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Enrichment of vegetable oils with phenolic antioxidants for food applications

Maria del Pilar Garcia Mendoza

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Par Maria del Pilar GARCIA MENDOZA

**Enrichissement d'huiles végétales par des antioxydants de
type phenolique en vue d'applications alimentaires**

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Soutenue le 23 Novembre 2020

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Enrichissement d'huiles végétales par des antioxydants de type phenolique en vue d'applications alimentaires

Les huiles végétales comme les huiles de cameline et tournesol sont des sources d'acides gras polyinsaturés bon pour la santé mais également très sensibles à la dégradation oxydative. Ce travail vise à améliorer la stabilité oxydative d'huiles alimentaires (principalement l'huile de cameline) via l'incorporation de composés phénoliques antioxydants soit comme composé pur (quercétine) soit comme un mélange plus complexe extrait à partir d'un coproduit de la noix. La faible solubilité de la quercétine dans l'huile a été contournée avec succès par le développement d'une voie d'enrichissement sans solvant en présence de phospholipides. Ainsi, la formulation à base de quercétine-phospholipides a permis d'augmenter significativement à la fois la solubilité de la quercétine and la stabilité oxydative de l'huile. Cette stabilité oxydative, mesurée par vieillissement accéléré à chaud, s'est révélée variable selon les concentrations en quercétine et phospholipides. L'hypothèse du rôle central des associations colloïdales dans ces observations a été formulée. Des données sur la solubilité de la quercétine dans différents solvants d'intérêt industriel ont également été générées. Pour les extraits phénoliques à base de tourteau de noix, en plus de l'amélioration de la stabilité oxydative, les paramètres influençant l'extraction ont été identifiés et une modélisation des procédés batch et semi-continu proposée. L'effet protecteur contre l'oxydation de l'huile d'un extrait de noix présentant une haute activité antiradicalaire s'est révélé dose-dépendant et a permis un accroissement significatif de la durée de conservation des huiles enrichies.

Mots clés : Enrichissement, huiles comestibles, antioxydants, phospholipides, oxydation des lipides

Enrichment of vegetable oils with phenolic antioxidants for food applications

Vegetable oils like camelina and sunflower oils are sources of healthy polyunsaturated fatty acids that are however highly susceptible to oxidative degradation. This work aimed at enhancing the oxidative stability of edible oils, mostly camelina oil by incorporating phenolics antioxidants, either as a pure component, quercetin, or as a more complex mixture extracted from a walnut by-product. The low solubility of quercetin in oils was successfully circumvented by developing a solvent-free route of enrichment in presence of phospholipids, so that quercetin-phospholipids formulation allowed to significantly increase both quercetin solubility and the oxidative stability of the oils. The enhanced oxidative stability, monitored under accelerated conditions of heating, was found to vary according to quercetin-phospholipid concentrations and ratios, and it was assumed that colloidal associations played a key role in the enhancement. Data of quercetin solubility in various solvents of industrial interest were also provided. For phenolic extract recovered from a walnut press-cake in addition to oxidative stability enhancement, modelling of batch and semi-continuous extractions was performed and influent parameters were identified. The protective effect against lipid oxidation of a walnut extract exhibiting high antiradical activity was dose-dependent and significantly extended the shelf life of enriched oils.

Keywords : Food enrichment, edible oils, antioxidants, phospholipids, lipid oxidation

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Dissemination of the results

Articles

M.D.P Garcia-Mendoza, F.A. Espinosa-Pardo, R. Savoire, C. Harscoat-Schiavo, M. Cansell, P. Subra-Paternault. Improvement of the oxidative stability of camelina oil by enrichment with phospholipid-quercetin formulations, Food Chem, accepted on September 25th, 2020.

Oral and poster presentations

- 23/08/2020: 24th International Congress of Chemical and Process Engineering CHISA 2020 (Prague, Czech Republic, postponed due to coronavirus outbreak to 2021) – oral presentation
- 30/01/2020: Lipids & Cosmetics Congress 2020: Top of innovations and research initiatives (Bordeaux, France) – oral presentation
- 07/06/2019: 21^{ème} Journée Scientifique de l'EDSC (Bordeaux, France) – poster presentation
- 28/05/2019: CBMN day (Bordeaux, France) – poster presentation
- 24/05/2019: 12th Young Scientist Symposium (Bordeaux, France) – oral presentation
- 14/05/2018: 14th International Symposium on Biochromatography and Nanoseparations (Bordeaux, France)

Résumé étendu

Les lipides en général constituent une part importante de notre alimentation et entrent dans de nombreux produits alimentaires consommés quotidiennement (vinaigrettes, margarine, pâtes à tartiner, produits de boulangerie, glaces, soupes, sauces, aliments en poudre, produits de nutrition infantile, poisson en conserve, bonbons, céréales pour petit-déjeuner, etc.). Les lipides influencent considérablement l'appétence, le goût et la texture des produits finis. Selon Hammond, (2003), lorsque les aliments contiennent des quantités importantes de matières grasses ajoutées, trois caractéristiques principales de l'aliment sont affectées ; i) l'aptitude au traitement pendant la préparation, ii) la qualité sensorielle (liée au goût et à la saveur) et iii) la durée de conservation, qui peut être de nature physique ou chimique (oxydation). De nos jours, dans les produits alimentaires, le gras d'origine animale tend à être remplacé par des lipides d'origine végétale et donc, la consommation de ces lipides est en augmentation. Les production et consommation annuelles d'huiles et de graisses d'environ 200 millions de tonnes augmentent régulièrement à un rythme de 2 à 6 millions de tonnes par an (FAO, 2015). Par ailleurs, les huiles végétales sont également largement utilisées pour des applications cosmétiques et pharmaceutiques. Par exemple, en tant que véhicule de médicaments dans les produits pharmaceutiques topiques, les injectables intramusculaires et les formes posologiques orales solides, et dans l'industrie cosmétique pour la fabrication de produits capillaires, produits nettoyants, crèmes topiques, lotions, parfums, produits de maquillage. Comme pour les aliments, les huiles végétales incorporées dans les produits cosmétiques et pharmaceutiques influencent considérablement leur qualité, leur stabilité et leurs propriétés fonctionnelles.

D'un point de vue chimique, les huiles végétales sont principalement composées d'un mélange de triglycérides (95-99%, trois acides gras esterifiés avec le glycerol), avec quelques quantités mineures de diacylglycérols, de monoacylglycérols (< 5%), et d'autres composants mineurs tels que les acides gras libres, les tocophérols, tocotriénols, phospholipides, phytostérols, entre autres. Les acides gras (AG) qui contiennent plus d'une double liaison dans leur squelette (Acides Gras Poly Insaturés, AGPI) ont un intérêt nutritionnel majeur, en particulier ceux de la série n-3, qui affectent positivement plusieurs processus physiologiques, modulant ainsi l'état de santé et l'apparition de maladies chroniques (Rodríguez et al., 2019).

Parmi les huiles végétales les plus produites au monde, on trouve l'huile de tournesol avec environ 19 millions de tonnes par an (STATISTA, 2020). Néanmoins, une huile moins 'exploitée' comme celle issue de *Camelina sativa* attire de plus en plus l'attention en raison de

sa valeur nutritionnelle et de ses conditions de culture. La caméline présente des exigences minimales en matière d'irrigation et d'engrais et l'huile de caméline contient environ 50 à 60% d'AGPI dont 35 à 40% d'acide α -linoléique (C18 :3 n-3, ALA), acide gras essentiel, et 15 à 20% d'acide linoléique (LA). L'huile de caméline contient également des antioxydants naturels tels que les tocophérols (teneur totale d'environ 500 à 700 mg/kg d'huile) et des composés phénoliques (jusqu'à 130 mg/kg). L'huile de tournesol est l'une des huiles végétales les plus populaires et dans certains pays, elle est plus utilisée que les huiles de soja, de coton et de palme. L'huile de tournesol présente une teneur élevée en LA (60 à 70%) et sa teneur en tocophérol est d'environ 700 mg/kg.

Dans ce contexte, on peut affirmer que les huiles de tournesol et de caméline présentent une teneur élevée en AGPI et d'autres propriétés physiques intéressantes qui les rendent très utiles pour les applications alimentaires, nutraceutiques et cosmétiques. Néanmoins, les huiles riches en AGPI sont également très sensibles à la dégradation par l'action de l'oxygène (processus d'oxydation) ce qui peut limiter leur utilisation dans les applications industrielles.

L'oxydation est une série indésirable de réactions chimiques complexes qui dégrade la qualité de l'huile. Cette altération est responsable du rancissement des huiles, de la perte de qualité nutritionnelle et du développement de saveurs et d'odeurs désagréables qui rendent les produits impropres à la consommation. Le principal mécanisme impliqué dans l'oxydation des lipides est appelé autoxydation ou peroxydation dans laquelle les acides gras insaturés des triglycérides sont attaqués par l'oxygène atmosphérique. Retarder l'oxydation des lipides de façon à produire des huiles et/ou des produits gras plus stables tout en gardant leur qualité nutritionnelle est devenu un défi majeur pour l'industrie. La résistance à l'oxydation peut être obtenue par l'ajout d'antioxydants naturels ou synthétiques. L'hydroxytoluène butylé (BHT) et l'hydroxyanisole butylé (BHA) sont des antioxydants synthétiques couramment utilisés pour la protection des graisses alimentaires. Cependant, ces additifs ont été associés à des lésions hépatiques et à la carcinogenèse (Szydłowska-Czerniak et al., 2018). En raison de ces risques potentiels pour la santé, l'intérêt des industries alimentaire, pharmaceutique et cosmétique pour les antioxydants naturels, tels que les polyphénols, s'est accru. Les polyphénols sont des métabolites secondaires végétaux qui présentent un effet protecteur contre l'oxydation des lipides (Bakkalbasi et al., 2018; Wang et al., 2018). Parmi les polyphénols, les flavonoïdes sont des dérivés phénoliques à structure benzo-pyrone qui ont fait l'objet d'une attention particulière en raison de leur capacité à piéger les espèces réactives (Ramadan, 2012). Parmi les

flavonoïdes, la quercétine présente une grande capacité à inhiber les dommages oxydatifs induits par les métaux et les non-métaux, en raison de la présence de son groupe 3-OH libre (Heim et al., 2002). Ce flavonoïde, présent dans les oignons, les pommes, le thé, le vin rouge, exerce de nombreux effets (antioxydants, anti-inflammatoires, anticarcinogènes, cardioprotecteurs, neuroprotecteurs et antihypertenseurs) ce qui le rend bénéfique pour la santé (Xu et al., 2016). D'autre part, un mélange complexe de composés antioxydants que l'on peut trouver dans des extraits obtenus à partir de matières végétales représente une alternative intéressante à l'utilisation d'un composé isolé et pur. L'utilisation d'agro-résidus comme source d'antioxydants phénoliques se développe car ces co-produits représentent une source peu coûteuse et abondante.

Néanmoins, malgré les multiples avantages des polyphénols, l'enrichissement des systèmes huileux avec ces composés peut être limité par leur faible solubilité dans les lipides en raison de leur nature polaire (Kidd, 2009). La littérature fait état de quelques travaux montrant que l'ajout de lécithine à de l'huile permet d'incorporer davantage de composés phénoliques, avec comme bénéfice, une plus grande stabilité à l'oxydation (Fregapane et al., 2020; Yara-Varon et al., 2017; Li et al., 2015).

Dans ce contexte, **ce travail vise à évaluer l'enrichissement d'huiles végétales, principalement l'huile de caméline, avec des antioxydants phénoliques, soit sous forme de composant pur, la quercétine, soit sous forme de mélange plus complexe extrait d'un sous-produit de noix, et ce afin d'améliorer la stabilité oxydative des huiles.** La spécificité du travail est de chercher à augmenter la concentration ajoutée en se basant sur la complexation des composés phénoliques avec les phospholipides rapportée dans la littérature pour les milieux aqueux, et considérant que les phospholipides se solubilisent dans les huiles grâce à leurs chaînes grasses. Pour atteindre cet objectif, nous avons donc étudié l'incorporation de phospholipides avec la quercétine ou avec des extraits de polyphénols issus de noix pour à la fois augmenter la solubilité des polyphénols dans les huiles et prolonger la durée de conservation des huiles enrichies. Plusieurs méthodes de réalisation du processus d'enrichissement ont été évaluées afin d'optimiser la solubilité phénolique et la stabilité de l'huile. L'évaluation de la solubilité de la quercétine dans d'autres solvants d'intérêt industriel et les méthodes d'extraction pour optimiser la récupération d'extraits riches en polyphénols à partir de tourteaux de noix, ont également été au centre de ce travail. Les objectifs susmentionnés ont été divisés séparément en cinq chapitres comme décrit ci-dessous.

Tout d'abord, une étude bibliographique a été menée au *Chapitre I*. Ce chapitre rapporte et décrit les principaux concepts liés aux huiles végétales, à l'oxydation des lipides, aux composés antioxydants, aux sources phénoliques et aux méthodes d'extraction, aux phospholipides comme additif de vectorisation et enfin, aux méthodes de fabrication d'un système polyphénol-phospholipide.

Dans la première partie de ce chapitre, certaines propriétés générales des huiles végétales qui mettent en évidence leur importance dans les applications alimentaires ou cosmétiques, ainsi que certaines données relatives à la production mondiale d'huiles ont été fournies. L'huile végétale étant une matrice assez complexe, il était alors nécessaire de préciser les éléments clés impliqués dans leurs propriétés, leur fonctionnalité et leur stabilité à l'oxydation.

Considérant que ces travaux visant à améliorer la stabilité à l'oxydation des huiles alimentaires, le principe, le mécanisme et les facteurs d'influence de l'oxydation des lipides ont été décrits en se focalisant sur l'auto-oxydation qui est connue comme le principal mécanisme d'oxydation des huiles et des graisses. Le rôle des composés antioxydants de type phénoliques dans le retard de l'oxydation des lipides a été présenté. Leur origine, structure, classification et leur mécanisme d'action ont été décrits. De plus, ont été répertoriés plusieurs exemples de travaux antérieurs axés sur l'enrichissement en huile avec des extraits de polyphénols naturels obtenus à partir de diverses matières végétales. La structure, les propriétés principales et l'applicabilité des phospholipides en tant que composés de vectorisation ont ensuite été discutées. Enfin, ce chapitre décrit les méthodes utilisées pour fabriquer un complexe/un système polyphénol-phospholipide et présente les publications traitant d'huiles co-enrichies avec des phospholipides et des composés phénoliques.

Le Chapitre II a soigneusement décrit les matériaux et les méthodes utilisés dans ce travail pour atteindre l'objectif proposé. Dans un premier temps, l'origine, les principales caractéristiques et les éventuels prétraitements des matières et composés utilisés dans ce travail, à savoir l'huile végétale, les polyphénols purs, les phospholipides et le tourteau de noix, ont été présentés, suivi par la description des méthodes développées pour mesurer avec fiabilité la solubilité des polyphénols dans des huiles et autres solvants, des méthodes de fabrication des formulations quercétine – phospholipides ainsi que les procédures d'enrichissement des huiles. De plus, est présentée la méthode utilisée pour évaluer l'auto-assemblage dans l'huile des phospholipides en structures colloïdales, structures qui ont un rôle clé dans l'activité

antioxydante. Dans cette perspective, ont été décrites les conditions et les méthodes d'analyse utilisées pour étudier la stabilité à l'oxydation des huiles enrichies et non enrichies. L'évaluation de la stabilité à l'oxydation s'est concentrée sur la formation de produits d'oxydation primaires, c'est-à-dire des diènes conjugués et des hydroperoxydes. Enfin, ce chapitre détaille les techniques d'extraction utilisées pour récupérer les composés phénoliques polaires d'un tourteau de noix qui seront ultérieurement incorporés à l'huile de caméline afin d'améliorer sa stabilité à l'oxydation. Des méthodes visant à extraire les polyphénols de la noix directement dans de l'huile de caméline ont également été décrites ainsi que des informations liées à l'analyse statistique des données expérimentales.

Les Chapitres III, IV et V correspondent à la présentation des résultats obtenus à partir des différentes méthodologies, étant donné que chaque chapitre correspond à un objectif spécifique de ce travail. Ces chapitres ont été soigneusement décrits ci-dessous.

Au *Chapitre III*, il a été globalement discuté des stratégies d'enrichissement des huiles alimentaires avec des formulations polyphénols-phospholipides afin d'améliorer la solubilité des polyphénols dans les huiles. En raison de ses propriétés antioxydantes, la quercétine a fait l'objet d'une attention particulière dans ce travail. Les résultats d'analyses chimiques des huiles de caméline et tournesol sont présentés et discutés en terme de facteurs clés pouvant influencer la stabilité à l'oxydation de ces huiles. Il a également fallu mettre au point une méthode pour déterminer la solubilité de la quercétine dans les huiles, méthode qui sera utilisée pour évaluer la solubilité de ce composé dans différents solvants d'intérêt industriel tels que le diméthylsulfoxyde, le polyéthylèneglycol, l'éthanol, le propanol, le cyclopentanol, l'eau et l'aniline. Deux méthodes pour réaliser une formulation quercétine-phospholipide dans huile ont été développées. La première passe par la fabrication d'un complexe en solution selon le principe utilisé dans la littérature pour formuler des liposomes en suspension aqueuse. Le complexe quercétine-phosphatidylcholine est ainsi fabriqué par un procédé d'évaporation de solvant. L'efficacité de complexation a été évaluée à différents rapports massiques quercétine: phosphatidylcholine et en utilisant de l'éthanol absolu ou un mélange de méthanol: chloroforme (70:30 v/v) comme milieu de complexation. Après cette étape de préparation, le complexe quercétine-phosphatidylcholine est incorporé dans l'huile via un ajout du complexe dilué dans du chloroforme (voie solvant). La seconde voie d'enrichissement est une voie sans solvant basée sur une dissolution directe dans l'huile. Cette voie a été appliquée à l'enrichissement des huiles de Miglyol, de tournesol et de caméline avec plusieurs formulations de quercétine-

phosphatidylcholine. Enfin, l'auto-assemblage de la phosphatidylcholine dans l'huile de caméline a été étudié au moyen de la méthode au 7,7,8, 8-tétracyanoquinodiméthane (TCNQ).

La mesure de la solubilité de la quercétine et le protocole pour obtenir cet enrichissement ont été délicats à développer. Après avoir validé les méthodes de quantification (spectroscopie UV, chromatographie en phase liquide, dosage des polyphénols totaux par la méthode de Folin Ciocalteu), il a été constaté que la naringénine, l'acide chlorogénique, l'acide sinapique ne sont pas solubles dans les huiles. En revanche, la quercétine était soluble dans le Miglyol (783 $\mu\text{g/g}$), l'huile de caméline (166-393 $\mu\text{g/g}$) et l'huile de tournesol (390 $\mu\text{g/g}$). En ce qui concerne la solubilité de la quercétine dans divers solvants, il a été observé que les solvants amphiphiles tels que le diméthylsulfoxyde et le polyéthylène glycol 400 présentaient une capacité élevée à solubiliser la quercétine (392 mg de quercétine/g et 163 mg de quercétine/g, respectivement) alors que l'eau la solubilisait mal (0,005 mg/g). Les écarts entre nos données et celles de la littérature et entre les données de la littérature sont discutés.

Concernant le complexe quercétine-phospholipide, une efficacité de complexation élevée ($> 95\%$) a été obtenue lorsque l'éthanol absolu a été utilisé comme milieu de complexation, et un rapport molaire quercétine-phospholipide de 1:1 a fourni les meilleures conditions de complexation. Néanmoins, l'enrichissement d'huile avec le complexe quercétine-phospholipide par une voie solvant (complexe dilué dans du chloroforme) a été inefficace pour améliorer la solubilité de la quercétine dans l'huile de caméline. À l'inverse, l'enrichissement avec des formulations phospholipides-quercétine par une voie sans solvant s'est avéré être une bien meilleure méthode qui a permis d'évaluer la solubilité de ce flavonoïde dans les huiles de Miglyol, de caméline et de tournesol. La solubilité de la quercétine dans l'huile de caméline est passée de 166 $\mu\text{g/g}$ à 1 298 $\mu\text{g/g}$ grâce à l'incorporation de phospholipides à une concentration de 20 mg/g d'huile. La solubilité de la quercétine est directement corrélée à la concentration de phospholipides ajoutés à l'huile (5-20 mg/g) selon une loi linéaire. On a également constaté que la CMC des phospholipides dans l'huile est bien inférieure aux concentrations de phospholipides utilisées pour l'enrichissement, ce qui laisse penser que les phospholipides s'auto-assemblent en micelles inverses. Dans ce contexte, ces structures colloïdales agissent probablement comme des 'porteurs' de quercétine et ont donc joué un rôle clé dans la solubilité accrue de la quercétine dans les huiles végétales.

Le Chapitre IV consistait principalement à évaluer la stabilité à l'oxydation d'huiles enrichies. L'oxydation est réalisée dans des conditions de vieillissement accéléré à 60 °C.

L'évaluation de la stabilité à l'oxydation consiste à suivre la formation de produits d'oxydation primaires tels que les diènes conjugués et les hydroperoxydes. La corrélation linéaire entre l'indice de peroxyde et les diènes conjugués a permis d'estimer la valeur de peroxyde d'huiles enrichies en mesurant uniquement la valeur des diènes conjugués. Dans ce chapitre, l'oxydation des huiles de cameline et de tournesol pures a d'abord été suivie, avant d'étudier principalement la stabilité à l'oxydation d'huiles de caméline enrichies en différentes formulations de quercétine-phospholipide selon la voie sans solvant présentée au *Chapitre III*. L'enrichissement en huile a été réalisé en variant la concentration et le ratio des deux composants, dans une gamme entre 168 $\mu\text{g/g}$ et 1 298 $\mu\text{g/g}$ pour la quercétine, et de 5 mg/g à 20 mg/g pour les phospholipides. L'amélioration de la stabilité à l'oxydation a été quantifiée en estimant le temps de latence d'oxydation, défini comme le temps nécessaire pour atteindre un indice de peroxyde de 15 $\text{m}_{\text{eq}}\text{O}_2/\text{kg}$ qui correspond au seuil au-delà duquel une huile vierge est jugée non consommable (Codex alimentarius). L'oxydation de l'huile de caméline à température ambiante a également été étudiée. Enfin, la consommation de la quercétine durant l'oxydation a été suivie par prélèvements et quantification par HPLC. Quelques essais de stabilité de l'huiles de tournesol enrichies ont également été menés.

Concernant les résultats du *Chapitre IV*, on peut affirmer, d'après le suivi des diènes conjugués et le calcul des temps de latence d'oxydation, que les phospholipides (5-20 mg/g) incorporés à l'huile de caméline retardent l'oxydation des lipides, probablement grâce à leur capacité à chélater les métaux et à leur fonction amine qui peuvent interagir avec les radicaux libres et bloquer les réactions de propagation (Henna Lu et al., 2011). Cependant, l'activité antioxydante des phospholipides n'était pas dose-dépendante puisque le temps de latence a varié de 24h pour l'huile pure à 40h pour l'huile enrichie quelle que soit la concentration en phospholipides. La quercétine seule, enrichissant une huile à sa concentration de saturation (168 $\mu\text{g/g}$), n'exerce qu'un faible effet protecteur contre l'oxydation lipidique.

En revanche, l'enrichissement d'huile avec des formulations de quercétine-phospholipide a amélioré la stabilité à l'oxydation de l'huile de caméline. Les échantillons saturés de quercétine aux différentes concentrations de phospholipides (5-20 mg/g) ont fourni l'effet protecteur le plus élevé en augmentant le temps de latence d'oxydation jusqu'à ~ 115 heures. Notre hypothèse est que la quercétine, en interagissant avec la tête polaire de la phosphatidylcholine, se rapproche des micro-environnements micellaires constitués d'un cœur aqueux entourés de composés tensioactifs dont les phospholipides, micro-environnements

considérés comme étant les sites actifs de l'oxydation (Li et al., 2015). Par conséquent, un effet protecteur amélioré de la quercétine quand elle est formulée avec un phospholipide peut être obtenu. Concernant le suivi de la quercétine dans l'huile de caméline, on a observé une forte diminution de la concentration au cours des 120 premières heures d'oxydation lorsque la quercétine est ajoutée seule, avec 70% de quercétine consommée à ce stade. La présence de phospholipides dans l'huile de caméline permet d'incorporer de plus grandes quantités de quercétine (253-1 298 $\mu\text{g/g}$) qui peuvent alors jouer le rôle de réservoir lors de l'oxydation accélérée de l'huile. Enfin, la comparaison des temps de latence sous conditions d'oxydation accélérée à 60°C ou sous température ambiante montre que les formulations quercétine-phospholipides proposées permettraient d'étendre significativement la durée de conservation d'une huile, qui passerait de 28 jours à 134 jours.

A la différence du *Chapitre IV* qui étudie l'enrichissement d'une huile avec un polyphénol pur, le *Chapitre V* est dédié à l'obtention d'extraits phénoliques plus complexes, puis à l'enrichissement de l'huile de caméline avec ces extraits phénoliques, toujours dans le but d'améliorer la stabilité oxydative de l'huile. Dans un premier temps, la méthode de macération (extraction solide-liquide) a été utilisée pour extraire les composés phénoliques d'un tourteau de noix. Les essais ont été menés selon un plan factoriel fractionnaire afin d'optimiser le rendement d'extraction phénolique et l'activité antioxydante des extraits de noix, considérant les facteurs indépendants suivants comme variables : temps (15-60 minutes), température (28°C-60 °C), rapport solide/solvant (1/13-1/25) et présence ou non de phospholipides (0-1.5% m/v) dans le solvant d'extraction (éthanol 60%). Le dosage de Folin-Ciocalteu et l'activité de piégeage du radical libre 2,2-diphényl-1-picrylhydrazyl (DPPH) ont été utilisés pour la quantification des polyphénols totaux et la mesure de l'activité antioxydante, respectivement. Sur la base des résultats de l'extraction par macération, une extraction semi-continue des polyphénols du tourteau de noix a été réalisée à des fins de comparaison. De l'éthanol à 60% (EtOH60) ou de l'éthanol à 90% (EtOH90) ont été percolés à 1 ml/min à travers une colonne contenant 5 g de tourteau à la température d'extraction de 60 °C. Un modèle de cinétique de désorption à deux sites (Kubatova et al. 2002) a été ajusté à la cinétique expérimentale d'extraction des polyphénols. Après cela, l'extrait de noix obtenu par macération qui présentait le rendement d'extraction et l'activité anti-oxydante les plus élevés a été utilisé pour enrichir l'huile de caméline. L'enrichissement a été réalisé à deux concentrations différentes (150 et 1 500 mg d'extrait/kg d'huile) sur des huiles contenant ou non des phospholipides (20 mg/g), et l'oxydation des huiles enrichies a été suivie en conditions

accélérées par chauffage à 60 °C. De plus, d'autres méthodes de macération directe de tourteau dans l'huile assistée ou non par ultrasons ont été testées afin de proposer des techniques d'enrichissement d'huile sans solvant.

Globalement, les résultats du *Chapitre V* ont présenté le tourteau de noix comme une source bon marché, abondante et riche de composés antioxydants tels que les polyphénols polaires. L'extraction par macération a fourni des extraits riches en polyphénols (dans une gamme de 2,2 à 14,8 mg de GAE/g de tourteau). L'analyse statistique des data a montré que la température d'extraction et un ratio solvant/solide élevés influencent positivement le rendement d'extraction des polyphénols totaux et l'activité anti-oxydante. D'un autre côté, l'incorporation de phospholipides au solvant d'extraction était nuisible pour le processus d'extraction. L'extraction phénolique totale (TPC) par macération pourrait être prédite de façon satisfaisante par le modèle polynomial suivant : $TPC \text{ (mg GAE/g WAC)} = 8,9 + 0,7 * \text{ temps} + 2,4 * \text{ température} + 1,4 * \text{ rapport solide-solvant} - 3 * \text{ Concentration PL} + 1,0 * \text{ température} * \text{ rapport solide/solvant} + 1,3 * \text{ température} * \text{ concentration PL}$, avec un coefficient de détermination supérieur à 0,90. D'autre part, la valeur du DPPH variait entre 12 et 137 μmol d'équivalent Trolox/g de tourteau. Le TPC et le DPPH peuvent être maximisés par des niveaux les plus élevés de température et de rapport solide-solvant, et en l'absence de phospholipides.

L'extraction semi-continue des polyphénols a permis d'obtenir des extraits fractionnés qui présentent diverses quantités de masse extraite et de concentration phénolique, par conséquent, ils pourraient être utilisés séparément pour diverses applications industrielles. Le modèle de désorption cinétique à deux sites a été ajusté avec succès ($R^2 > 0,98$) à l'extraction cinétique expérimentale des polyphénols à partir du tourteau de noix. EtOH60 a montré une meilleure performance pour extraire les composés phénoliques alors que EtOH90, pas assez polaire pour les polyphénols, a favorisé l'extraction de l'huile contenu dans le tourteau.

Concernant la stabilité à l'oxydation des huiles enrichies, les extraits hydroalcooliques de tourteau de noix ont montré une capacité significative à retarder l'oxydation des lipides de manière dose-dépendante, en augmentant le temps de latence d'oxydation jusqu'à 84 heures. Nos résultats sont en accord avec d'autres travaux (Fregapane et al., 2020; Bakkalbasi et al., 2018). L'effet protecteur apporté par les polyphénols polaires de l'extrait de noix peut être expliqué par la théorie du 'Polar paradox' (Bakkalbasi et al., 2018; Li et al., 2015). D'autre part, l'extraction directe des polyphénols du tourteau par l'huile de cameline par macération simple ou assistée par ultrasons n'a pas donné de bons résultats car la teneur totale en phénol

des huiles enrichies n'a pas augmenté de manière significative. Néanmoins, la dispersion du tourteau dans l'huile s'est avérée être un excellent moyen pour améliorer la stabilité de l'huile à l'oxydation puisque la formation de diènes conjugués dans l'huile de caméline enrichie en tourteau de noix était significativement plus lente que dans l'huile sans noix. La concentration en polyphénols étant restée quasi inchangée à l'issue de la macération, ce comportement pourrait être attribué à la solubilisation de composés antioxydants lipophiles de nature non polyphénolique. Néanmoins, ces composés n'ont pas été identifiés dans ce travail.

En conclusion finale, on peut affirmer que la stabilité à l'oxydation des huiles végétales alimentaires, principalement de l'huile de caméline, a été améliorée avec succès en les enrichissant avec des antioxydants phénoliques, soit sous forme de composant pur en utilisant de la quercétine, soit sous forme de mélange complexe utilisant des extraits phénoliques d'un co-produit de noix. L'incorporation de phospholipides et leur auto-assemblage dans les huiles ont joué un rôle clé dans l'augmentation de l'activité antioxydante de la quercétine et donc dans la plus grande stabilité à l'oxydation des huiles enrichies. De plus, l'enrichissement des huiles en phospholipides a permis d'augmenter considérablement la solubilité de la quercétine dans les huiles. Le tourteau de noix était une source riche et abondante de composés phénoliques dont les extraits polaires ont montré leur capacité à augmenter la stabilité de l'huile, mais qui peut lui-même retarder l'oxydation lipidique grâce à ses propriétés anti-oxydantes intrinsèques.

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Introduction

Vegetable oils are a group of fats that are derived from some seeds, nuts, cereal grains, and fruits. They are a source of several micronutrients such as fat-soluble vitamins, essential fatty acids and antioxidants which are essential for the human organism. In this context, vegetable oils are the basis of many food products such as food dressings, margarine, spreads, bakery products, soups, sauces, party dips, powdered foods, etc. When foods contain significant amounts of added fat, three main characteristics of the food are affected: i) the processability during preparation, ii) the sensory quality and, iii) the shelf-life, (oxidative stability) (Hammond, 2003). On the other hand, vegetable oils are also widely used for cosmetic and pharmaceutical applications. For instance, as a vehicle of drugs in topical pharmaceuticals, and in the cosmetic industry for fabricating shampoos, hair conditioners, topical creams, etc.

From a global point of view, plant oils are mainly composed of a mixture of triglycerides (95-99%), with some minor amounts of diacylglycerols, monoacylglycerols, free fatty acids, phospholipids, among others. Fatty acids (FAs) that form the triglycerides are responsible for the physical, nutritional and chemical properties of vegetable oils, being that polyunsaturated fatty acids (PUFAs) have a major nutritional interest. Among the most produced vegetable oils worldwide, it can be found sunflower oil whereas less “exploited” oils such as from *Camelina sativa* has been gained attention because its nutritional value and growing conditions. Camelina oil contains about 50–60 % of PUFAs among which 35–40% comprise α -linolenic acid (ALA) and 15–20% linoleic acid (LA) (Moslavac et al., 2014). On the other hand, sunflower oil commonly exhibits a high content of LA (60–70%) and a low content of oleic acid (20-30%). In this context, it can be stated that both sunflower and camelina oils exhibit a high content of PUFAs and other interesting physical properties which make them very useful for food, nutraceutical and cosmetics applications. Nonetheless, rich in PUFAs vegetable oils are also highly susceptible to degradation by the action of oxygen which may limit their use in industrial applications.

Oil oxidation is an undesirable series of complex chemical reactions that degrades the quality of the oil. This alteration is responsible for the rancidity of oils, the loss of nutritional quality and the development of unpleasant flavors and odors. From that perspective, the delay of lipid oxidation has become a major challenge for today's industry.

In this sense, an enhanced oxidative stability of oils can be accomplished by the addition of natural or synthetic antioxidants. For instance, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are common and effective synthetic antioxidants but they have been associated with liver damage and carcinogenesis (Szydłowska-Czerniak et al., 2018). As consequence, the interest of food, pharmaceutical and cosmetic industries for natural antioxidants, such as polyphenols, has increased. Polyphenols are secondary plant metabolites which exhibit protective effect against lipid oxidation (Bakkalbasi et al., 2018; Wang et al., 2018). Among polyphenols, quercetin exhibits a great ability to inhibit oxidative damage induced by both metals and nonmetals, due to the presence of its free 3-OH group (Heim et al., 2002). On the other hand, a variable mixture of polyphenols extracted from plant materials, i.e. not necessarily isolated and pure phenolics, also represents an important alternative to be used in the task of improving the oil stability. In this sense, the use of agro residues for obtaining polyphenol-rich extracts has been catching the attention of researchers.

Nonetheless, despite the multiple benefits of polyphenols, the enrichment of oily systems with these compounds may be limited by their low stability during processing, and the poor solubility that polyphenols exhibit in oils and fats due to their multi-ring and polar nature (Kidd, 2009). The above reduces the effectiveness of phenolic compounds to protect oils and fats against lipid oxidation. On the other hand, the solubility of polyphenols in water has been enhanced by the formation of polyphenol-phospholipids complex and therefore, these complexation/interaction between polyphenol and phospholipids can be also advantageous to increase the solubility of phenolics in oils thanks to the amphiphilic character of phospholipids. From that perspective, this phenol-phospholipid formulation incorporated to oils may also increase the antioxidant capacity of phenolics and thus, enhance the oxidative stability of enriched oils. Moreover, the incorporation of lecithin to vegetable oil has also showed to be beneficial for increasing the extraction of polyphenols from plant matrices (Li et al., 2015).

From that perspective, **this work aimed at evaluating the enrichment of edible oils, mostly camelina oil by incorporating phenolics antioxidants, either as a pure component, quercetin, or as a more complex mixture extracted from a walnut by-product in order to enhance the oxidative stability of oils.** For achieving this aim, we have studied the incorporation of phospholipids along with quercetin, or with polyphenol walnut extracts for both increasing the polyphenol solubility in oils and extending the shelf life of enriched oils. Several methods for carrying out the enrichment process has been evaluated in order to

optimize the phenolic solubility and the oil stability. The assessment of the quercetin solubility in other solvents of industrial interest and the extraction methods for optimizing the recovery of polyphenol-rich extracts from walnut cake, have been also part of the focus of this work. The aforementioned objectives have been divided separately in five chapters as described below.

Chapter I reported and described the main concepts related to vegetable oils, lipid oxidation, antioxidants compounds, phenolic sources and extraction methods, phospholipids as carrier molecules and finally, the methods for carrying out a polyphenol-phospholipid system. It has been described the main characteristics of both sunflower oil and camelina oil which has been the focus of this work. A literature review for describing the principle, mechanism and the influencing factors of lipid oxidation (autoxidation) has been also carried out. After that, the role of phenolic compounds for delaying the lipid oxidation has been detailed. In this context, the methods to extract phenolics from plant matrices was also approached. The structure, main properties and applicability of phospholipids as carrier compounds was then discussed. Finally, this chapter described the methods recently reported in literature for fabricating polyphenol-phospholipid system/complex which lead to an enhanced polyphenol solubility and antioxidant activity.

Chapter II carefully described the materials and methods used in this work for reaching the proposed aim. Firstly, the origin, main characteristics and pretreatments of the materials used in this work were detailed, followed by the description of the methods developed in this work for certainly measuring the solubility of polyphenols in oils and for fabricating a quercetin-phospholipid complex. Furthermore, it was described the methods for evaluating the self-assembly of phospholipids in bulk camelina oil and the analytical methods used for studying the oxidative stability of both enriched and non-enriched oils. Finally, this chapter detailed the extraction techniques used for recovering polar phenolics from walnut cake, in order to be later incorporated to camelina oil and therefore, enhance its oxidative stability.

Chapters III, IV and V are related to the report of the results obtained in this work. These chapters have been carefully described below.

In *Chapter III*, it has been globally discussed the strategies for enriching edible oil with polyphenols-phospholipid formulations in order to improve the solubility of polyphenol in oils. For that, it was firstly provided the chemical composition of sunflower and camelina oil, and

it was compared with that of reported in literature. After that, it was validated a method for determining the solubility of quercetin in oils and other solvents of industrial interest. In order to enhance the oxidative stability of enriched oils, we proposed and conducted some strategies for improving the solubility of quercetin in oils. In this sense, the complexation (solvent route) of quercetin with amphiphilic molecules such as phosphatidylcholine has been assessed. This chapter also described the development of a solvent free route for the enrichment of sunflower and camelina oils with several quercetin-phosphatidylcholine formulations. Finally, the self-assembly of phosphatidylcholine in colloidal structures in bulk camelina oil was studied through 7,7,8,8-Tetracyanoquinodimethane (TCNQ) method.

Chapter IV consisted mainly of the assessment of the oxidative stability of camelina oil enriched with several quercetin-phospholipid formulations by means of the solvent-free route. From that perspective, it has been firstly evaluated the oxidative stability of both pure camelina and sunflower oils, under accelerated conditions of heating (60 °C), in order to be compared with enriched oils. The assessment of the enhanced oxidative stability of enriched oils was carried out by estimating the oxidation lag time of various enriched oils. The oxidation lag time was defined as the time needed to reach a peroxide value of 15 m_{eq}O₂/kg (regulation of Codex Alimentarius for virgin oils). The oxidation of camelina oil at room temperature was also studied. Finally, in *Chapter IV* the degradation of quercetin under accelerated oxidation of enriched camelina oil was monitored through HPLC analysis.

Chapter V has focused on the extraction of polyphenol-rich extracts from walnut cake and on the enrichment of camelina oil with these phenolic extracts, aiming at enhancing the oxidative stability of camelina oil. Firstly, maceration extraction was carried out for recovering polar phenolics from walnut cake. For that, a fractional factorial design was conducted in order to optimize the phenolic extraction. Moreover, a semi-continuous extraction of polyphenols from walnut cake was also carried for comparison purposes. The two-site kinetic desorption model proposed by Kubatova et al. (2002) was adjusted to the experimental kinetic extraction of polyphenols by the semi-continuous method. After that, the oxidative stability of camelina oil enriched with walnut extracts was assessed. In addition, some methods, such as maceration and ultrasound assisted extraction, for enriching directly camelina oil with phenolics from walnut cake without using solvents were assessed.

Finally, it has been presented some general conclusions of main results as well as several perspectives which would be worth considering for future works.

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Chapter I: Literature review

1. Vegetable oils

Vegetable oils represent one of the main extracted products obtained from plants. Vegetable oils are present in cellular organelles of fruits or oleaginous grains, called lipid bodies. Depending on the quality of the raw material or the characteristics of the oil, this product can be obtained by mechanical methods such as, cold press and expeller press, as well as by solvent extraction methods that include solid-liquid extractions, supercritical fluid extraction, among others (Panadare & Rathod, 2017).

Vegetable oils are substances insoluble in water (hydrophobic) and belong to the chemical class of lipids, which form the group of greatest abundance in nature, consisting essentially of a mixture of different chemical compounds, the most important being fatty acids and their derivatives (Moretto & Fett, 1998).

Among their properties, vegetable oils can be a vehicle for fat-soluble vitamins and a source of energy and essential fatty acids, indispensable for the organism (Timilsena et al., 2017; Bockisch, 1998). In addition, oils are also responsible for the palatability, taste, and texture of several food products. They can also substitute animal fat and can be obtained from several plant species. This variety makes the oils widely consumed and produced worldwide, becoming an important component in our diet whose variety and consumption depends on the availability in each country or region. The global production of major oils is shown in Figure I-1, where oils from palm, soybean and rapeseed are the most produced.

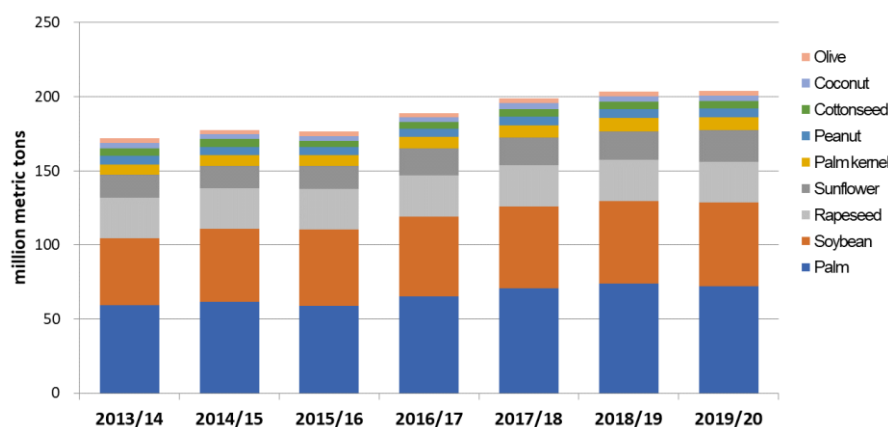


Figure I-1. Production of major vegetable oils worldwide (STATISTA)

In this context, the advantages of introducing oils into the diet have been extensively studied. Several studies have reported that oils play an important role in the prevention of cardiovascular diseases, improving cerebral function, and keeping healthy cholesterol levels (Rodríguez et al., 2016; Celia et al., 2013; Frankel et al., 2013). Other works stated that chemical, physical, and physiological properties of some compounds present in the oils make them suitable for several food, pharmaceutical and nutraceutical applications (Temelli, 2009). For instance, significant levels of bioactive compounds as tocopherols, phenolic compounds, phytosterols and carotenoids may contribute to the prevention of cardiovascular diseases. Particularly, the antioxidant properties of tocopherols and phenolics in oils are related to the protection of biomolecules from the action of free radicals (Rodríguez et al., 2016; Chen et al., 2011).

Furthermore, oils are also recognized as an important source of essential fatty acids which organism requires for basic functionalities and need to be supply only by ingestion (Saini & Keum, 2018). The composition of the oils varies considerably in the contribution of saturated, monounsaturated, and polyunsaturated fatty acids and particularly in the content of omega-6 and omega-3 fatty acids (Huerta-Yépez et al., 2016; Rodríguez et al., 2016).

In order to preserve the aforementioned benefits/characteristics of vegetable oils, so that they can be later used in food, pharmaceutical and cosmetic application, it is necessary to study their composition, functionality, and stability.

1.1. Oil composition

Regarding the composition, oils are mainly composed by triacylglycerols (approximately 95%) along with some free fatty acids, monoacylglycerols, and diacylglycerols. Oils also contain other composites in a variable proportion as proteins, phospholipids, free and esterified sterols, triterpene alcohols, carotenes, tocopherols, tocotrienols, chlorophylls, polyphenols, some pigments, hydrocarbons as well as traces of metals (Figure I-2). Globally, the oil composition is strictly dependent of the characteristics and quality of the raw material of origin (Shahidi, 2005).

