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RESEARCH ARTICLE

Incomplete lipid extraction as a possible cause for underestimation of lipid oxidation in emulsions

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Abstract

Lipid oxidation deteriorates the sensory and nutritional quality of food emulsions containing polyunsaturated fatty acids. Classically, different extraction solvents are used as a first step to measure lipid oxidation in emulsions. However, it is unclear how the applied extraction method influences the measured lipid oxidation values. In this work, we systematically examined the performance of common solvent mixtures such as chloroform, methanol, and hexane (or isooctane)–isopropanol on lipid extraction from emulsions stabilized with different emulsifiers (Tween 20 (T20), whey proteins, and pea proteins) and oxidation levels, and how this, in turn, affected the measured hydroperoxide concentrations. Chloroform–methanol was the most effective solvent (lipid yield >93 wt.%). When using hexane–isopropanol, extraction yields were consistently high for T20- and pea protein-based emulsions (>60 wt.%), but in whey protein-based emulsions, values as low as 26 wt.% were measured. In case of incomplete extraction, hydroperoxide concentrations measured by colorimetric methods need to be corrected for this effect. When using ^{1}H NMR to assess lipid oxidation, the actual amount of extracted lipids is intrinsically taken into account. This highlights not only the importance of the extraction method in determining lipid oxidation in emulsions but also that of the actual analysis method.

Practical application: This study highlights that the lipid extraction yield can vary depending not only on the emulsion composition (e.g., type of emulsifier) but also on the oxidative state of the emulsion and the extraction solvent used. If this is overlooked, errors can be made in the hydroperoxide determination. Although these effects can be corrected for, this is not standard procedure, which implies that awareness on this matter should be increased. It is also important to point out that depending on the solvent used, the different lipid classes (including various lipid oxidation products) may be extracted at different levels. Chloroform–methanol should be preferred for extraction of all lipid and lipid oxidation-derived molecules, including aldehydes.

Abbreviations: (s)PPI, ('soluble' fraction of) pea protein isolate; HIP, hexane–isopropanol; IOIP, isooctane–isopropanol; T20, Tween 20; WPI, whey protein isolate.

KEYWORDS
^{1}H NMR, hydroperoxide assay, lipid extraction, lipid oxidation, methodology

1 | INTRODUCTION

Food emulsions and other natural or manufactured products containing polyunsaturated fatty acids are prone to lipid oxidation, which deteriorates the quality of the food and thus is a concern in food production.[1] Lipids in emulsions are especially prone to oxidation due to the high interfacial area in emulsions where lipid oxidation is initiated, and a lot of work in the field has been dedicated to preventing this by various strategies.[2–8]

To measure lipid oxidation in emulsions, lipid extraction via organic solvents is a classical preliminary step for many methods used in literature.[9] One of the most frequently used methods to measure the hydroperoxide (primary oxidation products) concentration is the colorimetric method described by Shantha and Decker.[10] The original method used hexane–isopropanol (HIP) as an extraction solvent (ratio 3:2 v/v) and chloroform–methanol (7:3 v/v) for further measurements. Over time, the method was extended to the use of other solvent mixtures, for example, isooctane–isopropanol (IOIP), chloroform–methanol in the ratio of 2:1 v/v (Folch), or a ratio of 3:1 instead of 3:2.[11–13] As most of these methods assume a total extraction of the lipids (any lipid molecules present in the oil, including lipid oxidation products), the results may be skewed for incomplete extraction since they are expressed per kg of oil.[9,14] Besides, extraction solvents differ in polarity, and depending on the solvent used, classes of lipids that vary in polarity may be extracted more preferentially, and thus be under or overrepresented in the extract.

In addition, emulsifiers may influence the extraction of lipids by interacting with the extraction solvents, for example, by forming a network of denatured proteins thereby limiting the extraction of lipids.[15] From this, we suspect that both the choice of the extraction method as well as the emulsifier may have pronounced effects on the amount of extracted oil (and composition), and thus on the determined concentration of oxidation products that depend on the extracted amount of oil. Surprisingly, to date, this has not been investigated systematically, and that is therefore the goal of the present work.

To shed light on this matter, we measured the lipid extraction yield for different commonly used solvents, namely, HIP, IOIP, and Folch, for emulsions stabilized by Tween 20 (T20), whey protein isolate (WPI), or pea protein isolate (PPI), freshly prepared and under oxidizing incubation conditions. The extracts were analyzed for oxidation products using the colorimetric method described by Shantha and Decker and the ^{1}H NMR method described by Merkx et al.[10,16] from which concentrations of oxidation products can be determined independently of the extraction yield. Results were compared and put into perspective for possible implications of the extraction method.[3,8] On the basis of our outcomes, we advise on methods to obtain accurate lipid oxidation results for different extraction solvents considering also the emulsifier used.

