ESR spin trapping for *in situ* detection of radicals involved in the early stages of lipid oxidation of dried microencapsulated oils

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ABSTRACT

Different studies have shown that detection of free radicals by ESR spin trapping provides useful information on the susceptibility to oxidation of bulk oils and accordingly on the oxidative stability of different samples for comparative purposes. With the same goal, ESR spin trapping was evaluated in this work for *in situ* detection of radicals in dried microencapsulated oils (DMOs). By testing different oils, encapsulation matrices and oxidation conditions, results showed that ESR spin trapping can be useful to evaluate the oxidative susceptibility of DMOs, but ESR data should be interpreted cautiously, as the great complexity of the reactions involved may lead to data misinterpretations. Conditions for oxygen availability can have important impacts on the rates of both spin trapping and spin-adduct quenching affecting the levels of radicals observed. The kinetics of oxidation, spin trapping and spin-adduct decay should be known first in bulk oils for correct data interpretation in DMOs.

KEYWORDS: *ESR; spin trapping; lipid oxidation; microencapsulated oils; PBN*

1. Introduction

Lipid oxidation is one of the main causes of quality deterioration of foods. Lipids react with atmospheric oxygen through complex reactions producing hydroperoxides and therefrom a myriad of non-volatile and volatile compounds that impart the characteristic off-flavors known as oxidative rancidity. The reactions involve free radicals as central and highly reactive intermediates. The direct detection of lipid-derived radicals by Electron Spin Resonance (ESR) spectroscopy is not possible due to their very short life-times and very low steady state concentrations. They can, however, be indirectly detected by ESR spin trapping, where radicals are stabilized through reactions with an added diamagnetic molecular trap, normally nitrones or nitroso compounds. Through these reactions, new and more stable radicals (spin adducts) are formed in steady state concentrations that can be measured by ESR (Andersen & Skibsted, 2018; Andersen, Velasco & Skibsted, 2005).

Detection of radicals by ESR spin trapping has been used to evaluate early oxidation events in oils and in a variety of other foods (Andersen et al., 2018). Changes caused by the spin trap in the rate and pathway of lipid oxidation and losses of spin adducts by interactions with other radicals when oxidation progresses limit ESR experiments to the detection of the radicals involved in early stages of lipid oxidation (Velasco, Andersen & Skibsted, 2005). In this regard, the resistance of radical formation in bulk oils by ESR spin trapping has shown good correlation with the oxidative stability index provided by the Rancimat test (Velasco, Andersen & Skibsted, 2004). Other studies have displayed the utility of spin trapping to evaluate the efficacy of antioxidants (Thomsen, Vedstesen & Skibsted, 1999; Merks, Plankensteiner, Yu, Wierenga, Hennebelle & Van Duynhoven, 2021) and to determine the most important factor involved in the initiation of lipid oxidation in mayonnaise (Thomsen, Jacobsen & Skibsted, 2000). In addition, the spin trapping technique has also been applied to evaluate oxidative changes in milk (Kristensen, Andersen & Skibsted, 2002).

42 Although ESR spin trapping has been applied to liquid emulsions like mayonnaise and milk,
43 little information is available on its application to solid or dehydrated emulsions such as dried
44 microencapsulated oils (DMOs). Oil microencapsulation consists in the transformation of liquid
45 oils of nutritional interest, mainly oils containing long-chain polyunsaturated fatty acids, into
46 powdery food ingredients for food supplementation purposes (Bakry et al., 2016). Through
47 drying of oil-in-water emulsions containing proteins and/or carbohydrates, the emulsified oil is
48 encapsulated into powder particles that physically protect it with a solid wall from atmospheric
49 oxygen.

50 The evaluation of lipid oxidation in DMOs is normally approached by analyzing the oil fraction.
51 The oil extraction is sometimes tedious and it can be critical if the total extraction of lipids is not
52 obtained (Márquez-Ruiz, Velasco & Dobarganes, 2003). Also, it can affect the composition of
53 lipids as a result of extraction conditions or co-extraction of components other than lipids may
54 occur interfering with the subsequent analyses (Velasco, Marmesat, Holgado, Márquez-Ruiz &
55 Dobarganes, 2008). When the oil droplets are extracted together as a single phase, valuable
56 information is lost. In this respect, if oxidation takes place predominantly in the surface oil,
57 which quantitatively constitutes a minor fraction, the oxidation compounds are diluted with the
58 major non-altered encapsulated oil in the extraction and rancid samples may be found with
59 apparently a low degree of oxidation (Márquez-Ruiz et al., 2003). In this respect, due to its high
60 sensitivity, the direct detection of radicals by ESR spin trapping would be of great interest to
61 evaluate oxidative changes in DMOs. The oil extraction would not be necessary, and the product
62 would keep its structure. As a result, such a non-destructive approach would give clearly a more
63 realistic insight of the oxidative changes in DMOs.