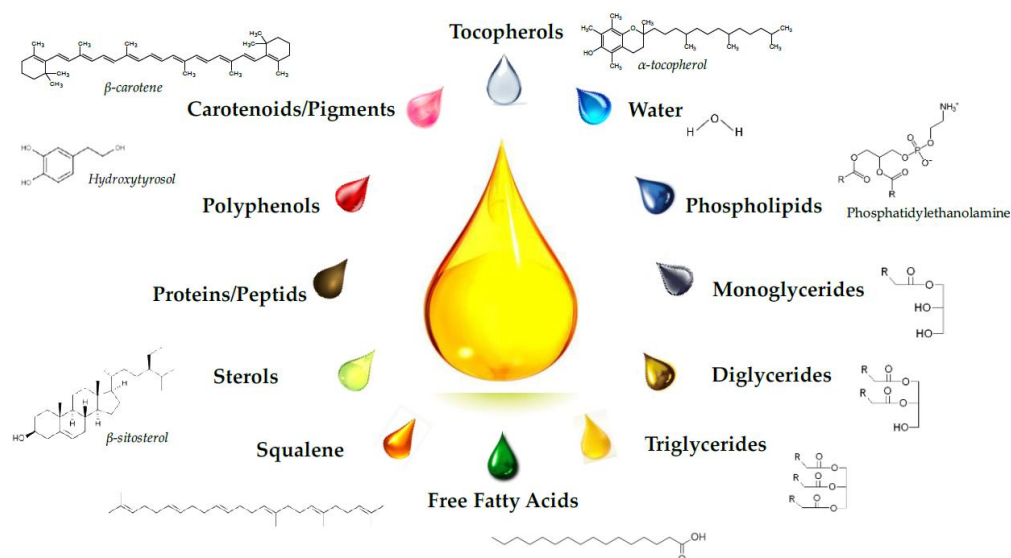


Figure I-2. General composition of vegetable oils (Source: Yara-Varón et al., 2017)

A triacylglycerol is consisted of three molecules of fatty acids esterified to a glycerol (three-carbon sugar alcohol) backbone at specific location identified as sn-1, sn-2, or sn-3 (Figure I-3). In nature, triacylglycerols are synthesized by enzyme systems and since triacylglycerols exist in different enantiomeric forms, i.e. with different fatty acids in each position, a ‘stereospecific numbering’ system has been recommended to describe all the different forms (Christie & Han, 2012). This means that when the letters "sn" appear in the nomenclature, the OH of the second carbon of glycerol (sn-2) is on the left on a Fischer projection. The numbering follows the one of Fischer's projections, being sn-1 the carbon at the top and sn-3 the one at the bottom as shown in Figure I-3.

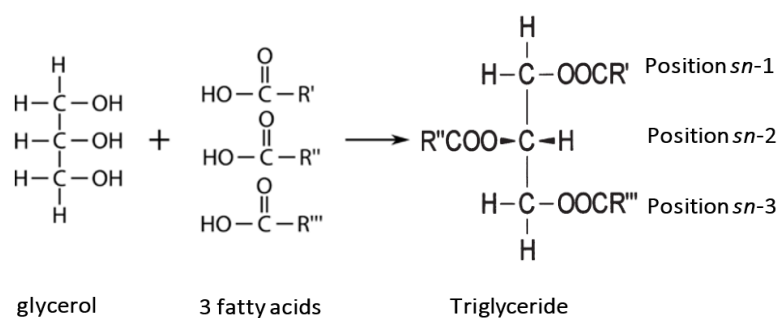


Figure I-3. Molecule precursors and structure of triacylglycerols

As mentioned previously, fatty acids exist in different forms in the triacylglycerol molecule; in saturated and unsaturated form, as short and long chain; odd or even carbon number; cis- or trans- configuration; branched or linear; as well as any combination thereof

(Güner et al., 2006; Sato & Ueno, 2005). All those characteristics and multiple combinations make the triacylglycerol variety considerable large and complex in a fat system.

Considering that triglycerides are constituted by three molecules of fatty acids which play a key role in the oil functionality and physicochemical properties, it is necessary to approach them more widely.

1.1.1. Fatty acids in vegetable oils

Globally, fatty acids are linear organic acids comprising a hydrocarbon chain, and a carboxyl group. The number of carbon atoms can vary from 4 to 24.

Fatty acids are found in nature as the associated form in which they are esterified with glycerol (1,2,3-trihydroxypropane), forming triglycerides and in the non-associated form known as free fatty acids (Reda & Carneiro, 2007). This latter occurs in small quantities and are natural components of oils and fats. In the associated form of glycerides, the fatty acids represent up to 96% of mass of the whole molecule (Moretto, 1998). Fatty acids are naturally distributed in a different way among plant oils as shown in Figure I-4, for instance.

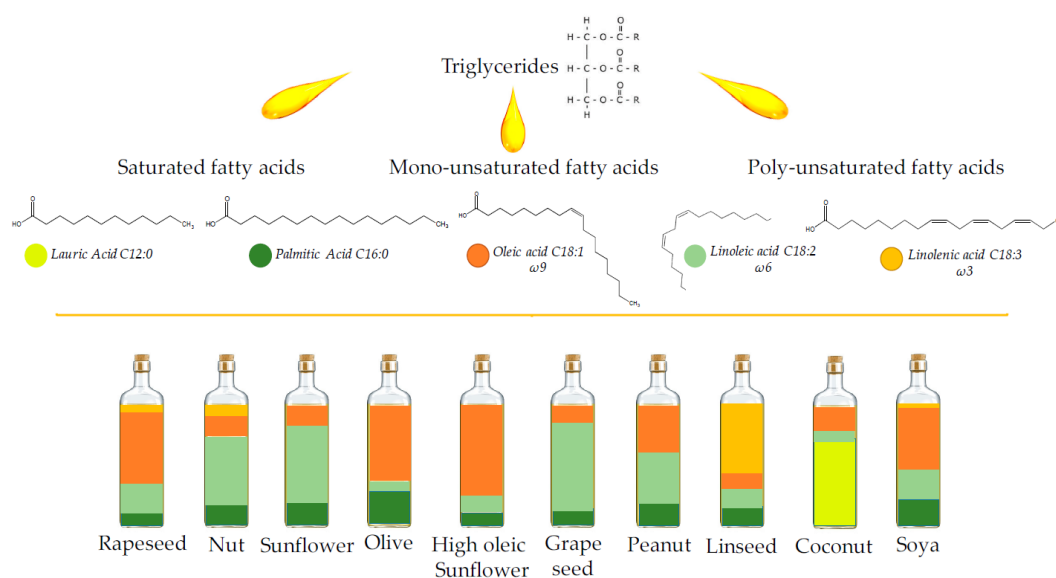


Figure I-4. Distribution of fatty acids in common vegetable oils (Source: Yara-Varón et al., 2017)

In oils and fats of commercial importance, over 1 000 fatty acids are known, but 20 or less are the most common. Globally, three fatty acids are dominant in vegetable oils: palmitic

(C16:0), oleic (C18:1), linoleic (C18:2), and occasionally stearic acid (C18:0) and linolenic acid (C18:3). The other fatty acids, found in special oils, include myristic, lauric, erucic, hexadecenoic, γ -linolenic, eleostearic, ricinoleic and vernolic acids (Gunstone, 2005). Moreover, the most common fatty acids have a 16 or 18 carbon-chain. Below this range, they are categorized as short or medium chain and above it as long-chain acids as shown in Table I-1.

Table I-1. Nomenclature and structure of the most common fatty acids in oils

Fatty acid ¹	Common name	Formula	Chain length
4:0	butyric	$\text{CH}_3(\text{CH}_2)_2\text{CO}_2\text{H}$	short
6:0	caproic	$\text{CH}_3(\text{CH}_2)_4\text{CO}_2\text{H}$	short
8:0	caprylic	$\text{CH}_3(\text{CH}_2)_6\text{CO}_2\text{H}$	short/medium
10:0	capric	$\text{CH}_3(\text{CH}_2)_8\text{CO}_2\text{H}$	medium
12:0	lauric	$\text{CH}_3(\text{CH}_2)_{10}\text{CO}_2\text{H}$	medium
14:0	myristic	$\text{CH}_3(\text{CH}_2)_{12}\text{CO}_2\text{H}$	medium
16:0	palmitic	$\text{CH}_3(\text{CH}_2)_{14}\text{CO}_2\text{H}$	
18:0	stearic	$\text{CH}_3(\text{CH}_2)_{16}\text{CO}_2\text{H}$	
18:1 9c	oleic	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{CO}_2\text{H}$	
18:2 9c12c	linoleic	$\text{CH}_3(\text{CH}_2)_4(\text{CH}=\text{CHCH}_2)_2(\text{CH}_2)_6\text{CO}_2\text{H}$	
18:3 9c12c15c	α -linolenic	$\text{CH}_3\text{CH}_2(\text{CH}=\text{CHCH}_2)_3(\text{CH}_2)_6\text{CO}_2\text{H}$	
22:1 13c	erucic	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_{11}\text{CO}_2\text{H}$	long
20:5 5c8c11c14c17c	EPA*	$\text{CH}_3\text{CH}_2(\text{CH}=\text{CHCH}_2)_5(\text{CH}_2)_2\text{CO}_2\text{H}$	long
22:6 4c7c10c13c16c19c	DHA*	$\text{CH}_3\text{CH}_2(\text{CH}=\text{CHCH}_2)_6\text{CH}_2\text{CO}_2\text{H}$	long

*Abbreviations of the systematic names eicosapentaenoic acid and docosahexaenoic acid (Adapted from: Gunstone, 2005)

Structurally, when fatty acids have only simple bonds between the carbons of the hydrocarbon chain, they are called saturated fatty acids. On contrary, unsaturated fatty acids are characterized by having one to six reactive double bonds in the molecule and are known as monounsaturated fatty acids in the first case, and polyunsaturated when they have more than one double bond (Heimann, 1982). In general, it can be stated that unsaturated fatty acids have

¹ **Notation Y X Zc:** Y is the number of carbon atoms in the fatty acid chain, X the number of unsaturations and Z the position of cis(c) or trans(t) unsaturation (counted from the methyl end of the fatty acid).

received more attention due to the health benefits associated to their consumption, particularly, of omega 3 and omega 6 fatty acids (Lupette & Benning, 2020). Moreover, since unsaturated fatty acids are highly susceptible to degradation reactions (oxidation) which significantly influence their stability in food and cosmetics products, even more attention has been given to study and improve their stability. The main mechanisms of lipid oxidation as well as the alternatives for delaying the oxidation of unsaturated fatty acids are discussed later in this manuscript.

Table I-2 shows the composition profile of fatty acids of some commercial vegetable oils. The proportion of the different saturated and unsaturated fatty acids in vegetable oils depends on its origin and within a plant species, the variation is influenced by climatic and growing conditions.

Table I-2. Fatty acids compositions of different vegetable oils

Oil	Composition (%)									
	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C20:1
Cottonseed	-	-	-	1.5	22	5	19	50	-	-
Peanut	-	-	-	0.5	6-11.4	3-6	42-61	13-34	-	-
Babassu	2.6-7	1.2-7.6	44-45	15-17	5.8-8.5	2.5-5.5	12-16	1.4-2.8	-	-
Rapeseed	-	-	-	-	1.5-6	1-2.5	52-67	16-31	6.4-14	-
Palm	-	-	-	0.6-2.4	32-45	4-6.3	38-53	6-12	-	-
Linseed	-	-	-	-	6	4	13-37	5-23	26-58	-
Corn	-	-	-	-	7	3	43	39	-	-
Olive	-	-	-	1.3	7-16	1.4-33	64-84	4-15	-	-
Soybean	-	-	-	-	2.3-11	2.4-6	24-31	49-52	2-10.5	-
Rice bran	-	-	-	-	20	2	42	32	-	-
Safflower	-	-	-	-	7	3	14	75	-	-
Sesame	-	-	-	-	9	6	41	43	-	-

Adapted from: (Rinaldi et al., 2007 and Shahidi, 2005)

In foods containing naturally oils, the most common saturated fatty acids are lauric, myristic, palmitic, and stearic acids with 12, 14, 16 and 18 carbons, respectively. Palmitic acid is the main fatty acid in palm oil, and lauric and myristic, which are saturated fatty acids with a shorter chain, are predominant in coconut and palm kernel oils (Nettleton, 1995).

Saturated fatty acids are involved in many biochemical processes, for instance, they are generally oxidized to provide energy for cellular and physical activities. Nevertheless, when their consumption exceeds the requirements, they may be stored in adipose tissue. In general, the main source of saturated fatty acids comes from animals and the unsaturated fatty acids from plants, with some exceptions.

In this chapter, a special attention to essential fatty acids has been given since the focus of this work is related to improve the oxidative stability of vegetable oils, composed mainly by polyunsaturated and essential fatty acids, i.e. omega 3 and omega 6, which are known to exhibit several nutraceutical properties, and therefore widely used in food, cosmetic and pharmaceutical applications.

1.1.2. Essential fatty acids

Although several fatty acids can be synthesized by the human body, there is a group of polyunsaturated fatty acids, known as essential fatty acids, that the organism cannot synthesize: omega-3 (n-3) and omega-6 (n-6) fatty acids. The precursor of the n-3 series of fatty acids is α -linolenic acid (C18:3 n-3, commonly known as ALA) and the precursor of the n-6 series of fatty acids is linoleic acid (C18:2 n-6, commonly known LA) (Rubio Rodríguez et al., 2010). In Figure I-5 is shown the structure of omega-3 and omega-6. The position of the carbon of the first double bond in n-6 series starts beyond the 6th carbon atom from the methyl end, whereas in n-3 series, the first double bond starts beyond the 3rd carbon atom.

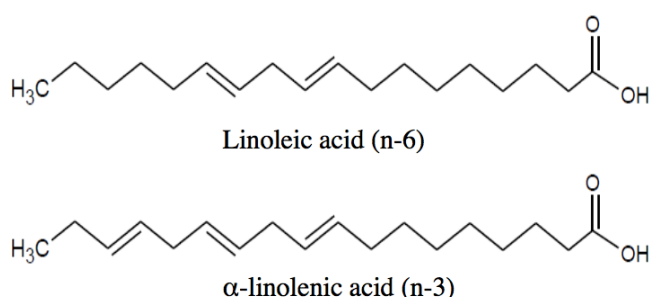


Figure I-5. Chemical structure of linoleic acid and α -linolenic acid

Depending on the location of the last double bond, Polyunsaturated Fatty Acids (PUFAs) control a great variety of biological functions, which range from blood pressure and

appropriate coagulation to the proper functioning of the nervous system and brain functions (Patterson et al., 2012). LA and ALA for instance, exhibit a hypocholesterolemic effect by decreasing the concentration of the low-density lipoprotein (LDLc) cholesterol fraction in the blood and, ALA may also help to reduce the levels of plasma triglycerides (Rajaram et al., 2009; Thomas et al., 2004).

The major dietary sources of LA include plant oils such as sunflower, safflower, and corn oils whereas rich dietary sources of ALA include green leafy vegetables, pumpkin seeds, walnuts, flax seeds, and oils (linseed, camelina, soybean and rapeseed) (Gammone et al., 2019).

Omega-3 fatty acids are constituted by more than one double bond in their backbone and their first double bond is between the third and fourth carbon atoms from the tail end. Globally, α -linolenic (ALA), eicosapentaenoic (C20:5 n-3; EPA) and docosahexaenoic (C22:6 n-3, DHA) acids are the three most abundant omega-3 fatty acids in foods. These PUFAs are stored in membrane phospholipids and are responsible for many of the cellular roles including the maintenance of the cell membrane structure, fluidity, signaling, and cell-to-cell interaction (Gammone et al., 2019).

Thus, the consumption of omega-3 may help to reduce the risk of developing chronic diseases such as cancer, heart disease, arthritis, and diabetes (Gammone et al., 2019; Saini & Keum, 2018; Wall et al., 2010). Hence, nowadays several strategies to increase the intake of omega-3 fatty acids are considered, such as the enrichment of food products with fish oil or ALA supplements, the fortification of omega-3 in oilseed crops by genetic engineering and increasing the content of omega-3 of food products from animal sources by feeding with omega-3 PUFAs-rich diet sources (Saini & Keum, 2018).

On the other hand, linoleic acid (LA) is the shortest-chained omega-6 fatty acid. It is the major polyunsaturated fatty acid in most human diets. Structurally, the first of the two double bonds of LA, is positioned six carbons away from the methyl carbon. Among its derivatives, arachidonic acid (C20:4 n-6; ARA) is the most unsaturated molecule since is constituted by 4 double bonds and 20 carbons. LA and ALA are also required for the biosynthesis of various classes of eicosanoids which are bioactive signaling lipids that regulate diverse sets of homeostatic and inflammatory processes linked to numerous diseases, including infection, inflammation, and cancer (Saini & Keum, 2018).

Commonly, Omega-6 fatty acids are mainly found in plants, especially in certain seeds such as from sunflower. However, ARA is also found in meats and fish. Several vegetable oils and crop seeds, including canola, soybean, corn, and sunflower oils, are the major sources of omega-6 in the form of LA with lower proportion of ALA.

Finally, it is important to highlight that although the composition in fatty acids of edible oil influence significantly their nutritional value, functionality and oxidative stability, other factors such as the position of individual fatty acids within the triacylglycerol molecule, the presence and composition of minor compounds such as tocopherols, phenols, carotenoids sterols, among others, also influence the properties and the oxidative stability of plant oils (Merrill et al., 2008). From this perspective, we think important to describe in the following section the main properties and chemical composition of both sunflower and camelina oils which are the focus of this work.

Nonetheless, what can be certainly stated about vegetable oils is that even though they represent an important source of energy for human body and exhibit several functional properties in food products such as taste, flavor and texture, or act as a carrier of active compounds in pharmaceutic and cosmetic applications, they are also highly susceptible to degradation by the action of oxygen. In consequence, many novel techniques for both study the mechanisms involved in lipid oxidation and for producing more stable oils are nowadays considered. Later in this chapter is carefully described the enrichment of edible oils with natural antioxidants such as polyphenols and/or phospholipids formulations as an interesting alternative for producing oil with a higher oxidative stability.

1.2. Camelina oil

Recently there is a growing interest for alternative low-input oilseed crops with the potential for food and nonfood utilization of the seed oil. In this context, *Camelina sativa* (L.) plant, originated in the Mediterranean area and Central Asia and member of the Brassicaceae family, has attracted the attention in research, mainly due to its exceptional oil composition and several properties for production of several chemical products as biofuels, biobased-products, feed, and foods (Przybylski, 2005).

Camelina, commonly known as false flax, linseed dodder, and gold-of-pleasure, is also recognized because of its ability to adapt to vast areas of the world. In this sense, the interest for developing sustainable agriculture systems with low demand of pesticides, fertilizers and

energy is achieved since *Camelina* plant produces oilseeds with important nutritional value, cultivated in extreme conditions (Berti et al., 2016).

Morphologically, camelina plants are heavily branched, growing from 1- to 3-feet tall producing seed pods (usually 5 mm in diameter) containing many small 10-25 oval shaped oily seeds (Figure I-6). Generally, these seeds are pale yellow-brown oblong, rough, and with a ridged surface and exhibit a small size ranging from 0.7 mm to 1.5 mm. Their color turns dark brown or reddish on ripening and under storage.



Figure I-6. a) Raceme, b) flower and c) seed pod of *Camelina sativa* (L.) (Source: Singh et al., 2013)

Camelina oil is the most important product obtained from camelina seeds. The oil content of camelina seeds ranges from 37% and 44% (Shahidi, 2005). This product has been obtained using the following methods or combinations: solvent extraction (Stroescu et al., 2015), cold pressing or mechanical warm (Raczyk et al., 2016; Zhao et al., 2014; Shukla et al., 2002) or supercritical CO₂ extraction (Belayneh et al., 2015; Moslavac et al., 2014). Industrially, to release the oil, two steps of crushing and pressing are required. The temperature during pressing can reach up to about 100°C and the crude oil obtained from camelina seed by pressing, corresponds to a golden yellow color liquid with a mild nutty and characteristic mustard aroma. Camelina oil is available in two forms; crude or unrefined, and refined obtained after refining, bleaching, and deodorizing process. When the oil is obtained by cold pressing and is not refined, the oil is appreciated for the aroma and flavor of the original raw material. Globally, cold pressing process method is initiated by a cold extraction (less than 27 °C) of the

seeds or cakes through a convectional pressure technique (hydraulic press), followed by a single filtration without any chemical treatment or refining.

Some of the general physical properties of camelina oil are reported in literature such as refractive index 1.4756 (at 25 °C), density 0.91 g/mL (at 25 °C), iodine number 105 (g I₂/100 g oil) and saponification value 187.8 (mg KOH/g oil) (Abramovic & Abram, 2005). Regarding the fatty acid composition, camelina oil could be considered a high-quality edible oil due to its high contribution of polyunsaturated fatty acids, mainly LA and ALA, and the relatively low content of saturated fatty acids (about 12%). Generally, approximately 54% of the fatty acids of camelina oil are polyunsaturated and 34% are monounsaturated, mainly oleic and eicosenoic acids. Most of camelina oils have a common fatty acid composition in which the content of ALA ranges from 30% to 40%, of eicosenoic acid is about 15%, and of erucic acid is less than 4%. In comparison with other oils, camelina oil is less unsaturated than flax oil but more than canola or sunflower oils.

According to Abramovic & Abram, (2005), the shelf life of camelina oil is 12–24 months and it is strictly dependent to the storage conditions since this oil is highly prone to oxidation due to its high content of PUFAs. The chemical composition of camelina oil may vary according to plant variety, growing conditions, oil extraction method, etc. In Table I-3 is shown a typical chemical composition of camelina oil.

1.3. Sunflower oil

Sunflower oil is one of the most popular vegetable oils and in some countries, it is more used than soybean, cottonseed, and palm oils. Sunflower (*Helianthus annuus* L.) is a plant grown worldwide, domesticated, and cultivated by natives of Central and North America and introduced to Europe by Spanish explorers in the 16th century (Guo et al., 2017). This plant belongs to the Asteraceae family and is commonly known by a variety of nutritional and medicinal benefits.

It is an annual herb with an erect, hard, hairy stem, 1-2 m high, and branched at the top. It is constituted by a large yellow inflorescence consisting of a circular head (*capitulum*). These flower heads are usually 8-15 cm in diameter but can attain 30-60 cm under cultivation and they are housing hard compartments called achenes (Pal, 2011) (Figure I-7). Each fully developed achene has a hard-outer shell composed mostly of cellulose and lignin. It contains a

single seed with a cylindrical, obovoid-compressed morphology and 1 cm of length which accounts for the 80% of the total weight of the seed and is rich in oil (up to 55%) (Pal, 2011).



Figure I-7. Morphology of the sunflower plant and seeds (Source: Mühlbauer & Müller, 2020)

Sunflower seeds contain valuable antioxidant, antimicrobial, anti-inflammatory, antihypertensive, and cardiovascular benefits due to the content of phenolic compounds such as caffeic, chlorogenic, gallic ferulic and sinapic acids, flavonoids (heliannone, quercetin, kaempferol, luteolin, and apigenin), polyunsaturated fatty acids (approximately 31%), and vitamins such as vitamin E (37.8 mg/100 g) (Guo et al., 2017). Although they are commonly used as a snack, combined with vegetables or in some bakery preparations, sunflower seeds are primarily collected for oil production, standing in 4th position at world level (8% of world production) after palm (29%), soybean (22%) and rapeseed (13%) (Guo et al., 2017; Salas et al., 2015).

Sunflower oil is extracted from the seeds mainly through two extraction methods, single pressing or prepressing followed by solvent extraction. In both methods, some initial steps of cleaning, drying, and flaking are carried out for conditioning the seeds that are later extruded to extract most of the oil. Pressing technique is based on a high pressure applied on the prepared sunflower seed to remove the oil from the solids fraction. This process allows to recover about 25% of oil (Le Clef & Kemper, 2015). In the prepress solvent extraction, a moderate pressure

is exerted on the prepared seed to remove the major quantity of oil, followed by a solvent extraction with hexane to deplete the remaining oil from the extruded solids. This latter method allows to recuperate around 40% of oil and nowadays it is an extraction technique highly used industrially (Gupta, 2002).

After extraction, the crude sunflower oil is submitted to a refining process in which it is degummed, neutralized, bleached, winterized (dewaxed) and deodorized to remove free fatty acids, phospholipids, pigments, volatile compounds, and waxes, respectively. The main purpose of the crude oils refining process is removing the undesirable non-triglyceride components of this oil, to meet the regulatory standards of quality and to provide a product with a high oxidative stability. In Table I-3 is shown a typical chemical composition of sunflower oil.

Table I-3. Chemical composition of camelina and sunflower oils

	Camelina oil	Sunflower oil
Main fatty acid composition (%)		
16:0	5.7	3.6 - 6.6
18:0	2.4	1.3 - 3
18:1 n-9	15.7	14 - 43
18:2 n-6	18.5	44 - 68
18:3n-3	32.8	-
20:0	-	-
20:1	15	-
20:2	-	-
20:3	-	-
22:1	3.5	-
Vitamin E (mg/kg)		
α-tocopherol	< 30	~ 600
β-tocopherol	< 30	< 30
γ-tocopherol	~ 600	< 30
δ-tocopherol	< 30	< 30
Total Vitamin E	500-800	600-700
Phenolics (mg/kg)	26 - 63.7	5 - 45
Total Sterols (mg/kg)	2000 - 5900	1000 - 4500

Source: Belayneh et al. (2015); Rinaldi et al. (2007); Shahidi, (2005); Gupta, (2002)

2. Oxidation of lipids

Although vegetable oils exhibit many health benefits and functionalities for developing several food, pharmaceutical and cosmetic products, they are also highly susceptible to various chemical reactions that may result in modifications of their original characteristics such as loss of nutrients, change in odor and taste, and economic losses. These chemical reactions are undesirable and mainly caused by the oxidation of lipids which also involve biological and physical modifications of oils (Namiki, 1990; Nawar, 1985).

Oxidative deterioration of lipids is one of the most important and frequent reactions in the foods where they are present. This alteration is responsible for the development of unpleasant flavors and odors, making food unfit for consumption, as well as causing other changes that will affect not only its nutritional quality - due to the degradation of fat soluble vitamins and essential fatty acids - but also its organoleptic properties. An extensive lipid oxidation of a food product may also affect the health of consumers due to the formation of potentially toxic compounds (Silvagni et al., 2012; Silva et al., 1999). Moreover, it has been reported that the peroxidation also may conduct to the formation of Maillard compounds, and other oxidized by-products, which are capable of reacting with biomolecules such as proteins, and then reducing their absorption (Kirk, 1984). The products of lipid oxidation are also antagonists of various other nutrients such as thiamine, calcium pantothenate, riboflavin, ascorbic acid, vitamin B12, tocopherols, vitamin A, proteins, lysine, and sulfur amino acids (Kirk, 1984).

For all this, the food and cosmetic industries are highly interested on developing new alternatives for significantly delaying the lipid oxidation in their products in a more natural way than using synthetic antioxidants.

The literature reports that the mechanism of lipid oxidation depends on the medium and the initiators involved in it, and it can be classified in enzymatic oxidation, photooxidation and autoxidation. Since the main mechanism of oxidation involved in this study was autoxidation, both enzymatic oxidation and photooxidation were described roughly.

- Enzymatic oxidation is catalyzed by lipoxygenase, cyclooxygenase, and peroxidase. These enzymes act on the polyunsaturated fatty acids, catalyzing the addition of oxygen to the polyunsaturated hydrocarbon chain which result is the formation of peroxides and

hydroperoxides (Silva et al., 1999). This oxidation occurs in plant and animal tissues, during the crushing of seeds and fruits, or directly in the bulk oil (Decker, 2002).

- Photooxidation is an important factor in the transformation of crude oil after its release into the environment and it is promoted essentially by ultraviolet (UV) radiation in the presence of photosensitizers/chromophores (chlorophyll, myoglobin, riboflavin, and others) (Shankar et al., 2015). Chromophores in the oil absorb sunlight and initiate a chain of photochemical reactions resulting in a wide range of products. This type of oxidation occurs by two principal mechanisms: direct photolysis and reaction with reactive oxygen species produced by solar radiation acting on a variety of photosensitizers (Shankar et al., 2015).

2.1. Autoxidation

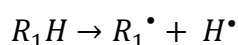
The autoxidation of lipids is initiated by temperature, metal ions or free radicals and it is known as the main mechanism of oxidation in oils and fats (Weng & Wang, 2000). This mechanism of oxidation of oils and fats is associated with the reaction of the unsaturated fatty acids with oxygen (Jadhav et al., 1996). This reaction occurs in three steps: initiation, propagation, and termination. Globally, during the initiation free radicals are generated from the substrate. Then, the methylenic H atom is abstracted from the unsaturated lipid molecule to form a lipid alkyl radical and once initiated, the chain reaction continues and ends only when the reserves of unsaturated fatty acids and oxygen are exhausted (Kirk, 1984). In addition, this reaction can be accelerated by submitting the lipids to light and heat conditions (Frankel, 1980). The three steps of autoxidation are carefully described as follows.

2.1.1. Initiation

The initiation is marked by the formation of free lipid radicals which occurs due to the removal of an atom of hydrogen from the allylic carbon (from the double bond) of the fatty acid molecule (RH) in the presence of trace metals, light or heat. The initiation can take place by direct action on the unsaturated fatty acid in which case lipid loses one hydrogen atom to form a free radical R[•].

Initiation:

Lipid free radical formation



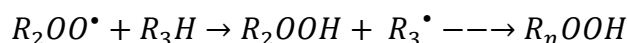
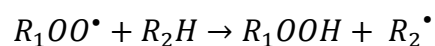
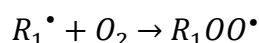
2.1.2. Propagation

In this step, the free radical (R_1^\bullet) binds rapidly the molecular oxygen (triplet state) and forms a peroxy free radical. The interaction between the radical R^\bullet with oxygen (O_2) at its normal state (triplet), requires a very low activation energy (Min & Boff, 2002), therefore the reaction rate is especially high when the oxygen content is not limited in the system (Ivanov, 1985). On contrary the direct reaction of the triplet oxygen with the unsaturated fatty acids (RH) requires a high activation energy. In this context, the free radicals formed act as propagators of the reaction, resulting in an autocatalytic, self-accelerating and self-propagating process.

The formed lipid radical (R_1^\bullet) reacts rapidly with oxygen (triplet state) to form a peroxy radical (R_1OO^\bullet), which reacts with another unsaturated lipid molecule (R_2H), to form a lipid hydroperoxide (R_1OOH), and a new unstable lipid radical (R_2^\bullet), which starts again the propagation sequence. Thus, the hydroperoxides are the main initial products of lipid oxidation. The formation of the hydroperoxide is commonly initiated in the chains of the most unsaturated fatty acids. As a new free radical is generated, more oxygen is necessary in the system for propagating the reaction (Choe & Min 2006; Wanasundara & Shahidi, 2005). In this way, the newly propagated lipid radical (R_2^\bullet) will then react with oxygen to produce another peroxy radical (R_2OO^\bullet), which result in a self-catalyzed and cyclical mechanism (R_nOOH).

Propagation:

Free radical chain reaction



The mechanism of action of antioxidant molecules is based on their ability to break the chain reaction of lipid oxidation by reacting with ROO^\bullet to form stable radicals that are either too unreactive or form nonradical products (Frankel, 1984).

After hydroperoxides formation, in the case of natural unsaturated lipids, the reaction is complemented by an electronic rearrangement of the methylene-interrupted double bonds in PUFAs which pass from the non-conjugated position to the conjugated position. This reorganization produces the isomeric hydroperoxides containing diene conjugated bonds which are used as an indicator of lipid oxidation (Shahidi & Zhong, 2010). In Figure I-8 is schematically shown the formation of conjugated dienes.

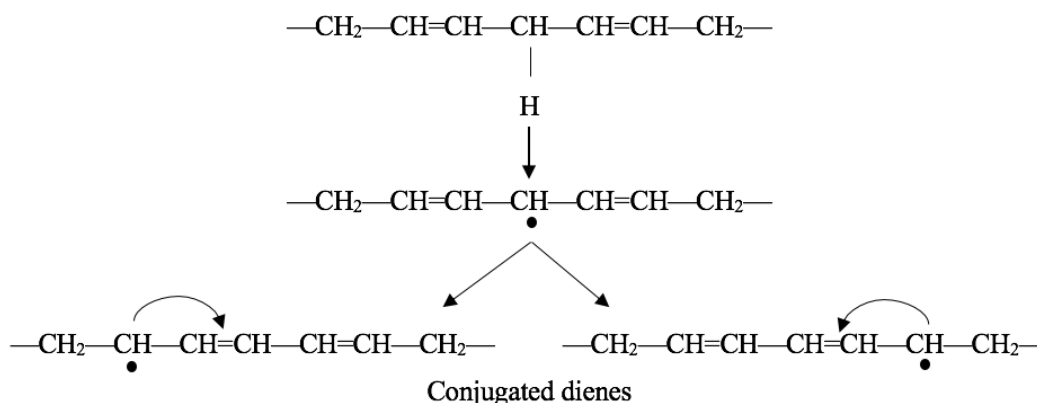
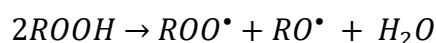


Figure I-8. Formation of conjugated dienes as primary products of lipid oxidation

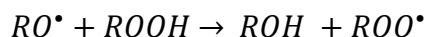
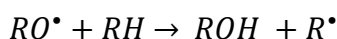
Peroxides formed can be also measured in lipid matrices as an indicator of lipid oxidation, however, since peroxides are unstable and reactive, their quantification is limited to the initial phase of lipid oxidation (Sevanian & Hochstein, 1985).

Being unstable, hydroperoxides (ROOH) can undergo various reactions including a decomposition by homolytic cleavage of the O-O bond, leading to two new free radicals: the oxy alkoxy radicals (RO[•]) and peroxy radicals (ROO[•]) which are involved in the initiation of new chain reactions (Frankel, 2005; Schaich, 2005). Lipid hydroperoxides (ROOH) can also react with oxygen to form secondary products (epoxyhydroperoxides, ketohydroperoxides, dihydroperoxides, cyclic peroxides and bicyclic endoperoxides) (Frankel, 2005).

Free radical chain branching (initiation of new chain reaction)



The radical $RO\cdot$ formed is much more reactive than $ROO\cdot$. It propagates the oxidation phenomenon by stripping a hydrogen atom from lipids (RH) and hydroperoxides (ROOH).



As an example, in Figure I-9 is schematically shown the oxidation of linoleic acid by autoxidation mechanisms.

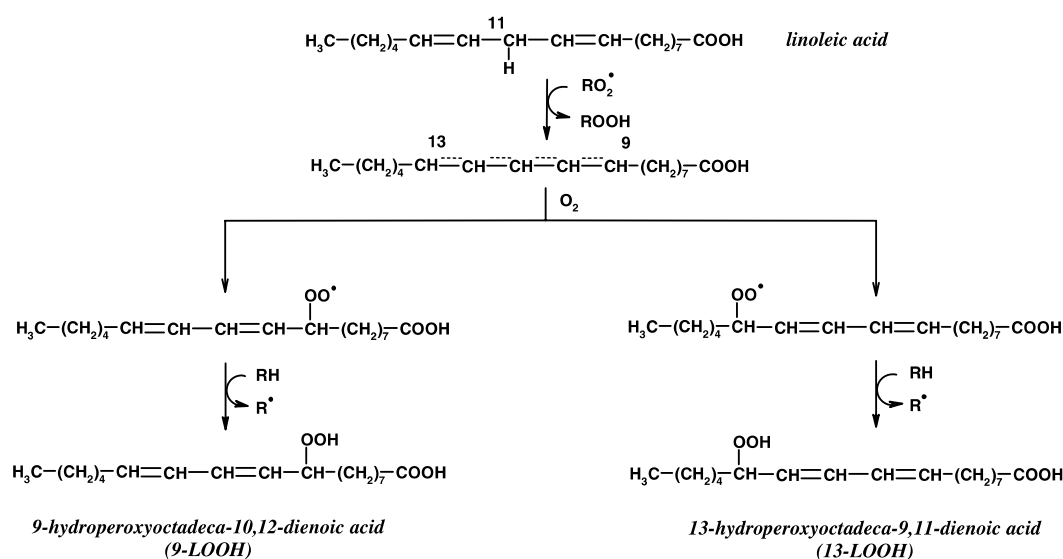


Figure I-9. Formation of the hydroperoxy fatty acids 9-LOOH and 13-LOOH by autoxidation of linoleic acid (Source: Vanderhaegen et al., 2006)

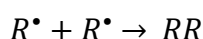
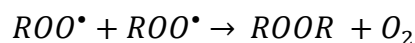
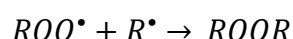
Finally, due to the exhaustion of the substrates, the propagation cycle is interrupted by the termination reactions where polar materials and polymers are formed.

2.1.3. Termination

Corresponds to the final phase where the triglyceride oxidation ends. In this stage the radicals $R\cdot$, $RO\cdot$ and $ROO\cdot$ are combined (peroxide and hydroperoxide accumulation) to form stable or non-reactive products ($ROOR$, RR). These final products are obtained by cleavage and rearrangement of the peroxides (Melo & Guerra, 2002). Free radicals finally form nonradical products by four major mechanisms: radical recombinations, a variety of cleavage reactions when proton sources are present to stabilize products, co-oxidations of other molecules (radical transfer) and eliminations (Schaich, 2005).

Termination

Formation of non-radical products



In the termination stage, the alkoxy radicals RO^{\bullet} also suffer scission reactions which corresponds to a rupture of the C-C bond in the α position of the carbon carrying the alkoxy function. It leads to the formation of a vinyl radical and an alkyl radical. These radicals then react with oxygen, hydrogen or with the hydroxyl radical which leads to the formation of carbonyl compounds such as alcohols, aldehydes, and ketones. This reaction takes place at room temperature, but the stripping process is favored when the temperature increases.

In this context, due to these additional scission reactions and some independent rearrangements of the peroxy radicals (ROO^{\bullet}) which are in competition with hydrogen abstractions in radical chain reactions, the complete lipid oxidation mechanism is stated as a quite complex process. For instance, secondary products of oxidation can be originated from the early stages of the lipid oxidation without going through hydroperoxides, hence some products different to hydroperoxides can also accumulate in the early stages of oxidation (Schaich, 2005). In this sense, a new integrated scheme for lipid oxidation has been proposed by Schaich, (2005), in which the major alternative pathways are added to the classic free radical chain (Figure I-10), whereas the kinetic evolution of the various compounds is summarized in Figure I-11.

As it can be observed in Figure I-11, the three stages of oxidation are characterized by the formation or consumption of different types of compounds. The time of each stage is influenced by many parameters such as the nature of the oil, oil composition, the availability of oxygen, the temperature, etc.

The lag phase or also known as initiation phase is characterized by the formation of free radicals in small amounts by the reaction of unsaturated fatty acids and oxygen. The duration of this phase is mainly influenced by the content of antioxidants initially present in the matrix, the presence of metal traces or other of pro-oxidant species, as well as by the

physical state of the lipid phase. The active stage of chain propagation comes later and is characterized by the rapid formation of primary products of oxidation such as hydroperoxides. Simultaneously a significant decreasing in the content of polyunsaturated fatty acids is observed in this stage. During the last phase or chain termination, secondary products of oxidation are formed as consequence of the decomposition of hydroperoxides (whose content decrease). Both nonvolatile and volatile oxidation compounds are formed from hydroperoxides. Volatile compounds are responsible for the unpleasant odor of oxidized foods and contribute to the development of their rancid taste.

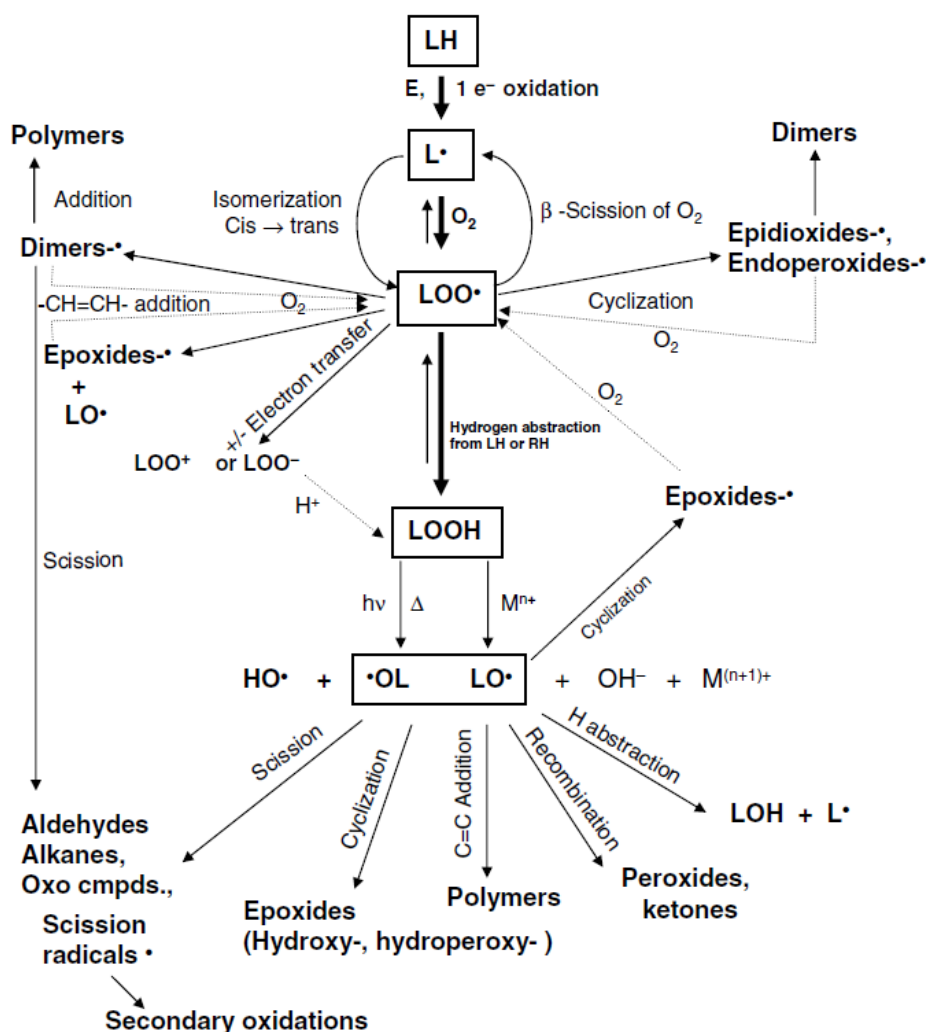


Figure I-10. Integrated scheme for lipid oxidation accounting for multiple reactions pathways competing with the classic hydrogen abstraction (Source: Schaich, 2005).

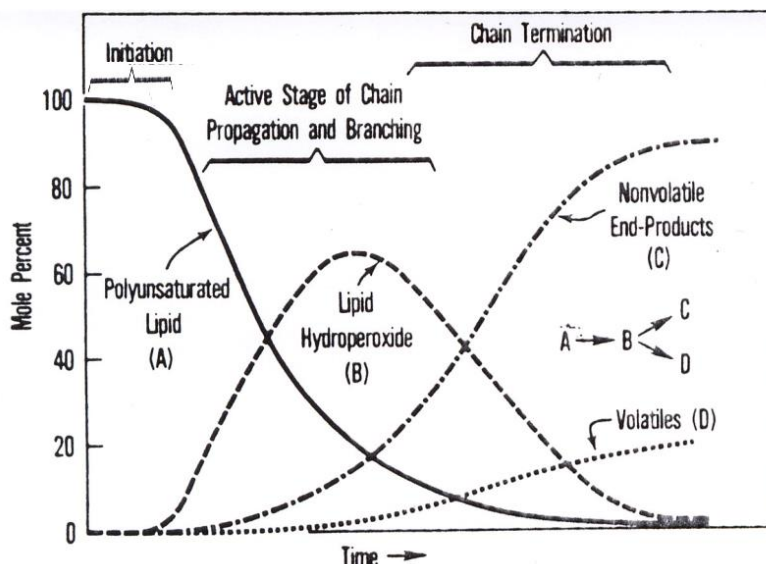


Figure I-11. Kinetic of autoxidation of polyunsaturated lipids (Source: Sampels, 2013)

In order to prevent the autoxidation of oils and fats, it is necessary to decrease the incidence of all factors that favor it: minimize energy levels (temperature and light); avoid the presence of metal ions traces in oils and decrease contact with oxygen since the solubility of oxygen is higher in oils than in water, and specifically higher in crude oil than refined oil (Choe & Min 2006). All aforementioned factors are responsible for initiating and accelerating the oxidation reactions; therefore, it is worth describing them carefully.

2.2. Catalysts of lipid oxidation

2.2.1. Temperature

Temperature is one of the main factors involved in lipid oxidation during food processing and preservation processes. For instance, temperature can be a critic parameter in lipid oxidation as it can accelerate the oxidation rates in a different level depending on the studied system. For example, Tan et al. (2001) stated that lipid oxidation rates were highly correlated (positively) with temperature during the oxidation of 10 different vegetable oils at 110 °C to 140 °C. According to Johnson & Decker, (2015), an increase in 10 °C of the storage temperature of most oily foods, it will duplicate the oxidation rate. Nevertheless, all the modifications that high temperature can promote in food products, due to the radical formation, may not result necessarily in an immediate alteration on the food quality, since some foods also contain endogenous antioxidants that may mitigate lipid oxidation.

According to Ingold, (1969), the tearing of a hydrogen atom from the fatty acid by the peroxy radical (ROO) is favored by high temperatures. Globally, a high temperature can increase the instability of lipids, due to the hydroperoxides breakdown which generates free radicals. At room temperature, for instance, the rate constant for stripping hydrogen by ROO is less than $1 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($\text{L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$) while at $65 \text{ }^\circ\text{C}$, it significantly increases up to $103\text{-}104 \text{ M}^{-1} \cdot \text{s}^{-1}$. From that perspective, it can be stated that temperature influences both the induction period of oxidation and the hydroperoxide content; at low or moderate temperature the formation of oxidation products is slow, and therefore a long induction period is observed as shown in Figure I-12.