2 | MATERIALS AND METHODS

2.1 | Materials

Rapeseed oil (kindly supplied by Unilever) was stripped from impurities and tocopherols by using alumina powder (MP EcoChrome ALUMINA N, Activity: Super I, Biomedicals). This procedure has proved useful to accelerate lipid oxidation in model systems and holds the advantage of standardizing the properties of the oil.[17,18] PPI, protein content 78% (N x 5.6; NUTRALYS S85F, Roquette), WPI, protein content 97.0%–98.4% (BiPro, Davisco), and Tween 20 (T20, Sigma Aldrich) were selected as emulsifiers. Methanol, 2-propanol, and *n*-hexane were supplied by Actu-All Chemicals. 2,2,4-trimethylpentane (isooctane) was obtained from Alfa Aesar. Deuterated chloroform ($CDCL_3$) with 0.03% tetramethylsilane, deuterated dimethylsulfoxide, and deuterated 4 Å mol sieves were purchased from Euriso-top. Hydrochloric acid (2 N) was obtained from Merck. Butanol, ethyl-acetate, chloroform, sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), sodium chloride, sodium azide, sodium dodecyl sulfate, 2,4-dinitrophenylhydrazine (DNPH), thiocyanate (NH_4SCN), guanidine hydrochloride, trichloroacetic acid (TCA) 40%, 0.144 M iron(II)sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), 0.132 barium chloride ($BaCl_2$), ascorbic acid, and a bicinchoninic acid (BCA) kit (BCA1-1 KT) were purchased from Sigma-Aldrich. Ultrapure water obtained from a Milli-Q system (Millipore Corporation) was used throughout all experiments.

2.2 | Preparation of continuous phase solutions

The pea protein solution was prepared by dispersing 10 wt.% PPI in 10 mM phosphate buffer (pH = 7.0) and hydrated for at least 48 h at 4°C. The soluble protein fraction was obtained by centrifuging (16 000 × *g*, 30 min) the protein suspension and collecting the supernatant, which was then centrifuged again under the same conditions. The protein concentration of the supernatant was determined with the Dumas method.[19] The supernatant, further referred to as "soluble pea protein" (sPPI), was then diluted to a concentration of 1 wt.% protein in the final emulsion. For WPI and T20, a solution was directly prepared in 10 mM phosphate buffer (pH = 7.0) in order to reach 1 wt.% in the final emulsion and was stirred for 2 h before being stored for 48 h at 4°C prior to usage.

TABLE 1 Overview of the solvents used for the different emulsions and time points and the measurements performed at those time points.

Emulsifier	Time points (days)	Used extraction solvent	NaCl	Measurements
T20	All	HIP 3:1	-	Lipid extraction yield; NMR
WPI	All	HIP 3:1	-	Lipid extraction yield; NMR
	0; 9/10	HIP/IOIP 3:2; Folch	+	Lipid extraction yield; NMR; colorimetric
sPPI	All	HIP 3:1	-	Lipid extraction yield; NMR
	0; 9/10	HIP/IOIP 3:2; Folch	-	Lipid extraction yield; NMR; colorimetric

Note: Ratios v/v: 3:1/3:2.[3,8]
Abbreviations: Folch, chloroform–methanol; HIP, hexane–isopropanol; IOIP, isooctane–isopropanol; sPPI, "soluble" pea protein isolate fraction; T20, Tween20; WPI, whey protein isolate.

2.3 | Preparation of the emulsion

A coarse emulsion was prepared by mixing 10 wt.% stripped rapeseed oil with the aqueous phase (sPPI/WPI/T20, final emulsifier concentration in the emulsion 1 wt.%) using a high speed blender (S18N-19G, Ultra-turrax R, IKA-Werke GmbH & Co.) at 11 000 rpm, for 1 min. The coarse emulsion was then passed through a high pressure homogenizer (M-110Y Microfluidizer equipped with a F12Y interaction chamber, Microfluidics) at 400 bar, which was repeated to a total of five times to obtain the final emulsion. The coil of the system was cooled throughout homogenization by ice water to prevent heating up of the emulsion during preparation. The emulsions were stored in 20-mL vials (6 mL/vial) with 0.02 wt.% sodium azide against microbial growth and horizontally rotated in an oven at 40°C in the dark. Samples were taken at days 0, 1, 3, 6/7, and 9/10 and stored at −80°C till further analysis; at least two emulsions were prepared independently.

2.4 | Measurements

Table 1 summarizes all the measurements and solvents used for the different emulsions (T20, WPI, or sPPI) and time points considered.