64 To the best of our knowledge, the application of ESR spin trapping to DMOs has only been
65 performed in two studies, one of them reported recently (Orlien, Andersen, Sinkko & Skibsted,
66 2000; Boerekamp, Andersen, Jacobsen, Chronakis, & García-Moreno, 2019). Orlien et al.

67 (2000) applied ESR spin trapping to confirm *in situ* generation of radicals by two radical
68 initiators of different polarity in rapeseed oil microencapsulated in a matrix formed by sucrose,
69 maltodextrin 10 and gelatin. Radicals were not observed in the absence of initiators at the
70 conditions applied. Boerekamp et al. (2019) evaluated the oxidative stabilities of two
71 encapsulation matrices by monitoring the formation of radicals during incubation of samples at
72 50°C during 16 days. Less accumulation of the radicals detected was attributed to less oil
73 oxidation and so to greater oxidative stability. The direct association of greater levels of radicals
74 detected by ESR spin trapping with greater oxidation or *vice versa* is generalized and accepted
75 without exceptions in the literature. However, failure to observe spin adducts does not prove the
76 absence of radicals. It may simply reflect low rates of spin trapping or the formation of short-
77 lived spin adducts (Marriott, Perkins & Griller, 1980). The detection of radicals depends on a
78 number of factors rather than on the formation rate of the radicals to be trapped only. These
79 factors also include the stability of the radicals to be trapped, the rate of trapping and the
80 stability of the spin adducts. Further research is required to get to know the real analytical
81 information on lipid oxidation provided by such an ESR technique that depends on kinetic
82 factors.

83 The objective of the present work was to investigate the possibilities of the ESR spin trapping
84 technique to evaluate *in situ* the formation of radicals involved in the early stages of lipid
85 oxidation of DMOs. The starting hypothesis of this research was that valuable information on
86 the oxidative susceptibility of DMOs can be provided by the ESR spin trapping technique.
87 DMOs obtained by freeze-drying of oil-in-water emulsions containing respectively three types
88 of oils with very different oxidative susceptibilities, namely, rapeseed oil (RO), high linoleic
89 sunflower oil (SO) and fish oil (FO), were studied. The spin trap α -phenyl-*N*-*tert*-butylnitrone
90 (PBN) was used because of the high stability of its spin adducts (Velasco et al., 2005).
91 Following a previous study (Boerekamp et al., 2019), the spin trap was added to the oils prior to

microencapsulation. Two encapsulation matrices were evaluated in the present work as study models. One of them comprised sodium caseinate and D-lactose, which will be referred to as matrix A, whereas the other one was obtained from sucrose, maltodextrin 10 and gelatin, referred to as matrix B. The physicochemical characteristics and oxidative stabilities of these matrices have been previously determined in a recent report (Holgado, Márquez-Ruiz, Ruiz-Méndez & Velasco, 2020). Formation of radicals was evaluated at mild oxidation conditions (40 °C).

2. Materials and methods

2.1. Chemicals

α -Phenyl-*N*-*tert*-butylnitron (PBN) (purity $\geq 97\%$) and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (purity approx. 98%) were respectively acquired from Fluka Chemie GmbH (Buchs, Germany) and Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and used as received. Saturated medium-chain triacylglycerol (MCT) oil was provided by Brøste A/S (Lyngby, Denmark). All other chemicals and reagents were of analytical grade and used without further purification.

2.2. Oils

The RO and SO were refined oils acquired from a local supermarket, whereas the FO (sand eel oil) was non-refined and kindly supplied by TripleNine Fish Protein a.m.b.a. (Esbjerg, Denmark). The compositions of main fatty acids were 5.1% C16:0, 1.7% C18:0, 59.6% C18:1, 20.8% C18:2 and 9.4% C18:3 for RO; 6.1% C16:0, 3.5% C18:0, 27.3% C18:1, 61.6% C18:2 and 0.1% C18:3 for SO; and 5.2% C14:0, 15.1% C16:0, 5.9% C16:1, 1.1% C17:0, 1.0% C17:1, 2.0% C18:0, 15.8% C18:1, 2.2% C18:2, 2.0% C18:3, 5.4% C18:4, 12.6% C20:1; 6.7% C20:5, 8.1% C22:1 and 9.0% C22:6 for FO. The tocopherol contents expressed in mg/kg oil were 232 (α -) and 316 (γ -) for RO, 489 (α -) for SO and 83 (α -) for FO. The peroxide values of the oils

were 2.5($\pm$ 0.3), 3.6($\pm$ 0.5) and 1.5($\pm$ 0.1) meq/kg oil for RO, SO and FO, respectively. The spin trap was dissolved in the oils at a concentration of 1 mg/g oil (5.64 mmol/kg oil) by stirring at room temperature. When indicated, the concentration of PBN was increased to 10 mg/g oil (56.4 mmol/kg oil).