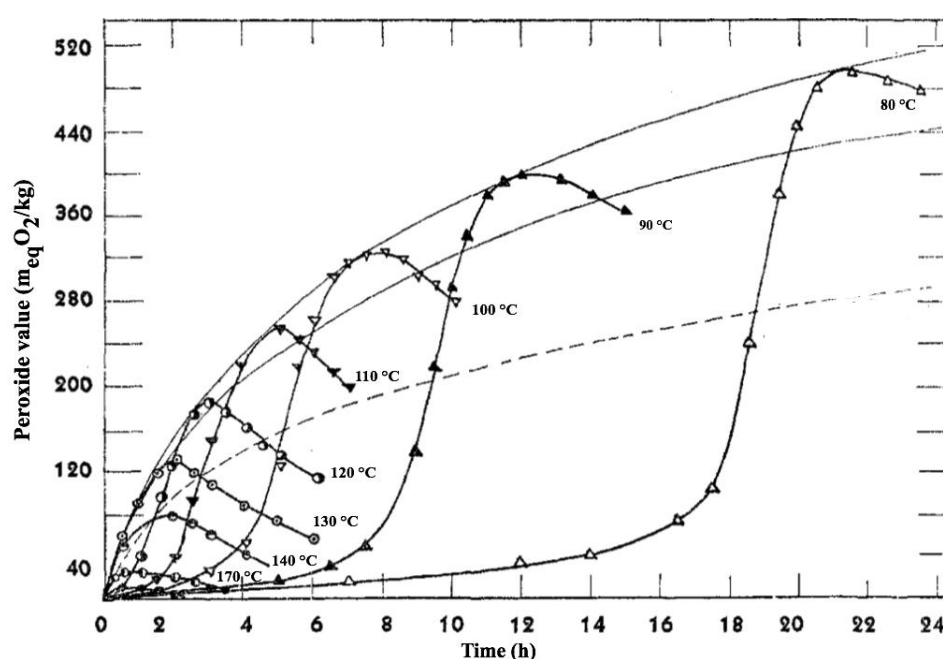


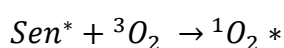
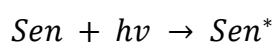
Figure I-12. Formation of hydroperoxides during oxidation of linseed oil at different temperatures (Adapted from: Hess & O'Hare, 1950)

In oils oxidized at temperatures above $150 \text{ }^\circ\text{C}$, the formation of hydroperoxides is practically absent since they are rapidly decomposed to secondary products of oxidation (polymeric compounds) (Figure I-12). On the other hand, it has been reported that increasing the temperature from $100 \text{ }^\circ\text{C}$ to $125 \text{ }^\circ\text{C}$, the induction period of rapeseed oil is reduced from 11 hours to less than 2 hours (Šimon & Kolman, 2001). In this context, by lowering the temperature is possible to limit lipid oxidation and thus, extend the shelf life of oil products. According to Ke et al. (1977), temperatures below $-40 \text{ }^\circ\text{C}$ are necessary to completely stop the oxidation since at temperature of $-15 \text{ }^\circ\text{C}$, the formation of peroxides is still possible.

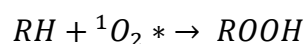
2.2.2. Oxygen

Thermodynamically, oxygen in its ground state is a triplet while double bonds of fatty acids are in singlet electronic state (1O_2). This fact implies that they cannot react directly to form another singlet, such as hydroperoxides, because of the different spin states. Hence, interactions between triplet oxygen and unsaturated fatty acids require that either oxygen is converted to a singlet state or double bond be excited into a triplet state. This latter seems unfeasible due to the requirement of extremely high amounts of energy (Chen et al., 2011). The activation energy for the direct oxidation of fatty acids is extremely high (150-270 kJ.mol⁻¹) therefore, such reaction is improbable (Frankel, 1984).

Furthermore, the triplet oxygen cannot switch to singlet state itself, nonetheless several reactions can occur, with the assistance of other minor components present in bulk oils, that lead to switch to a singlet state. Particularly photosensitizers (Sen) such as hemoproteins, riboflavin, or other pigments such as chlorophyll, which are activated (Sen*) by light (hv) can convert the triplet oxygen (3O_2) to singlet oxygen (1O_2) (Frankel, 2005).



Then, the singlet oxygen which is highly reactive ($^1O_2^*$) and excited may directly attack the unsaturated lipid molecule (RH) to propagate the lipid oxidation through the formation of lipid hydroperoxides (ROOH) (Schaich, 2005).



On the other hand, as explained previously, triplet oxygen is also involved in another type of reactions related to the lipid oxidation propagation. It can react directly with alkyl radicals via diffusion controlled radical-radical reactions to form peroxy radicals (Frankel, 2005).

In oils, the oxidation reactions occur when oil, oxygen, and catalysts are in contact. Therefore, the oxidation rate depends on oxygen concentration in the oil, the presence of catalysts and oxygen concentration at headspace. Regarding the oxygen concentration, several authors reported that at low oxygen contents, the oil oxidation rate is controlled by oxygen

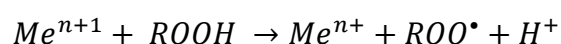
concentration and is independent of lipid concentration (Choe & Min, 2006). On contrary, at sufficiently high oxygen concentrations the oxidation rate is independent of oxygen concentration. The influence of oxygen concentration on oil oxidation increases at high temperature due to low solubility of oxygen in the oil at high temperature (Choe & Min, 2006).

In general, the concentration of oxygen in oil is influenced on the penetration of oxygen into the oil from the surface which is favored by a high oxygen partial pressure in the headspace (Min & Wen 1983), a high surface-to-volume ratio (Kanavouras et al., 2005) and the presence of mechanical agitation in the system that enhance oxygen transfer by convection mechanisms. Considering the key influence of oxygen in lipid oxidation, the removal or diminishing of the dissolved oxygen increases the oxidative stability and shelf life of bulk oils.

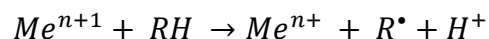
2.2.3. Metals

Lipid matrices generally contain traces of metal ions, which may come from the refining equipment, the hydrogenation process, the packaging, or different protein components such as enzymes. Heavy metals that increase the oxidation rate of fatty acids are those with two or more oxidation states and an intermediate redox potential, such as copper, iron, manganese, cobalt, magnesium, and vanadium (Nelson & Labuza, 1992). On contrary, metals that oxidize by two-electron transfers, i.e., Sn^{2+} and T^{1+} , are not active (Schaich, 2005).

The initiation of lipid oxidation by metals can be conducted by electron transfer or by the formation of transition complexes or complexes with hydrogen peroxide which catalyze the autooxidation and the decomposition of hydroperoxides by redox reactions. In lipid oxidation initiated by metals, several factors such as the redox potential, the presence of chelating agents, the metal localization phase, the availability of oxygen and presence of hydroperoxides determine the oxidation mechanism and its rate. Metal ions (Me^{n+1}) can initiate autoxidation only when hydroperoxides are present, since they decompose them, breaking down into alkoxy (RO^{\bullet}) and peroxy radicals (ROO^{\bullet}) according to the following reactions (Choe & Min 2006; Frankel, 2005):



However, among the multiple mechanisms that are feasible when metals are involved in lipid oxidation, it can be also found the direct attack of metal ions (Me^{n+1}) to unsaturated fatty acids (RH) which results in the formation of an alkyl radical (R^\bullet) as follow:



In this context, many processes are industrially conducted for removing traces of metal from edible oils. Some oils produced without refining, as sesame oil and extra virgin olive oil, contain relatively high quantities of transition metals. For instance, copper and iron content of crude soybean oil are 13.2 ppb and 2.80 ppm, respectively, but refining processes reduces their contents. Among the catalysts, copper is reported as the most active metals for inducing oxidation in oils, followed by iron (Andersson & Lignert, 1998). Copper accelerates hydrogen peroxide decomposition 50 times faster than ferrous ion (Fe^{2+}), and ferrous ion acts 100 times faster than ferric ion (Fe^{3+}).

3. Antioxidant compounds for delaying lipid oxidation

The use of exogenous antioxidant substances, in either food products or bulk oils, constitute a current alternative for avoiding the lipid oxidation and therefore the formation of all undesirable and deleterious products of oxidation already described above. Globally, antioxidants are molecules that at low concentration are capable of blocking the formation of free radicals, and therefore prevent or delay the lipid oxidation. These molecules are potent agents in eliminating oxidation chain reactions by getting oxidized themselves and inhibiting other oxidation reactions such as peroxidation (Balasubramaniam et al., 2019). The delay or prevention of lipid oxidation helps to maintain the quality and nutritional value of lipid systems and extend the shelf life of food products. The antioxidants used for protecting oils from rancidity can be natural or synthetic compounds, and exhibit a different range of action depending on dose, lipid composition, mechanism of action, etc.

Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are common and effective synthetic antioxidants for fat food protection, however these additives have been associated with liver damage and carcinogenesis (Szydłowska-Czerniak et al., 2018). These potential health risks have recently increased the interest of food, pharmaceutical and cosmetic industries for natural antioxidants such as polyphenols. In this sense, the protective effect of polyphenol compounds against lipid oxidation has been widely reported (Mikołajczak et al., 2020; Martínez et al., 2020; Wu et al., 2019; Bakkalbasi et al., 2018; Wang et al., 2018; Lue et al., 2017; Martín et al., 2012; Xu et al., 2011; Maqsood & Benjakul, 2010; Suárez et al., 2010). Other natural antioxidants such as carotenoids (Dimakou & Oreopoulou, 2012) or tocopherols (Alizadeh et al., 2019) are also able to exert a protective effect against lipid oxidation however, this work has focused on assess the enrichment of plant oils with phenolic compounds. Thus, in the following sections the nature, main classification, and mechanism of action of phenolic compounds, as antioxidants, has been carefully described.

3.1. Phenolic compounds

Plant metabolism is naturally divided into primary and secondary processes. Substances such as carbohydrates, lipids, proteins, and nucleic acids, which are essential for the development of the organism, have their origin in the primary metabolism. On the other hand, substances that are limited to certain functional groups, originating from different metabolic routes, are the result of secondary metabolism.

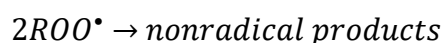
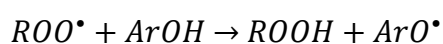
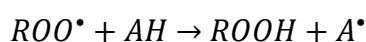
Phenolic compounds are one of the broadest groups of secondary metabolites in plants. These compounds are known for the great variety of functions in plants, being important in their development and defense. In addition, they include pigments and substances with reproductive and protective functions against insects, fungi, viruses, and bacteria (Vermerris & Nicholson, 2008). They are synthesized by plants both in normal conditions of development and under stress conditions.

These compounds also influence significantly the sensory and nutritional quality of foods derived from plant sources. For instance, polyphenols of vegetables and fruits contribute to sensory properties of food products, conferring attributes of color, flavor, and aroma. Furthermore, phenolic compounds have attracted the interest of many researchers due to their attractive nutritional properties on human health, mainly by reducing the risk of chronic diseases such as cancer and cardiovascular diseases (Wu et al., 2019). The importance of phenolic compounds for human health is due to their multiple biological effects, such as antioxidant, antimutagenic, anticancer activity and anti-inflammatory action (Vermerris & Nicholson, 2008).

According to their antioxidant capacity, phenolic compounds can act as primary or secondary antioxidants. Basically, primary antioxidants can directly inhibit oxidation reactions, and secondary antioxidants act in an indirect way thanks to their ability to scavenge oxygen or react with prooxidant compounds (Amarowicz & Pegg, 2019).

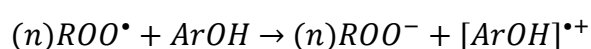
In this context, two mechanisms are considered when phenolic compounds are acting as primary antioxidants: hydrogen-atom transfer (HAT) or single-electron transfer (SET).

The HAT mechanism occurs when an antioxidant compound scavenges reactive oxygen species (ROS) (e.g., peroxy radical, ROO^{\bullet}) by hydrogen donation (Heim et al., 2002). In this sense, the HAT reaction mechanism includes the transfer of hydrogen atom (H^{\bullet}) from the antioxidant (AH, ArOH) to a radical ROO^{\bullet} in order to give stable free radicals (A^{\bullet} , ArO^{\bullet}). In this mechanism, the lower the bond dissociation enthalpy of the H-donating group in the potential antioxidant, the easier it will be for the reaction of free radical inactivation. This process is summarized by the following reaction scheme:

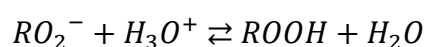
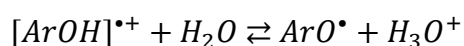


The radicals A^\bullet and ArO^\bullet obtained by the action of the antioxidants AH and $Ar-OH$, are more stable than ROO^\bullet species. A potent phenolic antioxidant ($ArOH$) needs to react more quickly with the oxidant (ROS) than the target to be protected, and A^\bullet must be rapidly converted to less reactive species (Apak et al., 2016).

On the other hand, the single-electron transfer (SET) mechanism occurs in cases where an antioxidant transfers a single electron to assist in the reduction of potential target composites. In SET mechanisms, the antioxidant ($ArOH$) provides an electron to the free radical ($nROO^\bullet$) and itself then becomes a radical cation ($ArOH^{\bullet+}$) (Liang & Kitts, 2014). In this mechanism, the ionization potential of the antioxidant is the most important energetic factor in evaluating the antioxidant action.



The resultant radical-cationic antioxidant compound is then deprotonated by interacting with water:



When phenolic compounds are acting as secondary antioxidants in chelation processes, they can inhibit the oxidation without directly interact with oxidative radicals or species (Amarowicz & Pegg, 2019; Mira et al., 2002). A high chelation activity is often characteristic of phenolic compounds that have a 5-OH and/or 3-OH moiety with a 4-oxo group in the A/C ring structure. Chemical structure of flavonoids is shown in Figure I-13 to identify the nomenclature of different rings and the aforementioned positions of hydroxyl and oxo groups.

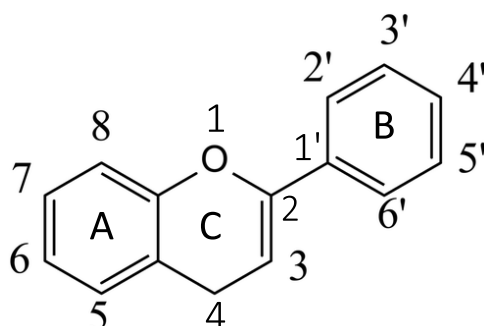


Figure I-13. Basic chemical structure of flavonoids (Van Acker et al., 1996)

Structurally, phenolic compounds have a particular structure, with one or more hydroxyl groups attached directly to an aromatic ring. Phenol is the basic structure for the entire group and the aromatic ring corresponds to benzene. The main classes of phenolic compounds, according to the nature of their carbon structure, are mainly phenolic acids, flavonoids, stilbenes and to a lesser extent, lignans (Figure I-14). In Table I-4 is shown the chemical structure of various classes of phenolic compounds. In this work, a special attention to flavonoids has been given since quercetin, the compound mostly used in this work belong to that group.

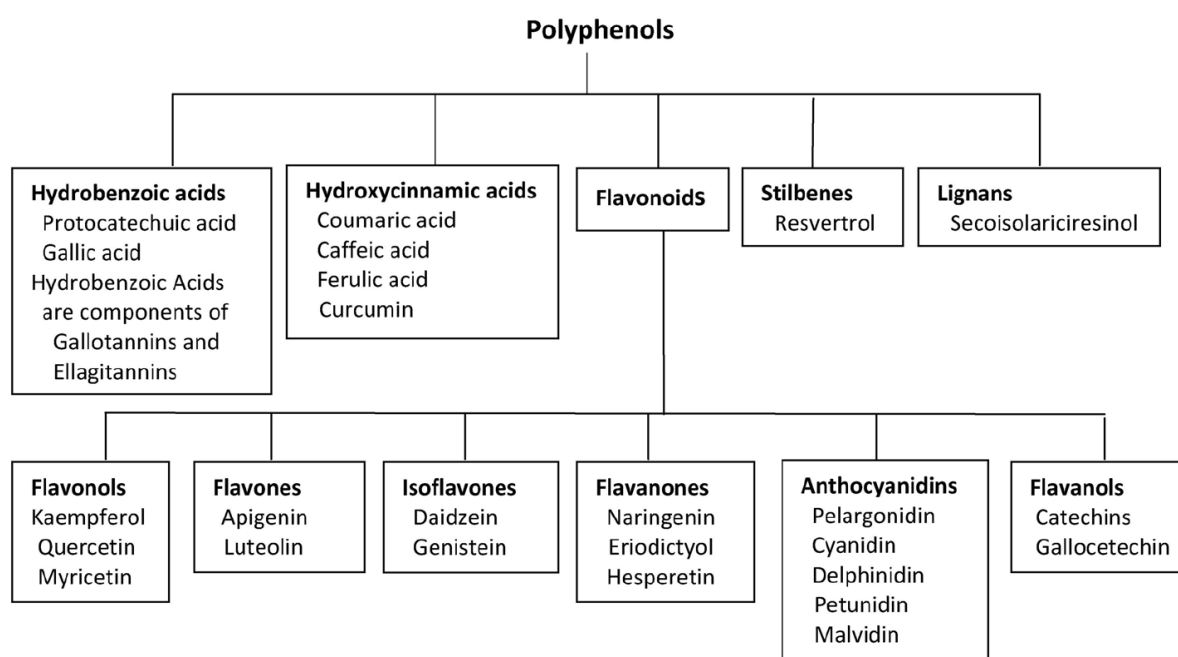
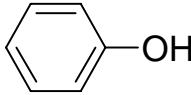
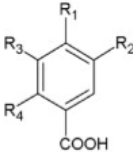
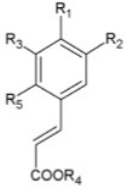
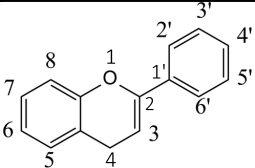
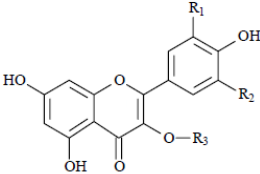
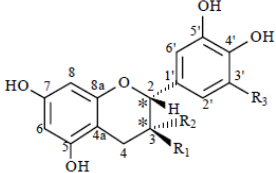
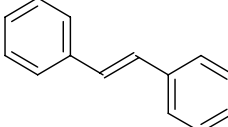


Figure I-14. Schematic diagram of subdivisions of phenolic compounds (Source: Hardman, 2014)

Table I-4. Chemical structure of different classes of phenolic compounds (Adapted from: Garcia-Salas et al., 2010; Turk, 2010)

Class	Basic structure
Simple phenols (basic structure)	
Hydroxybenzoic acids	
Hydroxycinnamic acid	
Flavonoids	
Flavonols (Flavonoid)	
Catechins (Flavonoid)	
Stilbenes	

3.2. Flavonoids

3.2.1. Structure

Flavonoids belong to the group of phenolic derivatives soluble in water with significant antioxidant properties and which are distributed in the vegetable kingdom, being more

concentrated in flowers, fruits, vegetables, wines and teas (Heim et al., 2002; Van Acker et al., 1996). They show colorings from purple to yellow and can be found in the free form (aglycone) according to the oxidation state of the C3 unit in the molecule. The molecule also appears in conjugated form, appearing together with a sugar (glycoside). These compounds are characterized by a 15-carbon skeleton that has a C6-C3-C6 cyclic structure, containing a basic flavylum nucleus (2-phenylbenzopyryl cation) composed of two aromatic rings (A and B), united by a three-carbon unit (heterocyclic ring C), which is condensed by an oxygen (as already shown in Figure I-13). The second aromatic ring (B) can be found in position 2, 3 or 4 of the three-carbon unit (C). (Malacrida & Motta, 2006; Delgado-Vargas & Paredes-López, 2003).

The variation in the heterocyclic C ring forms the basis of the classification of flavonoids in various subclasses; flavones, isoflavones, flavonols, flavanols, flavanones, anthocyanidins and chalcones (Boots et al., 2008).

Most of the beneficial health effects of flavonoids are attributed to their antioxidant and chelating abilities. Furthermore, flavonoids have been widely studied due to their cardioprotective effects, their ability to inhibit lipid peroxidation and to attenuate other processes related to reactive oxygen species (Roleira et al., 2015). Anti-inflammatory, antiviral activity, effects on capillary fragility, inhibition of platelet aggregation and activity against cancer, are some of the properties exhibited by these compounds (Roleira et al., 2015; Benavente-García et al., 2007; Marin et al., 2002).

From a food perspective, flavonoids are quite interesting antioxidants and functional ingredients for being used in several food products (Neri-Numa et al., 2020), emulsions (Yang et al., 2015) and even in pharmaceutical products (Alencar Filho et al., 2020). The use of flavonoids in food products is intended to enhance their shelf life and maintain their nutritional quality by delaying lipid oxidation and also for acting as functional ingredients by providing health benefits to consumers (Neri-Numa et al., 2020; Heim et al., 2002). In Table I-5 is shown some studies that used flavonoids in food systems as antioxidant and nutraceutical molecules. Food application of flavonoids and other phenolic compounds specifically in edible oils are carefully listed later in this chapter.

Table I-5. Flavonoids applications in food systems (Adapted from Neri-Numa et al., 2020)

Food product	Bioactive compound	Food application	Major findings	Reference
Sorghum powdered drink mix	Flavonoids: tannins	Functional and health promoting food ingredients	The tannin did not negatively influence the acceptance of drinks and improved their functional properties.	Queiroz et al., 2018
Biscuit	Flavonoids	Functional ingredient	The microencapsulation of green tea extract showed a protective effect in thermal treatment but did not protected in a biscuit-model.	Gomez-Mascaraque et al., 2017
Biscuit	Anthocyanin	Functional ingredient	The polyphenols of Artemide black rice spray-dried with maltodextrin and gum resulted an ingredient most stable to the storage and partially protected from thermal damage during the baking in a model biscuit.	Papillo et al., 2018
Bread	Flavonoids	Functional ingredient	The introduction of the flavonol extract of black tea in the dough can be used for the development of functional bakery since compounds were stable under processing conditions.	Souza et al., 2020
Bread	Flavonoids	Functional ingredient	Addition of green coffee promoted antioxidants potentially bioaccessible and bioavailable and protected lipids against oxidation.	Swieca et al., 2017
Beverage	Anthocyanin	Functional ingredient	Chitosan nanoparticles increased anthocyanin storage stability and delayed anthocyanin degradation in simulated gastrointestinal fluids.	He et al., 2017
Gelatine	Anthocyanin	Natural colorant and functional ingredient	The incorporation of inulin-coated microcapsules into the gelatine model-system provided greater protection to anthocyanins and presented best gelatine colour parameters throughout the storage.	Bernardes et al., 2019
Kefir and carbonated water	Anthocyanin	Natural colorant and functional ingredient	The stability of the anthocyanins followed the kinetics of the first-order reaction. Exposure to light of carbonated beverage significantly affected colour and anthocyanin stability during storage.	Montibeller et al., 2018

3.2.2. Antioxidant capacity related to molecular structure

Globally it can be stated that the protective effects of flavonoids are related to their capacity to transfer electrons to the free radicals, chelate redox-active metals and attenuate processes involving reactive oxygen species (Heim et al., 2002). According to Sanchez, (2009) the flavonoids with the following characteristics exhibit a particular ability for acting as antioxidants:

1. The presence of the 3', 4'-o-dihydroxy structure in the aromatic ring B, which confers greater stability to the radical formed.
2. The existence of hydroxyl groups on carbons 5 and 7 of ring A.
3. The double bond located in position 2,3 in conjunction with the 4-oxo group and the presence of 3-OH in ring C.

Flavonoids are hydrogen donor molecules due to the reducing properties of the hydroxyl groups present in their aromatic rings, in addition, they have the ability to delocalize the resulting radical within their structures, chelate transition metals through their catechol structure, and regenerate α -tocopherol through reduction of α -tocopheryl radicals (Sanchez, 2009). Structurally, the antioxidant activity of flavonoids depends upon the arrangement of functional groups around the nuclear structure since both the total number of hydroxyl groups and their configuration influence significantly several mechanisms of antioxidant activity (Heim et al., 2002). For instance, the free radical scavenging activity of flavonoids is mainly attributed to the high reactivities of hydroxyl substituents as follow.

- Hydroxyl groups on the B-ring of flavonoids donate hydrogen and an electron to hydroxyl, peroxy, and peroxy-nitrite radicals, which lose their reactivity as a result of their stabilization, and a relatively stable flavonoid radical is also formed. For that reason, The B-ring hydroxyl configuration is the most significant determinant for the scavenging of reactive oxygen and nitrogen species (Heim et al., 2002).

- Catechol structure in 3', 4' of the B ring greatly favors the inhibition of lipid peroxidation. On the other hand, the influence of the arrangement of other hydroxyl groups on the antioxidant activity of flavonoids is less prominent, especially regarding the A ring (Sanchez, 2009).

- Free radical scavenging by flavonoids is also largely dependent on the presence of a free OH at carbon 3 in ring C. Flavonoids with an OH group at carbon 3 and a catechol group at 3', 4' are 10-fold more potent than ebselen against peroxy-nitrite (Sanchez, 2009). Ebselen is a powerful reactive nitrogen species scavenger.

- The torsion angle of ring B with respect to the rest of the molecule, plays a major role in eliminating free radicals. Flavonols and flavanols with a 3-OH group adopt flat structures, while flavones and flavanones, which lack 3-OH group, adopt slightly curved structures. The planar shapes of some flavonoids allow for conjugation, better electronic delocalization, and increased stabilization of the phenoxy radical. Specifically, in quercetin, the relatively planar structure of quercetin allows the conjugated π -system of the AC-ring and that of the B-ring to efficiently interact (Moalin et al., 2011). Many polyphenols can act as antioxidants in chemical systems and food matrices as their extensive conjugated π -electron systems facilitate the donation of electrons from the hydroxyl moieties to oxidizing radical species (Duthie & Morrice, 2012).

In summary it can be said that the protection of lipids against oxidative deterioration allowed by flavonoids can be ascribed mainly to i) scavenging of hydroxyl, peroxy, or synthetic radicals, ii) termination of chain reactions in the lipid phase, involving peroxy radicals and hydroperoxides, and iii) chelation of divalent cations (metal traces) able to initiate oxidative reactions.

3.2.3. Quercetin: structure and antioxidative characteristics

The flavonoid focused on this work was quercetin due to its convenient chemical structure for acting as an efficient antioxidant molecule. Therefore, it is important to describe deeper key characteristic related to its plant sources, structure, and antioxidant properties.

Quercetin (3, 3', 4', 5, 7-pentahydroxyflavone) (Figure I-15) is an abundant flavonoid which satisfies the structural requirements that a flavonoid must meet to have maximum antioxidant potential. Quercetin is mostly found in natural sources as onions, apples, tea, red wine among others and it occurs mainly as glycoside, which means that a sugar group such as glucose, galactose, rhamnose, rutinose or xylose is bound to one of the hydroxyl groups of the flavonol (Boots et al., 2008). Evidence suggest that among its biological properties, quercetin exhibits antioxidant, antibacterial (Wang et al., 2016), anti-inflammatory (Boots et al., 2008), anticarcinogenic (Kumari et al., 2010), cardioprotective, antiviral (Anandam & Selvamuthukumar, 2014), neuroprotective and antihypertensive effect (Xu et al., 2016; Zhang et al., 2016). The antioxidant mechanism of quercetin is particularly related to its capacity to scavenge reactive species (Singh et al., 2011; Ramadan, 2008), partially ascribed to the

presence of its free 3-OH group in ring C, which increases stability of flavonoid radical (Heim et al., 2002).

Quercetin antioxidant effect on bulk oils has been reported previously by some authors. Palma et al. (2017) showed that the addition of 200 mg/kg of quercetin to bulk methyl linoleate increased its oxidative stability about 10-fold. They attributed this behavior to the presence of ortho-dihydroxy structure in the B ring and the 3-hydroxyl group in the C ring of quercetin. Huber et al. (2009) found a higher effectiveness of quercetin (at a concentration of 100 to 1000 μ M or \sim 30.2 to 302 mg/kg) for inhibiting lipid oxidation of bulk fish oil and of oil-in-water emulsion systems, in comparison with BHT added to bulk fish oil and α -tocopherol added to the oil-in-water emulsion. The oxidation of fish oil was induced by heat, light, peroxy radical or ferrous ion and the antioxidant activity of quercetin in the food system was dose-dependent. Naz et al. (2008) reported that quercetin at a concentration of 200 mg/L effectively reduced the oxidation rate of corn oil submitted to deep frying at 180 $^{\circ}$ C for 30, 60 or 90 minutes, since a significant decreasing in peroxide value, and p-anisidine value as well a relatively low reduction rate in iodine value was observed in the oils previously enriched with quercetin.

Structurally, quercetin has 2 benzene rings (A and B) connected by an oxygen containing a pyrene ring (C) (Figure I-15). Quercetin is comprised of five hydroxyl groups and is commonly found in its glycoside form, in which one or more of these hydroxyl groups are replaced by different types of sugar groups. Most of these groups of quercetin derivatives are quercetin O-glycosides. Quercetin and its derivatives normally exist in the form of yellow colored powder or crystals (Wang et al., 2016).

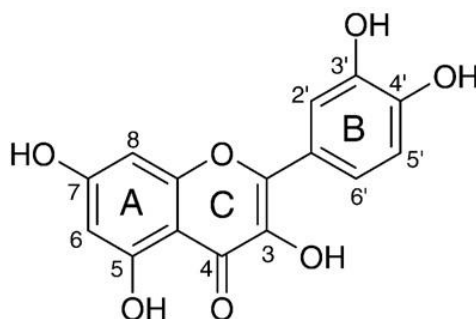


Figure I-15. Molecular structure of quercetin. Source: Boots et al., 2008

Regarding the antioxidative capacity of quercetin, several authors stated that within the flavonoid family, quercetin is the most potent ROS scavenger (for instance of superoxide species), with also scavenging activity of reactive nitrogen species (RNS) such as nitric oxide (NO[•]) (Cushnie & Lamb, 2005).

Basically, these antioxidative functions of quercetin are attributed to the presence of two antioxidant pharmacophores (relevant groups of a molecule responsible for its biological activity); the AC-ring and the B-ring. Their optimal functionality and configuration for the free radical scavenging activity are related first, to the 3-OH group which is the active center in the AC-ring, and its activity is positively influenced by the OH groups at the 5 and 7 position in the AC-ring (Moalin et al., 2011) and second, the high activity of the catechol group in the B ring that can be explained by the strong electron-donating capacity of its two adjacent phenolic OH groups and their intramolecular H-bonding interaction (Boots et al., 2008).

Despite the several advantages of phenolic compounds as inhibitors or retarders of lipid oxidation, their use in oily systems may be limited by their low stability during processing and storage and their poor solubility in oils and fats due to the multi-ring and polar nature of polyphenols (Ghanbarzadeh et al., 2016; Kidd, 2009). On one side, the chemical structure of phenolic compounds, related to the presence of aromatic structures and hydroxyl groups provide them a high capacity for acting as antioxidant, but on the other side, this structure also reduces their solubility in oils and thus their effectiveness to protect oils and fats against lipid oxidation (Szydłowska-Czerniak et al., 2018).

From a global point of view, many sources of polyphenols are currently investigated aiming at providing natural antioxidants with a high protective effect than can be used in different industrial applications. The next section focused on describing the current sources of polyphenols, in particular for being used in oily systems.

3.3. Current sources of polyphenols for oil enrichment

Recently, the use of agro residues for obtaining natural extracts has been catching the attention of researchers for food, pharmaceutical and cosmetic applications. This growing interest is due to the fact that plant by-products represent an un-expensive, abundant and rich source of natural and antioxidant molecules, that may be further used for enriching other

products aiming at providing color properties (pigments such as anthocyanins) or improving their lipid stability (phenols, carotenoids, etc.), for instance.

Moreover, since the use of synthetic antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), propyl gallate (PG) and tertbutyl hydroquinone (TBHQ) are regulated and even associated with liver damage and carcinogenesis (Szydłowska-Czerniak et al., 2018), the recovery of natural antioxidants has gained more attention. In particular, the use of natural active substances can be potentially used to enrich edible oil in order to enhance its oxidative stability, extend the shelf life, maintain its nutritional quality and improve the health of many consumers by preventing cardiovascular, immune frailty and degenerative diseases through a higher incorporation of polyphenols in human diet (Fregapane et al., 2020; Reboredo-Rodríguez et al., 2017). In this sense, in Table I-6 are listed several works aiming at enriching edible oils with natural antioxidants, mainly obtained from agro-residues.

Table I-6. Enrichment of edible oils with polyphenol extracts obtained from different residues

Reference	Antioxidants in extracts	Applied edible oil	Solvent of extraction	Materials (Residue)
Bakkalbasi et al., (2018)	Phenolic compounds	Walnut oil	Methanol:water (80:20, v/v)	Walnut press-cake extracts/ Walnut press-cake
Goula et al., (2017)	Carotenoids	Sunflower and soy oil	Hexane-isopropanol (60:40, v/v)	Pomegranate peels/extracts (<i>Punica granatum</i> L.)
Khemakhem et al., (2015)	Phenolic compounds	Virgin olive oil	-	Sweet lemon and sweet orange peels
Purohit & Gogate, (2015)	Carotenoids	Sunflower oil, coconut oil, soybean oil, rice-bran oil, and groundnut oil	-	Carrot residue (<i>Daucus carota</i> L.)
Sekhon-Loodu et al., (2013)	Phenolic compounds	Fish oil	Ethanol	Apple peel extracts
Zribi et al., (2013)	Phenolic compounds	Virgin olive oil	Ethanol-water (70:30, v/v)	Olive pomace extracts
Chemat et al., (2012)	Carotenoids	Sunflower oil, rapeseed oil, olive oil, and soybean oil	-	Sea buckthorn pomace (<i>Hippophae rhamnoides</i>)
Poiana, (2012)	Phenolic compounds	Refined sunflower oil	Ethanol 70% (v/v)	Grape seed extracts
Rubió et al., (2012)	Phenolic compounds	Virgin olive oil	Water	Thyme and olive pomace extracts

Sánchez de Medina et al., (2012)	Phenolic compounds	Refined edible oils	Ethanol	Olive pomace and leaves extracts
Orozco et al., (2011)	Phenolic compounds	Refined sunflower oil	Ethanol	Olive pomace extracts
Orozco-Solano et al., (2011)	Phenolic compounds	High oleic sunflower oil	Ethanol	Olive pomace extracts
Suárez et al., (2011)	Phenolic compounds	Virgin olive oil	Ethanol-water (50:50, v/v)	Freeze-dried olive cakes extracts
Abd-ElGhany et al., (2010)	-	Sunflower oil	Ethanol	Olive waste cake extract
Karoui et al., (2010)	Limonene	Refined corn oil	-	<i>Citrus aurantium</i> peel
Pu et al., (2010)	Astaxanthin	Flaxseed oil	-	Shrimp by-product (<i>Litopenaeus setiferus</i>)
Suárez et al., (2010)	Phenolic compounds	Virgin olive oil	Ethanol-water (50:50, v/v)	Olive cakes extracts
De Leonardis et al., (2007)	Hydroxytyrosol tyrosol, Caffeic acid and Ferulic acid	Lard	Ethyl acetate	Olive oil mill wastewater extract
Renuka Devi et al., (2007)	Oryzanols, Tocols and Ferulic acid	Soybean oil	Methanol	Defatted rice bran extracts

According to Fregapane et al. (2020) and Reboredo-Rodríguez et al. (2017), the general protocol for enriching edible oils with natural plant extracts involves two main steps: i) the extraction of the target compounds from the plant material and then, ii) the enrichment of the edible oil with the natural extract. After the addition of liquid herbal extracts, the solvent should be removed from the oil, unless the extracts have been previously dried.

From those works that first extracted the bioactive substances from different raw materials and then enriched the oils, can be inferred a general extraction procedure consisted of a solid– liquid extraction using mixtures of methanol or ethanol with water, as solvent. Nowadays, intensification techniques such as ultrasound, pressurized liquids, microwave, electric field, etc. can be also accoupled to conventional solid-liquid extractions in order to enhance extraction yields and shorten process time by modifying solvent physical properties. From that perspective, it is worth describing some of the aforementioned extraction methods which are of high interest for this work.

3.3.1. Conventional solid-liquid extraction (maceration)

Solid-liquid extraction is a separation process that occurs with the selective dissolution of one or more solutes from a solid matrix by a liquid solvent. Hence, solid-liquid extraction can be defined as a multicomponent, multiphase, un-steady state mass transfer operation

(Aguilera, 2003). During maceration extraction a solid matrix is placed in contact with a solvent for several hours, so that the soluble compounds are transferred from the plant material to the fluid phase. Aiming at increasing the mass transfer rate, some kind of stirring can be accoupled to the system, since agitation favors the gradient of concentration between solid surface and solvent and also avoids the bed compression. On the other hand, excessive agitation may cause the disintegration of particle solids. This unit operation (solid-liquid extraction) is extensively used to recover industrially many important food components such as carbohydrates, lipids, proteins, phytochemicals, among others. From an industrial production point of view, it is required (for economic reasons) that solid-liquid extraction achieve an adequate balance between extraction efficiency, extraction yields of the target and co-extracted compounds, and concentration of target compounds, in order to minimize costs. The above requires the understanding of the extraction technique principles, and to know the properties of both target compounds and extraction solvent since the interactions between solute and solvent are determined by properties such as the vapor pressure of the solute, the solubility of the solute in the solvent, the hydrophobicity, and the acid/base properties of both solute and solvent (Palma et al., 2013). In general, the compatibility between solvent and solute is based on assessing the polarity of the molecular structure to predict their solubility and miscibility, by assuming that non-polar solutes are dissolved by non-polar solvents while polar solutes are dissolved by polar solvents.

From that perspective, it can be stated that the solubility of target compounds in the solvent is a key parameter of the whole extraction process. Nonetheless, solid-liquid extraction is a complex process in which several factors are simultaneously affecting the system. For instance, the possible sites where target compounds are located into solid matrix have also a strong influence on extraction yields. According to Palma et al. (2013), the compounds present in natural products may be dissolved in the pore of the matrix and/or adsorbed on the pore surface (1), adsorbed on the surface of the matrix (2), dissolved/adsorbed in a micro/nano pore (3), chemically bounded to the matrix (4), or dissolved in the bulk solution (5) as shown in Figure I-16. Hence, depending on the solute location in the solid matrix, it is more easily accessed (for instance positions 1, 2 and 5 of Figure I-16) or accessed with more difficulty (positions 3, 4 of Figure I-16) by the solvent and extracted by it.

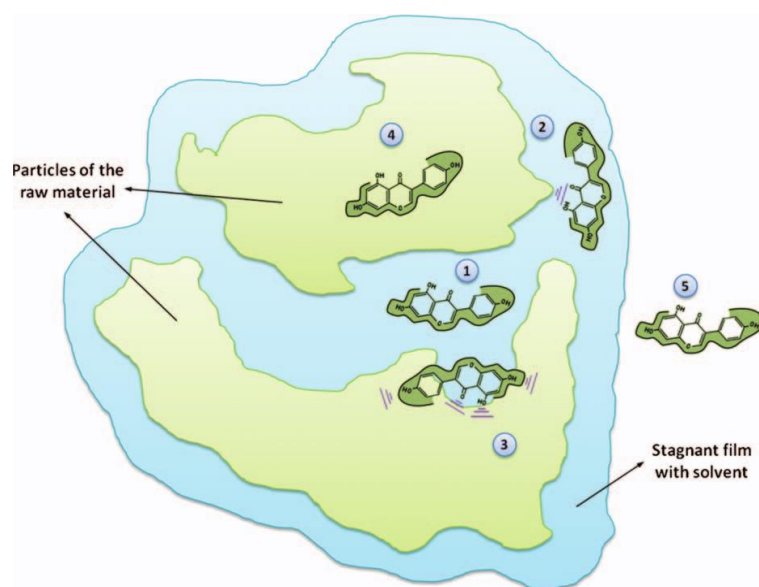


Figure I-16. Schematic conceptualization of location of target compounds in a natural matrix
(Source: Palma et al., 2013)

According to Palma et al. (2013) and Aguilera, (2003) the solid-liquid extraction is comprised by the following steps:

1. the solvent is transferred from the fluid phase to the solid surface and pervades it;
2. the solvent penetrates into the solid matrix by molecular diffusion;
3. the soluble material is solubilized by desorption from the matrix and solvation into the extraction solvent – the breakage of chemicals bonds may be required for desorption of target analytes from the solid matrix;
4. the solution containing the solutes returns to the surface of the solid by molecular diffusion;
5. the solution is transferred from the solid surface to the bulk fluid by natural or forced convection.

In this context, the maceration extraction can be influenced by several factor that play a key role in the rate of extraction. This influence is based on the fact that solid-liquid extraction can be considered as a diffusion process in which the solutes are transported from one part of the system to another by random movement as a result of a concentration gradient. Hence, the other factors that influence the maceration are (Takeuchi et al., 2009):

- **Preparation of the solid:** the plant cell structure is an important factor in extraction process since most of the solutes are stored in intracellular spaces, capillaries, or cell

structures. Some solutes (but in less proportion) can be also on the surface of the cell. In this sense, pretreatments such as grinding of the raw material are carried out in order to break the cell structures and increase the contact area between the solvent and the solid matrix. By releasing the solutes from cell structures and providing a greater surface area of the solid material, the extraction yield can be enhanced.

- **Diffusion rate:** the effective diffusivity of biological materials is based on the cell structure and the existence of porous and different compartments in the cell. Therefore, the diffusivity of solutes depends on the composition and their position in the solid material. Diffusion rate increases when solutes are more accessible to solvent.
- **Temperature:** in general, higher temperatures increase the solute's solubility in the solvent, increasing thus the solute diffusion rate into the solvent bulk which leads to a higher mass transfer rate. However, in the extraction of bioactive compounds, the use of elevated temperatures can generate undesirable reactions such as the degradation of thermolabile compounds.
- **Solvent choice:** the selection of the extraction solvent is based on several factors, such as its selectivity and capability of dissolving the solute, as well as its interfacial tension, viscosity, stability, reactivity, polarity, toxicity, and cost. In terms of green technologies, solvents such as ethanol, water, 2-propanol and ethanol-water mixtures have gained attention.
- **Solvent to feed ratio:** the extraction yield increases with solvent to feed ratio (S/F) since a higher gradient of concentration in the system is favored. However, a high S/F also implies the use of larger volume of solvent and higher energy consumption for purification/concentration steps.
- **Moisture of solid material:** the water in the solid material can compete with the extraction solvent for the solute's dissolution, and therefore affects the mass transfer. Drying of raw materials is usually performed before extracting process for facilitating other pretreatments such grinding and to provide homogeneous materials. On the other hand, drying conditions should not cause significant degradation of target compounds.

On the other hand, as the extraction process is described by mass transfer mechanism, it usually follow a kinetic curve which consist of three distinct phases: constant extraction rate period (CER), falling extraction rate period (FER), and diffusion controlled period (DC) as shown in Figure I-17. In the CER period the easily accessible solute that surrounds the particle is removed at an approximately constant rate. In this step, the main mechanism of mass transfer

is convection; therefore, providing agitation enhances the efficiency of the process. In the FER period some gaps begin to appear in the solute superficial layer that covers the solid particle; therefore, the extraction rate decreases rapidly as a result of the decrease of the effective mass transfer. In the DC period the easily accessible solute layer is depleted. Hence, the extraction rate depends exclusively by the diffusion rate of the solvent into the solid material and of the solvent+solute from the solid particle to the bulk solvent. DC is a slow stage of the extraction process.

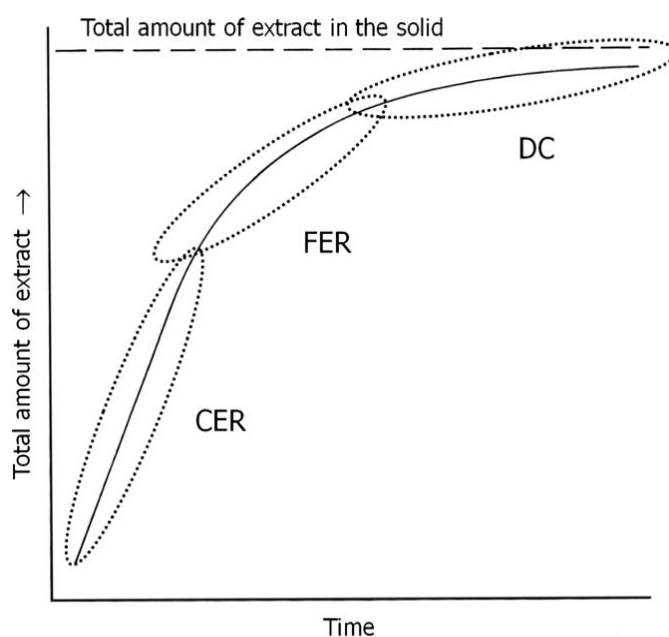


Figure I-17. Typical extraction kinetic of natural compounds from plant matrices

3.3.2. Intensified and green techniques for extraction of phenolics

Due to the current environmental concerns that require a more efficient use of energy and natural resources, the chemical and food industries are challenged to find new extraction technologies that allow to reduce energy, solvent and water consumption. By developing these green technologies of extraction, it becomes possible to meet legal requirements on emissions, have product/process safer, reduce costs as well as increase quality and functionality of final products. The challenges require novel extraction techniques that use extreme or non-conventional conditions, so that processes could be completed in minutes instead of hours with high reproducibility, reduced consumption of solvent, simplified manipulation, higher purity of the final product, elimination of post-treatment of wastewater and consumption of only a fraction of energy normally needed for a conventional extraction method (Chemat et al., 2020). From that perspective, the intensified techniques aim to maximize output with minimum inputs.