2.4.1 | Lipid extraction yield

After thawing emulsion aliquots (0.3 mL) at room temperature (RT), lipids were extracted by adding 1.5 mL of a mixture of HIP (in a ratio of 3:1 v/v), solvent to emulsion ratio 5:1 v/v, as described by Shantha and Decker[10]. The emulsion and solvent were added to Eppendorf vials (2 mL), and the weight of both emulsion (before freezing) and solvent was noted. The mixtures were vortexed (Digital Vortex Mixer, Fisher Scientific) three times for 20 s and centrifuged (5424R, Eppendorf) at 4700 × *g* for 8 min. After centrifugation, the lipid-containing phase (hexane phase) was collected, added to another Eppendorf vial, and weighed. The Eppendorf vials were then placed in an extraction evaporator (Reacti-Therm, Thermo Scientific), flushing the tubes with nitrogen gas (25°C) for 1.5 h. The residual lipid mass was recorded and the lipid extraction yield was reported as the percentage of lipids present in the hexane phase compared to the total amount of oil present in the emulsion aliquot.

In addition, HIP was used in a ratio of 3:2 v/v, isopropanol–isooctane in a ratio of 3:2 v/v, and chloroform–methanol in a ratio of 2:1 v/v as extractions solvents. Furthermore, NaCl was added to the WPI-based emulsion before extraction with the different solvents to facilitate the extraction of lipids.[20] For all solvents and ratios, the procedure remained the same as described for the HIP 3:1 (Table 1), and samples were taken at representative points in time.

For each emulsion, all measurements were performed in triplicate for each solvent and time point.

2.4.2 | Lipid oxidation measurements

After extracting the lipids as described in Section 2.2, lipid oxidation was measured using the colorimetric method of Shantha and Decker and the ^{1}H NMR method of Merkx et al.[10,16]

Colorimetric assay

Briefly, 10 µL of the hexane or isooctane phase were mixed with 1.4 mL methanol–butanol (2:1v/v) and 15 µL of assay reagent ($Fe(SCN)_2$). After 20 min incubation at RT, the absorbance was measured (510 nm) and the hydroperoxide concentration was calculated using a cumene peroxide calibration curve (0–160 µM). The measurements were performed in triplicate for each emulsion. The chloroform extracts were not analyzed by this method as chloroform gives a high absorbance at 510 nm (Figure S1) due to the enhanced formation of Fe^{3+} via trichloromethyl radicals.[21]

^{1}H NMR

The dried lipid extract (see Section 2.2) was re-solubilized in a NMR-compatible solvent (deuterated chloroform-deuterated dimethylsulfoxide; 5:1 v/v) in a ratio of 3:1 v/v. The measurements were performed in duplicate for all samples (HIP 3:1 solvent).

For both lipid oxidation methods, the hydroperoxide concentration was expressed as mmol per kg of oil. In the case of the colorimetric method, it is common practice to assume that 100% of the lipids present in the sample are extracted to the nonpolar phase. In the present work, we checked whether this assumption is sound by comparing with the mass of extracted lipids. For the NMR method, the total lipid content can be determined by integration of the triglyceride signal (4.46–3.93 ppm) and using it as reference.

2.4.3 | Protein oxidation measurements

Protein oxidation in the sPPI- and WPI-based emulsions was determined by measuring the carbonyl content according to the DNPH method described by Levine et al. and Sante-Lhoutellier et al.[22,23] Proteins were first precipitated by mixing an emulsion aliquot with isopropanol (1:10) followed by a centrifugation step at 15 000 × *g* (5 min, RT) to obtain the protein pellet, which was next dispersed in either 500 µL of 10 mM DNPH in 2 N HCL or only in 2 N HCL (blank). After incubation for 60 min in the dark, proteins were precipitated again with 500 mL of 40 wt.% TCA solution (10 min on ice). The dispersion was centrifuged at 15 000 × *g* for 5 min at RT, and the pellet was washed with 1 mL of ethanol/ethyl acetate 1/1 v/v (2×), then once with 1 mL of 2-propanol, and finally dissolved in 1 mL of 6 M guanidine hydrochloride at 37°C. Centrifugation under the same conditions removed the insoluble matter, if any, and the absorbance of the supernatant was measured at 370 nm. A molar absorption coefficient of 22 000 M^{-1} cm^{-1} was used to calculate the protein-bound carbonyl content. The soluble protein content in the supernatant was determined by the BCA-assay using sPPI or bovine albumin standard (for WPI-based samples) as calibration curve.[24] The results were expressed in mmol carbonyl per kg soluble protein for two independent measurement replicates.

2.4.4 | Statistics

Standard deviations were calculated from triplicate measurements of the same emulsion (lipid extraction, lipid oxidation via calorimetric method) or from duplicate measurements (relative middle phase height, protein oxidation, and lipid oxidation via NMR method). Significant differences between samples were determined using IBM SPSS statistics software with one-way ANOVA and post hoc with the Tukey HSD method to compare means. Significance was established with $p < 0.05$.

3 | RESULTS

In the following sections, the results are described for the different solvents and emulsifiers. The average droplet sizes ($d_{3,2}$) varied between 0.07 and 0.30 µm and did not change over time, with WPI-emulsions showing the smallest ($d_{3,2}$: 0.07–0.08 µm) and PPI-emulsions the largest values ($d_{3,2}$: 0.20–0.30 µm) (see Table S1).