2.3 Encapsulating components

Sodium caseinate, D-lactose monohydrate, gelatin from bovine skin and sucrose (99%) were obtained from Sigma Chemical Co. (St. Louis, MO). Maltodextrin (10 dextrose equivalents) was purchased from Fluka (Sigma-Aldrich Chemie Steinheim, Germany).

2.4 Dried microencapsulated oils

The DMO compositions expressed in w/w were respectively 25% oil, 35% caseinate and 40% D-lactose (matrix A); and 25% oil, 5% gelatin, 25% maltodextrin and 45% sucrose (matrix B). The elaboration conditions were those used in a previous study (Holgado et al., 2020) with the exception of the emulsification step. The coarse emulsions were also prepared in a DI-25 Ultraturrax homogenizer (IKA, Germany), but at 641 g (9500 rpm) for 1 min. These were also refined in an Emulsi-Flex-C5 high-pressure homogenizer (Avestin Inc., Canada), but at 150 MPa, passing the emulsions 4 times through the homogenizer (4 passes). In addition, the free oil or non-encapsulated fraction of DMO samples was removed with n-hexane to evaluate the isolated contribution of the encapsulated oil to the levels of radicals detected by ESR. An amount of 20 g of intact DMO was gently stirred in 100 mL n-hexane in the dark for 10 min. The solvent was removed using a paper filter and the sample was washed three times with 30 mL n-hexane each. The washed samples were extended on a piece of filter paper and left open in the air (lights of the fume cupboard off) at room temperature during 30 min for evaporation of residual hexane. During the removal of the free oil fraction, the rest of the intact DMO samples remained at room temperature in the dark. The oil contents in the treated DMO were 18-20%

140 (w/w) for matrix A and 22-23% (w/w) for matrix B. These were determined according to
141 Holgado et al. (2020).

142 With the aim of evaluating the effect of the emulsification process, new samples of dried-
143 microencapsulated SO (DM-SO) containing PBN (1 mg/g oil) were prepared from emulsions
144 homogenized at different conditions. From coarse emulsions prepared at 641 g (9500 rpm) for 1
145 min in the Ultraturrax, emulsions homogenized at 150 MPa and applying 1, 2, 3 and 4 passes,
146 respectively, were elaborated.

147 2.5. Oxidation conditions

148 Both the oils and the DMO samples were oxidized at 40°C in the dark under 0% relative
149 humidity. Independent aliquots of 1.25 mL oil were placed into Eppendorf tubes (surface-to-
150 volume ratio of 0.42 cm⁻¹). In addition, when indicated, the oils were also oxidized in ESR tubes
151 with 4 mm i.d. and 25 cm length (700-PQ-7, heavy wall; Wilmad Glass, Buena, NJ). Three
152 aliquots of 1 g oil were incubated into the ESR tubes (surface-to-volume ratio of 0.11 cm⁻¹). The
153 DMOs were oxidized in the ESR tubes only. Three aliquots of 0.5 g of each DMO sample were
154 incubated in the ESR tubes (surface-to-volume ratio of 0.09 cm⁻¹). Unless indicated, both the
155 Eppendorf tubes and the ESR tubes were kept open during incubation. The tubes were placed
156 into a desiccator containing silica gel, and the desiccator was placed in an oven. After the ESR
157 measurements, the samples that had been incubated in ESR tubes were placed again in the
158 desiccator for further analyses.

159 2.6. ESR spectroscopy analyses

160 The oils oxidized in the Eppendorf tubes were transferred to ESR tubes and analyzed by ESR
161 immediately after sampling. The samples oxidized in the ESR tubes were measured directly. The
162 amount of oil or DMO in the tubes was chosen to make sure that the samples completely
163 covered the resonant cavity of the spectrometer. Thus, the analyses were performed at constant