3.3.2.1. Ultrasound assisted extraction (UAE)

This method enhances the extraction yield by increasing the mass transfer between the solvent and plant matrix. For that, ultrasonic waves (vibrations with frequencies exceeding 20 kHz.) are transmitted through the solvent medium which promote the phenomenon of acoustic cavitation when the pressure related to the acoustic waves breaks the cohesion of a liquid (Chemat et al., 2020). These cavities start as microbubbles, which grow under the sound wave effect until they become unstable and violently collapse creating extreme localized conditions (high temperatures and pressures) inside the collapsing cavity (Chemat et al., 2020; Martinez et al., 2020) (Figure I-18). This collapse produces mechanical effects such as cell wall disruption, particle size reduction, intensive mixing and hot spots which significantly enhance the extraction rate. The schematic representation of ultrasound effect is shown in Figure I-18; in the first stage, bubbles are formed (a); such bubbles undergo expansion and compression (b), which cause their collapse or implosion (c); when this collapse occurs near the solid surface, the cell wall of the matrix is disrupted, causing the release of the intramolecular material in the solvent (d). The process variables that influence UAE are ultrasonic energy, frequency, temperature, extractor configuration, solvents, time, matrix/solvent ratio, particle size and matrix structure. These factors can be optimized through experimental designs in order to obtain high extraction yields. Among the advantages of UAE are; lower amounts of solvent, enhanced solvent penetration, extraction at lower temperatures, reduced extraction time and higher extraction yield.

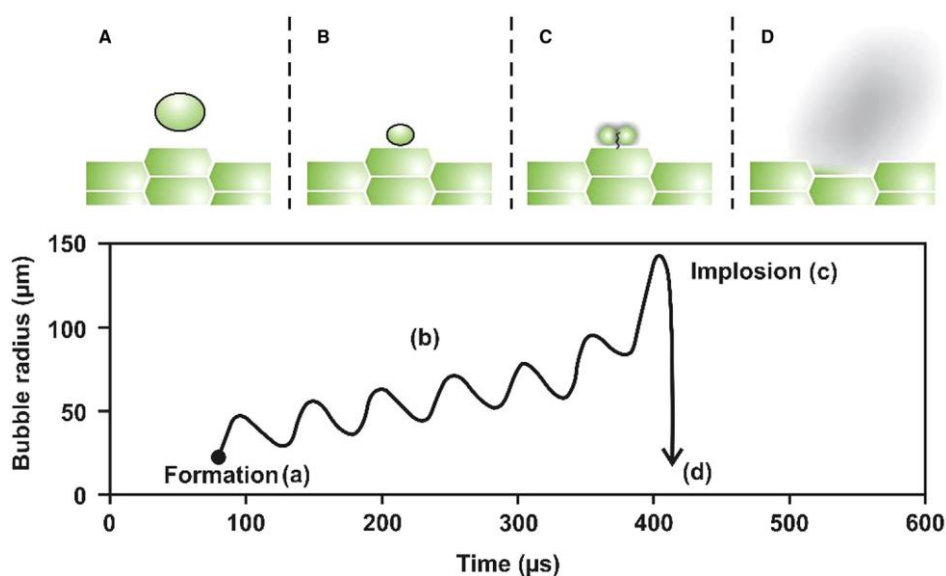


Figure I-18. Schematic representation of ultrasound assisted extraction on plant matrices

3.3.2.2. Pressurized liquid extraction (PLE)

This technique is based on the use of solvents at high temperatures and pressures, always below their respective critical points, so that the solvent is maintained in the liquid state during the whole extraction procedure. As a result of these particular conditions of pressure and temperature, a change in the solvent physicochemical properties occurs. For instance, the solvent surface tension and viscosity are decreased which allow the solvent to penetrate easier and deeper into the solid matrix being extracted (Alvarez-Rivera et al., 2020). As a consequence, significantly higher extraction yields are obtained compared with conventional extractions. Furthermore, PLE results not only in faster extraction processes but also in lower solvent consumption for the sample preparation of solids. This technique is influenced by factors such as temperature, pressure, flow rate, time and matrix condition. However, extraction temperature is by far the most important factor; theoretically, the higher the temperature, the greater the yield. Nonetheless, extraction at high temperatures may affect the extraction yield of thermolabile compounds. The pressurized liquid extraction can be performed in dynamic mode, static mode, or a combination of both. In the dynamic mode, the solvent continuously flows through the sample, while in the static mode, sample and solvent are maintained for a specific time at constant temperature and pressure (Alvarez-Rivera et al., 2020).

3.3.2.3. Supercritical fluids

This is a well-documented alternative to traditional organic solvents suitable for various extraction methodologies which is based in the use of any fluid above its critical point as solvent for extracting target compounds from plant matrices. A fluid is in its critical state when it is both heated above its critical temperature and pressurized above its critical pressure. In supercritical state, the physicochemical properties of the fluid can be modulated by changes in pressure and/or temperature, beyond their critical values. Moreover, supercritical fluids exhibit convenient characteristics for extraction process. For instance, they have a liquid-like density which induces a solvating power mimicking liquid-like characteristics, and their gas-like viscosity results in higher mass transfer. Carbon dioxide is the most used supercritical fluid because it possesses moderate critical temperature (31.1 °C) and pressure (7.38 MPa), exhibit convenient intrinsic properties (inert, non- toxic, non-inflammable), and it is inexpensive, abundant, and easily separated from the extracted compounds (Rosa et al., 2009). Although

changes in pressure and temperature of the supercritical fluid may modify the selectivity of the solvent for the target compounds, supercritical carbon dioxide exhibits a low polarity and therefore, it is poorly efficient to extract polar compounds. However, this limitation can be overcome by the incorporation of polar co-solvents (such as ethanol and water) to the supercritical carbon dioxide.

3.3.2.4. Microwave-Assisted Extraction (MAE)

Microwaves are electromagnetic waves, such as light, consisting of a system with an electric and a magnetic field, oscillating orthogonally to each other. MAE consists of a non-ionizing radiation in the range of frequencies from 300 MHz up to 300 GHz with two effects; ionic migration and dipole rotation (Chemat et al., 2020; Martinez et al., 2020). The combination of both effects causes the heating of the system, accelerate the solvent penetration into the matrix and facilitate the migration of ions initially contained inside the matrix to the solvent. Hence, the extraction yield of target compound from plant matrices can be increased by MAE.

There are other intensification techniques such as ohmic, infrared and ultraviolet (UV) heating, pulsed electric field, high voltage electrical discharge, etc. However, the aforementioned techniques have been not described in this work since they are less common methods or are outside the focus of this work.

3.3.3. Nuts as source of phenolic compounds

Among the plant matrices suitable for being extracted to obtain bioactive substances, the agro residues from nuts (walnuts, pistachios and almonds) has gained attention since they are a rich source of antioxidants that can beneficially impact health outcomes, in addition may lower low-density lipoprotein-cholesterol levels (Fregapane et al., 2020). A very large number of phenolic compounds, linked to antioxidant activities, are present in common nuts; varying widely among nut species and including from simple (i.e. phenolic acids and flavonoids) to highly polymerized substances (i.e. tannins) in very different quantities.

In this context, large amounts of cake are currently produced as by-product of oil extraction (by pressing methods) from walnuts. The press-cake is often discarded without any economic use or used as animal feed or fertilizer (Moghadam et al., 2020). However, walnut press-cake still represents an important source of proteins, polysaccharides and particularly of

bioactive compounds, such as polyphenols than can be recovered for being used in several food and nutraceutical applications. Since this by-product is locally generated in Nouvelle-Aquitaine, this work has been focused on walnut press-cake as a source of polyphenols to later enrich edible oils. In Table I-7 is shown several works which have reported the extraction of phenolic compounds from several nuts using diverse extraction methods and solvents.

Table I-7. Extraction of phenolic compounds from nuts by several methods

Nut type	Extraction technique and solvent	Total Phenolic Content	Reference
Almond (skin)	MAE. water/ethanol (30:70, v/v)	86.4 mg of QE/g of skin	Valdeś et al., (2015)
Almond (kernel)	UAE. water/methanol (30:70, v/v)	0.20–1.39 mg GAE/g nut	Colic et al., (2017)
Hazelnut (defatted kernel)	Maceration. water/ethanol (20:80, v/v)	23.2 mg of CE/g of extract	Alasalvar et al., (2006)
Hazelnut (skin)	UAE. water/formic acid (99:1, v/v)	40.1–120.7 mg GAE/g nut	Del Río et al., (2011)
Macadamia (kernel)	MAE. Water	45 mg/g of skin	Dailey et al., (2016)
Peanut (skin)	Maceration. water/ethanol (20:80, v/v)	130.1 mg/g of skin	Yu et al., (2005)
Walnut (kernel)	UAE. water/acetone (40:60, v/v)	24.6 mg GAE/g nut	Regueiro et al., (2014)
Walnut (kernel)	UAE. water/methanol (40:60, v/v)	7.7 mg GAE/g nut	Slatnar et al., (2015)
Walnut (defatted press-cake)	Ultra-Turrax. methanol:water (80:20)	58.2 mg GAE/g	Bakkalbasi et al., (2018)

Walnut (kernel)	Vortex + UAE. water/methanol/metha noic acid (20:80:0.1, v/v/v)	10.8 mg GAE/g nut	Fregapane et al., (2020)
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GAE: gallic acid equivalent, QE: quercetin equivalent, CE: catechin equivalent

4. Phospholipids

As it has been previously stated, phenolic compounds exhibit a poor solubility in oils which may limit their antioxidant activity in these systems. Nonetheless, it has been reported that oils enriched with phospholipids (with lecithin, for instance) exhibit a greater capacity for extracting phenolic compounds from plant matrices than oil without phospholipids (Yara-Varón et al., 2017; Li et al., 2015). On the other hand, the complexation of phenolic compounds with phospholipids is also a known method for improving the solubility of phenolics (Ghanbarzadeh et al., 2016). From that perspective, this work has focused on using phospholipids for improving the solubility of phenolics in camelina and sunflower oil, and therefore enhance the oxidative stability of these oils.

4.1. Structure, main characteristics, and importance

Phospholipids are a class of lipids that are the major component of all cell membranes. They exhibit the ability of forming lipid bilayers and serving as a vehicle of other molecules thus, they have been used for designing, flexible and suitable for human body requirements, delivery systems (Van Hoogevest & Wendel, 2014). Phospholipids are also surface-active molecules and therefore are used in the preparation of many types of formulations, such as fat emulsions, mixed micelles, suspensions, and liposomal preparations. Phospholipids have an amphiphilic character characterized by a polar headgroup and two hydrophobic fatty acid tails, what gives phospholipids the ability to be miscible in both water and lipid environments, in which the oxygen atom of the phosphate group has a strong tendency to gain electrons and the nitrogen atom to lose electrons (Hou et al., 2013).

Phospholipids are comprised of a glycerol molecule connected to two fatty acids (charged tail groups), and the third remaining site in glycerol is occupied by a phosphate group (Figure I-19). Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine are the major phospholipids that are mostly used in chemical preparations and several applications. Among these phospholipids, phosphatidylcholine is more used than the others (Ghanbarzadeh et al., 2016).

Since industrially produced phospholipids are accessible, the main sources are sunflower, soya, rapeseed, and chicken eggs. However, the most used phospholipids are those obtained from soya bean containing higher proportions of phosphatidylcholine (over 76%) with

a high content of polyunsaturated fatty acids like LA (about 70%), ALA and oleic acid (Khan et al., 2013).

Phosphatidylcholine is a group of phospholipids with the phosphate group esterified with an additional alcohol (choline) and contains one saturated and one unsaturated chain in its structure (Figure I-19). Naturally occurring phospholipids integrate a saturated fatty acid such as palmitic acid or stearic acid in position 1 and an unsaturated fatty acid such as linoleic acid, oleic acid, or arachidonic acid in position 2 (Ghanbarzadeh et al., 2016).

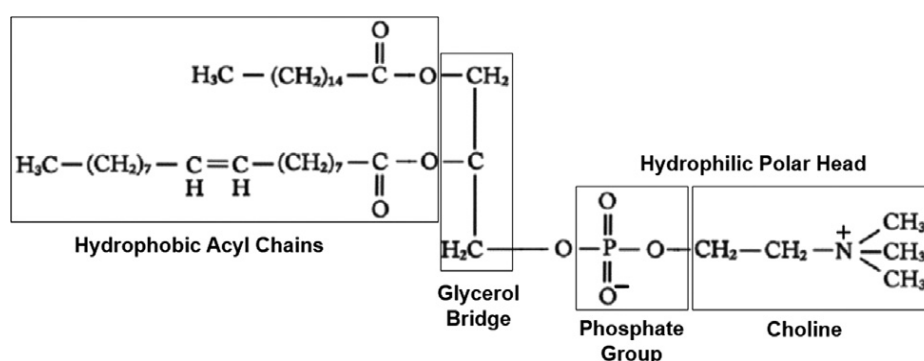


Figure I-19. Chemical structure of phosphatidylcholine. (Source: Ghanbarzadeh et al., 2016)

Among its biological properties, phosphatidylcholine from soybean, is generally used as a nutritional supplement to help support liver and brain health, however the importance of phosphatidylcholine is attributed to their main role in the biological membranes (Hou et al., 2013). Biodegradability, metabolic activity, and low toxicity and cytotoxicity are other outstanding advantages of using of phosphatidylcholine in comparison with its synthetic alternatives; unnatural emulsifiers such as polysorbates, polyoxyethylene castor oil derivatives, and sucrose fatty acid esters (Van Hoogevest & Wendel, 2014).

4.2. Self-assembly of phospholipids related to oil oxidation

Due to the amphiphilic character of phospholipids, they can self-assemble in water or oil into a variety of structures. The nature of those structures depends on the phospholipid structure and the dissolution medium. In oils, phospholipids generally form reverse micelles when their concentration exceeds the critical micelle concentration (CMC), with the polar phosphate headgroups pointing into the hydrophilic core of the reverse micelle and the nonpolar tails pointing toward the hydrophobic oil (Hancer et al., 2002; Gupta et al., 2001). This structuration of phospholipids in bulk oils is due to the natural water content (200 –

2000 ppm) of vegetable oils (Zhao et al., 2020). In the reverse micelles the nanoscale aqueous core is stabilized by a monolayer of surfactant molecules as shown in Figure I-20.

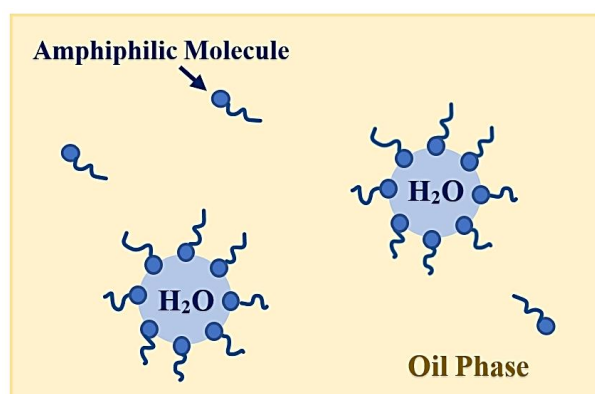


Figure I-20. Scheme of the reverse micelle assembly of phospholipids in oil

In other words, CMC is a measure that allows to determine the concentration at which surface-active molecules form association colloids, and more specifically, form reverse micelles in non-aqueous media (Kim et al., 2019). These formations are predominant in surfactants with low hydrophilic-lipophilic balance (HLB) as free fatty acids, diacylglycerols and monoacylglycerols and also in intermediate HLB surfactants such as phospholipids (Laguerre et al., 2011).

Globally, reverse micelles are dynamic, nanoscopic, roughly spherical aggregates which allow thermodynamically advantageous characteristics in comparison with surfactants monomers dispersed in oil or water (Zhao et al., 2020; Chaiyasit et al., 2007). They are efficient transport systems of bioactive molecules, model environments for biological membrane-bound proteins and nano-reactors that allow increased interactions between lipid and water-soluble components and thus may alter significantly chemical reaction rates (Chaiyasit et al., 2007). Moreover, reverse micelles also have the ability of increasing the solubility of different molecules and hydrophilic materials (Chen et al., 2010), and have influence on preservation of vegetable oils in food applications (Cui & Decker, 2016).

When reverse micelles are formed in a bulk oil, this latter begins to acquire properties of a multiphase system which can be convenient for improving the solubility of lipophobic molecules and for modifying the physical location of antioxidants in bulk oils bringing them closer to the site where oxidation reactions take place (Cui & Decker, 2016). On one hand, since some components of vegetable oils (as free fatty acids, monoacylglycerol, phospholipids)

and hydroperoxides are surface active, they participate in the formation of colloidal structures in which can interact with polar initiating species (as transition metals), causing them to decompose into free radicals that propagate the lipid oxidation reaction (Chaiyasit et al., 2007). On the other hand, it is also considered that surface active compounds enhance the formation of micelles that entrap amphiphilic antioxidants and bring them to the oil-water interphase to protect against lipid oxidation (Budilarto & Kamal-Eldin, 2015). Moreover, when polar antioxidants are incorporated to bulk oils, they are preferentially located at the interface of colloidal structures thus are more effective in inhibiting lipid oxidation than the nonpolar antioxidants that are dissolved in the lipid phase (*polar paradox theory*). In Figure I-21 is schematically represented the aforementioned statements related to the distribution of polar and non-polar antioxidants and the colloidal associations formed in bulk oils.

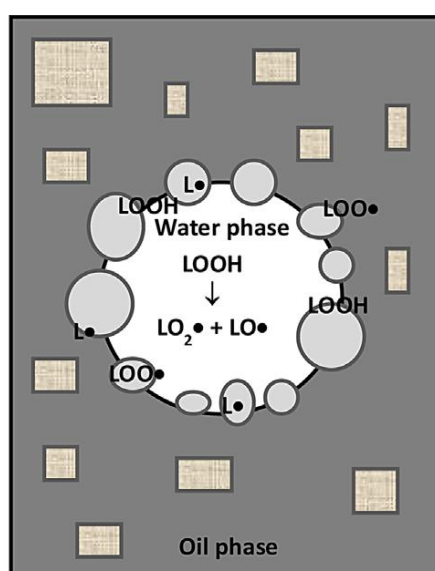




Figure I-21. Schematic representation of the distribution of hydrophilic  and hydrophobic  antioxidants in bulk oil and the microenvironment with hydroperoxides (LOOH) and water.

(Source: Budilarto & Kamal-Eldin, 2015)

Phospholipids such as phosphatidylcholine has been reported to have an antioxidant (Cardenia et al., 2011), prooxidant (Cui et al., 2014) or have no effect (Zhao et al., 2020) on the oxidative stability of lipid systems. From that perspective, the mechanism related to oxidative stability allowed by phospholipids in fat systems is controversial (Cui & Decker, 2016; Ramadan, 2012; Cardenia et al., 2011). The literature reports that the prooxidative effect of phospholipids is mainly related to the fact that: i) phospholipids themselves can be oxidation substrates owing to the presence of unsaturated fatty acids such as LA and ALA in their

structure, ii) micelles formed by phospholipids create oil-water interfaces where both hydrophilic prooxidants, such as iron and amphiphilic ones, such as hydroperoxides, come into close contact with triacylglycerides and therefore increase the lipid oxidation rate, iii) phospholipids may protect natural photosensitizers such as chlorophyll, which retard the decomposition of chlorophyll in the bulk oil and therefore extent its photooxidation effect (Cui & Decker, 2016).

Similarly, various mechanisms of action of phospholipid for exerting an antioxidant effect against lipid oxidation can be also found in literature (Cui & Decker, 2016; Ramadan, 2012; Cardenia et al., 2011):

- A specific synergism between phospholipids and minor compounds of bulks oil such as tocopherols and antioxidants, thanks to their ability of regenerating tocopherol and/or alter the physical location of antioxidants.
- The ability of phospholipids for chelate metals due the presence of negative charges on the phosphate head groups.
- The antioxidative properties of phospholipid Maillard reaction products formed by the reaction of carbonyls and free amine groups.
- The scavenging of free radicals through the reaction of phospholipids with peroxy radicals and the decomposition of hydroperoxides.

4.3. Polyphenol-phospholipid complexation

Phospholipids can be convenient carriers by acting as a building material of structures which contain bioactive compounds, in order to enhance the absorption across lipid-rich biological barriers and therefore improve the nutraceutical and therapeutic effects of the bioactive (Hou et al., 2013). Moreover, carriers made of phospholipids such as phosphatidylcholine also exhibits important benefits in food applications: i) protection of bioactive molecules from harsh conditions of processing, ii) increase the solubility of bioactive ingredients in an aqueous or lipophilic medium, iii) control the release rate of the antioxidants compounds for delaying lipid oxidation and, iv) improve the bioavailability of the carried ingredient (Ghanbarzadeh et al., 2016; Patel et al., 2014).

The alternative of complexing plant compounds with amphiphilic molecules such as phospholipids has been considered as one of the most successful methods for improving the

solubility, bioavailability, stability, and therapeutic efficacy of several plant constituents (Khan et al., 2013). Globally, this method is based on the entrapment, linking or incorporation of a nutraceutical constituent of plants in the phospholipid structure. Phenolic compounds, or any other bioactive molecule, are linked to the phospholipids, such as phosphatidylcholine, by means of the H-bonds from the polar sections. The choline head of the phosphatidylcholine is a polar group and make links to phytoconstituent by forming hydrogen bonding, while the lipophilic and nonpolar phosphatidyl group, then wraps the choline-bioactive complex (Ghanbarzadeh et al., 2016; Khan et al., 2013). Khan et al. (2013) have also reported that in a phyto-phospholipid complex, the encapsulation of bioactive molecule takes place in the polar head of phospholipids since experimental studies have revealed the fact that molecules possessing conjugated systems of π electrons, such as flavonoids, are capable of forming different complexes with cellular phospholipid molecules. In addition, because phospholipids have the ability to unify themselves into several assemblies where, the phytoconstituent could be entangled, structures such as shown in Figure I-22 are obtained.

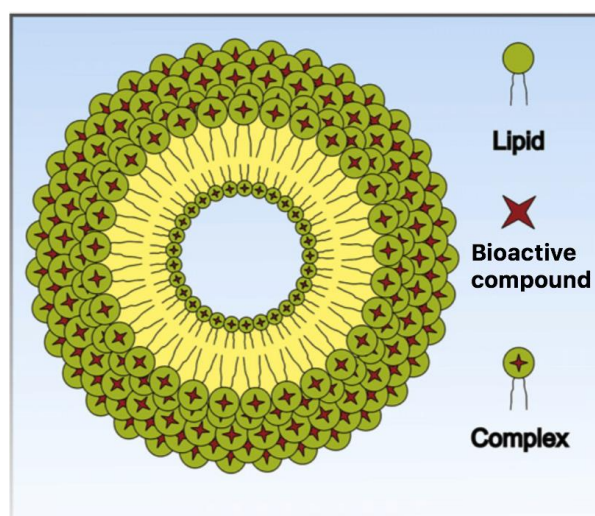


Figure I-22. Structure of a phyto-phospholipid complex micelle (Adapted from Khan et al., 2013)

4.3.1. Methods for forming a polyphenol-phospholipid complex

Firstly, for producing a polyphenol-phospholipid complex three principle ingredients are involved: phospholipids, phyto-active compounds (phenolic compound), and solvents. Generally, the complex between specific active compounds with phospholipids has been performed at different molar ratios ranging from 0.5:1 to 3:1 (Ghanbarzadeh et al., 2016).

However, in most of the research works a stoichiometric ratio of 1:1 has been considered most suitable for formulating a complex (Khan et al., 2013). Globally, phyto-phospholipid complexes are fabricated by mixing either pure bioactive compound or natural plant extracts and phospholipids in an organic solvent, then, after creation of complex, it should be separated from solvent. The complex can be prepared by different techniques which are carried out individually or are combined in a unique process; solvent evaporation, anti-solvent precipitation and/or mechanical dispersion techniques (Gnananath et al., 2017, Khan et al 2013). The most common technique for the phyto-phospholipid complexation found in literature include the solvent evaporation method and it was the technique used in this work.

Solvent evaporation method

This technique comprises the addition of both the phytoconstituents and the phospholipids source in a flask containing organic solvent as reaction medium. This reaction mixture is maintained for a specific time interval (about 1 hour) in order to achieve the maximum entrapment of the compound into the complex formed (Ramadan, 2016). During complexation process the mixture is maintained at temperature between 40 and 45 °C which offer a proper dissolution of both polar lipids and phenolic compounds (Ramadan, 2016). Nonetheless, complexation time and temperature can be optimized for the different phyto-phospholipid complexes in order to obtain maximum yield and drug entrapment. After reaching the complexation time, the organic solvent is then removed under vacuum using a rotary evaporator. This allows the formation of a polyphenol-lipid thin film which can be latter lyophilized and stored (Ramadan, 2012) or sieved (Gnananath et al., 2017). In the salting out anti solvent precipitation method, an anti-solvent like n-hexane is added to the complex film for precipitating it and recovering by filtration.

In Table I-8 it has been listed several works which have formed a phyto-phospholipid complex by several techniques and for a wide range of phytoconstituent. Although the studies shown in Table I-8 are mainly focused on pharmaceutical applications, what it is important to highlight is that the enhanced solubility of bioactive compounds by complexation with phospholipids has been rarely assessed in bulk edible oils. Nevertheless, the listed works are useful as precedents of the ability of phospholipids to i) enhance the solubility of polyphenols both in aqueous and lipid mediums (biological barriers/membranes can be considered mainly lipid systems), ii) improve the antioxidant activity of bioactive compounds both *in-vitro* and

in-vivo, iii) enhance the bioavailability/absorption of biological compounds which is key factor for producing nutraceutical or enriched foods.

Table I-8. Different applications of polyphenol-phospholipid complexation using various techniques.

Author	Different phospholipid complexes	Technique	Solvents	Application	Results
Saoji et al., (2017)	Bacopa extract + soy phosphatidylcholine	Solvent evaporation	Ethanol, n-hexane	Improve the solubility and permeability of standardized Bacopa extract	The complexed Bacopa exhibited a significantly higher aqueous solubility, ex-vivo permeation and in-vitro dissolution (release rate)
Zhang et al., (2016)	Quercetin-soy phosphatidylcholine	Antisolvent precipitation	Anhydrous ethanol, n-hexane (precipitation)	Bioavailability and protection effects against carbon tetrachloride-induced hepatotoxicity in SD rats	A significant increase of the solubility of complexed quercetin in water (13-fold). A greater absorption of the complex suggested the improved bioavailability and provided better protection to rat liver than free quercetin at the same dose
Saoji et al., (2016)	Phospholipid-Based Complex of Standardized Centella Extract	Antisolvent precipitation	Ethanol, n-hexane	Preparation and evaluation of phospholipid complex for the enhanced delivery of Centella extract phytoconstituents	The complex significantly improved the releasing, solubility in water (12-fold) and permeation of the extract, compared to the pure extract, or the physical mixture
Shalini et al., (2015)	Phytosome complex (phosphatidylcholine) of methanolic extract of <i>Terminalia arjuna</i>	Antisolvent precipitation	Methylene chloride and methanol, n-hexane (precipitation)	Antiproliferative effect of Phytosome complex on Human Breast Cancer Cell Lines	The complex is active pharmacologically and exerted more antiproliferative effect on cells as compared to pure methanolic extract of plant
Das & Kalita, (2014)	Rutin-phosphatidylcholine (egg yolk)	Solvent evaporation	Dichloromethane (phospholipids), methanol (rutin)	Transdermal application	The complex showed higher aqueous solubility than pure rutin. Higher permeability of the complex in rat abdominal skin over pure rutin

Chapter I: Literature review

Khan et al., (2014)	Luteolin-hydrogenated soy phosphatidylcholine	Solvent evaporation	Ethanol	Improvement in solubility and in-vitro diffusion profile of luteolin by luteolin-phospholipid complex and in-vivo anti-inflammatory potential of the complex	The complex was more effective than the pure component against inflammation. The solubility of the complex was higher than the solubility of pure luteolin in water
Shaji & Iyer, (2012)	Quercetin- β -cyclodextrin-phosphatidylcholine and quercetin- β -cyclodextrin--cholesterol	Solvent evaporation	Methanol for quercetin and chloroform for lipids	Improvement of quercetin entrapment and bioavailability	The antioxidant activity of liposomes was 50-times more than only quercetin. The entrapment of quercetin was enhanced and therefore, its <i>In-vitro</i> release was higher
Singh et al., (2012)	Quercetin-soy phosphatidylcholine	Antisolvent precipitation	Dichloromethane, n-hexane (precipitation)	Antioxidant activity	The water solubility of quercetin was improved by 12 folds in the prepared complex. No statistical difference between the quercetin complex and quercetin in the <i>In vitro</i> antioxidant activity
Nasibullin et al., (2002)	Quercetin-phosphatidylcholine (egg yolk). 0.005 M of phosphatidylcholine and various concentrations of quercetin	Non-specified	Non-specified	Study of the interaction between both components by NMR and quantum chemistry	The authors highlighted the ability of quercetin and phosphatidylcholine to form chain structures linked by hydrogen bonds

4.3.2. Polyphenol-phospholipid formulations for enriching oils

This section aims at describing the works found in literature related to the enrichment of edible oil with polyphenol-phospholipid formulations for enhancing oil oxidative stability. From that perspective, we have separated this section in two parts; i) works dealing with the incorporation of phospholipids (lecithin) to edible oil either to enhance the extraction of phenolics from plant matrices, or increase dissolution and stabilization of phenolic extract in the oil and, ii) works related to the formation of polyphenol-phospholipid complex (by methods such as solvent evaporation or anti-solvent precipitation method) for being later added to edible oils.

I) Lecithin for enhancing phenolic extraction and stabilization

Several authors have reported the improvement of the solubility of phenolic compounds in oils when rich-phospholipids additives were added to the oil (Yara-Varón et al., 2017; Li et al., 2015; Delgado-Adámez et al., 2014).

Delgado-Adámez et al. (2014) evaluated strategies for the development of a virgin olive oil enriched with extracts of olive leaf /cake and lecithin (6 mg/mL) in order to increase the necessary dose of the diet in phenolic compounds. The enriched oil significantly increased the content in phenolic compounds, antioxidant potential and oxidative stability 40, 4 and 1.5-fold more, respectively, than the control oil. Furthermore, the addition of lecithin had a positive effect both on the phenolic compounds content (2.2-fold), and on the oxidative stability of olive oil (increasing approximately 40%) assessed by Rancimat method. These authors (Delgado-Adámez et al., 2014) attributed this behavior to the ability of lecithin to stabilize the phenolic compounds in the bulk oil.

Li et al. (2015) studied the use of lecithin, and other surfactants, for improving the extractability (solubility) of phenolic compounds from olive leaves in refined sunflower oil. The authors reported that the addition of lecithin (5% w/w) could increase the phenolic extraction yield by a factor of 5 thanks to stronger interaction between oil and phenolic compounds when colloidal structures were formed. Li et al. (2015) stated that the self-assembled micellization in vegetable oils may help to develop an in-site direct extraction of hydrophilic antioxidants. According to Yara-Varón et al. (2017), in the oil enrichment, the solubilization efficiency, selectivity and enrichment factors are variable depending on the oil type and composition, as well as the amount and type of surfactant such as the phospholipids.

The simulation of Yara-Varón et al. (2017) showed that the solubility of polyphenols in plant oils can be enhanced by the incorporation of food additives such as surfactants (soy lecithin, diglycerides, etc.). Suárez et al. (2011) evaluated the use of lecithin (3 mg/mL) in a nutraceutical olive oil enriched with phenolic compounds from freeze dried olive cake (7 mg/mL) to improve the dissolution and stabilization of the phenolic extract in the oil. They reported a significant improvement (12% of increment) on the oxidative stability of the enriched oil evaluated by means of Rancimat method. The authors attributed this behavior to the ability of lecithin to stabilize the added phenolic compounds in the oil matrix due to its amphiphile behavior by forming reverse micelles.

On the other hand, Günal-Köroğlu et al. (2019) evaluated the enhancement of sunflower oil stability during deep-frying by enriching it with extracts from olive oil by-products (1 mg/g oil) and soy lecithin (5 mg/g oil). According to their results, the addition of olive extracts combined with lecithin inhibited lipid deterioration, retarded lipid oxidation and showed a slight darkening of sunflower oil. A lower formation rate of conjugated dienes and secondary products of oxidation were observed in oil enriched with polyphenol-lecithin formulations. In addition, Günal-Köroğlu et al. (2019) observed a slight protection against peroxidation of sunflower oil when the extracts were individually applied without the lecithin addition. Similarly, Günal & Turan, (2018) studied the effect of olive wastewater and pomace extracts (1-2 mg/g), lecithin (1-5 mg/g), and ascorbyl palmitate (0.2 mg/g) on the oxidative stability of refined sunflower oil under accelerated conditions of oxidation. Their results showed that the addition of polyphenol-lecithin formulation significantly increased the induction period of oxidation in a dose-dependent way. Lecithin (from 1 to 5 mg/g) alone also exhibited protection against lipid oxidation and a significant synergic action was observed between phospholipids and phenolic antioxidants.

II) Polyphenol-phospholipid complex for oil enrichment

In this context, Ramadan, (2008) has evaluated the antioxidative activities of native soy lecithin and mixtures of quercetin and lecithin (1:1 w/w) in the protection of triolein models stored under accelerated oxidative conditions for 15 days in the dark at 60 °C. The author reported that oxidative stabilities of quercetin–lecithin-enriched models were better than in models containing lecithin or quercetin alone, most likely as a consequence of synergism between polar lipids and quercetin. Similarly, Ramadan, (2012) studied the antioxidant and antiradical characteristics of native soy lecithin and phenolipids (complexes of quercetin and

lecithin, 1:99 and 3:97, w/w) in the protection of triolein and sunflower oil models stored under accelerated oxidative conditions for 15 day at 60 °C. This author reported that oxidative stabilities of models enriched with the complex were better than in models containing lecithin or quercetin alone, and that increases in concentration of quercetin in the complex resulted in an increase in its antioxidative potential. Chen et al. (2015) reported a strong antioxidant activity exhibited by an epigallocatechin gallate-soybean lecithin complex which effectively inhibited the oxidation of soybean oil and Yang et al. (2015) found an enhanced oral bioavailability and prophylactic effects on oxidative stress and hepatic damage of an oil solution containing a rosmarinic acid–phospholipid complex. Yang et al. (2015) observed that the complex markedly increased the antioxidative properties compared with the non-complexed rosmarinic acid. Laguerre et al. (2011) studied the antioxidant properties of chlorogenic acid and its alkyl esters in stripped corn oil in combination with dioleoylphosphatidylcholine (DOPC). They found that a combination of DOPC and phenolic compounds leads to an antagonistic effect with hexadecyl ester, but it does not significantly change the antioxidant capacity of chlorogenic acid. The authors (Laguerre et al., 2011) concluded that intrinsic parameters such as the hydrophobicity of lipophilized phenolics do not exert a strong influence on antioxidant capacity, but they can be highly influential if potentialized by extrinsic factors such as physical structures in the oil.

It is important to highlight that the studies afore mentioned in this section used organic solvents to simultaneously dissolve the phytoconstituent and the phospholipid, prior to oil enrichment or as a previous step to fabricate a polyphenol-phospholipid complex. On the other hand, and in the best of our knowledge, few studies have been focused on the enrichment of oils, with various polyphenol-phospholipid formulations without the use of organic solvents.

Finally, other authors who are worth mentioning, have studied the antioxidant effect of enriching oily systems, mainly emulsions, with phenolipids, i.e. compounds formed by means of esterification of phenol acids with alkyl esters of several chain length. Although these synthesized compounds did not involve phospholipids, they exhibit similarities with the phenolic-phospholipid complex regarding the improvement of phenol solubility due their amphiphilic character. Among the synthesized phenolic esters already studied, it can be found sinapic acid (Da Silveira et al., 2020; Szydłowska-Czerniak et al., 2018), ferulic acid (Szydłowska-Czerniak et al., 2018; Sørensen et al., 2015), caffeic acid (Szydłowska-Czerniak et al., 2018), chlorogenic acid (Laguerre et al., 2009), epigallocatechin gallate (Nikoo et al.,

2018; Zhong et al., 2012) and rosmarinic acid (Laguerre et al., 2010). In general, these authors successfully enhanced the antioxidant activity of the phenolic esters in the lipid system with a marked influence of the chain length of alkyl esters.

5. Conclusion and addressed topics

Throughout this chapter it has been shown the main factors that influence the characteristic and properties of edible oils which are of high interest for food, nutraceutical and cosmetic industries. Since, sunflower and camelina oil were the oils used in this works to evaluate the solubility of quercetin (flavonoid) and the influence of phospholipids on quercetin solubility and on the oil oxidative stability, we have provided relevant information related to the plant origin of these oils, and the fatty acid and minor composition which play a key role in both the biological properties and the oxidative stability of each oil. The improvement of both quercetin solubility in oils and the oxidative stability of vegetable oils is the key task that has been targeted. Because of that, we have firstly described the mechanism involved in lipid oxidation (autoxidation), the catalytic factors and then, we presented the alternatives for enhancing the oxidative stability of oils (i.e. incorporating natural antioxidants as phenolic compounds). In this context, it has been also carefully displayed the mechanisms of phenolics (in particular of flavonoids) for acting as antioxidants. Such antioxidant effect is positively correlated with the chemical structure of these compounds, although phenolic structure is also correlated to their low solubility in oils which may limit the antioxidant action. In the same way, we have presented an overview of the natural sources from which phenolic compounds can be extracted (giving a special attention to nut residues), as well as the methods (maceration, UAE, among others) used to extract phenolics from plant matrices.

As phospholipids were of high interest in this work for enhancing both the solubility of polyphenols in oils and the oxidative stability of bulk oils, topics such as chemical structure, and self-assembly organization of phospholipids (and its influence on lipid oxidation), and polyphenol-phospholipid complexation were also considered and discussed in this chapter. Finally, we presented a literature review with those works which aimed/evaluated the enrichment of edible oils with formulations of various polyphenols and phospholipids incorporated by different ways, i.e. incorporation of lecithin in oils as co-solvent for enhancing the phenolic extraction, addition of phospholipids to oils for improving the solubility and stabilization of phenolic extracts and the complexation of polyphenols with phospholipids to increase the polyphenol solubility.

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Chapter II: Materials and Methods

1. Materials

1.1. Oil samples

In this study, two different camelina (*Camelina sativa* L.) oils were used; virgin camelina oil from Biocoop, France (called BCoil), and cold-pressed camelina oil provided by Olead, France (called Coil). Refined sunflower oil (called Sfoil) (Bouton d'or-Geprocor, Monaco, France) was purchased in a local market in Bordeaux as well as BCoil. Both Sfoil and BCoil were stored at -18 °C in their original packing (1 L PET bottles and 250 mL amber glass bottles, respectively). Coil samples were placed in 2.5 L amber flasks and stored at -18 °C. Miglyol 810 (Caprylic/Capric triglycerides) was purchased from Stéarinerie Dubois (Boulogne-Billancourt, France) and stored at room temperature.

1.2. Pure polyphenols

Pure polyphenols were purchased from Sigma Aldrich Chemical Co. (St. Quentin Fallavier, France): quercetin ($\geq 95\%$ HPLC, solid, molecular weight: 302.24 g/mol), sinapic acid ($\geq 98\%$, molecular weight: 224.21 g/mol), chlorogenic acid ($\geq 95\%$, molecular weight: 354.31 g/mol), naringenin ($\geq 95\%$, molecular weight: 272.25 g/mol) and gallic acid ($\geq 97\%$, molecular weight: 170.12 g/mol).

1.3. Phospholipids

In this study two different sources of phospholipids were used as carriers. Lipoid P100 (soybean phospholipids which comprises 95.5% of phosphatidylcholine and called PL) was purchased from Lipoid GmbH (Grasse, France) and Lecisoya P97 (68% of phospholipids, mainly phosphatidylcholine (19%) and called LC) was acquired from Novastell, France. The molecular weight of phosphatidylcholine was considered of 775.04 g/mol according to Wang et al., (2015).

1.4. Walnut cake

The walnut cake used in this work as a source of polyphenols was kindly donated by Moulin de la Veyssère (Dordogne, Nouvelle-Aquitaine) (www.moulinde laveyssiere.fr) after oil extraction by pressing under mild heating. The fruits (*Juglans regia* L) were grown locally

and processed on April 2018. The cake was collected the day after the pressing and stored in batches of 1 kg at -18°C . Prior to polyphenol extraction, the walnut cake was ground using a home mill (Essentiel, EMC 1 model, 150 W, France) and sieved below $600\ \mu\text{m}$ (Retsch, Germany). The composition of walnut cake was also determined as follow: $3.34\% \pm 0.13$ of moisture, $27.29\% \pm 0.25$ of protein, 4.57 ± 0.04 of ash, $37.25\% \pm 0.8$ of oil and $27.55\% \pm 0.93$ of carbohydrates (obtained by difference).

1.5. Chemical reagents and solvents

Absolute ethanol (analytical grade), methanol (HPLC grade), chloroform (reagent grade), hexane (96 %, reagent grade) and sodium thiosulfate (0.01 N) were purchased from Scharlab (Barcelona, Spain). Orthophosphoric acid (85 %) was provided by Alfa Aesar (Karlsruhe, Germany), glacial acetic acid by JT Baker (Illkirch, France) and Iotec (iodine indicator) by VWR Chemicals (Leuven, Belgium). The 7,7,8,8 -Tetracyanoquinodimethane was purchased from Merck KGaA (Darmstadt, Germany). Potassium iodide (high purity) and Folin-Ciocalteu reagent (Laboratory Reagent) were purchased from Sigma Aldrich Chemical Co. (St. Quentin Fallavier, France).

2. Methods

2.1. Characterization of edible oils

Chemical characterization of oils was provided by ITERG - Institut des Corps Gras, Canéjan, France. Miglyol, Sfoil, BCoil and Coil were characterized regarding the Vitamin E profile (mg/kg) which corresponds to the tocopherols and tocotrienols content, the phospholipids content, the metals content (mg/kg) comprising copper and iron amount, the total amount of sterols (mg/kg) and the fatty acids profile which comprises the percentage of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) present in the oils. Fatty acid composition was determined according to NF EN ISO 12966.

2.2. Solubility of polyphenol in oils and oil enrichment by quercetin-phospholipid formulations

2.2.1. Determination of the solubility of polyphenols in different oils

In this study the solubility of quercetin in Miglyol, Sfoil and BCoil and Coil was assessed according the following procedure. Although this work focused primarily on quercetin, other polyphenols as naringenin, chlorogenic acid and sinapic acid were also analyzed regarding their solubility in oil.

2.2.1.1. Selection of the most appropriate wavelength for the solubility analysis of quercetin

First, for the selection of the most appropriate wavelength to analyze the quercetin solubility in different oils, a stock solution of quercetin in Miglyol (200 $\mu\text{g}/\text{mL}$) was prepared. For that, 0.004 g of quercetin were weighed in a glass flask using an analytical balance (Model PA 124, Ohaus, Switzerland) and 20 mL of Miglyol were added to the flask with a graduated pipette.

The solution was stirred for 3 hours at room temperature in a magnetic stirrer (Model C-MAG HS 7, Ika, Germany). Less concentrated solutions were prepared from the stock solution (100, 50 and 20 $\mu\text{g}/\text{mL}$) and were stirred for 20 minutes. The four solutions were then filtered using a PTFE filter membrane, pore size 0.2 μm (Rotilabo-syringe filters, sterile, Carl Roth, Germany) and diluted in Miglyol (1:10) to read in a microplate reader (Model Spark 10M, Tecan, Switzerland).

After, aliquots of 200 μL of each solution were transferred to 96-well microplate and were analyzed in microplate reader at different absorbances (200, 250, 300, 350, 374, 400, 450, 500, 550, 600, 650, 700, 750 and 800 nm). As it is observed in Figure II-1, the maximum absorbance of quercetin was at 374 nm as also reported in literature (Razmara et al., 2010).