3.1 | Extraction system—opaque layer

For T20-stabilized emulsions, a clear phase separation was obtained upon extraction, whereas for protein-stabilized emulsions, an opaque layer (referred to hereafter as the middle layer) was observed between the upper (hexane/isooctane or methanol) and lower phases (isopropanol or chloroform). The thickness of the middle layer was similar for all extraction methods and time points for the sPPI-emulsion (data not shown), whereas it varied in size per time point, extraction method, and even between samples of one-time point for the WPI-emulsion (Figure 1). Overall, the thickness of the middle layer was larger for the WPI-emulsions than for the sPPI-emulsions. Figure 1(ii) shows that the addition of NaCl decreased the thickness of the middle layer for all solvents used and the layer seemed more compact.

3.2 | Lipid extraction yield for different emulsifiers

Figure 2 shows the obtained yields for HIP (3:1 v/v) extraction from emulsions stabilized by T20 (a), WPI (b), or sPPI (c) over time. The extracted lipids represented >75 and >60 wt.% of the oil present in the emulsions, for the T20- and sPPI-based emulsions, respectively, with no differences between time points. In contrast, the lipid extraction yields for the WPI-stabilized emulsion were high in the fresh emulsion (around 70 wt.%) but then decreased to values as low as 26 wt.% on days 1 and 3 (Figure 2). At prolonged incubation, large variations were found (Figure 2, ii) indicating difficult lipid extraction from such aged systems.

3.3 | Effect of extraction method on lipid extraction yield

Figure 3 summarizes the lipid extraction yields obtained for emulsions stabilized by WPI or sPPI at day 0 or at the end of the incubation period (10 days) with different extraction methods, including the addition of a saturated NaCl solution (0.05 vol.% of total extraction system) (Figure 3, striped blue bars). For all solvents and emulsions, the lipid extraction yields were between 75 and 100 wt.%. The Folch 2:1 method led to significantly higher lipid extraction yields in all cases (75–86 vs. 95–100 wt.%). Similar lipid extraction yields were obtained with HIP 3:1, HIP 3:2, and IOIP 3:2 for both emulsions (WPI$^{(*)}$ and sPPI) and time points (0 and 10 days). The sPPI-emulsion generally showed a higher lipid extraction yield compared to the WPI-emulsion. In most cases, the addition of salt had no significant effect; only for the HIP solvent (3:2 v/v) the lipid extraction yield was significantly lowered, albeit the difference remained limited.

3.4 | Influence of the lipid extraction yields on the measured lipid oxidation levels

Figure 4 shows the measured hydroperoxide concentration in the protein-based emulsions for the different extraction methods and hydroperoxide assays. For all emulsifiers and solvents, the hydroperoxide concentration was the highest when measured with the ^{1}H NMR method, whereas it was substantially lower when measured with the colorimetric method. When these values were corrected for the actual lipid extraction yields (i.e., using the actual mass of extracted lipid instead of assuming complete extraction; see Section 2.4.2) the values