volume of sample, determined by the height of the cavity (maximum sample access: 11.5 mm, according to technical specifications) and the inner diameter of the ESR tube. The ESR measurements were carried out placing the base of the ESR tubes just some millimeters out of the cavity, i.e. in the lower part of the tubes. An ECS 106 ESR spectrometer (Bruker, Rheinstetten, Germany) with an X-band resonator (ER 4103TM) was used. Analyses were performed at room temperature. The parameters used in all ESR measurements were kept constant in each determination. Only the receiver gain was adjusted and measurements were accordingly corrected. The parameters applied were 3475.5 G (center field), 100 G (sweep width), 1024 points (resolution), 9.76 GHz (microwave frequency), 20 mw (microwave power), 1.1 G (modulation amplitude), 100 kHz (modulation frequency), 41 ms (conversion time), 328 ms (time constant), 42 s (sweep time) and 2 accumulated scans. The peak-to-peak amplitude of the low-field line ($M_I = +1$) of ESR spectra (first derivative) and the area under the curve of the absorption spectra (double integration) were determined by the Winepr software program (Bruker, Rheinstetten, Germany). Determination of the area was performed over the entire sweep width (100 G). The area values were taken when the signal-to-noise ratio (S/N) of the absorption spectra was ≥ 10 as indicated by the software program. When this did not occur, the peak-to-peak amplitude of the low-field line in the ESR spectra (first derivative) was taken instead and converted into area value using the equation reported elsewhere for the same bulk oils of the present study ($Area = 4014(\pm 35) \text{ Amplitude}$) (Velasco et al., 2005). This equation was confirmed in the range of concentrations applied for both bulk and microencapsulated oils (not shown). Samples presenting ESR signals with an amplitude value (peak-to-peak height of the low-field line) lower than ten times the baseline noise in the first-derivative spectra were considered to have non-quantifiable levels of spin adducts.

Quantitative ESR determination was approached by external calibration using TEMPO dissolved in MCT oil, as described elsewhere (Velasco et al., 2005). As to the bulk oils, it was

considered that the three types of oil had the same density as the MCT oil. In the case of the DMOs, the concentrations of the radicals observed were expressed on oil mass considering the bulk density of the products in the ESR tubes, the volume of sample in the resonant cavity and the oil contents determined in the DMO samples.

2.7. Determination of hyperfine splitting constants

Hyperfine splitting constants were obtained by computer simulation of the experimental ESR spectra. This was performed using the Public EPR Software Tools (PEST) from the National Institute of Environmental Health Sciences, available on the Internet (<http://epr.niehs.nih.gov/>). Optimization parameters were obtained when the correlation coefficient was above 0.995.

2.8. Oxidative stability index

The oxidative stability index (OSI) of the bulk oils was determined applying the Rancimat test (AOCS, 1997). A Rancimat instrument, model 679, from Metrohm Nordic ApS (Glostrup, Denmark) was used. Samples of 2.00 ± 0.01 g oil were analyzed at 100°C applying an air flow of 20 L/h.

2.9. Statistical analysis

Three independent aliquots of each oil were analyzed in each sampling when the experiments were carried out in Eppendorf tubes. The same applied for experiments in ESR tubes, but the same samples, oils and DMOs, were analyzed over time. The bulk oils were also analyzed in triplicate in the Rancimat test. Statistical analyses were made using SPSS 11.8 software (SPSS Inc., Republic of Ireland). Student's *t* test and ANOVA were applied for comparisons between two and three mean values, respectively. Tukey's test was applied after ANOVA to establish differences between means. Significance was considered at $p < 0.05$.

3. Results and discussion

3.1. Oil oxidative stability of the bulk oils

The selection of the bulk oils used for oil microencapsulation was based on their different degrees of unsaturation and their different oxidative stabilities as a result. The stability values obtained in the Rancimat test were $19.0(\pm 0.3)$, $11.9(\pm 0.2)$ and $1.45(\pm 0.04)$ h for RO, SO and FO, respectively. As the spin trapping technique is based on kinetic factors (Andersen et al., 2005), it was thought that it would be of interest to evaluate lipid substrates with different oxidation rates.

3.2. ESR spectra

The DMO samples showed broad three-line ESR spectra that were similar to those found in the bulk oils (Figure 1SM) and to those reported elsewhere (Velasco et al., 2005). Thus, the hydrogen splitting was not clearly observed due to line broadening effects caused by restriction in rotational mobility (Figure 1SM). Yet, computer simulation showed characteristic spectra of PBN spin-adducts, whose ^{14}N and ^1H hyperfine splitting constants were $14.89(\pm 0.04)$ G and $1.83(\pm 0.02)$ G, respectively. No significant differences were found between the coupling constants of the three oils nor between oils and DMO samples and neither were significant changes observed along oxidation time. Therefore, the radicals detected in the DMOs seemed to be of similar nature to those detected in bulk oils.