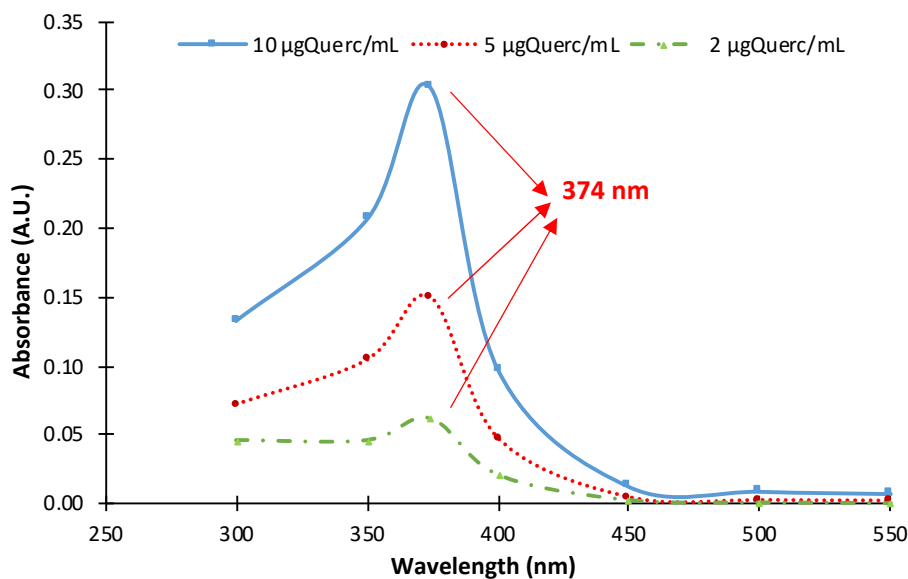


Figure II-1. Wavelength scan of quercetin in Miglyol

2.2.1.2. Preparation of calibration curves of quercetin in oils

A calibration curve was used for the spectrophotometric determination of polyphenol concentration in oil samples. Figure II-2 shows globally the schematic protocol followed for the preparation of calibration curves of quercetin in Miglyol, Sfoil and BCoil.

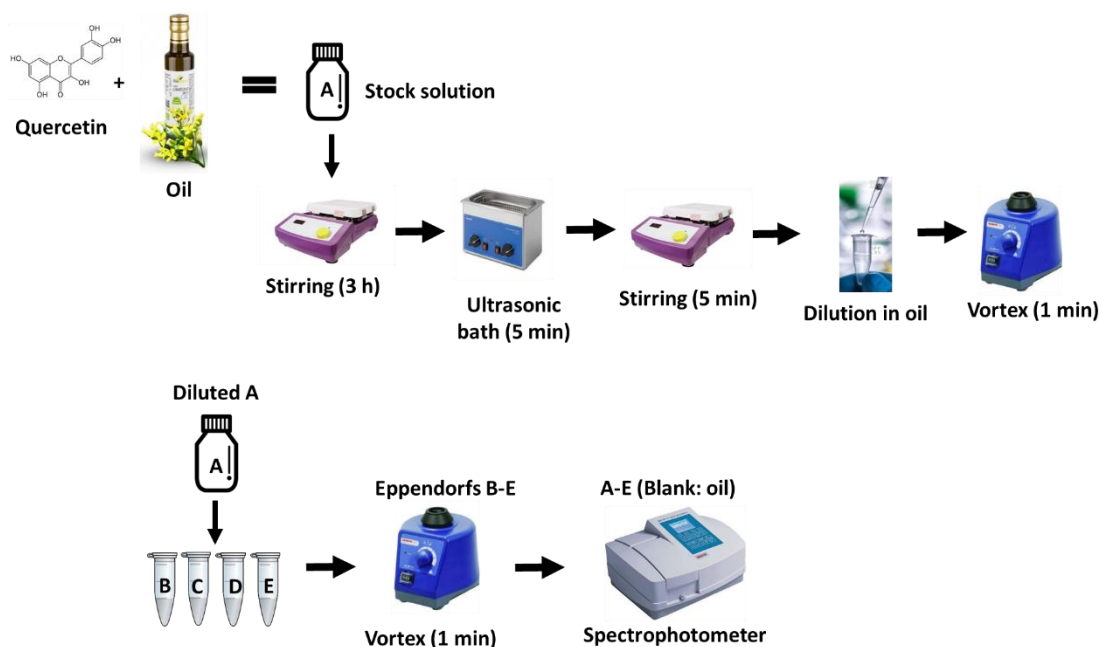


Figure II-2. Procedure to obtain a quercetin-in-oil calibration curve

Firstly, stock solutions of quercetin in BCoil (400 µg/mL), Sfoil (400 µg/mL) and Miglyol (300 µg/mL) were prepared. For that, corresponding amounts of quercetin were

weighed in individual glass flask using an analytical balance (Model PA 124, Ohaus, Switzerland), and 10 mL of oil were added.

The solutions were stirred for 3 hours at room temperature in a magnetic stirrer (Model C-MAG HS 7, Ika, Germany), followed by a sonication for 5 minutes in an ultrasonic bath (Model FB15049, Fisherbrand, Germany) to destroy agglomerates and, stirred again for 5 minutes. The stock solutions were diluted (1:10) and vortexed for 1 minute. Less concentrated solutions from the stock solutions were then prepared ranging a quercetin concentration from 5 to 40 $\mu\text{g/mL}$. The less concentrated solutions were prepared in aluminum covered Eppendorf's and vortexed for 1 minute before reading the absorbance.

Then, aliquots of 200 μL of each solution were transferred to 96-well microplate and the absorbance was read in a microplate reader at 374 nm, according to what was previously reported in section 2.2.1.1. Pure oils were read as a blank. The known quercetin concentration was plotted versus the absorbance at 374 nm and then, the equation obtained from the linear regression was used to calculate, by interpolation, the saturation concentration of quercetin in the different oils.

2.2.1.3. Preparation of oil samples oversaturated with polyphenols

Firstly, two different oversaturated solutions (5 000 and 6 000 $\mu\text{g/mL}$) of each polyphenol (quercetin, naringenin, chlorogenic and sinapic acid) in 10 mL of oil were prepared. After centrifuging and filtrating the oversaturated oil samples, the in-excess solute should be removed and therefore the same polyphenol concentration in both solutions was expected. The flowchart followed for the solubility estimation is presented in Figure II-3. As can be observed, the oversaturated solutions were stirred for 3 hours at room temperature using a magnetic stirrer followed by sonication for 5 minutes in an ultrasonic bath and, again stirred for 5 minutes. The solutions were left in 24 hours of settling time at room temperature to allow in-excess polyphenol to settle in the bottom of the flask. The supernatants of each solution were then transferred separately to 15 mL falcon tubes and centrifuged (Rotanta 460 RF, Hettich, Germany) at 13 300 g for 10 minutes. The supernatants were collected and submitted to a second centrifugation at same conditions.

The supernatant from the second centrifugation of both saturated solutions was collected and filtered on a 0.2 μm PTFE syringe filter (Rotilabo-syringe filters, Carl Roth, Germany). The filtrates were then diluted in oil (1:15 for quercetin in BCoil, 1:20 for quercetin

in Sfoil and 1:40 for quercetin in Miglyol) in order to obtain absorbances values in a range of 0.5 to 0.999. Aliquots of 200 μL of each diluted samples were used to read the absorbance in a microplate reader at 374 nm.

For naringenin, chlorogenic acid and sinapic acid, the filtrates were transferred to quartz cuvettes for reading the absorbance (spectrophotometer Model UH5300, Hitachi, Japan) at 290, 325 and 322 nm, respectively.

Finally, the polyphenol concentration was calculated interpolating the absorbance with the calibration curve previously obtained. The interpolated concentration was normalized by the dilution factor and the solubility of each polyphenol was expressed in μg polyphenols/g oil. Solubility measurements were performed in duplicate.

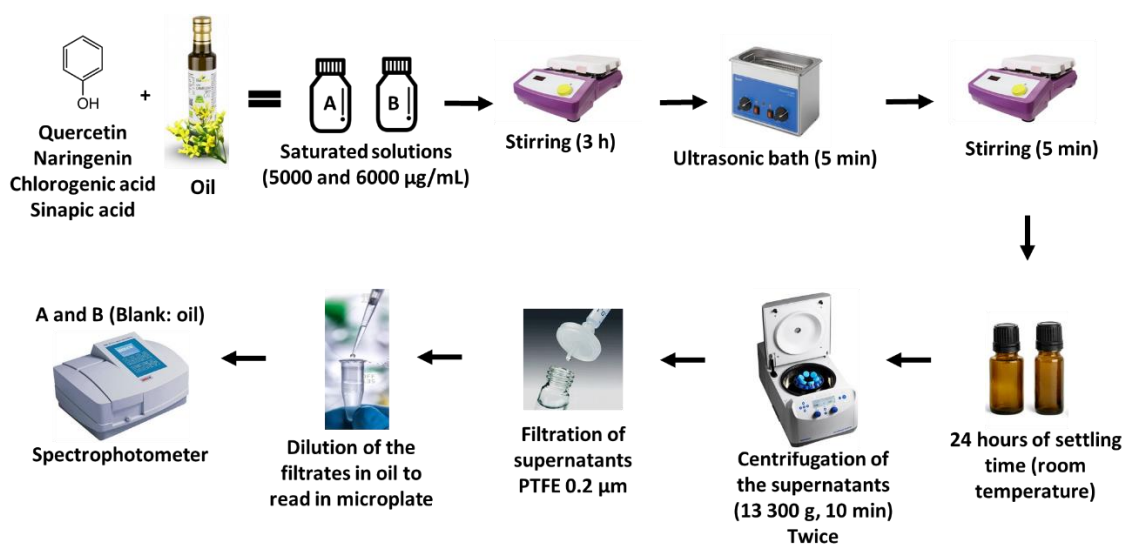


Figure II-3. Protocol for estimating the polyphenol solubility in different oils

Nevertheless, in the course of the progressive enrichment of oil with quercetin described below in section 2.2.5.1, we observed significant discrepancies between the quercetin concentration measured by the previous spectrophotometric method, and the theoretical quercetin concentration. Thus, it is feasible that the overestimation in the results was due to the presence of non-soluble quercetin particles in the stock solution prepared to build the calibration curve already described in section 2.2.1.2.

In this sense, it was necessary to repeat the quercetin quantification in the saturated solutions by several methods in order to validate the quercetin solubility measurements. Hence, two different methods of spectrophotometric analysis were set. Firstly, a new calibration curve

of quercetin in Miglyol was prepared by using a stock solution in a much lower concentration of quercetin than the previous 300 $\mu\text{g/mL}$. This curve was read at 374 nm in spectrophotometer and then used to quantify quercetin in saturated oils. Secondly, quercetin in saturated oils was quantified by Folin-Ciocalteau method at 750 nm (Singleton et al., 1999).

Moreover, High-Performance Liquid Chromatography (HPLC) analysis was also carried out for quercetin quantification. All these methods used for validating quercetin quantification in oil samples are carefully described in the following sections.

2.2.2. Validation of various methods for quercetin quantification in saturated oils

2.2.2.1. Quercetin quantification by using a calibration curve of quercetin in Miglyol

The solubility of quercetin in BCoil, Sfoil and Miglyol was calculated using a calibration curve of quercetin in Miglyol. The protocol used for the preparation of calibration curves is explained in Figure II-4.

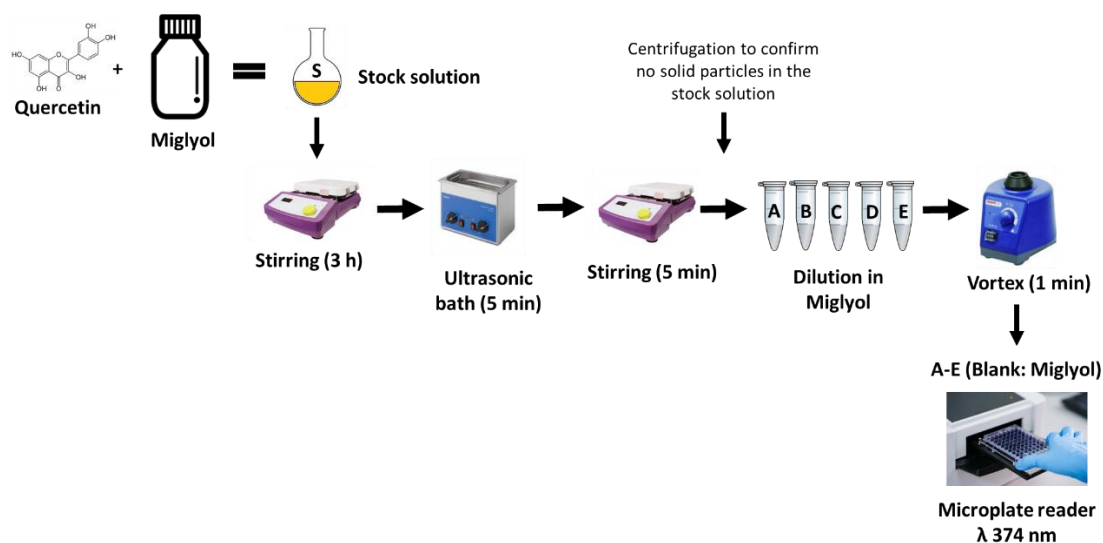


Figure II-4. Procedure to obtain a quercetin-in-Miglyol calibration curve

This protocol consisted of preparing a stock solution of quercetin in Miglyol at a concentration of approximately 40 $\mu\text{g/mL}$. For that, 10 mg of quercetin were weighed in a 250 mL-round bottom flask using an analytical balance which was further gauged with Miglyol. This solution was stirred for 3 hours at room temperature in a magnetic stirrer followed by a sonication for 5 minutes in an ultrasonic bath and stirring for 5 minutes. The stock solution was then centrifuged to ensure that there were no insoluble particles. After

checking that no insoluble particles remained in the bottom of the flask after centrifugation, less concentrated solutions from the stock solution were prepared in a range of 8 to 25 $\mu\text{g/mL}$, using Miglyol as diluent. Aliquots of 200 μL of each solution were then transferred to 96-well microplate and the absorbance was read in a microplate reader at 374 nm using pure Miglyol as a blank. The solutions in those ranges of quercetin concentration exhibited an absorbance at 374 nm below 0.999 and behaved linearly depending on the quercetin concentration, as expected. Therefore, the calibration curve (quercetin concentration versus absorbance at 374 nm) was plotted using Microsoft Excel and the straight-line equation was obtained to calculate the quercetin concentration in the saturated oils.

For preparing the oil samples saturated with quercetin the same protocol described in section 2.2.1.3 was maintained. After diluting properly, the saturated oil samples, for obtaining absorbances between 0.5 and 0.999, aliquots of 200 μL of each sample were read at 374 nm using a microplate reader. The quercetin concentration was calculated interpolating the absorbance of saturated samples (previously diluted) with the calibration curve. Solubility measurements were performed in duplicate and results were expressed in μg quercetin/g oil.

2.2.2.2. Total Phenolic Content (TPC) of oil samples saturated with quercetin

Polyphenol extraction from oil samples

Prior to the TPC analysis, quercetin from the saturated oil samples (BCoil, Coil, Sfoil and Miglyol) was extracted following the protocol described by Rombaut et al. (2014). For comparative purposes, the quercetin of the stock solution of Miglyol prepared in section 2.2.2.1 with a concentration of ~ 40 μg quercetin/mL, was also extracted.

The protocol of Rombaut et al. (2014) consisted of weighing 2.5 g of oil sample in a 10 mL glass flask and add 0.1 g of Tween 20. After, 5 mL of a methanol/water solution (80:20 v/v) were added. The mixture was stirred using a magnetic stirrer for 5 minutes, followed by a sonication for 15 minutes in an ultrasonic bath and, stirred again for 5 minutes. The mixture was then centrifuged at 3 000 g for 10 minutes and then the methanol-water phase (supernatant) was collected in an amber glass flask.

The same extraction cycle, from the addition of 5 mL of a methanol/water solution (80:20 v/v), was repeated twice more. After centrifugation, the three supernatants were pooled

in the same amber glass flask, sealed and stored at 4 °C until TPC analysis. Polyphenols from the pure oils were also extracted. All extractions were performed in duplicate.

TPC analysis by Folin-Ciocalteu method

The TPC of the methanol-water extracts previously obtained from i) the saturated samples of quercetin in BCoil, Coil, Sfoil and Miglyol, ii) the stock solution of quercetin in Miglyol and, iii) the pure oils (BCoil, Coil, Sfoil and Miglyol), were quantified using the method described by Singleton et al. (1999), with some modifications.

Firstly, 800 µL of deionized water were added to 50 µL of methanol-water extract in a 2 mL Eppendorf. After, 50 µL of Folin-Ciocalteu reagent was added to the mixture and vortexed. After 3 minutes in the dark, 100 µL of sodium carbonate (1 N) were added. The solutions were left to stand for 2 hours in the dark and then, aliquots of 200 µL of each solution were transferred to 96-well microplate and the absorbance was then read at 750 nm.

For the quantification of the TPC of the extracts, a calibration curve was built using gallic acid as standard. TPC was determined by interpolating the absorbance of the samples based on a calibration curve built using gallic acid as standard in a concentration range of 0–350 mg/L. The coefficient of determination of the calibration curve was always of 0.999. The TPC of the pure oils were subtracted to the TPC of saturated oil in order to estimate only the concentration of quercetin in each oil. The TPC analysis were performed in triplicate and expressed as µg of Gallic Acid Equivalents (GAE) per g of oil.

2.2.2.3. HPLC analysis for quercetin quantification

The oils saturated with quercetin, the stock solution of quercetin in Miglyol (~40 µg/mL), the pure oils, as well as the methanol-water extracts obtained from these oils in section 2.2.2.2, were analyzed by HPLC following the method described by Xu et al. (2016) with some modifications.

An Agilent 1260 high performance liquid chromatograph (Agilent Technologies, Santa Clara, USA) equipped with a quaternary pump solvent delivery system, an automatic injector and a diode array detector Agilent Infinity G1315D was used. The sample injection volume was adjusted from 2 to 10 µL and the chromatographic separation was carried out on C18 column (Eclipse XDB-C18 3.5 µm, 4.6 x 100 mm, Agilent Technologies France, France)

maintained at 30 °C. The mobile phase consisted of HPLC grade methanol (solvent A) and water with 0.03% phosphoric acid (solvent B) at a flow rate of 1 mL/min. The elution program was as follow: 0 to 5 minutes of 50% A, 5 to 8 minutes of 100% A and 8 to 12 minutes of 50% A. Total run time was set at 12 minutes and the retention time of quercetin was at 4 minutes. Quercetin was quantified at 370 nm using a calibration curve of quercetin in absolute ethanol ranging a concentration of 0 to 20 µg/mL. The stock solution of quercetin in ethanol was prepared at a concentration of 400 µg quercetin/mL. The coefficient of determination of the calibration curve was of 0.999.

Before HPLC analysis all solutions were filtered on 0.45 µm PVDF syringe filter (Rotilabo-syringe filters, Carl Roth, Germany). The oils saturated with quercetin were diluted (in the same oil of analysis) before injection as follows: 1:15, 1:7, 1:20 and 1:40 for saturated BCoil, Coil, Sfoil and Miglyol, respectively. The HPLC analyses were run in duplicate and results were expressed in µg quercetin/g oil.

2.2.3. Determination of the solubility of quercetin in other solvents

Once validated the methods for quercetin quantification described in section 2.2.2, the solubility of quercetin in other solvents such as dimethyl sulfoxide (DMSO), polyethylene glycol 400 (PEG 400), polyethylene glycol 20% (diluted in water and called PEG 400–20%), polyethylene glycol 40% (diluted in water and called PEG 400–40%), absolute ethanol, 1-propanol, cyclopentanol, aniline and water was also evaluated. For that, the protocol for the preparation of samples saturated with quercetin explained in section 2.2.1.3 was followed with some modifications.

Different oversaturated solutions of quercetin in the different solvents were prepared (PEG 400, 200 mg/g; PEG 400-20%, 1.5 mg/g; PEG 400-40%, 2.5 mg/g; DMSO, 400 mg/g; Cyclopentanol, 70 mg/g and 1-propanol, 50 mg/g) in glass flasks. All oversaturated solutions were stirred for 3 hours at 35 °C in a water bath (Model F12, Julabo, France) followed by sonication for 5 minutes in an ultrasonic bath and, again stirred for 5 minutes at 35 °C. The solutions were left for settling overnight at room temperature in 15 mL Falcon tubes. The upper phase was then filtered on 0.2 µm PTFE syringe filter (Rotilabo-syringe filters, Carl Roth, Germany), diluted in absolute ethanol and analyzed by HPLC as described in section 2.2.2.3. Solubility measurements were performed in duplicate and the results were expressed in mg quercetin/g solvent.

As validation analysis, concentrations under the saturation of quercetin in PEG 400 (60, 100 and 150 mg/g), PEG 400-20%, (0.01 mg/g), PEG 400-40% (0.1 mg/g), DMSO (40, 50 and 300 mg/g) and cyclopentanol (30 mg/g) were also analyzed by HPLC.

Due to the high toxicity of aniline, the solubility of quercetin in this solvent was estimate in a visual manner. For that, several concentrations of quercetin in aniline were prepared ranging a concentration of 2 to 25 mg/g. After the settling time, the solubility of quercetin was estimated visually as the concentration in which quercetin particles started to become insoluble.

2.2.4. Formation of a quercetin-phospholipid complex

2.2.4.1. Complexation by a solvent route

The complexation of quercetin with phospholipids by a solvent route was performed according to the protocol described by Ramadan, (2016) and Zhang et al. (2016) with some modifications. In the process, showed in Figure II-5, phospholipids (LC or PL) and quercetin were diluted separately in solvent A and solvent B, respectively. Firstly, chloroform was evaluated as solvent A and methanol as solvent B. After, with the aim of using only one solvent, absolute ethanol was used both as solvent A and solvent B.

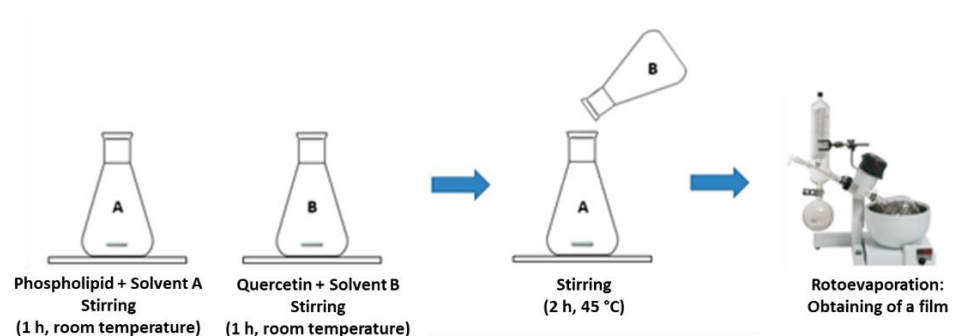


Figure II-5. Protocol for quercetin-phospholipid complexation by a solvent route

The complexation was carried out using a molar ratio quercetin-phospholipid of 1:1 (which corresponds to a mass ratio of 1:2.67). Approximately 0.0267 g of PL were diluted in 70 mL of solvent A, whereas 0.01 g of quercetin were diluted in 30 mL of solvent B. For the complexation with LC, approximately 1 g of LC and 0.09 g of quercetin were used. Solution A and B were stirred separately for 1 hour at room temperature and then, solution B was transferred to solution A. The mixture was stirred in a digital magnetic hotplate stirrer with temperature control (Model SC162, Stuart, UK) at 45 °C for 2 hours. According to

Ramadan, (2016), 45 °C is the most appropriate temperature to dissolve polar lipids and phenolic compounds together. Finally, the mixture was transferred to a 250 mL round bottom flask (previously weighed) and the solvent was removed using a rotary evaporator (Model RE 300DB, Stuart, UK). After obtaining a polyphenol-lipid film, the round bottom flask was flushed with nitrogen, covered with aluminum foil and stored at 4 °C until its further analysis. The complexation experiments were performed in triplicate.

2.2.4.2. Purification of the complex with chloroform and calculation of complexation efficiency (CE)

A purification step was carried out as an additional process aiming at removing the remaining free or non-complexed quercetin in the complex film. The protocol followed for the complex purification is shown in Figure II-6.

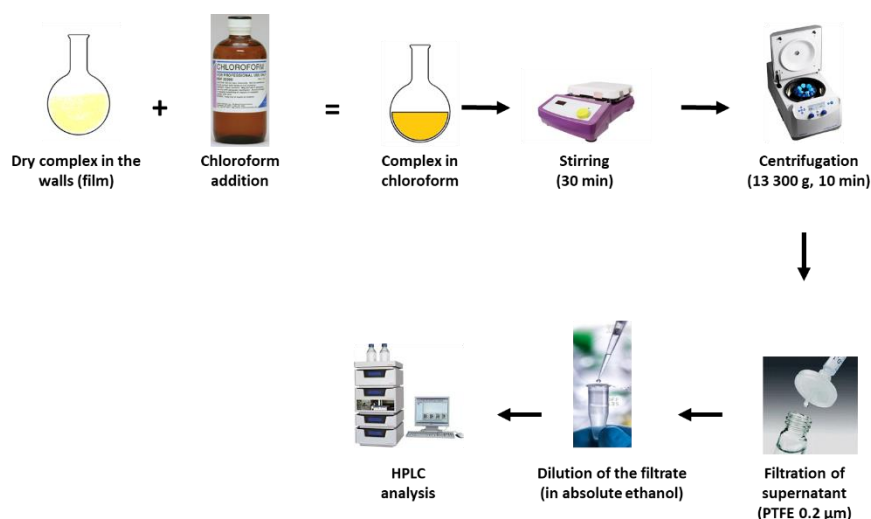


Figure II-6. Schematic protocol for complex purification and complexation efficiency calculation

The protocol consisted of diluting the complex film adhered to the walls of the round bottom flask by the addition of 20 mL of chloroform. Mostly the complexed quercetin solubilizes in chloroform, whereas the non-complexed or pure quercetin does not (Chebil et al., 2010). Therefore, the solution was stirred for 30 minutes and then centrifuged at 13 300 g for 10 minutes. After centrifugation, the free and non-complexed quercetin appeared as a precipitate form, although some particles stayed suspended in the supernatant. For that, the supernatant was filtered using a PTFE filter membrane with a pore size of 0.2 μm. An aliquot

of filtered solution was diluted 150-fold in absolute ethanol and analyzed by HPLC for the quantification of complexed quercetin. Finally, the complexation efficiency (CE) was calculated by the Equation II-1:

$$CE(\%) = \frac{\text{Complexed quercetin (g)}}{\text{Total quercetin (g)}} \times 100 \quad (\text{Equation II-1})$$

where *complexed quercetin* represents the mass of quercetin in the supernatant after filtration and, the *total quercetin* corresponds to the mass of quercetin initially used to fabricate the complex.

2.2.4.3. Assessment of different quercetin-phospholipid mass ratios for the complexation process

In addition to the complexation using a quercetin-PL mass ratio of 1:2.67, the following mass ratios were also evaluated: 2.4:1, 1:1.67 and 1:2. The other conditions of the complexation protocol detailed in section 2.2.4.1 were maintained, and absolute ethanol both as solvent A and solvent B was used. All complexes were also purified with chloroform and the complexation efficiency was then calculated as described in section 2.2.4.2.

2.2.5. Oil enrichment with quercetin-phospholipid complex by solvent route

Camelina oil (only BCoil) was enriched with quercetin-PL complex aiming at increasing the solubility of quercetin in oil. For that, two different solvent methods were carried out for enriching the BCoil with the complex diluted in chloroform (after the purification step). Then, the solubility of complexed quercetin was determined after enriching the oil. In the following sections, the two enriching methods were carefully described.

2.2.5.1. Progressive enrichment of Coil by solvent route

Firstly, a quercetin-PL complex was prepared following the protocol described in section 2.2.4.1, using a molar ratio 1:1 with 0.03 g of quercetin and 0.08 g of PL, employing chloroform as solvent A and methanol as solvent B.

This enrichment process consisted of the progressive addition of approximately 1.5 mL of complex, previously purified and diluted in chloroform as described in section 2.2.4.2, to 7 mL of BCoil. The progressive enrichment consisted of four addition of 1.5 mL of complex.

After each addition, the BCoil was vortexed for 5 minutes for facilitating the dissolution of the complex in the oil. The chloroform was then evaporated by nitrogen flux for 1-2 hours until constant weight of the enriched oil. After solvent evaporation, the samples were vortexed again for 5 minutes and left in settling time for 15 minutes. Finally, an aliquot from the upper phase of the enriched oil was diluted (if necessary) in pure BCoil and read at 374 nm for estimating the complexed quercetin dissolved in the oil. The absorbance of the enriched oil was interpolated by using the calibration curve obtained in section 2.2.1.2. This experiment allowed us to compare the measured and the theoretical quercetin concentration after each cycle of addition. The nomenclatures A1, A2, A3 and A4 were adopted to specify the number of additions of 1.5 mL of diluted complex to BCoil, i.e. A1 corresponds to 1.5 mL of diluted complex added to 7 mL of Coil, A2 to a second addition of 1.5 mL of diluted complex added to the same Coil, and so on. This process was performed in duplicate.

Due to the discrepancies observed between the measured quercetin concentration, by spectrophotometric method (section 2.2.1.2 and 2.2.1.3), and the theoretical quercetin concentration, an “one step enrichment method” was carried out once validated the quercetin quantification by HPLC analysis.

2.2.5.2. One step enrichment by solvent route

For this enrichment, a quercetin-PL complex was prepared at a molar ratio 1:1 using absolute ethanol both as solvent A and solvent B and following the protocol described in section 2.2.4.1.

The one step enrichment consisted of the addition of a single amount of complex, previously purified and diluted in chloroform as described in section 2.2.4.2, to 10 mL of BCoil. The volume of the single amount of complex added to BCoil varied in order to provide the following PL concentrations: 1, 2.5, 10 and 16 mg PL/g BCoil. It was guaranteed that by those additions of complex to BCoil, the quercetin concentration was higher than saturation (> 358 µg quercetin/mL BCoil or 393 µg quercetin/g BCoil).

After the complex addition, the enriched oil was mixed, and the chloroform was then removed. For removing the chloroform, both nitrogen flux and rotary evaporation were used, however no significant differences on quercetin solubility were observed between the two methods. After solvent evaporation, the enriched oil was stirred for 3 hours at room temperature, followed by a sonication for 5 minutes in an ultrasonic bath and, stirred again for

5 minutes. The sample was then transferred to 15 mL Falcon tube and left in 24 hours of settling time at room temperature. The upper phase of the sample was transferred to a new 15 mL Falcon tube and centrifuged at 13 300 g for 10 minutes. The supernatant after centrifugation was collected and filtered on 0.2 µm PTFE syringe filter. The quercetin quantification, i.e. the solubility of the complexed quercetin, was carried out by HPLC analysis following the protocol already described in section 2.2.2.3.

2.2.6. Oil enrichment by a solvent-free route using quercetin-phospholipid formulations

In order to develop a simpler process for enriching camelina oil, without using organic solvents, a solvent-free route using several quercetin-phosphatidylcholine formulations was adopted. Contrary to most studies of oil enrichment (Yang et al., 2016; Chen et al., 2015; Ramadan, 2012; Laguerre et al., 2011; Suárez et al., 2011; Laguerre et al., 2010; Laguerre et al., 2009; Ramadan, 2008), this method does not use an organic solvent to first dissolve the phenolics, the extracts or the phenolic-phospholipid system.

2.2.6.1. Estimation of the solubility of phospholipids in oils

Firstly, different amounts of PL were added to 30 g of Coil, Miglyol or Sfoil in order to provide concentrations between 0.3 and 100 mg PL/g oil. The samples were prepared in sealed glass flasks and flushed with nitrogen to avoid oxidation reactions during enrichment process. The oils were then stirred at 35 °C for 1 hour in a digital magnetic hotplate stirrer with temperature control followed by sonication in ultrasonic bath for 10 minutes and stirred again for 4 hours at 35 °C. After stopping the stirring, it was determined the maximum concentration of PL that can be solubilized in each oil without observing insoluble particles of PL. The results showed that at concentrations higher than 20 mg of PL per gram of Coil, 15 mg of PL per gram of Miglyol and 0.33 mg of PL per gram of Sfoil, cannot be dissolved.

Due to the poor solubility of phospholipids in Sfoil (0.33 mg/g), PL was diluted in methanol at a concentration of 0.5 g/mL and aliquots of this solution were directly added to Sfoil to provide a concentration of 20 mg PL/g oil. The mixture was gently mixed, and methanol was then removed using a rotary evaporator.

2.2.6.2. Solubility of quercetin in oils enriched with phospholipids

Once determined the maximum amount of PL able to be soluble in each oil, several phospholipid concentrations were assessed in Coil, whereas Sfoil and Miglyol were only enriched at their maximum PL concentration, i.e. 20 mg PL/g oil and 15 mg PL/g oil, respectively.

Firstly, different amounts of PL were added to 10 g of Coil for providing the following PL concentrations: 1, 5, 10, 15 and 20 mg/g oil. All samples were then over saturated with quercetin and flushed with nitrogen. The oils were then stirred at 35 °C for 1 hour in a digital magnetic hotplate stirrer with temperature control followed by sonication in ultrasonic bath for 10 minutes and stirred again for 4 hours at 35 °C. Finally, the samples were left for settling overnight at room temperature and the upper phase was then filtered on 0.45 µm PTFE syringe filter and analyzed by HPLC for quercetin quantification. The enriched oils were diluted if necessary, in Coil.

The same afore-mentioned protocol was followed for assessing the solubility of quercetin in Sfoil and Miglyol enriched at 20 and 15 mg of PL per gram of oil, respectively.

The nomenclature CQL1*, CQL5*, CQL10*, CQL15* and CQL20* was adopted to refer to Coil saturated with quercetin at 1, 5, 10, 15 and 20 mg PL/g, respectively. Whereas SQL20* and MQL15* refer to Sfoil and Miglyol saturated of quercetin at 20 and 15 mg PL/g, respectively.

On the other hand, samples labeled CL5, CL10, CL15 and CL20 corresponds to Coil enriched with only phospholipids at a concentration of 5, 10, 15 and 20 mg/g, respectively. SL20 refers to Sfoil enriched with only phospholipids at a concentration of 20 mg/g.

2.2.6.3. Enrichment of Coil and Sfoil with several quercetin-phospholipid formulations

Besides enriching Coil and Sfoil at various PL concentrations and saturated of quercetin, both oils were also enriched with PL below quercetin saturation as described in Table II-1. The same protocol described in section 2.2.6.2 was followed with the difference that after settling time overnight, the enriched oils were not filtered since quercetin content was

completely dissolved in the oil. The various quercetin-phospholipid formulations were carried out in order to study their effect on the oxidative stability of oils.

Table II-1. Quercetin-phospholipid formulations used for enriching Coil and Sfoil

Nomenclature	PL (mg/g oil)	Quercetin ($\mu\text{g/g oil}$)	Oil
CQL10-220	10	220	Coil
CQL15-220	15	220	Coil
CQL20-220	20	220	Coil
CQL20-440	20	440	Coil
CQL20-740	20	740	Coil
SQL20-220	20	220	Sfoil
SQL20-440	20	440	Sfoil

2.2.7. Critical Micelle Concentration (CMC) of PL in Coil

The self-assembly of phospholipids in Coil was investigated using the 7,7,8,8 - Tetracyanoquinodimethane (TCNQ) solubilization method according to Kanamoto et al. (1981). The CMC was determined for both PL in Coil and PL in Coil saturated with quercetin (168 $\mu\text{g/g}$), in order to verify any influence of quercetin on the CMC. In addition, CMC assay in Coil with only quercetin (168 $\mu\text{g/g}$) was also performed to confirm the inability of quercetin to form micelles in the oil.

2.2.7.1. CMC of only PL in Coil

Firstly, 70 g of a stock solution of Coil enriched with PL at 20 mg/g oil was prepared following the solvent-free route protocol. Then, different amounts of the stock solution were added to 10 g of pure Coil to provide solutions ranging a concentration of 4 to 26 000 $\mu\text{mol PL/kg oil}$ (which corresponds to 0.003 to 20 mg PL/g oil). 10 mg of TCNQ were then added to each solution and stirred for 1 hour at 55 °C into a thermostatic water bath. The samples were then centrifuged at 800 g for 20 minutes at room temperature and the supernatants were collected and read at 480 nm in a spectrophotometer using quartz cuvettes. Pure Coil was used as a blank. The CMC was estimated by the inflection point in the curve plotting absorbance as a function of PL concentration (semilog plot). For that, a numerical analysis of data was carried out to determine the intercepting of the two slopes that provided the highest R^2 .

In Figure II-7. can be observed the coloration changes of Coil enriched at various concentration of PL after addition of 10 mg of TCNQ and 1 hour of stirring at 55 °C.

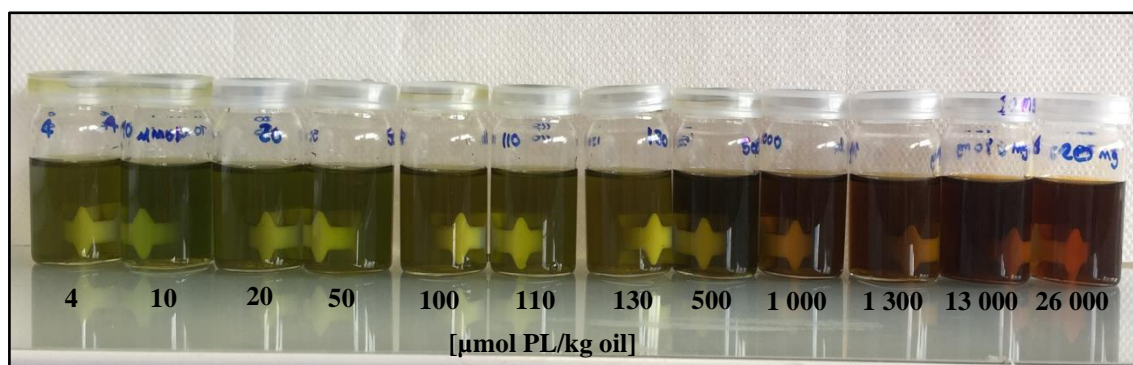


Figure II-7. Coil enriched at various concentration of PL after TCNQ probe

2.2.7.2. CMC of PL in Coil saturated with quercetin

The determination of the CMC of PL was assessed in Coil saturated with quercetin (168 $\mu\text{g/g}$), ranging a concentration of 4 to 26 000 $\mu\text{mol PL/kg oil}$.

Firstly, a stock solution of Coil saturated with quercetin (called stock solution A) was prepared following the protocol already described in section 2.2.1.3. Secondly, a stock solution of PL (20 mg/g) in Coil saturated with quercetin (called stock solution B) was also prepared following the solvent-free route protocol. Then, different amounts of stock solution B were added to 10 g of stock solution A in order to provide solutions at a fix quercetin concentration (168 $\mu\text{g/g}$) and PL concentration ranging from 4 to 26 000 $\mu\text{mol/kg oil}$. 10 mg of TCNQ were then added to each solution and stirred for 1 hour at 55 °C into a thermostatic water bath. The samples were then centrifuged at 800 g for 20 minutes at room temperature and the supernatants were collected and read at 480 nm in a spectrophotometer using quartz cuvettes. Coil saturated with quercetin (168 $\mu\text{g/g}$) was used as a blank. The CMC was also estimated by the inflection point in the curve plotting absorbance as a function of PL concentration (semilog plot).

2.2.7.3. CMC assay in Coil saturated with quercetin

For this experiment, a stock solution of Coil saturated with quercetin (168 $\mu\text{g/g}$) was prepared following the protocol already described in section 2.2.1.3. Different amounts of the stock solution were added to 10 g of pure Coil to provide solutions ranging a quercetin concentration from 8.5 to 168 $\mu\text{g/g oil}$ (which corresponds to 28 to 557 $\mu\text{mol/kg oil}$). 10 mg of

TCNQ were then added to each solution and stirred for 1 hour at 55 °C into a thermostatic water bath. The samples were then centrifuged at 800 g for 20 minutes at room temperature and the supernatants were collected and read at 480 nm in a spectrophotometer using quartz cuvettes. Coil was used as a blank and the absorbance was plotted as a function of quercetin concentration. The results confirmed the inability of quercetin to form micelles in the oil.

2.3. Assessment of the oxidative stability of enriched and non-enriched oils

The oxidative stability of both pure and enriched oils (by the solvent-free route) was assessed through the Schaal oven test (AOCS, 1998). In this sense, pure Coil and Sfoil, as well as their enriched counterparts were submitted to oxidative stability assessment. For that, approximately 7 g of oil were placed in 40 mL sealed amber flasks and submitted to accelerated oxidation at 60 °C in a laboratory drying oven (DRY-Line, VWR, UK) for 6 to 240 hours. An amber flask containing the 7g of oil was periodically collected from the oven and then stored at -80 °C until its analysis. All the enriched and non-enriched oils were oxidized in duplicate and the oxidation was monitored by the conjugated dienes formation following the method described in the following section.

It was previously verified that enough oxygen was available in 40 mL sealed amber flasks for oxidation of ~7 g of oil. For that, 7 g of Coil were placed in sealed and unsealed amber flasks and oxidized at 60 °C for several days.

2.3.1. Conjugated dienes (CD) analysis

Conjugated Dienes (CD) analysis was carried out according to AOCS standard method 2.501 (AOCS, 1998). The principle of the analysis is based on the fact that PUFAs suffer a shift in their double bond position when being oxidized. The results are mixtures of conjugated dienes hydroperoxide products that define disturbance in the structure of lipids when the oxidation takes place (Corongiu & Milia, 1983). This PUFAs oxidation is detected by increased UV absorption at approximately 233-235 nm due to the presence of CD. For that reason, the increase in absorbance is commonly known as an indicator of oxidation (Farhoosh et al., 2012; Chatha et al., 2006).

For CD analysis, approximately 0.05 g of oil samples were weighed in a 10 mL amber round bottom flask using glass micropipettes. Each flask was gauged with hexane using a

plastic pipette, stirred and reserved. The solution was then diluted in hexane in a hermetic vial to avoid solvent evaporation, stirred and transferred to quartz cuvettes for reading the absorbance at 233 nm in spectrophotometer (Model UH5300, Hitachi, Japan). Pure hexane was used as blank. The dilution is dependent on the oxidation state of the oil sample. Absorbance in a range of 0.600 to 0.999 was highly desirable for greater accuracy. CD value was expressed as specific absorbance (A.U. 233 nm/mg/mL) normalizing the optical density by the mass of lipids (mg) per volume of solution (mL).

2.3.2. Peroxide value (PV) analysis

In addition to the CD analysis, the PV of pure Coil and Sfoil submitted to accelerated oxidation was determined. For that, the standard method ISO 3960 (2017) was used. This method allows quantifying primary oxidation compounds (Rombaut et al., 2017; Shahidi & Zhong, 2005) and it is often used to characterize oil degradation (Reboredo-Rodriguez et al., 2017). This technique corresponds to an iodometric titration method, which is based on the oxidation of the iodide ion by hydroperoxides. The reaction of peroxides with potassium iodide releases iodine that is titrated at the end of the process (Antolovich et al., 2002). PV measures the concentration of hydroperoxides that are formed continuously in the initial phase of lipid oxidation.

For the procedure, approximately 2 g of oil sample were weighed in an Erlenmeyer flask previously flushed with nitrogen. After the addition of 10 mL of chloroform for dissolving the oil, 15 mL of glacial acetic acid were added to the sample using a 25 mL glass pipette. After, 1 mL of a saturated aqueous solution of potassium iodide previously prepared (1,5 g/mL) was added using an automatic pipette.

The solution was vigorously stirred and reserved in darkness for 5 minutes at room temperature and then, 75 mL of distilled water were added to stop the reaction. The solution is then stirred and an aliquot of Iotect (color indicator) was added to titrate the free iodine using a sodium thiosulfate solution (0.01 N). Two determinations were performed from the same sample.

Chloroform, glacial acetic acid and the potassium iodide solution were previously flushed with nitrogen to prevent reducing and oxidizing substances formation. In addition, a blank procedure (without oil sample) was also performed at the end of each PV determination.

PV was expressed in milliequivalents of active oxygen per kg of oil (meqO_2/kg) and was calculated by the Equation II-2:

$$\text{PV} = \left(\frac{V \times N}{m} \right) \times 1000 \quad \text{Equation II-2}$$

where, V is the mL of the standard sodium thiosulfate solution used for the titration step; N is the exact normality of the standard sodium thiosulfate solution; m is the mass (g) of oil sample.

2.3.3. Correlation between Conjugated Dienes (CD) and Peroxide Value (PV)

The oxidation kinetics at 60 °C of pure Coil and Sfoil were studied as a reference allowing furthermore to assess the correlations between conjugated dienes and peroxide value. The correlation has been studied previously by several authors in order to evaluate the proportionality and linear relationship between both oxidative indicators (Eliseeva et al., 2017; Marmesat et al., 2009; Wanasundara et al., 1995; Shahidi et al., 1994).

2.3.4. Assessment of the oil stability at room temperature

The oil stability of pure Coil and saturated with quercetin (168 $\mu\text{g/g}$) was assessed at room conditions in order to simulate the “home storage” and compare it with the accelerated oxidation at 60 °C. Therefore, 70 g of both enriched and non-enriched Coil were placed in a 100 mL glass flask, covered with aluminum foil, and stored at room conditions up to 74 days. Aliquots of the oils were withdrawn after 3, 10, 17, 24, 33, 41, 56 and 74 days to monitor the oxidative stability through CD measurements. The experiment was carried out in duplicate.