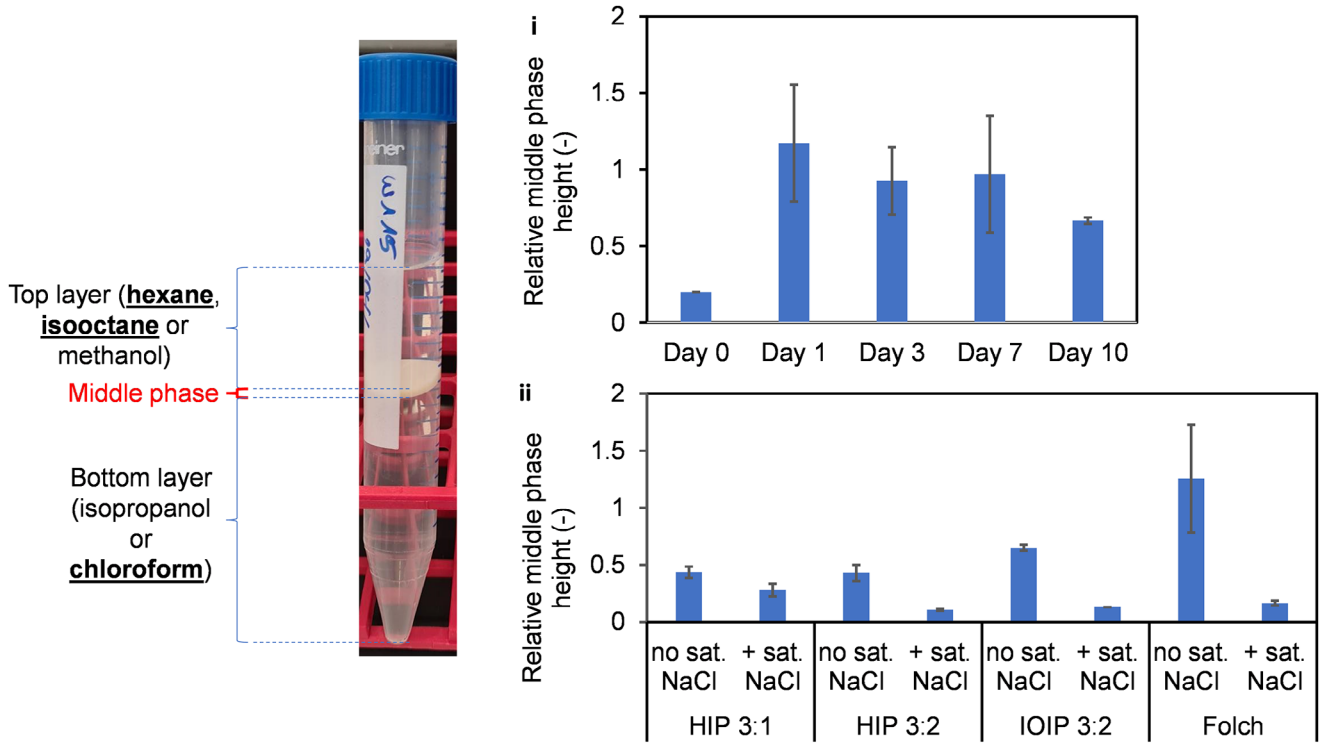

FIGURE 1 Relative middle layer height of the whey protein isolate (WPI)-emulsion extracted (i) with hexane–isopropanol (HIP) solvent at different time points and (ii) at day 10 with the different solvents (HIP/isooctane–isopropanol (IOIP)/Folch), ratios (3:1/3:2), and with or without the addition of a saturated NaCl-solution (0.05 vol.% of total extraction system). The error bars show the standard deviations of two analyzed tubes of the same emulsion (except day 0 where only one tube was analyzed; see Figure S2).

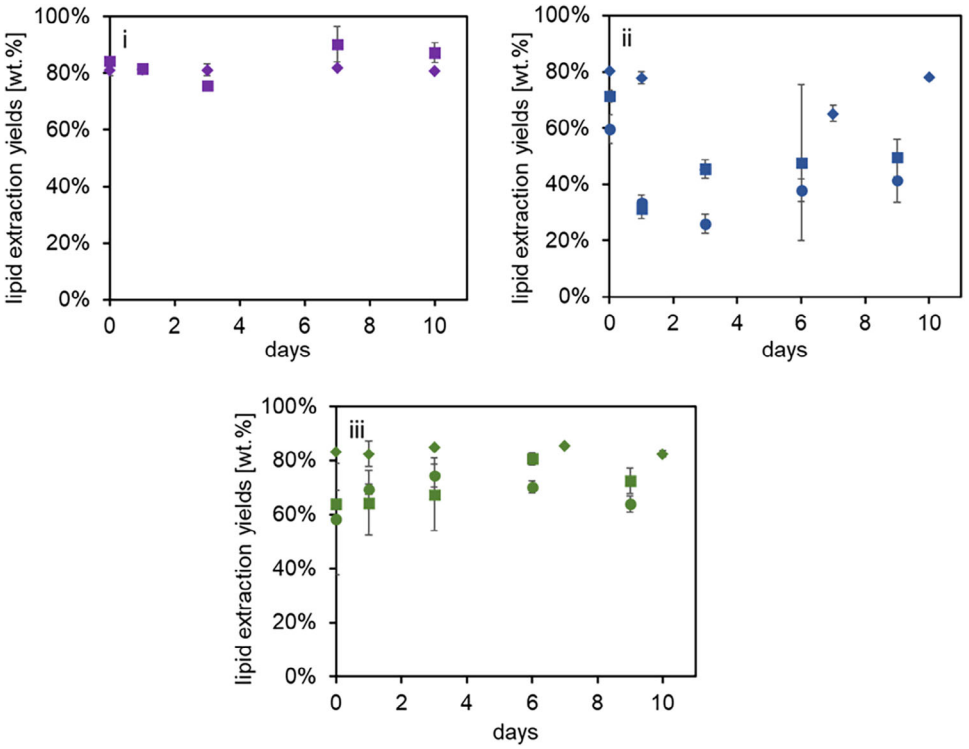

FIGURE 2 Lipid extraction yields for emulsions stabilized by (i) Tween 20 (T20), (ii) whey protein isolate (WPI), or (iii) soluble pea protein (sPPI), as function of incubation time. In each panel, the different symbols correspond to independently prepared emulsions. The error bars denote the standard deviations of three independent measurements of a given emulsion.