The coupling constants obtained were consistent with those reported for 2-methyl-2-nitrosopropane (MNP), i.e. 14.8-14.9 G (^{14}N) and 2.1 G (^1H) (Vicente, Empis, Deighton, Glidewell, Goodman & Rowlands, 1998; Merx et al., 2021), rather than with those for alkyl-PBN adducts, i.e. 14.7 G (^{14}N) and 3.26 G (^1H), found in fatty acid methyl esters oxidized in the presence of PBN (Vicente et al., 1998). Through ESR and ENDOR analyses, it has been found that MNP is a decomposition product of the highly unstable peroxy-PBN spin adducts, whose ^{14}N and ^1H hyperfine splitting constants were 13.7 G and 2.0 G, respectively. MNP is a spin trap and is also able to trap lipid alkyl radicals. The ^1H hyperfine splitting in the ESR spectra of MNP

adducts has been reported to be due to the H attached to the C of the lipid moiety attached to the N. The MNP spin adducts were the predominant radical species detected respectively in the methyl esters of oleic, linoleic and linolenic acids in the presence of PBN under aerated conditions (Vicente et al., 1998).

3.3. Detection of radicals in the initial DMO samples

The first ESR spectra after the preparation of the samples showed remarkable differences between the encapsulation matrices. While the ESR signals recorded in samples A were below the LOQ (4.0 $\mu\text{mol/kg}$ oil in DMO) (SO and FO) or even not detected (RO), samples B exhibited quantifiable (SO and FO) or detectable (RO) signals. The concentrations of spin adducts found for the SO and FO samples were $4.7(\pm 0.5)$ $\mu\text{mol/kg}$ oil and $4.9(\pm 0.4)$ $\mu\text{mol/kg}$ oil, respectively. No ESR signals were detected in the fresh bulk oils containing PBN. Therefore, these results suggested that oxidation events took place during the encapsulation process and that incipient oxidation was greater during preparation of samples B.

3.4. Detection of radicals during early oxidation

The presence of spin adducts in the intact samples of microencapsulated RO (DM-RO) and SO (DM-SO) during the first days of incubation was expected considering the oxidative stability values of the bulk oils obtained in the Rancimat test (**Figure 1A-B**). Thus, higher concentrations of spin adducts were recorded for the sample containing the less stable oil (SO). However, the FO sample showed very low concentrations of spin adducts compared to the other two samples containing more stable oils. This was observed with both encapsulation matrices. A decrease in the concentration of radicals in sample B containing FO was even observed after the fifth day of incubation (**Figure 1B**). In contrast, the detection of radicals in the bulk oils showed reasonable results, i.e. the most stable oil (RO) exhibited the lowest concentration of radicals during the first days of incubation. The spin adducts increased rapidly in the SO and FO, although no clear differences were observed between them (**Figure 1C**).

The amounts of radicals detected in samples A at the end of the period studied were clearly much higher compared to samples B (**Figure 1A-B**). The concentrations of spin adducts found for samples A on day 8 were 63-356 $\mu\text{mol/kg}$ oil, whereas they were 9.0-100 $\mu\text{mol/kg}$ oil in samples B. These results seemed not to be in agreement with those obtained for the initial samples, which suggested more incipient oxidation in samples B caused by microencapsulation. In fact, samples A showed quantifiable levels of radicals later (1-2 d) than samples B (0-1 d).

The concentrations of radicals detected in the DMOs prepared with matrix A were significantly higher compared to the parent bulk oils (**Figure 1**). As an example, the concentration of spin adducts detected in the DM-SO was as high as $356(\pm 14)$ $\mu\text{mol/kg}$ oil on day 8, while its bulk oil counterpart only showed $13.2(\pm 0.5)$ $\mu\text{mol/kg}$ oil. A similar observation was noted for the samples prepared with matrix B, except for the dried-microencapsulated FO (DM-FO), where the spin-adduct concentration was low (9.0 ± 0.8 $\mu\text{mol/kg}$ oil) and of the same level as that found in the bulk oil (13.5 ± 0.1 $\mu\text{mol/kg}$ oil).

The non-encapsulated or free oil fraction was removed from DMO samples prior to incubation to evaluate the contribution of the encapsulated oil. The levels of radicals detected in the samples devoid of the oil fraction more susceptible to oxidation were with no exceptions remarkably higher compared to their parent intact samples (**Figure 1A-B**). In addition, results confirmed the unexpected low concentrations of radicals found for the intact samples containing FO in both encapsulation matrices and also the higher levels of radicals in the samples containing SO compared to the samples of RO during the first days of incubation (**Figure 1A-B**). Samples A also displayed higher levels of radicals than samples B.

3.5. Detection of radicals during late oxidation

The unexpected results found made it necessary to extend the experiments over time to see whether it was possible to obtain further information that helped understand the results for the early stages of oxidation. Thus the DMO samples and bulk oils were analyzed for up to 42 and

287 71 days of incubation, respectively. Results showed characteristic curves of radical decay in the
288 DMOs (**Figure 2A-B**) (Qian, Wang, Schafer & Buettner, 2000; Velasco et al., 2005). In the bulk
289 oils, losses of accumulated radicals were only observed for the FO, although a decrease in the
290 radical accumulation rate clearly occurred from 8 and 14 days in SO and RO, respectively
291 (**Figure 2C**).