2.4. Recovery of polyphenols from walnut cake for oil enrichment

As a cheap and rich source of polyphenol compounds, walnut press-cake was submitted to both maceration and semi-continuous extraction. Each method was carefully described in the next sections.

2.4.1. Extraction processes

2.4.1.1. Maceration extraction

Globally, 3 g of walnut cake were extracted in a sealed glass flask with 39, 57 or 75 mL of a mixture ethanol:water 60:40 (v/v) (mixture called EtOH60) for providing the following mass-to-solvent ratios; 1:13, 1:19 or 1:25, respectively. Firstly, the solvent was heated in a digital magnetic hotplate stirrer with temperature control up to the desired temperature, i.e. 28 °C, 44 °C or 60 °C. The amount of walnut cake (3 g) was then added and an extraction time of 15, 37.5 or 60 minutes was counted. During extraction time, the mixture was continuously stirred, and the desired temperature was maintained.

Prior to extraction, various amounts of PL were dissolved in EtOH60 for 30 minutes, providing the following concentrations of phosphatidylcholine (wt/v); 0, 0.75% or 1.5%. EtOH60 containing PL was used as extraction solvent in order to evaluate the effect of phospholipids on the solubilization capacity of the solvent.

After extraction procedure, the samples were transferred to Falcon tubes and centrifuged at 13 300 g for 6 minutes. The supernatants were then filtered on a 0.45 µm PVDF syringe filter and stored in amber sealed flask at -18°C until further analysis.

2.4.1.2. Semi-continuous extraction (COL)

The semi-continuous extractions were carried out by flowing the solvent through a bed of walnut cake particles and collecting periodically the effluent in graduated tubes. Conditions of temperature (60 °C), solvent (EtOH60 or EtOH90 (mixture ethanol:water 90:10 v/v)) and volume of solvent (25 mL per gram of walnut cake) were selected from the maceration investigations. Approximately 5 g of walnut cake was mixed with glass beads (diameter of 0.5-0.75 mm or 2 mm) prior being loaded in a stainless steel column of 19.8 cm height x 0.9 cm internal diameter and overtopped by glass beads to fill completely the column (height of the inert filing of approximately 13 cm and 1 cm for beads of 0.5-0.75 mm and 2 mm, respectively). The solvent that flew from top to the bottom of the column, met first the inert filler. The column was placed in an oven (Model CTO-SA, Shimadzu, Japan) to control the temperature. The solvent, pre-heated at 60 °C by a magnetic hotplate stirrer with temperature control was delivered at 1.0 ± 0.05 mL/min by a dual pump (Model 515, Waters, USA). At the column exit, a capillary drove the effluent to a graduated tube immersed in ice. The graduated tube was

changed periodically during the extraction course to collect 6 fractions of 7 mL, 7 mL, 7 mL, 22 mL, 44 mL and 44 mL (V_i mL), respectively, over the 140 minutes of extraction. The breakthrough time was 9.05 ± 1.01 minutes and did not depend on the inert filler diameter. Three experiments were replicated to account for reproducibility deviations.

Once collected, the fractions were transferred to round bottom flasks by pipetting. An aliquot of 2 mL was sampled for TPC while the rest was desolventized using a rotary evaporator. The extracted amount in each fraction was quantified by the weight obtained after solvent evaporation multiplied by the volume factor $V_i/(V_i-2)$ to account for the aliquoted 2 mL.

The walnut cake after COL extraction was submitted to Soxhlet extraction in order to quantify its content in lipids. Since for COL extraction the raw material was mixed with glass beads, the whole mix (walnut cake+glass beads) was loaded in a Soxhlet thimble and extracted during 4 hours by hexane. The oil recovered by Soxhlet extraction was then weighted and the amount of oil previously extracted by COL was calculated as the difference between the amount of oil present initially in the walnut cake sample and the oil recovered by Soxhlet.

2.4.2. Characterization of polyphenol-rich extracts from walnut cake

Firstly, the mass of compounds extracted from walnut cake was quantified after rotary evaporation of a known volume of extract, normalized by the total volume of each fraction. Then, the following analysis were carried out on walnut extracts.

2.4.2.1. Total Phenolic Content (TPC)

The TPC of the extracts obtained by maceration and semi-continuous extraction was carried out following the protocol described in section 2.2.2.2 (TPC analysis by Folin-Ciocalteu method). The extracts from maceration were only filtered, prior to TPC analysis, using 0.45 μm PVDF syringe filters.

On the other hand, the extracts from semi-continuous extraction were submitted to a minor modified treatment before phenolic quantification. Firstly, 2 mL aliquots of extracts were vortexed for 1 minute followed by filtration (0.45 μm PVDF syringe filters) of those extracts that contained solid particles, i.e. the fractions recovered during the first stages of extraction. The extracts that contained co-extracted oil were then centrifuged at 10 000 rpm for

20 minutes and the supernatant (polar phase) was collected and diluted (if necessary) in deionized water for TPC analysis. The extracts that did not contain oil were directly diluted in deionized water for TPC analysis.

2.4.2.2. Antioxidant activity by DPPH method

The antioxidant activity of the extracts from maceration extraction was evaluated through the scavenging activity of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical according to method described by Espinosa-Pardo et al. (2017). In this method, the ability of an antioxidant molecule to neutralize a radical is evaluated. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) compound is a stable radical that has an intense purple color and absorbs radiation at 518 nm. Therefore, a decrease in the absorption of radiation is referred to the reduction in the concentration of DPPH by the transfer of electrons from the antioxidant species.

Solutions consisting of 50 μ L of extract or standard solution of Trolox[®] and 150 μ L of 0.2 mM DPPH in methanol, were analyzed on a multimode microplate reader with absorbance filters for a wavelength of 520 nm. The method consisted of monitoring the decoloring process of the afore-mentioned solutions during 35 minutes of reaction. The antioxidant activity was calculated by interpolating the absorbance of the samples with a calibration curve built with Trolox[®] solution at different concentrations (7–300 μ mol/L). Methanol 70% was used as a control and the antioxidant activity was expressed as μ mol of Trolox Equivalent/g cake. Measurements of antioxidant activity were performed in triplicate.

For preparing the 0.2 mM DPPH solution, 4 mg of 2,2-diphenyl-1-picrylhydrazyl DPPH were diluted in 50 mL of methanol using a volumetric flask.

2.4.3. Oil enrichment with polyphenol from walnut cake

In order to enrich Coil by polyphenols from walnut cake several methods were assessed. These methods include the enrichment of oil with hydroalcoholic extracts previously obtained from walnut cake, as well as the enrichment by direct solubilization of polyphenol of walnut cake in Coil. The purpose of this process was to enrich Coil using natural polyphenol obtained from plant residues such as walnut press-cake. In the following sections, all evaluated methods are carefully described.

2.4.3.1. Oil enrichment using extracts from maceration

This method consisted of adding a polyphenol extract obtained from walnut cake by maceration to Coil. First, Coil was enriched with dry extract at a concentration of 150 mg extract/kg oil. For that, approximately 50 mL of hydroalcoholic extract from experimental run No.10 (Table V-1, *Chapter 5*) was dried using a rotary evaporator, followed by 10 minutes at 75 °C in a laboratory drying oven. After that, 10.5 mg of dry extract was removed from the walls of the bottom flask and added to both 70 g of pure oil and oil previously enriched with PL (20 mg/g). The sample was then stirred at 35 °C for 1 hour followed by sonication in ultrasonic bath for 10 minutes and stirred again for 4 hours at 35 °C for dissolving the dry extract into the oil. However, after stirring the dry extract remained insoluble and the TPC of Coil was not enhanced. Therefore, it was decided to enrich the oil directly with the hydroalcoholic extract without the drying step.

For that, two levels of enrichment were assessed: 150 and 1 500 mg extract/kg oil. Therefore, 1.4 mL or 14 mL of extract were directly added to 70 g of oil (containing or not, 20 mg of PL/g oil) which provide concentrations of 150 and 1 500 mg extract/kg oil, respectively. The sample was gently homogenized, and the solvent was then removed in a rotary evaporator. The enriched oil was agitated at room temperature for 1 hour using a digital magnetic stirrer. For enriching the oil containing PL, the 14 mL walnut extract was previously concentrated to 1.5 mL under nitrogen flux before adding to the oil, in order to avoid PL precipitation.

After enriching Coil, its TPC was measured following the protocol already described in section 2.2.2.2. In addition, the enriched oils were submitted to the oxidative stability assessment under accelerated condition of heating at 60 °C. The enrichment of Coil was performed in triplicate as well as the oxidative stability assessment.

2.4.3.2. Direct enrichment of Coil with walnut cake

Maceration

The first method evaluated for the direct enrichment of Coil with walnut cake was maceration. In this sense, 14 g of walnut cake were directly stirred with 70 g of oil (containing or not, 20 mg of PL/g oil) at 35 °C using a digital magnetic hotplate stirrer with temperature control. The mixture was continuously agitated for 37.5 minutes (this is the intermediate time

used in maceration extraction with EtOH60 described in section 2.4.1.1). The samples were then transferred to Falcon tubes and centrifuged at 13 300 g for 6 minutes for removing walnut particles.

The TPC of enriched oils were measured however, an improvement in the TPC of Coil was not observed. Therefore, a direct enrichment assisted by ultrasound was evaluated.

Ultrasound Assisted Extraction (UAE)

For the direct enrichment of oil with walnut cake, assisted by ultrasound, 6 g of cake were directly added to 24 g of Coil (containing or not, 20 mg of PL/g oil). The mixture was then submitted to sonication using an ultrasonic processor (Sonifier digital 550, Branson, USA) provided with a 13 mm diameter probe and configured for 7 minutes of experiment with 50% of pulse and 50% of power (275 W). In order to avoid temperature rising during the enrichment, the container was kept into ice. Either way, the temperature of the oil after sonication was measured.

After sonication, the mixture was transferred to Falcon tubes and centrifuged at 13 300 g for 6 minutes. The supernatants were placed in amber glass flasks, flushed with nitrogen and maintained at room temperature until TPC analysis.

In order to increase the solubility of phenolic compounds from walnut cake in Coil, the addition of 2% of water and 20% of EtOH60 to the oil were evaluated. For that, and prior to sonication, 0.48 g of water were added to 23.52 g of oil or 4.8 g of EtOH60 to 19.2 g of oil. After, oil enrichment by sonication, the EtOH60 was removed using a rotary evaporator.

Incorporation of walnut particles in Coil

Looking for a method to improve the oxidative stability of oils, the incorporation of walnut particles to Coil submitted to accelerated oxidation was evaluated. This method consisted of incorporating walnut particles to oil in a ratio of 1:4. For that, 1.5 g of walnut cake was added to 6 g of oil (containing or not, 20 mg of PL/g oil), stirred overnight at room temperature and then submitted to accelerated oxidation at 60 °C. The walnut particles were not removed prior the oil oxidation and nor were stirred during oxidation time. This method of enrichment aimed at improving the solubility of antioxidant compounds in the oil, due to effect of high temperature (60 °C) applied during oxidation course.

The oxidative stability assessment of Coil with walnut particles was carried out for 6 to 240 h following the protocol carefully described in section 2.3 Before CD and TPC measurements, the samples were centrifuged at 13 300 g for 6 minutes for removing the walnut particles.

2.5. Statistical analysis

Results were expressed as mean \pm standard deviation (SD) and were statistically evaluated using the Software Minitab 17® (Minitab Inc.). The significant differences ($p < 0.05$) were analyzed through the Tukey's test.

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Chapter III: Polyphenol solubility and oil enrichment with quercetin-phospholipid formulations

Introduction

Polyphenols are known for exhibiting cardioprotective effects and for the capacity to delay the lipid oxidation in food systems (Heim et al., 2002). In this sense, polyphenols have attracted attention of food, pharmaceutical and cosmetic industries. In particular, quercetin is an abundant flavonoid which has gained attention due to its antioxidant, anti-inflammatory, anticarcinogenic, cardioprotective, neuroprotective and antihypertensive effect (Xu et al., 2016; Zhang et al., 2016). Nonetheless, as already discussed, the direct use of phenolic compounds, such as quercetin, in oily systems may be limited by their poor solubility and stability which may reduce considerably their effectiveness to protect food, cosmetic or pharmaceutical products against oxidation. From that perspective, this chapter globally discusses the strategies for enriching edible oil with polyphenols-phospholipid formulations in order to improve the solubility of polyphenols in oils. However, prior to develop these strategies, several analysis and discussions of industrial interest should be considered.

Therefore, this chapter consists of the following parts: i) a basic discussion concerning the chemical composition of the oils chosen for this study; camelina and sunflower oil, ii) the assessment of the solubility of various polyphenols in edible oils and in solvents of interest for food, pharmaceutical and cosmetic applications, iii) addressing the enrichment of edible oils - under the food fortification concept - through the incorporation of minor compounds such as polyphenols and polyphenol-phospholipid systems and iv) the assessment of the self-assembly of phospholipids in bulk oils.

First characteristics of the oils (camelina and sunflower oils) used along the thesis, are presented and key features related to their physical and oxidative properties are discussed. Then this chapter focused on the solubility estimation of quercetin in camelina and sunflower oils, and reports attempts for other phenolics (naringenin, chlorogenic acid and sinapic acid). A method for estimating the solubility of quercetin in camelina and sunflower oil was developed and validated through several analytical techniques including UV-spectrophotometric methods and HPLC analysis. Furthermore, the solubility of quercetin in other solvents of industrial

interest such as water, Miglyol, polyethylene glycol 400, dimethyl sulfoxide, cyclopentyl alcohol, propanol-1, ethanol, and aniline has been also assessed.

After that, we proposed and conducted some strategies for improving the solubility of quercetin in oils. In this sense, the interaction or complexation of polyphenols with amphiphilic molecules such as phosphatidylcholine has been assessed. For that we developed a quercetin-phosphatidylcholine complex by solvent evaporation method. The complexation efficiency was evaluated at different quercetin: phosphatidylcholine mass ratio and using absolute ethanol or a mixture of methanol: chloroform (70:30 v/v) as complexation medium. Then, we addressed the enrichment of camelina oil by adding the quercetin-phosphatidylcholine complex diluted in chloroform (solvent route). The addition of various amounts of complex to camelina oil was studied in order to estimate the solubility of complexed quercetin in camelina oil under various conditions of enrichment.

Later, this chapter describes the development of a solvent free route for the enrichment of camelina oil with several quercetin-phosphatidylcholine formulations. In this context, we evaluated the effect of phosphatidylcholine concentration on the solubility of quercetin in camelina oil, sunflower oil and Miglyol. Moreover, during this section several mechanisms reported in literature related to the interactions between quercetin and phospholipids in oily systems have been carefully discussed.

In order to evaluate the possible molecular organization of phospholipids and quercetin in oils, the critical micelle concentration (CMC) of phosphatidylcholine in camelina oil in absence and presence of quercetin (168 $\mu\text{g/g}$) was also addressed. For that, the 7,7,8,8-Tetracyanoquinodimethane (TCNQ) method was used in order to determine the concentration in which surface-active molecules (phospholipids) form association colloids as reverse micelles. The CMC assessment intended to get information related to the quercetin-phospholipids interactions which influenced the solubility of quercetin in oils.

1. Characterization of edible oils

The nutritional value of edible oils is mainly related to their composition of individual fatty acids. The characterization of this parameter allows us to estimate the oil nutritional value, its quality and stability. Moreover, fatty acids composition in oils are relevant for the production of food supplements and nutraceutical products. In this sense, Table III-1 shows the chemical characterization of Miglyol, camelina oil from Olead (Coil), camelina oil from Biocoop (BCoil) and sunflower oil (Sfoil).

Table III-1. Chemical composition of Miglyol, Coil, BCoil and Sfoil

	Name	Miglyol	Coil	BCoil	Sfoil
Fatty Acids Profile (%)	C4	< 0,05	< 0,05	< 0,05	< 0,05
	C6	0.4	< 0,05	< 0,05	< 0,05
	C8	56.6	< 0,05	< 0,05	< 0,05
	C10	42.7	< 0,05	< 0,05	< 0,05
	C12	0.2	< 0,05	< 0,05	< 0,05
	C14	< 0,05	0.1	0.1	0.1
	C16:0	< 0,05	5.7	5.5	6.6
	C16:1	< 0,05	0.1	0.1	0.1
	C18:0	< 0,05	2.4	2.3	3.3
	C18:1t	< 0,05	< 0,05	< 0,05	0.05
	C18:1 n-9	< 0,05	13.8	16.8	30.8
	C18:2t	< 0,05	< 0,05	< 0,05	0.4
	C18:2 n-6	< 0,05	17.3	18.9	57.2
	C18:3t	< 0,05	0.1	0.1	< 0,05
	C18:3 n-3	< 0,05	37.5	33.2	0.1
	C20:0	< 0,05	1.4	1.4	0.2
	C20:1	< 0,05	13.7	14.0	0.2
	C20:2	< 0,05	2.1	1.8	< 0,05
	C20:3	< 0,05	1.7	1.2	< 0,05
	C22:0	< 0,05	0.3	0.3	0.7
	C22:1	< 0,05	2.9	3.2	< 0,05
	C24	< 0,05	0.2	0.3	0.3
	C24:1	< 0,05	0.6	0.6	< 0,05
		Σ SFA	99.9	9.9	9.9
	Σ MUFA	< 0,05	31.1	34.7	31.1
	Σ PUFA	< 0,05	58.7	55.2	57.2
	PUFA n-3	< 0,05	37.5	33.2	0.1
	PUFA n-6	< 0,05	21.2	21.9	57.2
	Total Trans	< 0,05	0.1	0.1	0.5
Glyceride profile (%)	Free fatty acids	< 0,1	0.6	0.3	< 0,1
	MAG	< 0,1	< 0,1	< 0,1	< 0,1
	DAG	1	< 0,1	< 0,1	4.2

	TAG	-	99	99.3	95.6
	MCT	99	-	-	-
	Sterols	< 0,1	0.3	0.3	0.2
	Sterols esters	< 0,1	< 0,1	< 0,1	< 0,1
Vitamin E (mg/kg)	Acetate	< 5	< 5	< 5	< 5
	α -tocopherol	< 2	29.0	18.0	651.5
	β -tocopherol	< 2	< 2	< 2	21.8
	γ -tocopherol	< 2	685.5	725.8	3.7
	δ -tocopherol	< 2	11.9	11.4	< 2
	α -tocotrienol	< 2	< 2	< 2	< 2
	β -tocotrienol	< 2	< 2	< 2	< 2
	γ -tocotrienol	< 2	< 2	< 2	< 2
	δ -tocotrienol	< 2	< 2	< 2	< 2
	Total Vitamin E	< 2	726.4	755.2	677.0
Phospholipids (g/100g)	PE	< 0.05	< 0.05	< 0.05	< 0.05
	PI	< 0.05	< 0.05	< 0.05	< 0.05
	PC	< 0.1	< 0.1	< 0.1	< 0.1
	LPC	< 0.05	< 0.05	< 0.05	< 0.05
	LPE+PS+PA	< 0.1	< 0.1	< 0.1	< 0.1
	Total Phospholipids Content	< 0.35	< 0.35	< 0.35	< 0.35
Metals (mg/kg)	Copper	< 0,005	< 0,005	< 0,005	< 0,005
	Iron	0.03	1.3	0.1	0.01
Total Phenolic Content (mg GAE/kg)		-	30 \pm 2.6	44.8 \pm 3.1	33.1 \pm 1.9

Σ SFA: total saturated fatty acids, Σ MUFA: total monounsaturated fatty acids, Σ PUFA: total polyunsaturated fatty acids, MAG: monoacylglycerol, DAG: diacylglycerol, TAG: triacylglycerol, MCT: medium chain triglycerides, PE: Phosphatidylethanolamine, PI: Phosphatidylinositol, PC: Phosphatidylcholine, LPC: Lysophosphatidylcholine, LPE: Lysophosphatidylethanolamine, PS: Phosphatidylserine, PA: Phosphatidic Acid, GAE: gallic acid equivalents

As can be observed in Table III-1, Miglyol is composed by fractionated caprylic (56.6%) and capric (42.7%) fatty acids and thereby, it can be considered as medium chain fatty acid oil. Miglyol is a neutral and not oxidizable oil due to its composition in saturated fatty acids and therefore it was used in this study for comparison purposes and for developing the methods related to the assessment of the quercetin solubility.

Regarding the fatty acids profile of Coil and BCoil, they exhibited similar content of saturated fatty acids (SFA) (approximately 9.9%) with predominant amount of palmitic (16:0) (5.6%) and stearic (18:0) (2.3%) acids. The content of monounsaturated fatty acids (MUFA)

of Coil was 31.1% whereas of Bcoil was 34.7%, mainly comprised of oleic acid (18:1) and eicosenoic acid (20:1). On the other hand, Coil exhibited a higher content of polyunsaturated fatty acids (PUFA) than BCoil (58.7% against 55.2%), where α -linolenic acid (ALA, 18:3 n-3) was predominant in both oils followed by linoleic acid (LA, 18:2 n-6). The few variations between Coil and BCoil related to their content of PUFA and therefore of MUFA and SFA, may come from the plant variety or growing conditions.

What is important to highlight regarding camelina oil composition is the high content of ALA (> 37%), since ALA is an essential fatty acid whose intake is fundamental for the human health and the prevention of many degenerative diseases (Lunn & Theobald, 2006). Moreover, ALA is highly susceptible to oxidation, so it is important to promote alternatives for preventing its peroxidation. Globally, the fatty acid composition of both camelina oils shown in Table III-1, was consistent with those reported by Anderson et al., (2019), Ratusz et al., (2018), Raczyk et al., (2016), Ratusz et al., (2016), Belayneh et al. (2015), Abramovic & Abram, (2005) and Zubr & Matthaus, (2002).

On the other hand, the content of minor compounds such as tocopherols also plays a key role in the oxidative stability of bulk oils. Both camelina oils exhibited total tocopherol content in the same range; 726 mg/kg and 752 mg/kg for Coil and BCoil, respectively. These values were consistent with those reported by Ratusz et al., (2018) (560-752 mg/kg), Hrastar et al., (2012) (556-873 mg/kg) and Abramovic et al., (2007) (760 mg/kg). Coil and BCoil exhibited small differences regarding the composition of tocopherols, however both oils were mainly composed by γ -tocopherol (> 90% of total tocopherol content) which is also in agreement with Ratusz et al., (2018) and Hrastar et al., (2012). The total sterol content (unsaponifiable matter) of camelina oils was in the range of 4700-4800 mg/kg, similar to that reported by Ratusz et al., (2018) and Belayneh et al. (2015) and higher than refined oil such as Sfoil (3340 mg/kg) (Table III-A1).

Regarding Sfoil, it exhibited 11.1% of SFA, similarly to camelina oil with a predominant content of palmitic (6.58%) and stearic (3.31%) acids. Even though the MUFA content of Sfoil and camelina oils was similar (around 30%), oleic acid (18:1 n-9) was found to be higher in Sfoil (30.8%) than in camelina oils (around 15%). Sfoil is also an important source of PUFAs (57.2%), mainly of LA (99% of PUFAs). Author such as Guo et al., (2017) and Dunford, (2015) have reported that conventional sunflower oil exhibits a content of LA between 65% and 70% and of oleic acid in the range of 20% to 25%. However, discrepancies

in the fatty acid composition may be also due to plant variety, growing conditions or genetic modifications (hybrid procedures) made for modifying conveniently the fatty acid composition. Although both Coil and Sfoil exhibited similar amount of PUFAs (around 58%), the Coil exhibited higher proportion of ALA than Sfoil, therefore it can make Coil more susceptible to lipid oxidation than Sfoil.

Sfoil exhibited a tocopherol content of 677 mg/kg which is in the ranged reported by Gupta et al., 2001) for conventional sunflower oil. According to Gotor & Rhazi, (2016) and Gupta et al., 2001), the high content of α -tocopherol (652 mg/kg) in Sfoil provides a high protection against photo-oxidation, whereas due to low content of γ -tocopherol (3.7 mg/kg), Sfoil is theoretically poor protected against autoxidation.

On the other hand, as can be observed in Table III-1, Coil exhibited a higher metal content (1.28 mg/kg) in comparison with refined Sfoil (0.009 mg/kg). This could be explained by the fact that during the refining process, the traces of metals in plant oils are intentionally removed in order to avoid deleterious effects since metals are active catalysts of lipid oxidation (Schaich, 2005). The difference in metals content between Coil and BCoil (0.057 mg/kg) could be related to the growing and soil conditions or differences in the industrial process developed for each company for extracting the oil from camelina seeds. In this sense, it can be stated that Coil is more susceptible to peroxidation than BCoil because of the catalytic action of iron in bulk oils (Choe & Min, 2006).

A considerably low content of phospholipids was also observed in camelina and sunflower oils. The low content of phospholipids in Sfoil is explained by the fact that the phospholipids are usually removed during the refining process, specifically during the degumming step (Dunford, 2015). Regarding camelina oils, the low content of phospholipids may come from the oil extraction method. For instance, Bredan et al., (2000) have reported a significant lower content of phospholipid in mechanical pressed oils in comparison with hexane extracted oils. Cold pressed oils also have lower phospholipid content than hot pressed and/or cooked seed oils. The phospholipid content in plant oils can be enhanced by using solid-liquid extraction methods which involve polar solvents such as ethanol (Mas'ud et al., 2019).

Phenolic compound are other minor compounds in oils that may play a key role in the quality and stability of edible oils. The total phenolic content (TPC) of Coil, BCoil and Sfoil was also shown in Table III-1. BCoil exhibited the highest content of phenolic compounds

(45 mg GAE/kg), followed by Sfoil (33 mg GAE/kg) and Coil (30 mg GAE/kg). The content of phenolic in BCoil and Coil was in the range than that reported by Ergonul & Ozbek, (2018) (25.9 to 63.7 mg GAE/kg) for oil extracted from different varieties of camelina seeds from Turkey. Similarly, Terpinc et al., (2012) reported a TPC of 57 mg of CAE (chlorogenic acid equivalents)/kg camelina oil.

On the other hand, Laulloo et al. (2015) reported a TPC of refined sunflower oil of 45.3 mg GAE/kg, whereas Janu et al., (2014) and Siger et al., (2005), found lower values; 4.9 mg GAE/kg and 11.5 mg GAE/kg, respectively. Similar to other minor compounds, differences in the TPC in edible oils may be caused by several factors such as growing conditions (whether, soil composition, degree of environmental stress), plant variety, oil extraction methods and even by the analytical method used for quantify the phenolic content in oils (Siger et al., 2005).

2. Determination of the solubility of polyphenols in different oils

Solubility is a physicochemical property of great importance for the formulation of novel food and pharmaceutical products. It indicates the maximum amount in which a substance can be dissolved in a specific amount of solvent or mixture of solvents, forming a homogeneous solution at certain temperature, where the distribution of atoms, molecules and ions is uniform (Coltescu et al., 2020). Solubility is an important parameter because it establishes the maximum concentration of the bioactive compounds in a final product and also influences its future efficacy. For instance, for developing products with therapeutic effect or biological activity, it is necessary that the active compound reach a specific concentration and stability in the nutraceutical product for ensuring a minimum absorption in gastrointestinal tract (Ghanbarzadeh et al., 2016; Rein et al., 2012). From that perspective, polyphenols are natural antioxidant of great interest for developing food, cosmetic and pharmaceutical products due to their capacity to scavenge free radicals and delay lipid oxidation. Thereby, in the following section are presented the results related to the solubility of quercetin in edible oils which measures have been validated by several methods. After that, the solubility of quercetin in other solvents of industrial interest has been also presented.

2.1. Validation of various methods for quercetin quantification in saturated oils

2.1.1. Sample preparation

In order to be clear about the different methods (Table III-2) used for validating the assessment of the quercetin solubility in oils, follow a reminder about the different analytical protocols (methodologies were also described in section 2.2.2, *Chapter II*).

Quercetin quantification by using a calibration curve of quercetin in Miglyol

This method consisted basically in prepare a stock solution of quercetin in Miglyol at a concentration of approximately 40 $\mu\text{g/mL}$ from which less concentrated solutions were prepared in a range of 8 to 25 $\mu\text{g/mL}$, using Miglyol as diluent. The aforementioned solutions were read in a microplate reader at 374 nm in order to build a calibration curve. Aliquots of oils saturated with quercetin (prepared as described in section 2.2.1.3, *Chapter II*) were then read at 374 nm and the quercetin concentration was estimated using the already built calibration curve.

Quercetin quantification by measuring the Total Phenolic Content (TPC)

This method consisted first of a methanolic water extraction (80:20 v/v) of quercetin from oils saturated with quercetin followed by the total phenolic quantification of the methanol-water extracts by Folin-Ciocalteu assay. After 2 h of reaction of polyphenols with Folin-Ciocalteu reagent, the samples were read at 750 nm in a microplate reader.

HPLC analysis for quercetin quantification

In addition to spectrophotometer analysis previously detailed, a High-Performance Liquid Chromatography Analysis (HPLC) was also carried out. This analysis consisted of: i) the injection of the methanol-water extracts obtained previously and, ii) the direct injections of the different oils saturated with quercetin. Both type of samples (methanolic-water extracts and oils saturated with quercetin) were injected using a mobile phase consisted of methanol (solvent A) and water with 0.03% phosphoric acid (solvent B) at a flow rate of 1 mL/min. Quercetin was quantified at 370 nm using a calibration curve of quercetin in absolute ethanol ranging a concentration of 0 to 20 $\mu\text{g/mL}$.

2.1.2. Estimation of quercetin in oils saturated with quercetin by different methods

In Table III-2 has been shown the solubility of quercetin in Miglyol, Sfoil, BCoil and Coil, assessed by methods previously described. These data were statistically analyzed by the comparison of means using a Tukey post hoc test and the results showed that there are no significant differences between the methods for quantifying quercetin concentration in oils saturated with quercetin. In this way, it was possible to stated that the results shown in Table III-2 are confident values and that the protocol for measuring quercetin solubility in oil has been successfully validated.

Table III-2. Solubility of quercetin in oils estimated by different methods

Oil	Microplate-UV		HPLC	
	Calibration curve in Miglyol ¹	TPC ²	Methanol-water extracts ¹	Saturated oils ¹
Miglyol	830 ^a ± 45	813 ^a ± 37	832 ^a ± 72	783 ^a ± 43
Sfoil	367 ^b ± 22	381 ^b ± 36	429 ^b ± 41	390 ^b ± 28
BCoil	381 ^b ± 36	389 ^b ± 35	424 ^b ± 30	393 ^b ± 38
Coil	154 ^c ± 10	162 ^c ± 3	177 ^c ± 2	168 ^c ± 5

¹ read at 374 nm and expressed in µg quercetin/g oil, ² read at 750 nm and expressed in µg GAE/g oil. Equal letters in the same column indicate no significant difference at level of 5% (p < 0.05)

As observed in Table III-2, the solubility of quercetin varied with the type of oil being that Miglyol > Sfoil > Coil. Differences in solubility or miscibility of phenolics are influenced by multifaceted behaviors attributed mainly to polar interactions and hydrogen bonding (Kim et al., 2018a), therefore the solubility of quercetin in different oils could be affected by their different composition in minor compounds. Miglyol is made of triglycerides by fractionation of saturated oils and thus contains low amounts of non-glyceridic compounds, Sfoil is refined so depleted in pigments, free fatty acids, phospholipids, wax, etc., whereas Coil is a virgin oil thus all minor compounds remain in the oil. From that perspective, the content of minor compounds seemed to negatively influence the capacity of oils to solubilize quercetin, however BCoil (which is also a virgin oil) exhibited a similar capacity to solubilize quercetin than Sfoil. In this sense, the endogenous water content of oils could also play a key role in the

facility to dissolve quercetin, since it can help create a local hydrogen environment (Kim et al., 2018a) that allows the formation of hydrogen bonding and van der Waals interactions with quercetin. Unfortunately, the amount of endogenous water in the oils was not measured in this work.

Our hypothesis for explaining the differences in the quercetin solubility is also related to the structure of the fatty acids in the different oils. For instance, Miglyol is composed of triglycerides with medium chain fatty acids (C8, C10) whereas the other oils (Sfoil, BCoil and Coil) are composed mainly of a 18 chain length and therefore exhibited lower ability to dissolve quercetin compared to Miglyol. A shorter fatty acid carbon chain confers to the oil a lower viscosity than those of long chain (Wahyudi et al., 2018) and hence a higher diffusivity of quercetin in Miglyol can be expected. Among sunflower oil and camelina oil, the latter exhibited about 14% of fatty acids with a 20-carbon chain length that are not present in Sfoil. Similarly, Shahba et al. (2016) have reported that the solubility of cinnarizine significantly increased upon decreasing the fatty acid chain length.

Comparing our results with literature, similar ranges of solubility of quercetin in other plant oils have been previously reported by Chen et al. (2016) for corn oil (< 220 µg/g), and Rich et al. (2017) for olive oil (around 330 µg/mL). On the other hand, Rich et al. (2017) have stated that the oil/water partition coefficient of quercetin favors partition into oil (log P = 1.8), whereas Van Dijk et al. (2000) estimated that the percentage ratio of quercetin partitioning between water and olive oil was 53%. Therefore, quercetin can be considered as neither fully lipophilic nor hydrophilic.

Regarding other polyphenols such as naringenin, chlorogenic acid and sinapic acid, they were not soluble, or at least at concentrations no measurable by spectrophotometric method, in neither Sfoil nor Coil. When samples of Sfoil and Coil were saturated with the aforementioned polyphenols and then read in a spectrophotometer, it was not observed any light absorption different to that observed for pure Sfoil and Coil, indicating that the oils were not able to solubilize these phenolic compounds.

For naringenin this was expected since the preference of this aglycone for polar organic solvents has been already reported. For instance, Zhang et al. (2015) found a poor solubility of naringenin in non-polar solvents as hexane and petroleum ether, whereas Chuang et al. (2017) related a higher solubility of naringenin in polar solvents such as PEG 400 due the poor

lipophilicity nature of naringenin. Although the predicted octanol-water partition coefficient of sinapic acid ($\log P = 1.5$, PubChem) and naringenin ($\log P = 2.4$, PubChem) indicated some hydrophobicity of these compounds, this is not necessarily correlated with a good solubility in oils as observed previously for quercetin. Furthermore, the solubility of polyphenols in oils cannot be predicted merely by the $\log P$, since oils are complex matrixes composed by fatty acids of diverse carbon chain length and many other minor compounds which may significantly influence their solvating power. Whereas the $\log P$ is a parameter only defined by the ratio between the solute concentration in octanol and in water, at a certain temperature (Rothwell et al., 2005).

Regarding chlorogenic acid, it was expected a poor solubility of these compound in oils first due to its marked poor lipophilic potential ($\log P = -0.5$, PubChem) and second, due to the structural characteristic of chlorogenic acid which have a hydrophilic quinic acid moiety (Rui et al., 2017). Chlorogenic acid is structurally an ester of caffeic acid and quinic acid (Rui et al., 2017). The fact of having only one aromatic ring and multiple hydroxyl groups, makes chlorogenic acid a marked hydrophilic compound, in particular because it has been reported that the hydrophilicity of a compound increases with the number of hydroxyl groups (Chen et al., 1996).

2.2. Determination of the solubility of quercetin in other solvents

The analysis of the solubility of quercetin in other solvents different than oils was based on the suggestions from the group of prof. Mohamed Benali of University of Technology of Compiègne, who is dedicated, among other topics, to the prediction of solubility of compounds in different solvents by means of Hansen solubility parameters. In this context, Hansen approach estimated that for instance solvents such as aniline, DMSO and ethanol are better mediums to solubilize quercetin than other solvents as water. The experimental assessment of the solubility of an organic compound in different solvents plays an important role in processes of separation and purification of valuable compounds. In the assessment of the solubility of compounds in different solvents both experimental data as well as the analysis of the chemical structure of compounds are important to predict the solubility of the composites in different solvents. For instance, literature reported that the most representative parts of the structure of flavonoids that may influence the solubility are the OH or OCH₃ substituent on ring B, the ring B connection position, the bond order between C2 and C3, and an OH substituent at 3 position (Zhang et al., 2017). On the other hand, there is a lack of experimental data, or at least of

consistent experimental data, related to the solubility of polyphenols such as quercetin in different solvents. From that perspective, a study of solubility of quercetin in solvents of interest for food, pharmaceutical, cosmetic, or chemical applications are shown in Table III-3. The assessment of the solubility of quercetin in these solvents was carried out by the protocol schematically represented in Figure III-1.

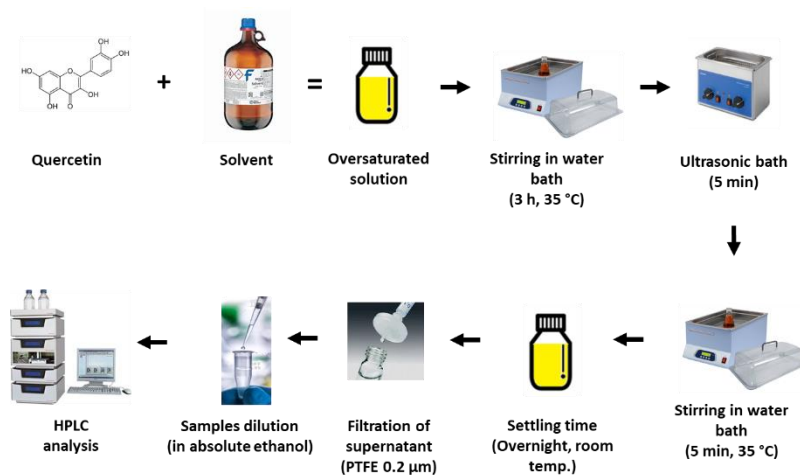


Figure III-1. Schematic protocol for the assessment of the solubility of quercetin in different solvents.

As quercetin concentrations in various solvents was progressively increased until reach the saturation, in Figure III-2 are shown some plots that correlates the theoretical (prepared) concentration of quercetin in the solutions with the quercetin concentration measured by HPLC analysis. From that perspective, quercetin concentrations in the straight line corresponds to the unsaturated samples (in which measured quercetin concentration is equal to the prepared quercetin concentration); data far away from the straight line corresponds to samples saturated in quercetin (the measured concentration corresponds to the concentration of quercetin solubility).

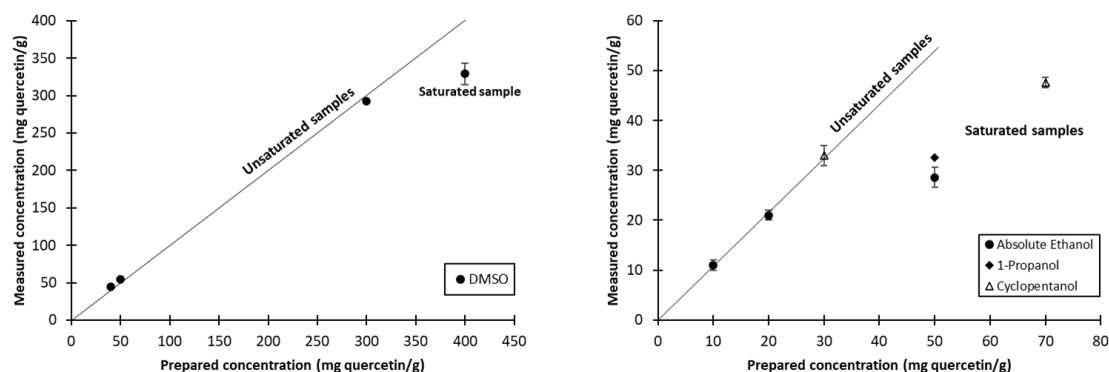


Figure III-2. Correlation between measured concentration and theoretical concentration of quercetin in different solvents

As it can be observed in Table III-3, the highest solubility of quercetin was exhibited in dimethyl sulfoxide (DMSO) among all assessed solvents. DMSO has been widely used to increase the solubility of quercetin in water for many chemical and pharmaceutical applications (Kim et al., 2009; Lavoie et al., 2009; Wu et al., 2008; Braganhol et al., 2007). Ferry et al., 1996 reported a lower value of 136.4 mg/g compared to the one obtained in this work for the solubility of quercetin in DMSO, which supposes the existence of discrepancies in the results.

Table III-3. Solubility of quercetin in several solvents

Solvent	Solubility (mg/g)
DMSO	329.2 ± 14.4
PEG 400	162.8 ± 7.8
PEG 400 – 20%	0.040 ± 0.001
PEG 400 – 40%	0.424 ± 0.018
Absolute ethanol	28.58 ± 1
1-Propanol	32.6 ± 1.6
Cyclopentanol	47.6 ± 0.6
Aniline	$2 < \text{Solubility} < 5$
Glycerol	2.47 ± 0.19
Water	0.005

In this solubility study, it was expected that quercetin exhibited a poor solubility in water as has been widely reported in literature (Zhang et al., 2017; Abraham & Acree, 2014;

Althans et al., 2014; Chebil et al., 2010), however, the existence of several inconsistencies in the results of the solubility of quercetin in water has been also observed and discussed before (Zhang et al., 2017; Abraham & Acree, 2014). For example, Lauro et al. (2002) estimated in 0.0077 mg/g the solubility of quercetin in water at room temperature. However, these authors informed that for this calculation they prepared a calibration curve of quercetin in water in a range of 0.005-0.02 mg/g, which is above the solubility of quercetin estimated by the authors. Other authors such as Kim et al. (2009), reported that the solubility of quercetin in water was 0.015 mg/g, nevertheless, the authors incorporated a small amounts (1%) of DMSO in order to increase the solubilization of this compound in water. Xu et al. (2016) and Kendre et al. (2014) also indicated different quercetin solubility values at room temperature of 0.0125 mg/g and 0.16 mg/g, respectively. Zhang et al. (2017) stated that the discrepancies among the solubility data of quercetin and other flavonoids in water already reported in literature, are frequently observed due to the low solubility of these compounds in water. These authors (Zhang et al., 2017) hypothesized that the possible reasons of this discrepancies might be related to the methods of analysis or the experiment environments. Furthermore, the solubility of compounds in water can be also influenced by the pH value and ionic force.

On the other hand, quercetin also exhibited a high solubility in polyethylene glycol 400 (PEG 400), commonly used as hydrophilic carrier for solid dispersions (Savjani et al., 2012). However, this solubility was negatively influenced when water was added to this solvent, at PEG400:water ratios (v/v) of 40:60 (PEG 400-40%) and of 20:80 (PEG 400-20%). The solvating power of PEG400 markedly decreased with the addition of water so much so that the solubility of quercetin in PEG 400-40% (0.424 mg/g) and PEG 400-20% (0.040 mg/g) was closer to that of pure water than that of pure PEG 400. On the other hand, we observed some discrepancies in the solubility of quercetin in PEG 400-20% and PEG 400-40% with the results reported by Kendre et al. (2014), who obtained higher solubility values for this solvent mixtures; 1.56 mg/g for PEG 400-20% and 2.3 mg/g for PEG 400-40%. Other discrepancies were also observed related to the solubility of quercetin in absolute ethanol found in this work (28.58 mg/g) with that reported by Razmara et al. (2010) (8.78 mg/g). Althans et al. (2014) stated that one of the best solvents for diluting quercetin is ethanol, but also reported discrepancies in the experimental data related to the solubility of quercetin in this solvent.

The significant discrepancies among the experimental data reported for quercetin solubility in different solvents elucidate the complex tasks that these measurements entail.

Moreover, several environmental and methodological conditions may significantly influence the measured concentration of saturation such as temperature, purity of solvents and of composites, estimated time for reaching saturation, conditions of dissolution (the use of agitation, for instance), analytical methods, etc.

Regarding quercetin solubility in 1-propanol, it was slightly higher than in ethanol (32.6 mg/g against 28.58 mg/g). In this sense, Taraba & Szymczyk, (2020) have reported that the strongest interactions of quercetin and rutin (glycoside form of quercetin) with short chain alcohols are observed between rutin-ethanol and quercetin-propanol. Furthermore, the authors also stated that both short chain alcohols are good and commonly used solvents for solubilize quercetin because of their low toxicity and polarity.

Globally, it was observed that PEG 400 and DMSO exhibited the highest solvating power for dissolving quercetin (Table III-3). This was expected under the consideration that these solvents are recognized as amphiphilic solvents (Parray et al., 2020; Gurtovenko & Anwar, 2007). According to Chebil et al. (2010) amphiphilic solvents proved to be effective mediums for solubilizing quercetin. This can be explained by the fact that water molecules cannot interact with the hydroxyl moieties of quercetin due to the bulky hydrophobic core of the solute, whereas amphiphilic solvents are able to interact with both hydrophobic and hydrophilic moieties of quercetin, which are reflected in a more stable organization of the solvent molecules around the structure of quercetin (Chebil et al., 2010). Although, it is also considered that ethanol has amphiphilic character (Gurtovenko & Anwar, 2009), it exhibited lower solvating power than DMSO and PEG400.