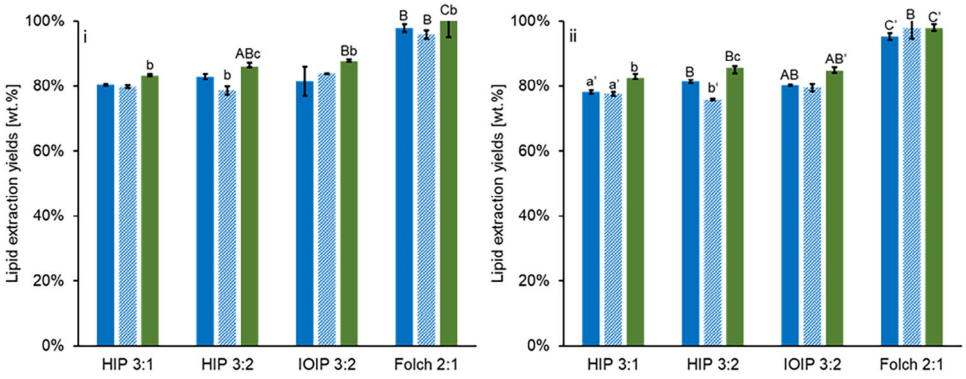

FIGURE 3 Lipid extraction yields for emulsions stabilized by whey protein isolate (WPI) (blue) and soluble pea protein (sPPI) (green) extracted with the different indicated solvents. For WPI-based systems, the extraction was conducted as such (plain blue bars) or with added NaCl (striped blue bars). Data for freshly prepared emulsion (panel (i)) or after 10 days of incubation (panel (ii)) are reported. The error bars represent the standard deviations ($n \geq 2$) and significant differences are indicated with letters ($p < 0.05$; small letter: significance between WPI (with and without the addition of NaCl) and sPPI; capital letter: significance of solvent; apostrophe: significance between measured time points).

obtained with NMR for the sPPI-stabilized emulsions remained higher, whereas rather similar values to those determined by NMR method were obtained for the WPI-stabilized emulsions (see Section 4).

In addition, the aldehyde concentrations in the extracted lipids were measured with the NMR method after extraction with different solvents (Figure 5). The largest dependency on the used extraction solvent was found for the long *n*-alkanals ($n > 5$), of which the concentration varied significantly between 0.21–0.64 mmol/kg oil and 0.31–0.96 mmol/kg oil (IOIP < HIP 3:2 < HIP 3:1 < Folch) for the sPPI- and WPI-based emulsions, respectively. Folch extraction led to significantly higher aldehyde concentrations compared to the IOIP extraction for both protein-stabilized emulsions and compared to HIP (3:1 or 3:2 ratio) for the sPPI-emulsion.

3.5 | Effect of lipid and protein oxidation on the lipid extraction yield

To investigate if lipid and protein oxidation are connected to the lipid extraction yield, we plotted both in Figure 6. For sPPI-based emulsions (Figure 6i), the lipid extraction yields were relatively high for all oxidation levels, whereas the lipid extraction yields for the WPI-based

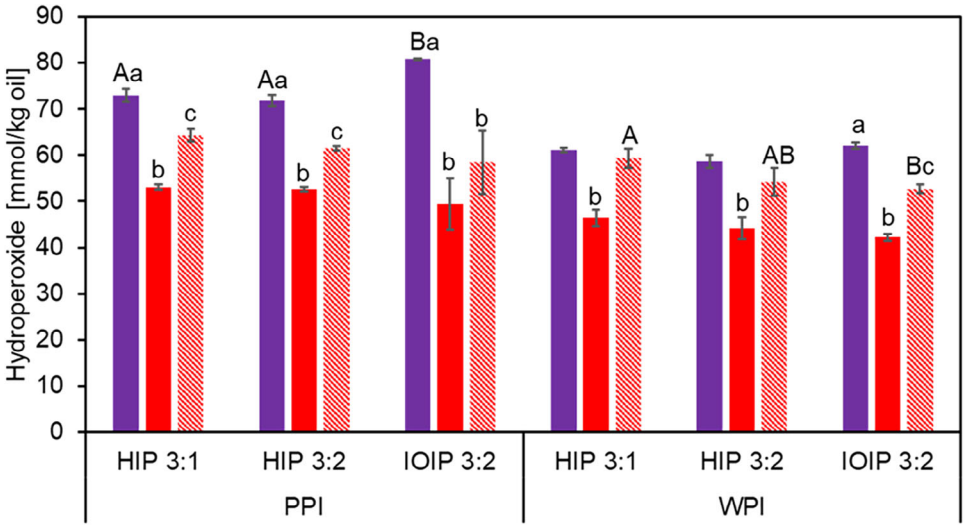

FIGURE 4 Hydroperoxide concentration after 10 days of incubation of the soluble pea protein (sPPI) and whey protein isolate (WPI)-emulsions measured with the NMR (purple) or colorimetric (red) methods, with (hashed) or without (filled) correction for the lipid extraction yield for different solvents (hexane–isopropanol [HIP] 3:1 or 3:2, isooctane–isopropanol [IOIP] 3:2). The error bars represent the standard deviation from two (NMR) or three (colorimetric method) independent replicates. Capital letters indicate significant ($p < 0.05$) differences between the data obtained by the individual measurement methods after extraction with different solvents; small letters indicate per solvent and emulsifier significant ($p < 0.05$) differences between the NMR and calorimetric method (with and without correction).