292 As the drop of accumulated spin adducts took place at different concentrations (**Figure 2**), the
293 loss of ESR signal can not only be attributed to interactions of the spin adducts between each
294 other (Janzen & Haire, 1990). Instead, losses of spin adducts were more likely due to their
295 interaction with the oxidation chain reactions, e.g. by addition to other lipid radicals forming
296 diamagnetic products (Qian et al., 2000). In this regard, from a previous study on bulk oils it was
297 reported that the rate of radical accumulation decreased when the oxidation measured by the
298 peroxide value increased substantially, suggesting a faster decomposition of spin adducts
299 (Velasco et al., 2005). Therefore, valuable information can be extracted from the changes
300 observed in the rate of radical accumulation and from the maximum concentrations of radicals
301 accumulated in the DMOs. If losses of spin adducts are observed when oxidation progresses, it
302 can be assumed that the more rapid oxidation, the earlier the radical decay will be observed with
303 a lower maximum amount of radicals accumulated. This is in agreement with results reported for
304 different fish oils incubated at 40°C in the presence of PBN (Falch, Velasco, Aursand &
305 Andersen, 2005). The less stable sample showed lower resistance to radical formation and
306 accumulated lower levels of spin adducts over time. Accordingly, the low concentrations of spin
307 adducts detected in the DM-FO samples of the present study can be attributed to fast oxidation,
308 even during very early incubation. Thus, the decomposition rate of spin adducts dominated over
309 their formation at early stages in the samples B containing FO. The expected high free acidity in
310 the crude FO may also have contributed to decreasing the stability of the PBN spin adducts and
311 so to the levels of radicals observed (Jerzykiewicz, Cwiela-Piasecka & Jezierski, 2013).

The greater amounts of radicals accumulated in the DMOs compared to their parent bulk oils could therefore be attributed to slower oxidation in the capsules (**Figure 2**). In this respect, it is worth mentioning that the DMOs were, for convenience, oxidized in the ESR tubes, in which the sample surface-to-volume ratio was 0.09 cm^{-1} , whereas the bulk oils were incubated in Eppendorf tubes, with a greater sample surface-to-volume ratio (0.42 cm^{-1}). As a result, not only did the encapsulation wall limit the diffusion of oxygen to reach the oil phase, but also the oxygen diffusion through the head space of the ESR tube and the lower surface-to-volume ratio of sample contributed to limiting oxidation even more in the DMOs compared to the bulk oils.

Applying the same reasoning, the greater amounts of radicals accumulated in the DMOs devoid of the free oil fraction suggested slower oxidation compared to the intact samples. In addition, the rate at which spin adducts were accumulated prior to the decay observed was also indicative of faster decomposition and indirectly of faster oxidation in the samples that accumulated less levels of radicals (Figure 2).

Likewise, the results in Figure 2 also suggest that oxidation was faster in the oils encapsulated with matrix B (note the different concentration scales), which would be in agreement with the results found for the initial samples. These results are also coherent with those obtained in a recent report, in which the same encapsulation matrices as those used in the present work were studied (Holgado et al., 2020). It was found that an encapsulation matrix constituted by sodium caseinate and D-lactose, named matrix A in the present study, provided greater protection against lipid oxidation than a matrix formed with gelatin, maltodextrin (10 DE) and sucrose, referred to as matrix B in the present work.

3.6. Changes induced in the oxidation rate as a consequence of oxygen restrictions

Given that oxidation may have been slowed down when using the ESR tubes as incubation vessels compared to Eppendorf tubes, further experiments were run to evaluate these hypothetical differences. The bulk oils were also oxidized in the ESR tubes at the same

conditions as those used for the Eppendorf tubes. It was observed that the levels of spin adducts accumulated in the oils were clearly higher when oxidation took place in the ESR tubes for the RO and SO samples (**Figure 3A-B**), confirming the hypothetical slower oxidation as a result of oxygen limitations. However, the results obtained for the bulk FO were just the opposite, i.e. much lower radical levels were detected when oxidation was slowed down in the ESR tubes (**Figure 3C**).

The unexpected results obtained for the FO could have been related to a remarkably faster oxygen consumption for such a polyunsaturated oil. It was observed that rancidity was clearly perceived in the FO just after two days of incubation in both recipients. In contrast, rancidity was not detected in the vegetable oils during the entire assay. Insufficient oxygen supply in the ESR tube when oxidation was very fast, such as it was for the FO, could have had important consequences in the spin trapping reactions and also in the stability of the spin adducts formed.