3. Quercetin-phospholipid complexation by solvent evaporation method

The results related to the complexation of quercetin with phospholipids have been described in terms of complexation efficiency, i.e. quercetin entrapment, first when different solvents were used as complexation medium and secondly, when different quercetin-phospholipid mass ratios were evaluated.

The complexation efficiency (CE) can be considered as a measure of the entrapment of a compound/molecule in a carrier system, and it is calculated as the ratio between the mass of compound that has been complexed and, the initial mass of free compound used to prepare the

complex. In Figure III-3 and Figure III-4 have been summarized the protocols for quercetin-phospholipid complexation and complex purification, respectively.

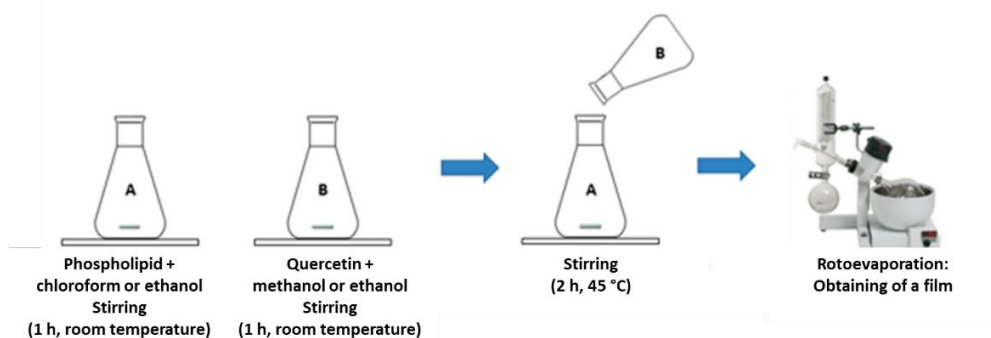


Figure III-3. Protocol for quercetin-phospholipid complexation by a solvent route

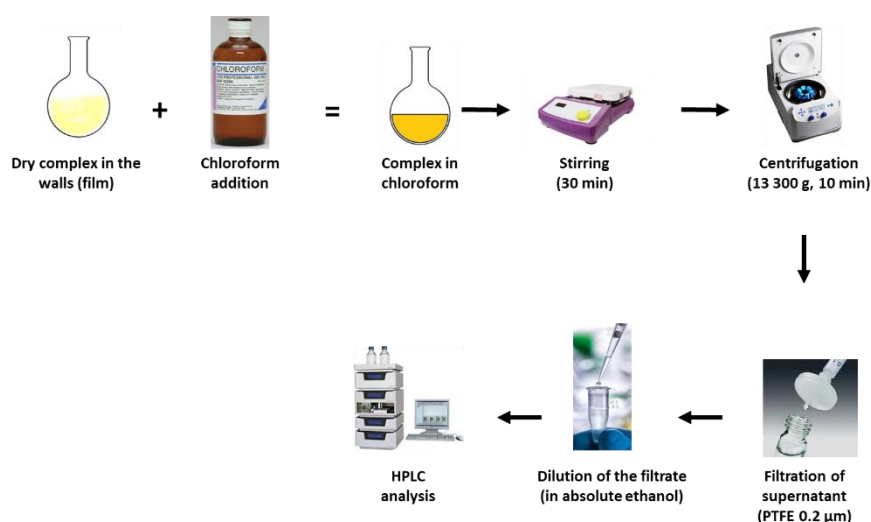


Figure III-4. Schematic protocol for complex purification and complexation efficiency calculation

3.1. Complexation using methanol:chloroform solution or absolute ethanol

During the attempt for complexing quercetin with Lecisoya P97 (LC) as carrier system, it was observed a poor solubility of LC both in chloroform and absolute ethanol. Even after 2 hours of stirring at 45 °C, it was not possible to solubilize 1 g of LC neither in 70 mL nor 100 mL of ethanol. A poor solubility of LC was probably due to it is comprised of only 68% of phospholipids with 19% of phosphatidylcholine. In this sense, Lipoid P100 (PL) was chosen as more suitable carrier for complexation process since it is comprised of 95.5% of phosphatidylcholine and, showed high solubility both in chloroform as absolute ethanol.

Khosrowaninia et al., (2015), have stated the importance of the concentration/purity of phosphatidylcholine in the formation of phospholipid carriers such as liposomes. For these authors, a purity of phosphatidylcholine ranging from 80 to 100% is required for fabricating more stable carries.

After choosing PL for its complexation with quercetin, two different complexation mediums were evaluated. Firstly, a mixture methanol:chloroform 30:70 (v/v) and secondly, absolute ethanol (complexation protocol carefully described in chapter II, section 2.2.4.1), and the result are presented in Table III-4. As it can be observed, absolute ethanol was the most appropriate solvent for quercetin-PL complexation process, since it allowed to obtain 97% of efficiency against 75% of efficiency obtained when the mixture methanol:chloroform was used as complexation medium. Furthermore, the use of absolute ethanol for the complexation process was also more appropriate than the mixture methanol:chloroform, to the extent that ethanol was appropriate for the solubilization of both quercetin and phospholipids whereas chloroform solubilized properly PL but poorly solubilized quercetin (experimental observations).

Table III-4. Efficiency of the quercetin-PL complexation using different solvents for preparing the complex

Solvents	CE (%)
Methanol:Chloroform 30:70 (v/v)	75 ± 1%
Absolute ethanol	97 ± 1%

Our results were consistent with several authors that obtained a high complexation efficiency (between 86-97%) of polyphenols and bioactive phytoconstituents with phosphatidylcholine when they used ethanol as a solvent medium (Saoji et al., 2017; Saoji et al., 2016; Maryana et al., 2015). Whereas other author such as Shaji & Iyer, (2012) obtained a quercetin-phosphatidylcholine complexation efficiency of 78% when a chloroform-methanol mixture was used as complexation medium. These authors (Shaji & Iyer, 2012) used a higher quercetin-phospholipid ratio than this work (1:10 w/w against 1:2.67 w/w used in this work).

Furthermore, we can consider that the high complexation efficiency obtained using ethanol as a solvent medium can be sustained to the fact that this solvent is considered for several authors (Gurtovenko & Anwar, 2009; Zhang et al., 2006; Klemm, 1998) as an

amphiphilic molecule with chemical and physical properties associated with hydrophilic and hydrophobic molecules. For instance, Chebil et al., (2010) found that a better solubilization of quercetin is obtained when dissolved in amphiphilic solvents. Moreover, the amphiphilic character of this solvent also implies greater interactions with the phospholipid molecule. For instance, Barry & Gawrisch, (1994) stated that ethanol is able to form strong interaction with the lipid-water interface of phosphatidylcholine bilayers. They (Barry & Gawrisch, 1994) presumed that because of its amphiphilic properties, ethanol is capable to be attracted simultaneously to both hydrophobic and hydrophilic targets; this phenomenon involves a hydrophobic interaction of the ethyl moiety with a glycerol domain and a hydrophilic interaction with a carbonyl or phosphate group. Gurtovenko & Anwar, (2009) and Klemm, (1998) also argued that the strong interactions between ethanol molecules and the phospholipid head groups are related to hydrogen bonding.

On contrary, although chloroform allows a good solubilization of phospholipids, it does not allow a good solubilization of quercetin. Chebil et al., (2010) reported the incapacity of chloroform to solubilize quercetin. Their studies revealed that there is no specific organization of chloroform around the hydrophilic groups of quercetin due to its inability to interact with quercetin.

Therefore, it can be stated that ethanol could have allowed greater interactions between quercetin and phospholipid by making it closer to each other, thanks to the ability of ethanol to solubilize both molecules. On the other hand, the interactions between quercetin and phospholipid in a methanol:chloroform mixture could be weaker due to the limited solubility of quercetin in chloroform.

3.2. Complexation with different quercetin-PL mass ratios

Although several works (Xu et al., 2016; Zhang et al., 2016; Khan et al., 2013; Ramadan, 2008) related to the formation of polyphenol-phospholipid complex have reported a molar ratio of 1:1 as the most suitable condition for the complexation process, in this work other quercetin-PL ratios have been studied. In particular, a quercetin-PL molar ratio 1:1 corresponds to 1:2.67 mass ratio, therefore the other mass ratios assessed in this work aimed at evaluating the feasibility of using lower proportion of PL for the complexation process. Lower amounts of PL for complexation may represent economic savings. Table III-5 shows the efficiency of complexation obtained for the different quercetin-PL mass ratios (2.4:1, 1:1.67,

1:2.). It is important to highlight that those complexations were carried out using absolute ethanol (100 mL) as complexation medium since this solvent provided the greatest complexation efficiency, as already discussed in the previous section. In this section, in addition to the calculation of the complexation efficiency at the different quercetin-PL mass ratios, it has been detailed several important experimental observations or difficulties related to the complexation process.

Table III-5. Complexation efficiency (CE) using several quercetin-PL mass ratios

Quercetin:PL mass ratio	Quercetin:PL molar ratio	Mass of quercetin (mg)	Mass of PL (mg)	CE (%)
2.4:1	2.4:0.37	60	25	NM
1:1.67	1:0.63	60	100	NM
1:2	1:0.75	20	40	20 ± 1
1:2.67	1:1	10	26.7	97 ± 1

NM: Not measurable

The experimental results showed that when the quercetin-PL complex was formed at mass ratios of 2.4:1 and 1:1.67, the estimation of the complexation efficiency could not be conducted. It was firstly because during the purification step, the polyphenol-phospholipid film (complex) was poorly soluble in chloroform; quercetin particles remained attached to the walls of the round-bottom flask and it did not dissolve in the liquid phase. Secondly, because after centrifuging the chloroform solution, a large amount of non-complexed quercetin remained insoluble and suspended in the solution as observed in Figure III-5. Therefore, during the filtration of the supernatant, the syringe filter rapidly clogged due to the large amounts of suspended quercetin that remained insoluble. It is worth remembering that the process for calculating the complexation efficiency leads to the solubilization of the complex in chloroform, followed by a centrifugation and filtration steps (this protocol has been carefully described in chapter II, section 2.2.4.2).

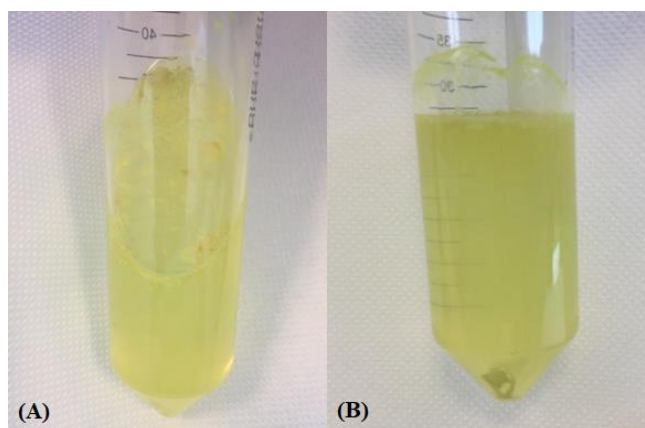


Figure III-5. Quercetin-PL complex formed using mass ratios 2.4:1 (A) and 1:1.67 (B) after purification in chloroform

On the other hand, when the quercetin-PL complex was formed at a mass ratio of 1:2, the complex film exhibited a greater solubility in chloroform than 2.4:1 and 1:1.67 quercetin-PL complexes. In addition, a lower amount of non-complexed quercetin remained suspended in the chloroform solution after centrifugation. Thereby, an aliquot, mostly free of insoluble particles, of the complex diluted in chloroform could be taken from the falcon tube and then filtered on 0.2 μm PTFE syringe filter. The filtered solution was then analyzed by HPLC in order to quantify the complexed quercetin. Nonetheless, the calculation of the complexation efficiency of this quercetin-PL complex (mass ratio of 1:2) exhibited a quercetin entrapment of only 20%.

Finally, when the quercetin-PL complex was formed at a mass ratio of 1:2.67 (which corresponds to a molar ratio 1:1), a high solubilization of the complex in chloroform was observed. Later, in the centrifugation, a very low amount of insoluble or non-complexed quercetin appeared at the bottom of the falcon tube. In this sense, and as already discussed, this quercetin-PL complex allowed to complex 97% of quercetin. Therefore, a quercetin-PL molar ratio of 1:1 was confirmed as the most appropriate condition for obtaining a high complexation efficiency. This results are in agreement with Xu et al., (2016); Zhang et al., (2016); Khan et al., (2013); Ramadan, (2008) and now it can be argued with certainty that decreases in the proportion of PL in the complex will conduct to a significant decreasing in the complexation efficiency of quercetin, and that increases in the amount of PL above this ratio are unnecessary.

4. Oil enrichment with quercetin-phospholipid complex by solvent route

The results obtained for the two different solvent methods for enriching BCoil, with the quercetin-PL complex diluted in chloroform after the purification, has been described in the following sections.

4.1. Progressive enrichment of BCoil by solvent route

For this enrichment, it was first prepared a quercetin-phospholipid complex as described in section 2.2.5.1 (*Chapter II*) using 0.03 g of quercetin and 0.08 g of PL (which corresponds to a molar ratio 1:1). After complex purification with 20 mL of chloroform and considering a complexation efficiency of 75%, the concentration of complexed quercetin in the chloroform solution was then 1125 $\mu\text{g/mL}$. The progressive enrichment consisted of four additions of 1.5 mL (each addition) of complex diluted in chloroform to 7 mL of BCoil with an evaporation step (nitrogen flux) between each addition (Figure III-6). Thereby, it was first calculated the theoretical quercetin concentration of enriched oil after each addition of diluted complex, and it was then compared to the measured quercetin concentration by spectrophotometric method, as shown in Table III-6.

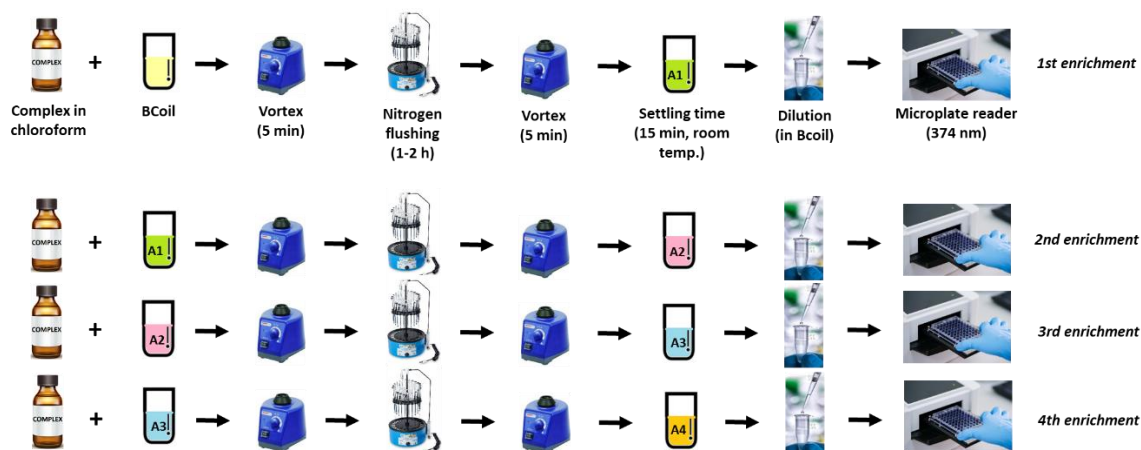


Figure III-6. Schematic representation of the progressive enrichment of BCoil by solvent route

As displayed in Table III-6, significant discrepancies between the concentration of quercetin in the oil measured by spectrophotometric method after each addition of complex and the theoretical concentration of quercetin were obtained. As it was previously mentioned in chapter II, it is feasible that the overestimation on the results was due to the presence of non-soluble quercetin particles in the stock solution prepared to build the calibration curve.

Table III-6. Theoretical and measured quercetin concentration in BCoil progressively enriched with quercetin-PL complex

	Progressive addition of complex			
	A1	A2	A3	A4
Volume (mL) of diluted complex added to BCoil	1.5	1.5	1.5	1.5
Accumulated volume (mL) of diluted complex added to BCoil	1.5	3	4.5	6
Theoretical quercetin concentration $\mu\text{g/mL}$ BCoil	241	482	723	964
Measured quercetin concentration $\mu\text{g/mL}$ BCoil	1256 ± 81	1758 ± 90	2494 ± 101	2189 ± 139

After observing the discrepancies in the progressive enrichment of BCoil, it was necessary to hardly work on developing an accurate method for quantifying quercetin concentration in saturated solutions. Therefore, this work developed several methods (described above in section 2.1) for validating the measures of quercetin solubility in oils in order to obtain reliable and reproducible results.

Once validated the methods for measuring the solubility of quercetin in oils, it was evaluated the quercetin solubility in other solvents and the “one step enrichment method” was carried out.

4.2. One step enrichment of BCoil by solvent route

For this enrichment, the quercetin-PL complex was prepared in absolute ethanol as solvent medium. After purifying the complex by the addition of chloroform, a single amount of diluted complex was added to BCoil. The volume of the single amount of complex added to the oil varied in order to provide PL concentrations of 1, 2.5, 10 and 16 mg PL/g BCoil. After removing the chloroform, the enriched oil was stirred, left to settle, centrifuged and filtered for quercetin quantification. The solubility of complexed quercetin in BCoil at various PL concentrations has been shown in Table III-7.

Table III-7. Solubility of complexed quercetin at different PL concentrations by the one step enrichment method

PL (mg/g BCoil)	Theoretical quercetin concentration ($\mu\text{g/g BCoil}$)	Quercetin solubility ($\mu\text{g/g BCoil}$)
16	5 800	121 \pm 1
10	3 600	66 \pm 4
2.5	900	58 \pm 2
1	390	37 \pm 1
0	5 000	393 \pm 38

Results in Table III-7 showed that the enrichment of BCoil through the one step enrichment process did not lead to increase the solubility of quercetin in oil, since lower values of quercetin solubility were found in oils enriched with quercetin-PL complex than that saturated with pure or non-complexed quercetin (393 mg/g BCoil). Although as concentration of PL in Bcoil increased from 1 to 16 mg/g, more quercetin could be dissolved in BCoil (it passed from 37 to 121 μg quercetin/g BCoil), the complexation of quercetin by this method was deleterious for quercetin solubility.

Even though this result was unexpected, in the best of our knowledge there are no reports related to the solubility estimation of complexed quercetin in seed oils that allow us to compare these results. The solubility of complexed quercetin has been merely assessed in water (Xu et al., 2016; Singh et al., 2012; Suárez et al., 2011), chloroform (Xu et al., 2016) and octanol (Suárez et al., 2011).

Our hypothesis for explaining these results is that although the quercetin-PL complex allowed the solubilization of quercetin in chloroform, the complexation, or the entrapment of quercetin in PL was not sufficiently stable over time. We are based on the fact that approximately after 1-2 hours, precipitates and suspended particles began to appear in the chloroform solution. Furthermore, more insoluble, and suspended particles were observed when diluted quercetin-PL complex was added to BCoil. In Figure III-7 can be observed the macroscopic aspect of quercetin-PL complex right after its purification with chloroform and after 1 hour of storage at room temperature. From this perspective, it can be hypothesized that the bonding of quercetin with PL was not sufficiently stable over time since some quercetin began to separate from PL and therefore it lost the capacity to stay soluble in chloroform.

In this context, some deeper analysis of the quercetin-PL complex by means of infrared spectroscopy, differential scanning calorimetry or x-ray diffraction techniques are suggested in order to certainly establish the formation of a complex between quercetin and PL. It is worth mentioning that the direct enrichment of BCoil with the quercetin-PL film (complex) formed after rotary evaporation of ethanol was attempted however, the film remained attached to the walls of the round bottom flask without dissolving in the oil.

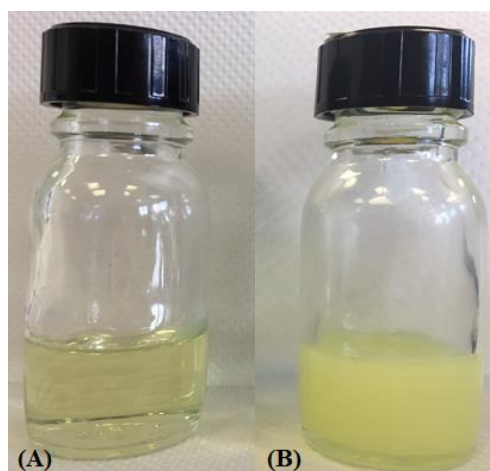


Figure III-7. Macroscopic aspect of quercetin-PL complex right after its purification with chloroform (A) and after 1 hour of storage (B)

From the previous results, we could conclude that the enriching methods using the quercetin-PL complex diluted in chloroform are inefficient for improving the solubility of quercetin in oil and also time-consuming techniques. Thereby, a new method for enriching oil with quercetin-PL formulation by a solvent-free route was proposed, aiming at increasing the solubility of quercetin in edible oils and hence improve their oxidative stability.

5. Oil enrichment by a solvent-free route using quercetin-PL formulations

During this section, the results obtained related to the oil enrichment with several quercetin-PL formulations, by a solvent-free route, are discussed. The schematic representation of the solvent-free protocol is displayed in Figure III-8.

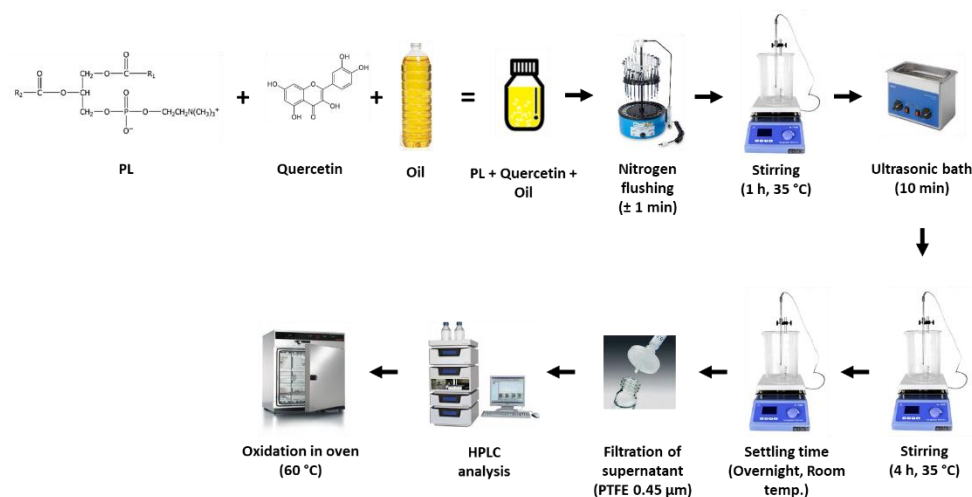
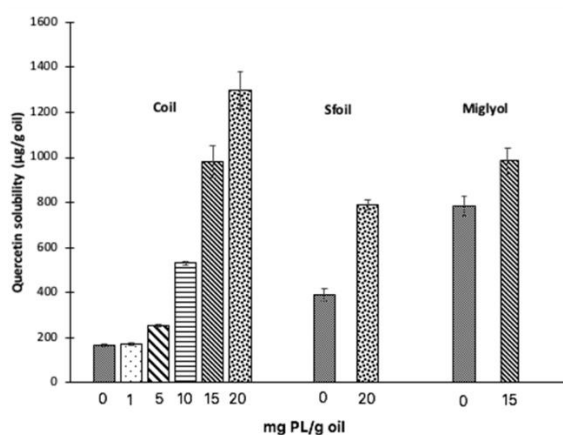


Figure III-8. Schematic representation of solvent-free route steps

As it can be observed in Figure III-9 the enrichment of edible oils with PL allowed a significant improvement of quercetin solubility in Miglyol, Sfoil and Coil. Compared with the systems free of PL, the solubility of quercetin in Miglyol, Sfoil and Coil increased 1.2-fold (MQ* versus MQL15*), 2-fold (SQ* versus SQL20*) and 7.7-fold (CQ* versus CQL20*), respectively. The presence of phosphatidylcholine overcame the limited solubility of quercetin in all oils, particularly in Coil (i.e. 168 µg quercetin/g without PL against 1 298 µg quercetin/g with 20 mg PL/g).



ID Sample	Oil	mg PL/g oil	Solubility (µg quercetin/g oil) ^a
CQ*	Coil	0	168 ± 6
SQ*	Sfoil	0	390 ± 28
MQ*	Miglyol	0	783 ± 43
CQL1*	Coil	1	173 ± 7
CQL5*	Coil	5	253 ± 4
CQL10*	Coil	10	530 ± 10
CQL15*	Coil	15	981 ± 70
CQL20*	Coil	20	1 298 ± 84
SQL20*	Sfoil	20	792 ± 22
MQL15*	Miglyol	15	984 ± 56

^aData expressed as mean values and standard deviations

Figure III-9. Solubility of quercetin in oils enriched with PL at various concentrations

Moreover, a significant linear correlation between quercetin solubility and the concentration of PL in Coil (CQL5*, CQL10*, CQL15*, CQL20*) was also observed (Figure III-10) (Pearson correlation $R^2 = 0.98$, $p \leq 0.05$). From linear correlation was observed that the solubility of quercetin was correlated with PL concentration by a factor close to 0.055 (in a concentration range of 5 to 20 mg PL/g), which corresponds approximately to a dissolution of 1 molecule of quercetin per 8 molecules of phosphatidylcholine (quercetin:PL mass ratio 1:20).

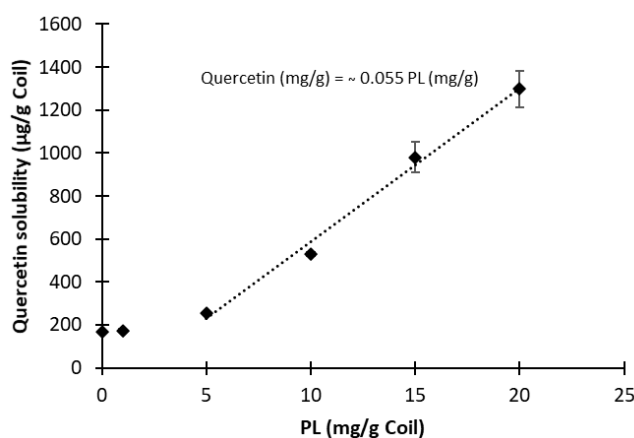


Figure III-10. Correlation between quercetin solubility and PL concentration in Coil

A higher quercetin solubility and its correlation with the PL concentration can be explained by the interaction between the amphiphilic molecules (PL) and the -OH groups of the phenolic rings of quercetin which are implicated in the formation of hydrogen bonds and/or van der Waals interactions. In this sense, quercetin-PL interactions have been previously identified. For instance, Nasibullin et al. (2002) studied the interaction of phosphatidylcholine with quercetin by NMR and highlighted the ability of quercetin and phosphatidylcholine to form chain structures linked by hydrogen bonds. In addition, they showed that the most probable interactions between PL and quercetin involve the π electron system of ring B of quercetin with the choline head of PL, and the formation of a hydrogen bond between the OH group (O19, H29) of ring A and the phosphate group of phosphatidylcholine (Figure III-11). On the other hand, the antioxidant activity of quercetin is related to the 3-OH group of the AC-ring and to the catechol group in the B ring. Therefore, the interaction of quercetin with PL might not hamper its antioxidant capacity since different OH groups are involved.

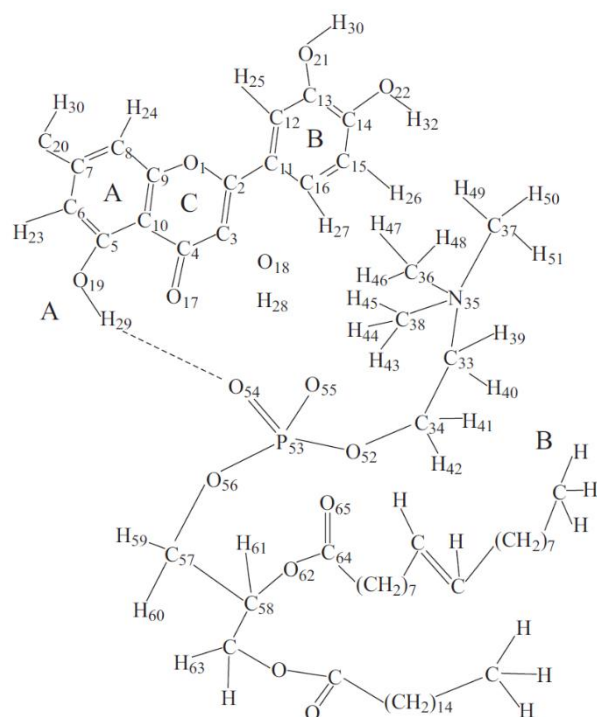


Figure III-11. Structure of the complex of (A) quercetin and (B) phosphatidylcholine
(Adapted from: Nasibullin et al., 2002)

On one side, the presence of hydroxyl groups denotes the preference of quercetin for polar domains (Khan et al., 2014; Li et al., 2013; Tan et al., 2012; Maiti et al., 2007; Ollila et al., 2002), whereas its phenolic aromatic rings could be involved in hydrophobic interactions (Pawlikowska-Pawłęga et al., 2007). In fact, Pawlikowska-Pawłęga et al. (2007) after determining the electronic absorption maxima, observed that in a phospholipid membrane system, quercetin was located in the polar head group region rather than in the hydrophobic zone of the lipid bilayer. By using a DSHA fluorescent probe to monitor possible perturbations of the lipid bilayers by the incorporation of flavonoids, Ratty et al., (1988), demonstrated that flavonoid aglycones could interact directly with the polar head group of phospholipids on the surface of the membranes.

The interactions between phospholipids and phenolics are the basis of the fabrication of phenolic-phospholipid complexes leading to an enhanced solubility in aqueous medium. However, the ability of phospholipids to enhance the solubility of bioactive compounds in other media is rarely quantified except for chloroform. For instance, Zhang et al., (2016) and Xu et al., 2016 observed a significant increase of the solubility of quercetin in water (13-fold and 3-fold, respectively) and in chloroform (more than 700-fold) (Xu et al., 2016) when quercetin

was complexed with phospholipids. Moreover, the physical mixture of quercetin and soy lecithin also significantly increased the solubility of quercetin in chloroform (14-fold), and to a lesser extent in water (1.3-fold) (Xu et al., 2016). Freag et al. (2018) attributed the increase of solubility of celastrol in aqueous medium to the solubilizing effect of the phospholipids when celastrol was complexed or only mixed with soy phosphatidylcholine. The ability of delivery systems (made of phospholipids) to enhance the solubility of bioactive compounds has been also reported by Ghanbarzadeh et al. (2016).

Several authors have observed that during the direct enrichment of seed oils, the solubility of phenolic compounds can be improved by the addition of phospholipids (Fregapane et al., 2020; Suárez et al., 2011). For instance, during the enrichment of virgin olive oil with olive leaves extracts, Delgado-Adámez et al. (2014) reported an increase in the TPC of the enriched oil from 800 to 1800 $\mu\text{g CAE/mL}$ by the addition of 6 mg/g of lecithin. These authors highlighted the ability of lecithin to stabilize the phenolic compounds in bulk oils. Similarly, Li et al. (2015) also observed that during the enrichment of refined sunflower oil with phenolics from olive leaves, the addition of lecithin (5% w/w) could significantly increase the phenolic extraction efficiency from 1.87 to 9.5 mg/g. They attributed this behavior to the fact that the presence of phosphate group in lecithin endows with an even more polar character and allows the formation of a larger interface and stronger interactions with phenolic compounds that lead to a higher phenolic extraction. Li et al. (2015) concluded that the significant increase in the extraction yield obtained by lecithin addition is related to the presence of well-defined colloidal aggregations formed by this amphiphilic molecule. Our results are also in agreement with the simulations of Yara-Varón et al. (2017) that showed that the theoretical solubility of bioactive compounds (polyphenols) in plant oils can be enhanced by the incorporation of food additives such as surfactants (soy lecithin, diglycerides, etc.). Some microscopes observations made in this work during solubilization of PL+quercetin in Miglyol showed that PL and quercetin seems to form aggregates in bulk oil during dissolution process (Figure III-12).

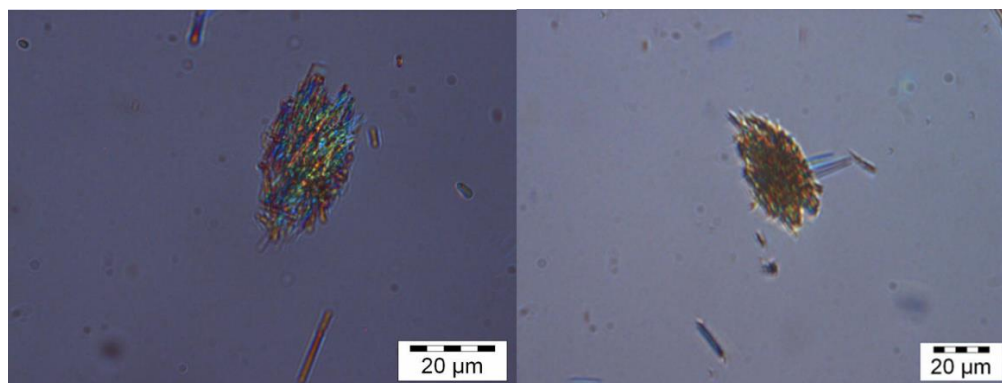


Figure III-12. Microscopic images after 30 min of solubilization of PL+quercetin in Miglyol

It is worth mentioning that we also attempted to observe the interaction between PL and quercetin in bulk Coil, related to increased solubility of quercetin, by means of infrared spectroscopy analysis. For that, pure Coil, Coil saturated with quercetin (168 $\mu\text{g/g}$), Coil + PL (20 mg/g) and Coil + PL (20 mg/g) + quercetin (168 $\mu\text{g/g}$, 500 $\mu\text{g/g}$) were analyzed by infrared spectroscopy using a Nicolet 6700 (Thermo Scientific) spectrometer equipped with an MCT detector. However, due to the nature of Coil (a complex matrix consisting of several compounds/molecules) and the low concentration of both quercetin and PL in the Coil, the infrared spectra was inefficient to observe modifications or shifts on the stretching bands of quercetin and PL, therefore the analysis was not conclusive. According to literature, the infrared spectroscopy analysis carried out for studying the interactions between PL and quercetin (in a formed complex) is done by analyzing the pure compounds in solid state (Freag et al., 2018; Zhang et al., 2016; Singh et al., 2012), and there are no reports of this type of analysis in bulk oils.

6. Critical Micelle Concentration (CMC) of PL in Coil

The interactions between quercetin and phospholipids could lead to molecular complexes or to supramolecular structures. As previously discussed, in bulk oils the structuration of PL in colloidal structures such as reverse micelles plays a key role in the interaction with phenolic compounds, since this association may improve the dispersion, solubilization and stabilization of phenolic in an oil matrix (Li et al., 2015). Moreover, the formation of these aggregation also plays a key role in the antioxidant activity of phenolic compounds in bulk oils and therefore in its oxidative stability, however this latter issue will be carefully discussed in next chapter.

From that perspective, it is essential to study the formation of colloidal structures in the oils enriched in this work. In this sense, CMC measurements allow to investigate the potential micelle formation and the concentration at which this formation occurs. On the other hand, more than only determining the CMC of PL in camelina oil, what is important in this section is to evaluate the micelle formation for understanding the structural organization of the PL-quercetin system around the water cores of bulk oils that may influence both quercetin solubility and antioxidant activity. To study the assembly of PL in camelina bulk oil the TCNQ method has been used (Zhao et al., 2020; Laguerre et al., 2011). The principle of the TCNQ method is that the reversed micelles solubilize the TCNQ dye leading to a rapid change of color around the CMC (Lehtinen et al., 2017).

In our case, CMC would reveal the formation of reverse micelles of phospholipids with the two hydrophobic fatty acid chains pointing towards the oil phase and the polar head groups (phosphoric based esters) pointing into the hydrophilic core (usually called “water pool”) of the micelles at a nano-scale (Hancer et al., 2002; Gupta et al., 2001). Indeed, phospholipid self-assembly into such colloidal structures has been reported in bulk oils by several authors (Kittipongpittaya et al., 2014; Chen et al., 2010; Danino et al., 2002; Gupta et al., 2001; Subramanian et al., 2001) which also stated that the micelles size, among other characteristics, are influenced by changes in the oil water content. Although the water content of Coil was not measured, plant oils naturally content 200–2000 ppm of water (Zhao et al., 2020) that plays a key role in the formation of colloidal structures since it affords an oil-water interface where phospholipids could associate into micelles. Moreover, exogenous water was indirectly added to Coil by its enrichment with PL, since PL contains 1-2% of water.

The result of the TCNQ method for determining the CMC of PL in Coil is shown in Figure III-13. As can be observed, at low concentrations of PL in Coil there was no significant modifications in the absorbance of TCNQ. By contrast, above 79 $\mu\text{mol PL/kg oil}$, the absorbance increased drastically. In this sense, after performing a numerical analysis of data to determine the intercepting of the two slopes that provided the highest R^2 ($R^2 > 0.83$), the CMC of PL (mainly phosphatidylcholine) in Coil was found to be around 79 $\mu\text{mol phospholipid/kg oil}$ (0.061 mg PL/g oil). According to Laguerre et al., (2011), this inflection point determines the CMC, in which a charge transfer occurs between TCNQ and phospholipids when they self-assemble in physical structures. This CMC value is in the same range as that found for dioleoylphosphatidylcholine in corn oil (65 $\mu\text{mol/kg oil}$) (Laguerre et al., 2011), but lower than

that reported for phosphatidylcholine in stripped peanut oil (122 $\mu\text{mol/kg}$ oil) (Zhao et al., 2020). These variations may account for differences in the FA chain length of the phospholipids, the presence of minor endogenous compounds (such as free fatty acids, partially hydrolyzed triglycerides that are still present in crude oils), and the content of endogenous water in oils (Kim et al., 2018b).

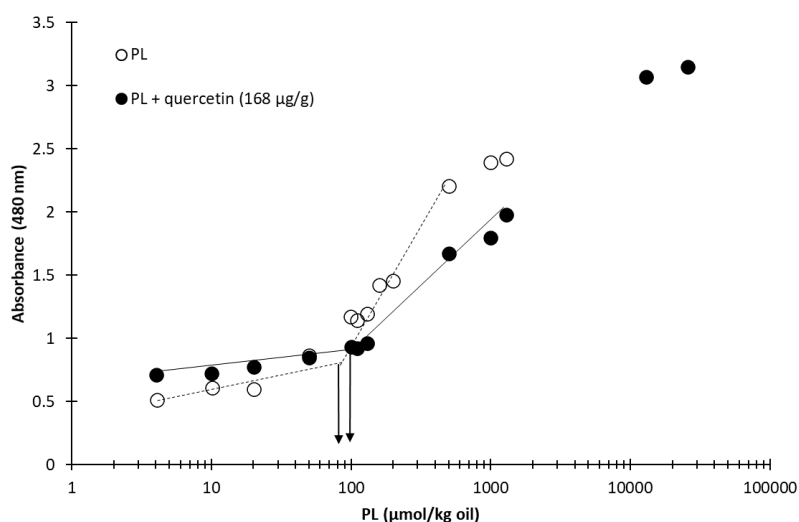


Figure III-13. Determination of the CMC by TCNQ probe of PL alone and PL + quercetin

Figure III-13 also showed that the CMC of phospholipid slightly increased to 98 $\mu\text{mol PL/kg}$ oil (0.076 mg PL/g oil) with the addition of quercetin at the saturated concentration of 168 $\mu\text{g/g}$ (0.016% wt). However, accounting for the model numerical errors, it is difficult to conclude about the significance of a 20 $\mu\text{mol PL/kg}$ oil difference. Some authors have reported that the addition of components to bulk oils, at certain concentrations, may modify the CMC of phospholipids, but it consists mostly in surface-active components. Lehtinen et al. (2017) studied the effect of the addition of oleic acid in the CMC of lecithin in rapeseed oil at room temperature and 70 °C. They observed for instance that only the addition of oleic acid above 5% (wt) significantly increased the CMC of lecithin (that is 0.055% wt.) in rapeseed oil either at room temperature or 70 °C. Similarly, Kittipongpittaya et al. (2014) studied the CMC of 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) in corn oil and confirmed that the addition of oleic acid at 0.5% did not affect the CMC value of DOPC however, the increase in the addition of oleic acid from 0.5 to 5% (wt) led to increase the CMC from 400 to 1 000 $\mu\text{mol/kg}$ oil. It was stated that, because of its surface-active capacity, oleic acid could reside at the oil/water interface, acting therefore as a co-surfactant that alters the assembly properties of the DOPC reverse micelle.

Moreover, we have also verified that the presence of quercetin in Coil within the concentration range of 8 to 168 $\mu\text{g/g}$ and without PL, did not form micelles when the TCNQ method was carried out. In other words, it has been confirmed that quercetin at 168 $\mu\text{g/g}$ did not interfere with the formation of micelles and did not significantly change the assembly behavior of phospholipids despite the strong interactions between the two components evidenced by the enhanced solubilization of quercetin.

The influence of quercetin concentrations above 168 $\mu\text{g/g}$ on the CMC of PL in Coil was not studied because higher concentrations of quercetin could not be dissolved in Coil, unless the Coil was enriched with PL above a concentration of 5 mg/g. When TCNQ dye was added to Coil enriched with PL at 5, 10, 15 and 20 mg PL/g and saturated with quercetin (at each PL concentration), the UV-absorbance read at 480 nm exceeded the value of 2.5. The high light absorption at 480 nm of these samples could be an indirect indicator of micelle formation at those PL-quercetin concentrations. Nonetheless, the confirmation of colloidal structuration in camelina oil enriched with PL (5, 10, 15 and 20 mg/g) and quercetin above 168 $\mu\text{g/g}$ by means of analysis such as small-angle X-ray scattering, is highly recommended.

In the way to understand the possible organization between quercetin and phosphatidylcholine, some authors (Laguerre et al., 2009) suggested that the phospholipid reverse micelles formed above the CMC may act as carriers for phenolic compounds due to the presence of co-micelles which transfer the phenolic compounds to the water phase. Similarly, Li et al. (2015) showed that the presence of phospholipids in bulk oils confers a more polar character and provide a larger interface for interacting with phenolic compounds. It is important to highlight that the enrichment of Coil carried out in this study has been done at significantly higher concentrations (5 to 20 mg/g or 6 451 to 25 805 $\mu\text{mol/kg}$) than the CMC of PL in Coil (79 $\mu\text{mol/kg}$), therefore, close proximity between quercetin and phosphatidylcholine is plausible.

It is worth mentioning that some preliminary tests of self-assembly of PL-quercetin system in bulk oils were performed by Dr. Nada Taib-Maamar (from CBMN institute) by means of molecular dynamics simulation technique, in particular coarse grain (CG) approach. For that, Martini force field (Marrink et al., 2007) and GROMACS 4.5 package (Oostenbrink et al., 2004) were used. These tests showed that PL tended to surround quercetin particles when incorporated to bulk oils (in presence of water). As can be observed in Figure III-14A, PL and quercetin are dispersed in the background, but with time (Figure III-14D), PL and

quercetin began to organize themselves, along with water molecules, in forms of aggregates. More analysis or molecular simulations are necessary to conclude that these aggregates correspond to micelle structures. This preliminary simulation just aimed at providing an idea about the organization of a PL-quercetin system in bulk oils.