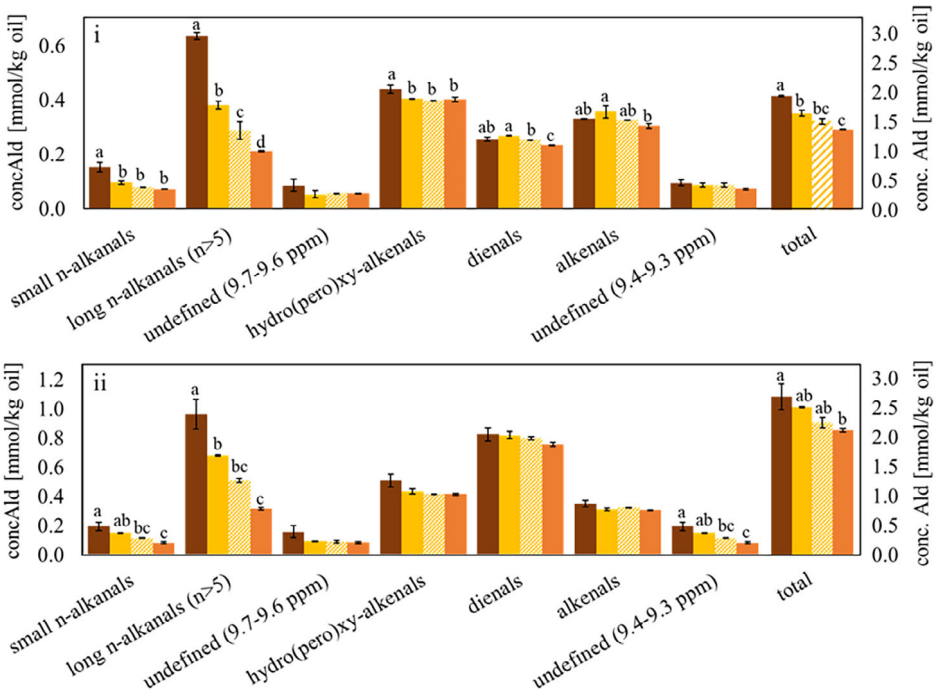

FIGURE 5 Concentrations of aldehyde classes (1: small *n*-alkanals ($n < 3$); 2: long *n*-alkanals ($n > 3$); 3: undefined (9.7–9.6 ppm); 4: hydro(pero)xy-alkenals; 5: dienals; 6: alkenals; 7: undefined (9.4–9.3 ppm); primary *y*-axis) and total aldehyde concentration (secondary *y*-axis) for the soluble pea protein (sPPI)-emulsion (i) and the whey protein isolate (WPI)-emulsion (ii) at day 10 extracted using Folch 2:1 (brown), hexane–isopropanol (HIP) 3:1 (yellow filled), HIP 3:2 (yellow striped), or isooctane–isopropanol (IOIP) 3:2 (orange).[16] The error bars represent the standard deviation of two NMR measurements. Letters indicate significant ($p < 0.05$) differences between the NMR measurements with different solvents.

emulsions (Figure 6ii) were much more scattered. They were found to be high (60–80 wt.%) at low and, sporadically, very high protein oxidation levels, whereas they were noticeably low (24–55 wt.%) at moderate (protein) to high (lipid) oxidation levels.

4 | DISCUSSION

It is first clear that both the extraction solvent used and the analysis method lead to differences in the amount of oxidation products measured (both hydroperoxides and aldehydes), which was herein exemplified for emulsions stabilized with a surfactant (T20) or protein ingredients (sPPI and WPI). Although the lipid extraction yield in T20-stabilized emulsions was not affected by the extraction solvent used, the situation was very different for protein-stabilized emulsions. There, the formation of a three-layered medium was observed upon extraction with a middle layer consisting of an organogel-like phase. This is most likely formed by a denatured protein network, which can possibly hamper the full extraction of lipids into the nonpolar phase of the system (in this study, chloroform, *n*-hexane, or isooctane).[14,25]

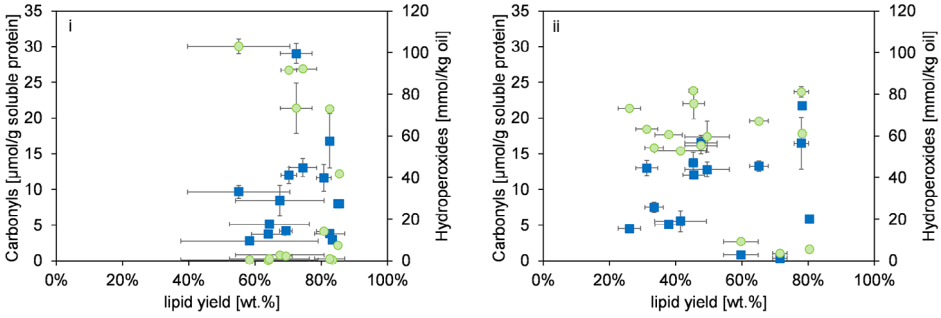

FIGURE 6 Influence of protein oxidation expressed as carbonyls (blue filled symbols) and of lipid oxidation expressed as hydroperoxides (measured with ^{1}H NMR; green striped symbols) on the lipid extraction yield for three independently prepared soluble pea protein (sPPI)-(i) and WPI-(ii) emulsions. Errors denote standard deviations of two (carbonyls and hydroperoxides) or three (lipid yield) independent measurements of the same emulsion.