3.7. Changes induced in the spin trapping rate by increasing the PBN concentration

The concentration of the spin trap was increased ten times, from 1 to 10 mg/g oil, in order to enhance the rate of spin trapping and to evaluate changes in the accumulation of radicals during oxidation of oils in both Eppendorf and ESR tubes. As expected, the levels of radicals detected in the oils oxidized under no oxygen restriction, i.e. in Eppendorf tubes, considerably increased as a consequence of faster trapping (**Figure 4A**). The FO only showed higher levels of radicals ($2.9 \pm 0.1 \mu\text{mol/kg}$) than the SO ($2.4 \pm 0.2 \mu\text{mol/kg}$) and RO ($1.5 \pm 0.1 \mu\text{mol/kg}$) on the first day of incubation. Then, the recorded concentrations in the FO were significantly lower, probably due to faster spin adduct quenching.

When oxidation took place under limited oxygen access, i.e. in ESR tubes, the PBN concentration effect on the accumulation of radicals in the vegetable oils was also similar to that observed in Eppendorf tubes, but losses of accumulated spin adducts were observed over time (**Figure 4B**). The effect of PBN concentration in the FO was however not observed when the

samples were incubated in ESR tubes (**Figure 4B**). Only a very slight increase of radicals was detected up to day 4 when the concentration of PBN was increased from 1 to 10 mg/g oil. Thus the level of radicals accumulated up to the four day of incubation increased from $3.1(\pm 0.2)$ $\mu\text{mol/kg}$ oil to $6.6(\pm 0.3)$ $\mu\text{mol/kg}$ oil. Then, the levels of radicals observed were very low in both cases and no significant differences were found. Consequently, the radicals detected rapidly dropped in the FO under oxygen restriction conditions.

3.8. Confirming results under oxygen restriction

To confirm the effect of oxygen depletion on the accumulation of radicals, oil samples that had been oxidized in Eppendorf tubes for 12 days were transferred to ESR tubes and oxidized simultaneously at the same oxidation conditions. The ESR tubes were incubated closed to achieve a pronounced effect. The results clearly confirmed the effects, as increased levels of radicals were observed in the vegetable oils just after incubation in the ESR tubes, whereas very fast depletion of radicals occurred in the FO (**Figure 5**).

The slight increase of radicals accumulated in the vegetable oils could be attributed to a decrease in the oxidation rate, as remarked above. Furthermore, the results found in the three oils as a whole suggested that when oxygen was consumed, depletion of the accumulated radicals predominated over further trapping. Therefore, the faster the consumption of oxygen, the faster was the depletion of spin adducts compared to further spin trapping. This conclusion would definitely explain the different levels of radicals accumulated at the end of the assay for each oil.

The radical decay observed when oxygen was depleted could be attributed to an extremely limited formation of peroxy radicals. According to studies by Vicente et al. (1998), adducts of PBN with peroxy radicals are the precursors of MNP, which in turn reacts with radicals forming the observed spin adducts. Furthermore, the greater availability of alkyl radicals when the level of oxygen was reduced could also have contributed to a more effective depletion of the observed spin adducts. In this regard, studies by Janzen, Krygsman, Lindsay and Haire (1990) showed

that the trapping of alkyl radicals by aminoxyls to produce alkoxyamines was the main decay reaction of PBN spin adducts in the thermolysis of azobis(isobutyronitrile) (AIBN).

The residual radicals detected in the FO after oxygen depletion gave ESR spectra that were not different from those observed in the rest of the samples analyzed in the present study (Figure 2SM). Only a very slight significant increase in the ^1H splitting constant was observed at the end of the assay (from 1.83 ± 0.02 to 1.92 ± 0.03 G), but this was still far from that reported by Vicente et al. (1998) for alkyl adducts of PBN observed under very limited oxygen conditions (3.26 G). In this respect, it is worth mentioning that even when the ^1H splitting in the ESR spectra was not clearly noticeable due to the line broadening caused by mobility restriction (Velasco et al., 2015), computer simulation provided quite precise values of the ^1H coupling constant.

3.9. Detection of radicals under different microencapsulation conditions

Given that radicals were detected as a result of oil microencapsulation, further experiments were carried out to go in depth in this respect. It was thought that the more critical step was emulsification, as the emulsions were dried by lyophilization, i.e. under vacuum and very low temperatures. Thus, DM-SO samples were obtained by using the same emulsions described above, but applying different passes at 150 MPa in the high-pressure homogenizer. No ESR signals were detected in samples encapsulated with matrix A (not shown). Only when 4 passes were applied a very slight signal whose intensity was below the LOQ ($\leq 4.0 \mu\text{mol/kg}$) was recorded, confirming the results presented above. In contrast, a gradual increase in the levels of radicals was observed in samples B with the number of homogenization passes (**Figure 6**). An ESR signal was detectable from 2 passes and this was quantifiable only when 4 passes were applied. These results suggest that lipid oxidation took place during the emulsification step and that oxidation was enhanced by the number of homogenization passes, probably due to the heating caused by the process.

