region; the optical polarizing microscopic images of the lipid layers of digested milk containing bile salts with and without lumefantrine (PDF)

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Notes

The authors declare no competing financial interest.

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