The addition of NaCl can decrease the volume of such a middle layer, as also described by Folch et al. or Radin et al.[20,25,26] Our results show that substantial variations in the lipid extraction yield occur in protein-stabilized emulsions, especially when they are subjected to oxidative conditions (i.e., WPI), and that such variations are also contingent upon the used lipid extraction procedure. For instance, lipid extraction yields were typically lower with a HIP- or IOIP-based extraction compared to a Folch extraction, independently of the observed middle layer thickness. This is expected to be due to the capacity of chloroform to efficiently extract lipids even when strongly interacting/bound with other molecules in complex multiphase systems, for example proteins.[9,25] Chloroform, which is more polar than hexane or isooctane, may also be particularly suited to extract aldehydes, which are more polar compared to triglycerides and hydroperoxides, especially when they are no longer connected to the triglyceride backbone. Accordingly, in oxidized emulsions ($t > 0$ days), higher concentrations of aldehydes were extracted with chloroform in comparison to the other solvents (Figure 5). Specifically, for methods such as the common TBARS and *p*-anisidine value, which are used to measure aldehydes after lipid extraction, any effect on the measured concentrations caused by the extraction method would be very relevant. In addition, solvents vary in their volatility (chloroform < hexane < isooctane),[15,27] which affects the time needed for evaporation, which could, in turn, modulate the extent to which volatile aldehydes may be lost upon this process, although the amount of volatiles is expected to be relatively low compared to the total amount of aldehydes present. Still, it could be an interesting point to further address in the future. Furthermore, for whey protein-stabilized emulsions, it will be interesting to investigate the cause for variations in the lipid extraction yield over time, which is probably related to a certain oxidative state of the proteins, as indicated also by Radin.[25] Plant proteins in such isolates are often already oxidized to a certain degree due to the applied protein fractionation processes, which is less of an issue for whey proteins.[28]

An important highlight of our work is the fact that the hydroperoxide concentrations measured by the colorimetric method substantially deviated from those measured by NMR. The NMR method inherently measures the oil content (i.e., the triglyceride concentration) for each extracted lipid sample simultaneously with the whole range of hydroperoxides and aldehydes, which makes it a more reliable method. The colorimetric method may further be affected by differences in reactivity between lipid hydroperoxides and the cumene hydroperoxides used for the calibration that are subject to chemical degradation (which needs to be regularly checked).[21] The correction of the oil yield will contribute to narrowing the disparity in outcomes between those two methods. However, it is crucial to acknowledge that additional factors (e.g., for the PPI-emulsion) may also play a role that needs to be further investigated and explored.

In the light of the experience gained, some practical advice may be shared. An overview of the advantages and disadvantages of both methods considered in the present work is given in Table S2. Although the **NMR** method has the advantage of taking into account the actual lipid amount in the assessed sample and simultaneously analyzing primary and secondary oxidation products, the related high costs and need for advanced equipment make the method not readily available for a majority of laboratories. Conversely, for the **colorimetric method,** the low costs and basic equipment involved are clear benefits, and this method can be considered robust as long as one would standardly determine the lipid extraction yield and take it into account in the analysis. For the time being, this method will remain a widely used means to measure lipid oxidation in emulsions.

5 | CONCLUSION

This study has shown that solvent-based cold extraction of lipids from emulsions is challenging. In general, higher lipid extraction yields were obtained when using chloroform–methanol (2:1 v/v) (>95 wt.%) than when using hexane (or isooctane)–isopropanol (3:1 or 3:2 v/v) (75–86 wt.%) as the extraction solvent. The extraction yield can vary depending on the emulsifier used and as function of time, dropping as low as to 26 wt.% for WPI-stabilized emulsions within a few days of incubation under oxidizing conditions. Fluctuating lipid extraction yields can substantially influence the determined hydroperoxide concentration when using colorimetric methods that assume total extraction of all lipids. This may lead to underestimating the hydroperoxide concentration when the actual lipid extraction yield is not determined nor compensated for. The NMR method measures the total lipid content of the lipid extract simultaneously with the lipid oxidation products and does not have the limitations raised for the colorimetric method. In general, a Folch extraction (chloroform/methanol) should be preferred to extract all lipid classes (including relatively hydrophilic ones), which is especially relevant for lipid oxidation products such as aldehydes.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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