4. Conclusions

The PBN spin trapping technique can be a useful tool to evaluate microencapsulation conditions and the oxidative protection provided by different encapsulation matrices during the process. When applied to evaluate the protection provided by the encapsulation matrix during storage, results confirmed the starting hypothesis, but experiments should be limited to few days or even hours, depending on the lipid substrate, and ESR data should be interpreted cautiously. The great complexity of the reactions involved in the formation and decomposition of the radicals detected may lead to data misinterpretation. In this regard, accumulation of radicals in DMO is the result of faster radical trapping compared to spin-adduct decomposition. Detection of higher levels of radicals does not necessarily mean a higher level of oxidation. Oxygen availability conditions can have important impacts on the rates of both spin trapping and spin-adduct quenching and accordingly on the levels of radicals observed. Thus, ESR spin trapping depends to a great extent on experimental conditions. The kinetics of oxidation, spin trapping and spin-adduct decay should be understood first in bulk oils for a correct data interpretation in DMOs.

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513 **FIGURE CAPTIONS**

514 **Figure 1.** Formation of radicals during early oxidation stages of oils microencapsulated in
515 matrix A (sodium caseinate and D-lactose) (**A**) and matrix B (gelatin, maltodextrin (10 DE) and
516 sucrose) (**B**), and in bulk oils (**C**) incubated at 40 °C. Squares, circles and triangles represent
517 levels of spin adducts detected in rapeseed oil, sunflower oil and fish oil, respectively. Solid
518 symbols represent intact samples, whereas open symbols refer to dried microencapsulated oils
519 devoid of the free oil fraction. Error bars show standard deviation (n=3).

520 **Figure 2.** Radical decay over oxidation time in oils microencapsulated in matrix A (**A**) and
521 matrix B (**B**), and in bulk oils (**C**) incubated at 40 °C. For symbol assignment see Figure 1. Error
522 bars show standard deviation (n=3).

523 **Figure 3.** Formation of radicals during the incubation of bulk rapeseed (**A**), sunflower (**B**) and
524 fish (**C**) oils in Eppendorf (oil surface-to-volume ratio of 0.42 cm⁻¹) (○) and ESR tubes (oil
525 surface-to-volume ratio of 0.11 cm⁻¹) (●) at 40 °C. Error bars show standard deviation (n=3).

526 **Figure 4.** Effect of the spin trap concentration on the accumulation of spin adducts during the
527 incubation of bulk rapeseed (squares), sunflower (circles) and fish (triangles) oils in Eppendorf
528 (oil surface-to-volume ratio of 0.42 cm⁻¹) (**A**) and ESR tubes (oil surface-to-volume ratio of 0.11
529 cm⁻¹) (**B**) at 40 °C. Open and solid symbols represent oils containing α -phenyl-*N*-tert-
530 butylnitrone at 1 mg/g and 10 mg/g oil, respectively. Error bars show standard deviation (n=3).

531 **Figure 5.** Influence of oxygen availability conditions on the accumulation of spin adducts
532 during incubation of bulk rapeseed (**A**), sunflower (**B**) and fish (**C**) oils containing 10 mg/g PBN
533 at 40 °C. Solid symbols represent samples oxidized in open Eppendorf tubes (oil surface-to-
534 volume ratio of 0.42 cm⁻¹), whereas open symbols refer to samples oxidized in closed ESR tubes
535 (oil surface-to-volume ratio of 0.11 cm⁻¹) that had been previously incubated in Eppendorf tubes
536 for 12 days. Error bars show standard deviation (n=3).

537 **Figure 6.** ESR spectra obtained in fresh samples of sunflower oil microencapsulated in matrix B
538 from emulsions homogenized 1 (A), 2 (B), 3 (C) and 4 (D) times at 150 MPa.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

SUPPLEMENTARY MATERIAL

FIGURE CAPTIONS

Figure 1SM. ESR spectra of samples of rapeseed (A), sunflower (B) and fish (C) oil incubated at 40°C during 4 days: bulk oils (A-C), oils microencapsulated in matrix A (sodium caseinate and D-lactose) (A'-C') and matrix B (gelatin, maltodextrin (10 DE) and sucrose) (A''-C'').

Figure 2SM. ESR spectra of bulk fish oil samples containing 10 mg/g PBN and oxidized at 40°C during 17 days. (A) Sample incubated in an Eppendorf tube. (B) Sample incubated in a closed ESR tube that had been previously incubated in an Eppendorf tube during the first 12 days.

Figure 1SM

Figure 2SM