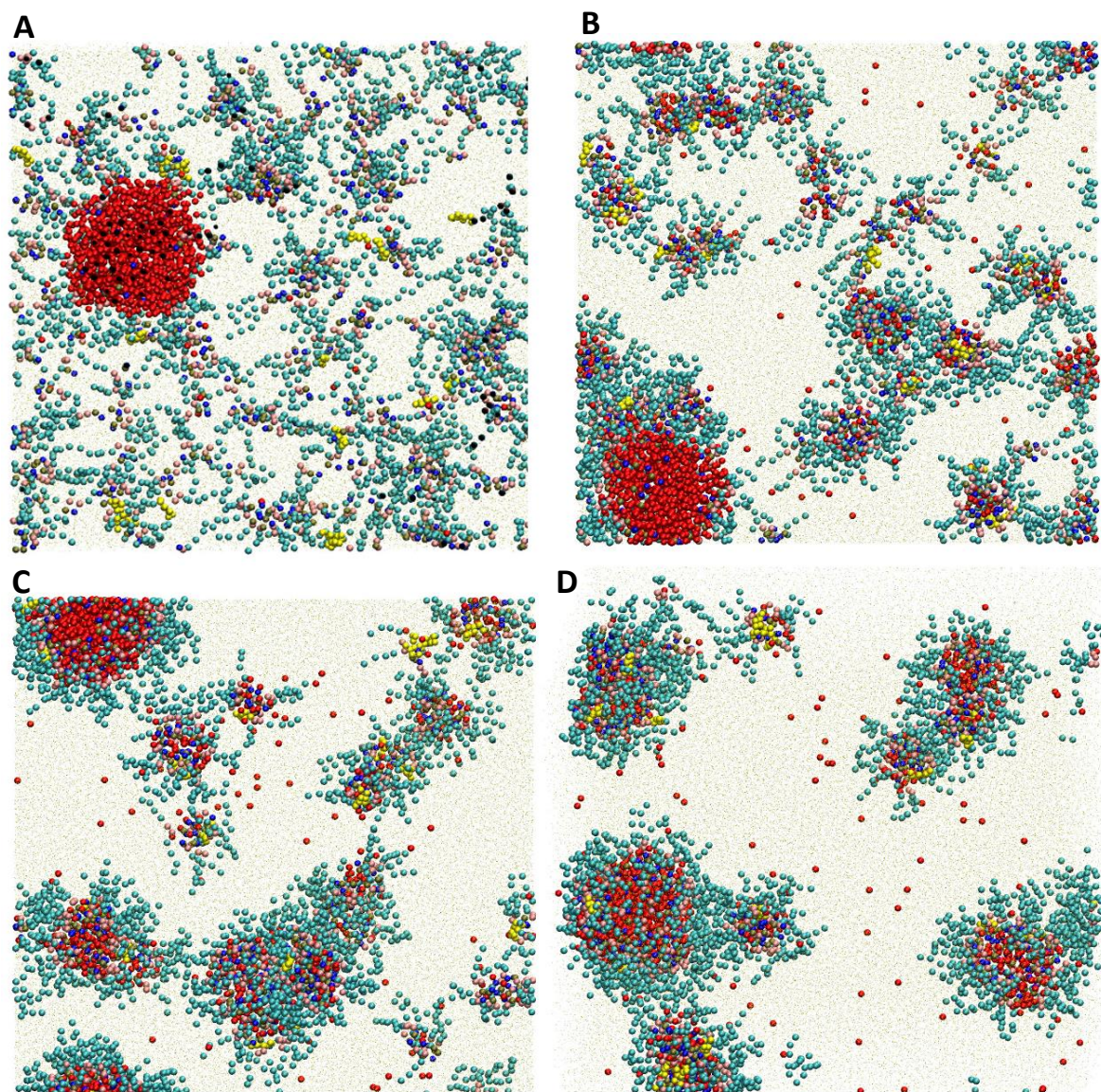


Figure III-14 : Snapshots of the self-assembly process at 0 μs (A), 1 μs (B), 2.5 μs (C) et at 5 μs (D). Quercetin is shown as yellow bead, water is shown as red bead, PL tails as cyan bead, PL phosphate headgroup (phosphorus) in green and choline (nitrogen) in blue, glycerol in pink and triglycerides as yellow dots

7. Conclusion and perspectives

From this chapter, it can be concluded that among the evaluated edible oils, they presented a different chemical and fatty acid composition due firstly to their plant origin and plant variety but also because of their industrial treatment (refined or no refined oils). For instance, lower content of pigments, free fatty acids, etc. were expected in refined Sfoil than in virgin camelina oils, moreover Coil exhibited a higher content of metals than the other oils. These variations in oil composition obviously played a key role in the dissolving of quercetin in bulk oils.

It has been also shown the significant discrepancies among the solubility data of quercetin in water that has been reported in literature. Moreover, discrepancies between the data reported in this work and the one found in literature regarding quercetin solubility in solvents such as DMSO, ethanol and PEG 400 was also found. These discrepancies can be related, among other factors, to the difficult task of developing a proper and accurate method or protocol for estimating the solubility of poorly soluble solutes in certain solvents. From that perspective, we have validated in this chapter, a reproducible and confident method for estimating the solubility of quercetin in oils. And thereby, we have been able to also provide several data related to the quercetin solubility in other solvents than oil which are of great interest for food, pharmaceutical, cosmetic, and chemical applications. In this sense, amphiphilic solvents such as dimethyl sulfoxide and polyethylene glycol 400 exhibited high capacity to solubilize quercetin whereas water poorly solubilized it.

On the other hand, phenols such as naringenin, chlorogenic and sinapic acids were not soluble neither in camelina oil nor in sunflower oil. The solubility of polyphenols in oils cannot be merely predicted by its coefficient of partition ($\log P$) since plant oil are complex matrices in which many compounds may exert significant chemical interactions with exogenous polyphenols. However, the molecular structure of phenolic compounds such as the presence of OH groups, glycosides, quantity of phenol rings, etc. may denote some preferences for lipophilic or aqueous mediums.

Regarding the quercetin-PL complex, a high complexation efficiency ($> 95\%$) was obtained when absolute ethanol was used as complexation medium, moreover proportions of phospholipid below a quercetin-phospholipid molar ratio of 1:1, were deleterious for complexation efficiency. Ethanol proved to be a more suitable solvent for complex quercetin

with phospholipid due to its higher capacity of dissolving together quercetin and phosphatidylcholine than the chloroform:methanol mixture. Nonetheless, the oil enrichment with the quercetin-phospholipid complex diluted in chloroform (a solvent route) showed to be inefficient for improving the solubility of quercetin in camelina oil. In this sense, the analysis of the quercetin-PL complex through infrared spectroscopy, differential scanning calorimetry or x-ray diffraction techniques are suggested for future works, in order to certainly establish or discard the formation of a complex between quercetin and phosphatidylcholine. In addition, pretreatments such as lyophilization of the complex film, prior to oil enrichment, may facilitate the formation of a powder-like complex (porous particles) with a greater capacity to solubilize in oils.

Finally, the oil enrichment with PL formulation by a solvent-free route proved to be an efficient method for significantly improving the solubility of quercetin in Miglyol, camelina and sunflower oils. The enhanced solubility of quercetin by the addition of PL was related probably to the interactions formed between the choline head of PL and the π electron system of quercetin or between the OH group of quercetin and the phosphate group of phosphatidylcholine. Minor compounds could also play a role in the enhancement of quercetin solubility since Sfoil, Coil and Miglyol exhibited differences in the improvement of quercetin solubility in presence of PL. In addition, the presence of well-defined colloidal aggregations of PL may act as carriers for quercetin and allows the formation of a larger interface for having stronger interactions with polyphenols.

The formation of reverse micelles in bulk Coils enriched with quercetin-PL was proved by means of the 7, 7, 8, 8- tetracyanoquinodimethane (TCNQ) solubilization method. Further analysis related to a deeper characterization (such as size and shape) of this colloidal structuration in bulk oil can be interesting for future works.

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Appendix

Table III-A1. Sterols profile of Miglyol, Coil, BCoil and Sfoil

Sterol	Miglyol	Coil	BCoil	Sfoil
Cholesterol	16.2	6.8	6.1	< 0,1
Brassicasterol	< 0,1	5.2	4.7	0.1
24-methyle cholesterol	< 0,1	0.1	0.1	0.1
Campesterol	< 0,1	23.9	24.5	8.7
Campestanol	< 0,1	0.3	0.2	0.1
Stigmasterol	< 0,1	0.9	0.8	8.3
D7 campesterol	< 0,1	0.3	0.4	2.5
D523 Stigmastadienol	< 0,1	< 0,1	< 0,1	0.4
Clerosterol	< 0,1	0.5	0.6	0.6
beta sitosterol	83.8	55.3	55.6	56.6
stigmastanol	< 0,1	0.2	0.2	0.3
D5 avenasterol	< 0,1	5.9	5.8	2.6
D524 Stigmastadienol	< 0,1	0.8	0.9	1.6
D7 stigmasterol	< 0,1	0.1	< 0,1	13.5
D7 avenasterol	< 0,1	< 0,1	< 0,1	4.7
Total Content (mg/kg)	< 25	4824.2	4791.0	3340.6

Chapter IV: Improvement of the oxidative stability of Coil enriched with quercetin-phospholipid formulations

Introduction

Polyunsaturated fatty acids (PUFAs) are widely used in food, pharmaceutical and cosmetic applications as carriers of active substances which are useful in the treatment of several diseases. Nevertheless, PUFAs are highly susceptible to be oxidized leading oils and fats to degradation. Camelina oil can be considered as a promising product for food, pharmaceutical and cosmetic applications related to its high content of omega-3 essential fatty acids and natural tocopherols that may prevent degenerative and inflammatory conditions (Krzyżaniak et al., 2019; Moslavac et al., 2014). As it has been discussed in *Chapter III*, camelina oil contains about 50–60% of polyunsaturated fatty acids (PUFAs) among which 35–40% comprise α -linolenic omega-3 fatty acid, and 15–20% linoleic omega-6 fatty acid, whereas sunflower oil comprises 50–60% of linoleic omega-6 fatty acid and 0–1% of linolenic omega-3 fatty acid. Since the above makes camelina oil theoretically more susceptible to lipid oxidation than sunflower oil, this chapter has mainly focused on the strategies for improving the oxidative stability of camelina oil. In this context, the approach of this chapter is related firstly to the assessment of the oxidative stability by Schaal oven test of pure Coil and sunflower oil and then, of Coil enriched with different quercetin-phosphatidylcholine formulations.

The first part of this chapter consists of the assessment of the oxidative stability of pure camelina and sunflower oil through the monitoring of the formation of primary products of oxidation: hydroperoxides (PV) and conjugated dienes (CD). In addition, a linear correlation between PV and CD is obtained for oxidation of both camelina and sunflower oil, in concordance with several authors (Eliseeva et al., 2017; Marmesat et al., 2009).

As already discussed in *Chapter I*, the delay or prevention of lipid oxidation can be accomplished by the addition of natural antioxidants to edible oils which maintain quality and extend their shelf life protecting from rancidity. Thus, in the second part of this chapter, the oxidative stability of camelina oil enriched with different quercetin-phospholipid formulations is studied. For that, we evaluated the protective effect of both quercetin and phosphatidylcholine in different concentrations incorporated to oil through the solvent-free

route developed in previous chapter, *section 5*. We address the possible interactions between quercetin and phosphatidylcholine which results in an enhanced oxidative stability of camelina oil.

The assessment of the enhanced oxidative stability of enriched oil is carried out by estimating the oxidation lag time of various oils enriched with different quercetin-phospholipid formulations. The oxidation lag time is defined as the time needed to reach the maximum value of PV (estimated through the correlation between CD and PV) that is permitted for edible virgin (15 m_{eq}O₂/kg) and refined oils (10 m_{eq}O₂/kg) according to regulation of Codex Alimentarius, (1981).

In addition, in this chapter the degradation of quercetin under accelerated oxidation of enriched camelina oil is also monitored by HPLC analysis, which allows us to follow the remaining quercetin in oils related to their initial content of phospholipids and therefore of quercetin. At the end of this chapter, we study the oxidative stability of camelina oil at room temperature in order to correlate the accelerated oxidation at 60 °C with the hypothetical stability of oil stored at simulated home conditions.

1. Preliminary tests of oxidation

Before performing the main oxidative stability assessment of enriched and non-enriched oils, many preliminary tests were carried out in order to confirm the reproducibility of the experiments and set the appropriate conditions of accelerated oxidation:

- Accelerated oxidation of bulk oils in sealed and unsealed flasks: As it has been discussed in *Chapter I*, the oxidation rate of lipids is significantly influenced by the oxygen available in the system. A low concentration of oxygen may decrease the oxidation rate of oils submitted to accelerated conditions of heating. From that perspective, we have verified that enough oxygen was available in the 40 mL-sealed amber flasks during oxidation of bulk oils. For that, 7 g of pure Coil and Sfoil was placed in sealed and unsealed amber flasks and oxidized at 60 °C for several days. As can be observed in Figure IV-1, the CD formation in oils placed in sealed or unsealed amber flasks was equal. Therefore, it can be concluded that enough oxygen is available in sealed flasks for the oxidative stability assessment. In this context the following oxidation experiments were carried out in sealed amber flasks as already described in *Chapter II*.

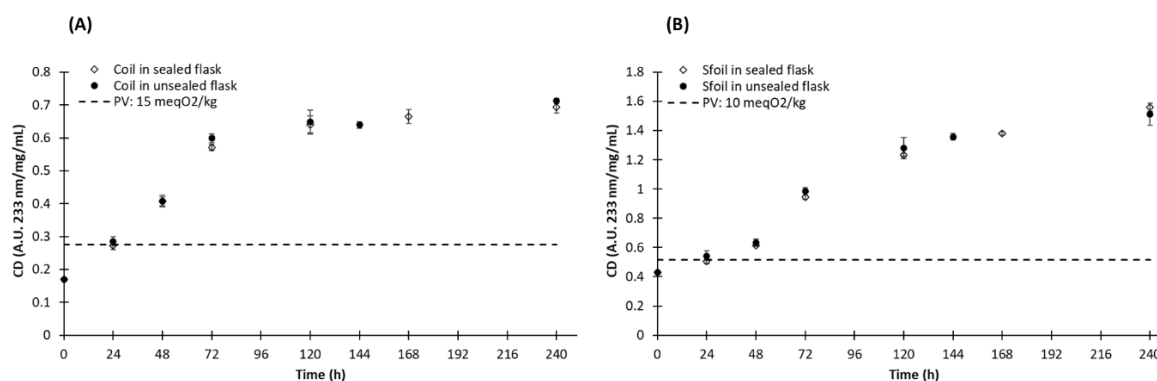


Figure IV-1. Time course monitoring of CD of (A) Coil and (B) Sfoil oxidized in sealed and unsealed amber flasks

- Accelerated oxidation of Miglyol: The time course formation of CD of Miglyol was also monitored during 10 days at 60 °C. The results of the monitoring showed that no oxidation reactions were taken place in Miglyol since the CD value varied few (< 5%) from 0.146 (A.U. 233 nm/mg/mL) along oxidation. This small variation corresponds to the analytical method deviation and not to a real oxidation of Miglyol. This behavior was expected since Miglyol is a neutral and not oxidizable oil due to its composition in saturated fatty acids (caprylic (56.6%)

and capric (42.7%) fatty acids). Miglyol was used mostly as model for the quercetin solubility assessment and for developing experimental protocols.

- Oxidation of non-enriched Coil after being submitted to all solvent-free route steps (Figure IV-2): In order to verify the influence of the heating treatment (5 hours at 35 °C), ultrasound (10 min) and filtration (0.45 μm PTFE syringe filter) on oxidative stability of oils, an aliquot of Coil was submitted to all aforementioned steps and then oxidized at 60 °C for 10 days. It is important to remind that during the solvent-free route, the oil samples were previously flushed with nitrogen in order to limit lipid oxidation.

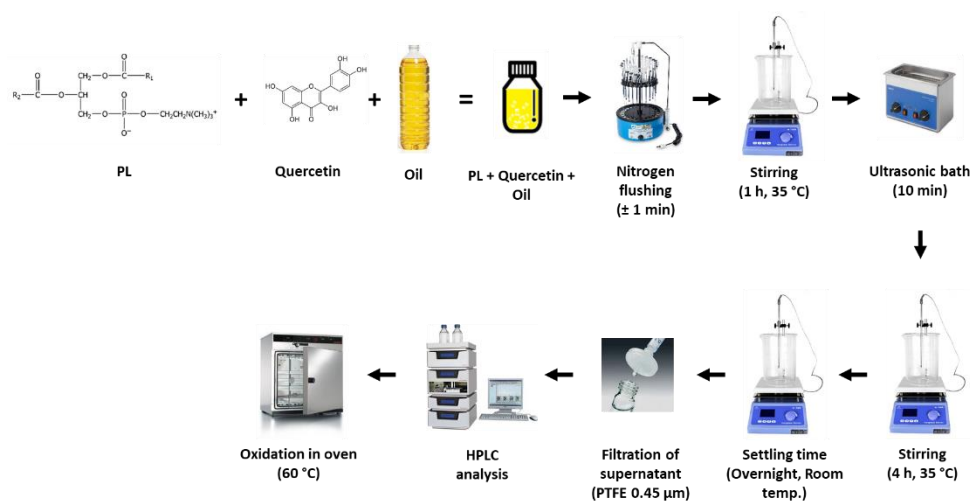


Figure IV-2. Schematic representation of solvent-free route steps (for samples saturated with quercetin)

The results of the oxidative stability assessment showed no significant differences in the formation of CD between raw Coil and Coil submitted to all solvent-free route steps shown in Figure IV-3. In this context, neither the temperature nor ultrasound treatment accelerated oxidation rate of Coil. The removal of oxygen from flasks by flushing nitrogen probably helped to avoid lipid oxidation during solvent-free route steps, moreover 35 °C can be considered a moderate temperature and ultrasound treatment was not so long. Hereinafter, the oxidative stability assessment of enriched Coils was compared with that of raw Coil (called pure Coil).

Considering that the samples enriched with PL and quercetin (at concentrations below solubility) were not filtered prior to oxidation, the oxidative stability of non-enriched Coil submitted to the same protocol of Figure IV-2 but without filtration was also verified. Nonetheless, it was not observed any significant difference on the oxidative stability of this latter sample (Figure IV-3). Although non-enriched Sfoil was not submitted to all solvent-free

route steps in order to evaluate any modification in the oxidative stability, we have assumed that similar to Coil, Sfoil was not affected by the steps shown in Figure IV-2.

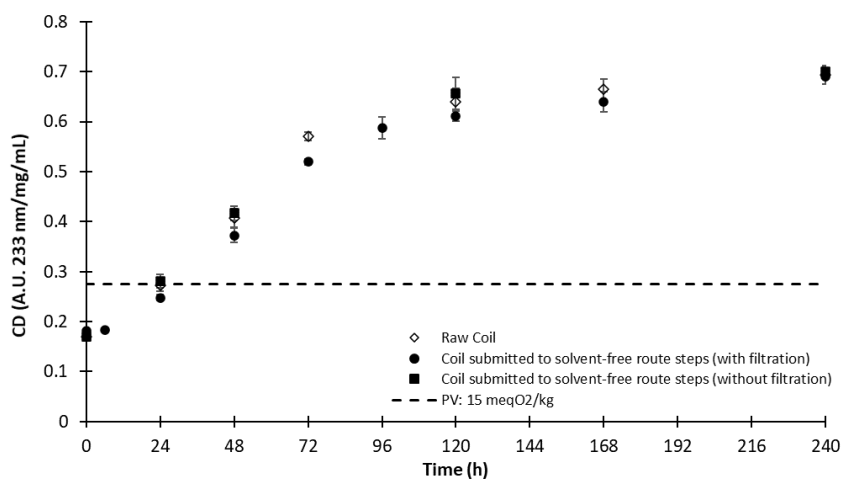


Figure IV-3. Time course monitoring of CD of raw Coil and Coil submitted to solvent-free route steps

2. Oxidative stability of non-enriched oils

To study the oxidative stability of vegetable oils is necessary to estimate the content of primary and secondary oxidation products over time, since the oxidative stability of a lipid system is a function of the formation rate of these compounds. It is also well known that lipid oxidation is more complex than that comprised in the three stages of oxidation; initiation, propagation and termination (Schaich, 2013), in which secondary oxidation products are theoretically formed after primary ones. Nonetheless, this work has focused on the monitoring of primary products of oil oxidation, i.e. conjugated dienes (CD) and peroxide value (PV), which are the criteria reported in current regulation (Codex Alimentarius) for considering an oil suitable for human consumption (food grade applications).

The principle of formation of CD is based on the fact that PUFAs suffer a shift in their double bond position when they are oxidized. This results in a mixture of conjugated dienes and hydroperoxide products that causes disturbances in the structure of lipids when the oxidation takes place (Corongiu & Milia, 1983). Thus, the oxidation of PUFAs can be detected by the increase in the UV absorption at approximately 233-235 nm due to the presence of CD. For that reason, the increase in absorbance is commonly known as an indicator of oxidation (Farhoosh et al., 2012). Among the numerous degradation products formed, hydroperoxides

are also associated to the primary oxidation products that are involved in propagation reactions (Eliseeva et al., 2017). These chemical species are classically quantified by PV and the Codex Alimentarius, (1981) fixes the PV of virgin oils (15 meqO₂/kg) and refined oils (10 meqO₂/kg) above which the oils are no more edible.

PV is defined as the number of micrograms of active oxygen peroxides contained in one kilogram of fat. This value is determined by an iodometric titration method, which is based on the oxidation of the iodide ion by hydroperoxides. PV measures the concentration of hydroperoxides that are formed continuously in the initial phases of lipid oxidation. However, PV measurement is rather time consuming, uses large volumes of organic solvents and needs quite high amount of raw materials, so quantification of CD is also used as a measurement of primary oxidation products especially since CD and PV are directly correlated (Eliseeva et al., 2017; Bachari-Saleh et al., 2013; Marmesat et al., 2009; Gómez-Alonso et al., 2004).

In this work, linear correlations between CD and PV of pure Coil and refined Sfoil oxidized at 60 °C were found and reported in Figure IV-4. The correlation between the primary oxidation products was given by the equation: PV (meqO₂/kg) = (CD (A.U. 233 nm/mg/mL) - 0.1127) / 0.0108 for Coil and, PV (meqO₂/kg) = (CD (A.U. 233 nm/mg/mL) - 0.3848) / 0.013 for Sfoil, with coefficients of determination of 0.99. A positive correlation between these two parameters allows to confirm that hydroperoxides behaved as supporting conjugated diene structures (Farhoosh & Pazhouhanmehr, 2009).

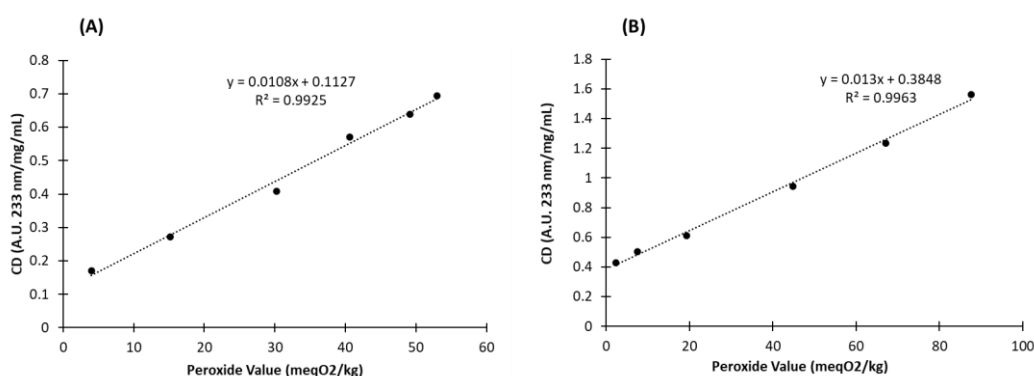


Figure IV-4. Correlations between CD and PV for (A) Coil and (B) Sfoil

A high correlation between these two factors indicates the possibility to use one of the factors if the other is not accessible during oxidation monitoring. In this sense, these correlations allowed us to estimate the PVs of enriched Coil and Sfoil submitted to accelerated

oxidation, through the measurement of the CD. We have established a correlation between CD and PV for each oil since this correlation is specie dependent (Sfoil and Coil).

Before oxidation, the PV of pure Coil and Sfoil were measured to be 4.01 and 2.24 $\text{m}_{\text{eq}}\text{O}_2/\text{kg}$, respectively, whereas the CDs of pure Coil and Sfoil were 0.170 and 0.429 (A.U. 233 nm/mg/mL), respectively.

As can be observed, Coil exhibited a higher PV value in comparison with Sfoil. The differences of PV in oils can be attributed to a mild oxidation that may occurs in the very earlier steps of oil processing. Edible oils are exposed to several factors as light, temperature, transition metals, that have a negative effect on the oil stability and may induce free radical production and subsequent lipid oxidation. According to Nogueira et al. (2019), the development of a mild oxidation not necessarily promote noticeable changes in the sensory aspects of the oils, however, it still can produce biologically reactive and toxic oxidation products. Moreover, levels of oxidation products can increase after the exposure of oil to conditions of transport, storage, and consumption. For instance, after purchase by the consumer, due to the opening of bottles, the development of oxidation reactions and consequently an increasing in the levels of lipid oxidation markers (as hydroperoxides, anisidine, malondialdehyde and volatile compounds) are observed because of the exposure of the oil surface to oxygen (Nogueira et al., 2019).

Another explanation for the differences in the PV values of Coil and Sfoil is related to the composition of each oil. According to Decker et al. (2010), a variation in content of peroxide value of different oils could be explained by the fact that the oxidation products formed from bulk oils vary depending on their composition of PUFAs and endogenous minor polar components. As shown in previous chapter, the Coil used in this study is comprised of a higher amount of ALA which is more prone to oxidize than Sfoil and also, Coil exhibited higher content of prooxidant metals compared to Sfoil (1.28 mg/kg against 0.009 mg/kg, respectively). The differences between Coil and Sfoil, in addition to the fatty acid composition, are also related to the fact that this latter is a refined oil whereas Coil is a virgin one. During refining process many prooxidant compounds (metals, phospholipids, pigments, waxes) are removed from bulk oils therefore, a lowered formation of oxidation products can be expected in refined oils. The age of the oil can also influence the initial content of hydroperoxides, since oils stored for longer times probably suffered oxidative reactions by oxygen, light and temperature action during storage.

On the other hand, from the linear regression shown in Figure IV-4, we deduced that for Coil, a CD value of 0.2747 (A.U. 233 nm/mg/mL) corresponded to a PV of 15 m_{eq}O₂/kg, which is the highest PV allowed in Codex Alimentarius, (1981) for virgin oils suitable for human consumption. And for Sfoil, a CD value of 0.5148 (A.U. 233 nm/mg/mL) corresponded to a PV of 10 m_{eq}O₂/kg, which is the highest PV allowed for edible refined oils.

In the following, we defined the oxidation lag time as the time needed for the different enriched Coils and Sfoil to reach CD values of 0.2747 and 0.5148, respectively, under accelerated conditions of oxidation (60 °C, in the dark, with no limitation of oxygen). Hence, this oxidation lag time was used in this work to study the effect of quercetin and PL concentration on the oxidative stability of oil.

Using the aforementioned criteria, it was found that the oxidation lag time of pure Coil and Sfoil are estimated in 24 ± 1 h and 26 ± 2 h, respectively.

3. Oxidative stability of enriched oils by quercetin-phosphatidylcholine formulations

3.1. Influence of PL on the oxidative stability of enriched oils

The oxidative stability of oils is influenced by both external factors and the composition of the oil system. In this context, the assessment of the oxidative stability of this work has been carried out giving a special attention to Coil as a non-stripped virgin oil, i.e. an edible oil preserving its minor compounds. Thus, the effect of PL concentration on the oxidative stability of enriched Coil was firstly assessed. For that, Coil was enriched with PL at different concentrations; 5, 10, 15 and 20 mg/g, without any addition of quercetin, and then submitted to accelerated oxidation at 60 °C. Figure IV-5 shows the positive influence of enriching Coil with PL by lowering the formation of CD during accelerated oxidation and therefore, enhanced the oxidative stability of Coil.

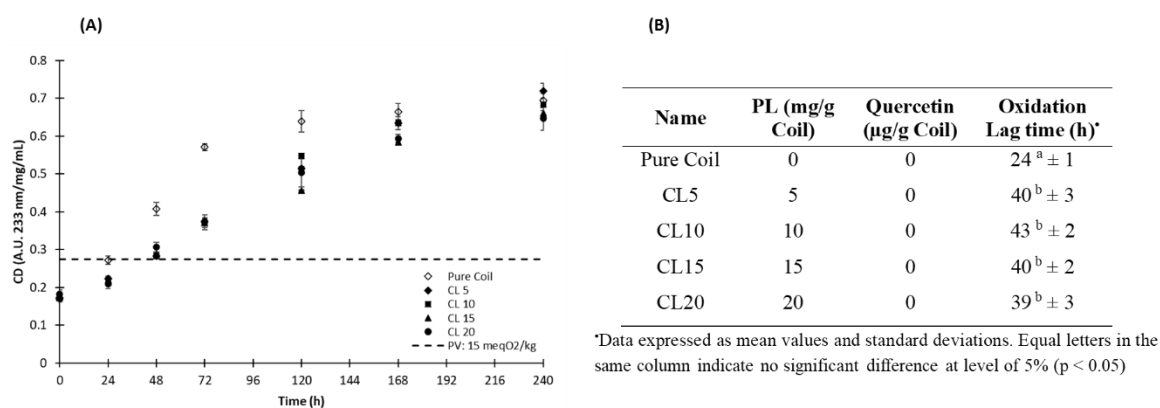


Figure IV-5. Oxidative stability of Coil enriched with PL at different concentrations by monitoring CD formation (A) and by estimating oxidation lag times (B)

More precisely, PL concentrations from 5 to 20 mg PL/g (0.5-2%) increased the oxidation lag time from 24 ± 1 h to 40 ± 2 h (Figure IV-5). Nevertheless, the protective effect against lipid oxidation was not correlated with the concentration of phospholipids in Coil since the maximum protective effect was already achieved at 5 mg/g Coil. The protective effect of PL against lipid oxidation observed in this work was consistent with that reported for other authors. For instance, Sugino et al. (1997) also reported a decrease in the autoxidation of a DHA-rich oil by the addition of egg yolk phospholipids. The protective effect was dose-dependent (in a range of 0.1-5%) for the egg yolk lipids that contained 31% of phospholipids. Furthermore, Lyberg et al. (2005) and Nwosu et al. (1997) reported the protective effect of phosphatidylcholine against lipid oxidation of DHA and salmon oil, when phospholipids were added at a concentration about 10 mg/g and 5 mg/g, respectively.

The mechanism related to oxidative stability due to phospholipids in oil systems is complex (Cui & Decker, 2016; Ramadan, 2012; Cardenia et al., 2011; King et al., 1992) since phosphatidylcholine has been reported to have an antioxidant (Cardenia et al., 2011), prooxidant (Cui et al., 2014), or no effect (Zhao et al., 2020) on oxidation reactions. Therefore, in order to discuss about the protective effect exhibited by PL in enriched Coil, some mechanisms of action of phospholipids in oils have been argued.

First, it is worth to discuss about the potential prooxidant character of PL. Phospholipids themselves can be oxidation substrates owing to the presence of unsaturated fatty acids. The fatty acid composition of PL used in this work evidenced C18:2 (6c) as the main PUFA contributor (65.6%) aside C18:3 (3) at 4.4%, the rest being C16:0, C18:1 (9c) and C18:0 at 15.4%, 11.4% and 3%, respectively. Both LA (C18:2 n-6) and ALA (C18:3 n-3) are

widely recognized to be highly susceptible to the free radical action due to their large number of double bonds and their position within the fatty acid chain, which induce the lipid peroxidation and the formation of reactive compounds. Moreover, as evidenced by the CMC measurement, the Coil enriched by PL at levels above 79 $\mu\text{mol/kg}$ is constituted by reversed micelles that create oil-water interfaces where hydrophilic prooxidants, such as iron, and amphiphilic compounds such as hydroperoxides, may come into close contact and therefore, increase the oxidation rate (Cui & Decker, 2016).

Although the water content of Coil was not measured, the addition of PL to the oil came along with the addition of water since PL contains 1-2% of water. From that perspective, by increasing the PL concentration in oil from 5 to 20 mg/g, the added water concentration increased from 50-100 ppm to 200-400 ppm. Though, this water content was a too modest amount to induce phase separation (Lehtinen et al., 2017), Laguerre et al. (2011) have reported that the addition of water to stripped corn oil at 400 ppm level maintain or decrease the oil stability, whereas in Kittipongpittaya et al. (2016), water itself added at 400 or 1000 ppm did not have effect on lipid oxidation of stripped corn oil but a combination of 1000 ppm of water plus minor compounds like oleic acid or sterols decreased the lag time significantly. Conversely, in the same study (Kittipongpittaya et al., 2016), the addition of water with the lipophilic α -tocopherol, that is not surface active, did not impact the antioxidant activity of the tocopherol. In our work, water added via the surface-active phospholipids could therefore have a negative effect though the small added concentration might minimize the effect.

On contrary, in this work, the addition of PL to Coil was found to exert a protective effect so, the prooxidant potentialities were offset by antioxidant properties that are mainly related to their polar head group as follow:

(i) Chelating metals: it has been widely reported that certain metals show deleterious effects on the organoleptic properties and oxidative stability of vegetable oils as oxidation process catalysts. The negatively charged phosphoryl group of PL is able to chelate metals especially iron and copper, thus limiting their reactions with PUFAs (Cui & Decker, 2016; Koga & Terao, 1995). Several studies have also reported the ability of phospholipids to chelate metals of high pro-oxidant activity (Cui & Decker, 2016; Koga & Terao, 1995; Yoshida et al., 1991; Jewell & Nawar, 1980). The presence of traces of metals as Fe, Cu, Mn and Ni in bulk oils depends on the crop's environment conditions and on the soil composition. The

concentration of these elements is an important criterion for the preservation of vegetable oils properties (Cabrera-Vique et al., 2012).

The chelating property of PL probably was of high importance for explaining the protective effect observed in this work, since Coil exhibited a high content of metals (1.28 mg/kg) as previously observed in Table III-1 (*Chapter III*). Virgin oils may exhibit a higher content of metal in comparison with refined ones, since during refining process many prooxidant compounds such as the traces of metals are usually removed (Daun & Unger, 2016). Hence, the hypothesis that PL acted as effective antioxidant by binding metals was highly considered.

(ii) the amine function of phosphatidylcholine which could directly interact with free radicals and block the propagation reactions (Henna Lu et al., 2011); (iii) the amino group of phosphatidylcholine could facilitate the transport of hydrogen or electron to oxidized tocopherols, favoring its regeneration and thus, delaying the formation of hydroperoxides (Samdani, 2018; Budilarto & Kamal-Eldin, 2015). Since Coil naturally contains tocopherols (726.4 mg/kg), a synergic effect between phospholipids and tocopherols could not be ruled out. Several studies in vegetable oils have indeed attributed the antioxidant properties of individual phospholipids to the synergism with tocopherols (Cui et al., 2015; Lee & Choe, 2011; Takenaka et al., 2007; Sugino et al., 1997; King et al., 1992; Kashima et al., 1991).

This synergism is due to the ability of phospholipids to regenerate tocopherols and/or alter their physical location, particularly by improving the accessibility of the functional group of tocopherol to the site where iron-dependent oxidation reactions take place (Cui & Decker, 2016). Koga & Terao, (1995) also studied the enhanced radical scavenging activity of Vitamin E (dl- α -tocopherol) by the addition of phospholipids in a model bulk oil. They found that when the oil was exposed to lipid-soluble radical initiator, the phospholipids exerted a slight effect on the action of Vitamin E. Nevertheless, when the system was exposed to a water-soluble radical initiator, the phospholipids enhanced the antiradical activity of Vitamin E. Thus, it can be attributed to phospholipids the capacity to improve the accessibility of tocopherol towards the aqueous environment, where the initiating oxidation reaction takes place and consequently, the capacity of tocopherols to scavenge free radicals is potentiated.

Since PL used in this work, contains 0.18% of tocopherols, its addition to Coil represents an addition of 0.009 to 0.036 mg/g of tocopherol that could be also implicated in the

synergism with phospholipids, acting together with the endogenous tocopherol of Coil for improving its oxidative stability. From that perspective and in order to evaluate if only the amount of exogenous tocopherol added to Coil through PL addition had an influence on the oxidative stability of Coil, a sample of Coil enriched with only 0.036 mg/g of α -tocopherol was prepared and then, oxidized at 60 °C. However, this sample did not exhibit any improved stability (Figure IV-6), therefore it can be stated that the antioxidant effect of PL was not due to the addition of exogenous tocopherol but to synergism between phospholipids and tocopherols. Protective effect against lipid oxidation in bulk oils by the incorporation of α -tocopherol at higher concentrations than 0.036 mg/g has been reported. For instance, Yin et al. (2012) found that α -tocopherol at a concentration of 0.11 mg/g was effective to protect high linoleic sunflower oil oxidized at 100 °C. Bakkalbaşı, (2018) significantly reduced the lipid oxidation rate of walnut oil when it was enriched with 0.1 mg α -tocopherol/g oil.

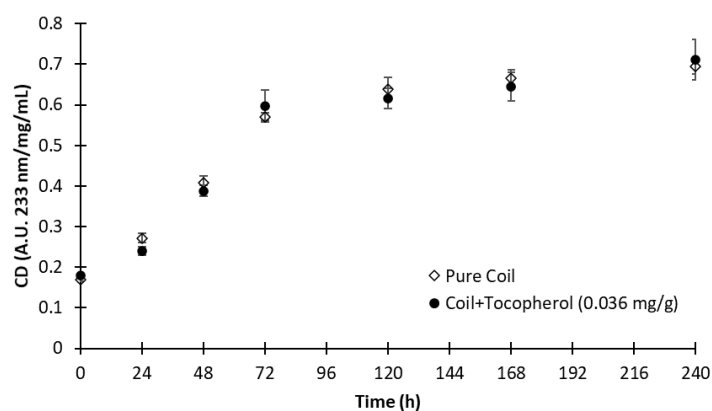


Figure IV-6. Time course monitoring of conjugated dienes of pure Coil and Coil + α -tocopherol (0.036 mg/g)

Regarding the influence of PL in the oxidative stability of Sfoil, it was not observed a significant increase in the oxidation lag time when PL was added to Sfoil at a concentration of 20 mg/g (31 ± 2 h), compared to the lag time of pure Sfoil (26 ± 2 h). Furthermore, when Sfoil was enriched with quercetin alone (at concentration of 230 and 390 μ g/g), or with PL (at 20 mg/g) plus quercetin at different concentrations such as 220 μ g/g, 440 μ g/g and 792 μ g/g, it was neither observed a significant increase in their oxidation lag times, since those samples exhibited an oxidation lag time between 26 and 31 h regardless the PL or quercetin concentration. From that perspective, it would be necessary to assess deeply the oxidative stability of enriched and non-enriched Sfoil between 0 and 31 h of accelerated oxidation (60 °C) as performed for Coil but not for Sfoil (as shown in Figure IV-7). Experimental data

of CD measurements after 6, 12, 24, 36 h is highly necessary to accurately determine the different oxidation lag times of various enriched Sfoils.

Although Figure IV-7 did not show protection effect of PL-querceetin formulations below the limit of edibility (i.e. $PV = 10 \text{ meqO}_2/\text{kg}$), it was observed a reduction in the formation of CD after 48 h of oxidation of enriched Sfoil. Nonetheless, this reduction of CD formation was the same regardless quercetin and PL concentration.

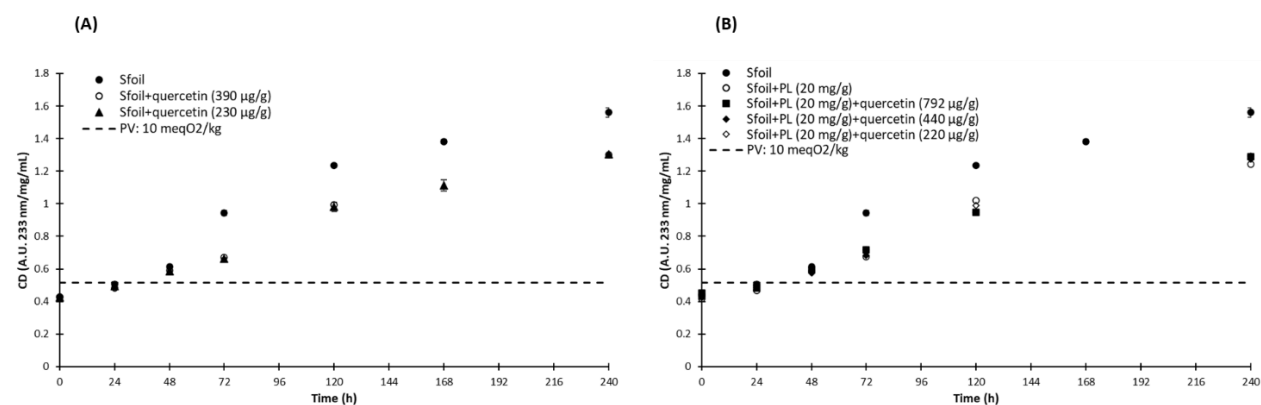


Figure IV-7. Time course monitoring of CD of **(A)** Sfoil with different quercetin concentrations (0, 230 and 390 $\mu\text{g/g}$) and of **(B)** Sfoil with PL (20 mg/g) and different quercetin concentrations (0, 220, 440 and 792 $\mu\text{g/g}$)

Considering that the various PL-querceetin formulations in Sfoil did not significantly increase the shelf life of enriched oils, this work focused on the enrichment of Coil with several PL-querceetin formulations and on the assessment of its enhanced oxidative stability. The fact that we have not observed significant variation in the protective effect of different PL-querceetin formulations in Sfoil could be because refined Sfoil exhibits naturally higher oxidative stability than virgin Coil since prooxidant endogenous compounds are removed during the refining process. For instance, oxidation of Coil might be catalyzed by the high content of metals (1.28 mg/kg), whereas in Sfoil the mechanisms involved in the oxidation should be different to metal catalyst, since Sfoil has a low concentration of metals (0.009 mg/kg) (Table III-1, Chapter III). These metals are probably the prooxidants mainly attenuated by PL, thus the protective effect of PL and probably of quercetin is likely to be less evident in a refined oil than in a virgin one which is plenty of prooxidant endogenous compounds.

In the next section, we have shown and discussed the effect of different concentrations of PL-querctetin formulations on the oxidative stability of Coil, that unlike Sfoil, the PL-querctetin formulations extended the shelf life of enriched oils.

3.2. Influence of PL-querctetin formulations on oxidative stability of enriched Coil

The influence of the interactions between PL and querctetin on the oxidative stability of enriched Coil was addressed in this section and it was summarized in Table IV-1 in terms of oxidation lag times. The monitoring of CD formations of different enriched oils was also provided below.

Table IV-1. Oxidation lag times of enriched and non-enriched Coil

Name	PL (mg/g Coil)	Querctetin (µg/g Coil)	Oxidation Lag time (h)*
Pure Coil	0	0	24 ^f ± 1
CQ*	0	168	28 ^f ± 1
CQL5*	5	253	117 ^{ab} ± 7
CQL10-220	10	220	63 ^c ± 4
CQL10*	10	530	109 ^{ab} ± 6
CQL15-220	15	220	57 ^{cd} ± 3
CQL15*	15	981	121 ^a ± 9
CQL20-220	20	220	58 ^{cd} ± 4
CQL20-440	20	440	74 ^c ± 4
CQL20-740	20	740	101 ^b ± 4
CQL20*	20	1298	115 ^{ab} ± 7

*Data expressed as mean values and standard deviations. *Samples saturated with querctetin. Equal letters in the same column indicate no significant difference at level of 5% ($p < 0.05$)

As displayed in Figure IV-8 and Table IV-1, the addition of querctetin to Coil at saturated concentration (168 µg/g) in absence of phospholipids, did not significantly protect the oil against lipid oxidation. This was unexpected considering that for instance, the addition of querctetin dihydrate in a range of 0.25 to 2 µmol/g oil (84.6 to 676.6 µg/g) significantly increased the induction period of high oleic sunflower oil (Becker et al., 2007). Similarly, Naz et al. (2008) reported that querctetin at a concentration of ~220 µg/g effectively reduced the oxidation rate of corn oil submitted to deep frying at 180 °C for 30, 60 or 90 min, since a

significant decreasing in peroxide value, and p-anisidine value was observed in the oils previously enriched with quercetin. On the other hand, other results closer to ours such as of Frenzel & Steffen-Heins, (2015) reported that quercetin in a concentration ranging from 750 to 4500 μM (226 to 1360 $\mu\text{g/mL}$) was not able to significantly reduce the free radical formation during oxidation of unsaturated soy phosphatidylcholine liposomes. They attributed this behavior to: (i) different solubilization sites for quercetin and lipid radicals, so that both reactants were not close enough to interact, and (ii) the low radical-scavenging activity of quercetin in the presence of α -tocopherol.

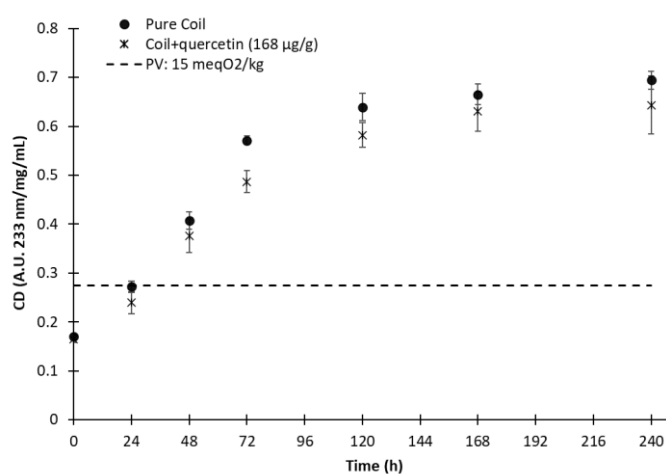


Figure IV-8. Time course formation of CD of Coil enriched with 168 μg quercetin/g oil

Although some comparisons related to the effect of quercetin against lipid oxidation have been given above, it is also important to highlight that the antioxidant activity of a molecule is significantly influenced by the lipid system in which this molecule is acting (bulk oils, emulsions, etc.) (Laguette et al., 2009), its composition and by the presence and nature of association colloids (Laguette et al., 2011). Therefore, antioxidants compounds may act differently depending on each lipid matrix.

The influence of enriching Coil at a low and fixed concentration of quercetin (220 $\mu\text{g/g}$, below quercetin solubility) while varying the concentration of PL from 10 to 20 mg/g was also evaluated (samples called CQL10-220, CQL15-220 and CQL20-220, respectively). Table IV-1 showed that the capacity to delay the oxidation was statistically equal regardless of the phospholipid concentration. This behavior was also observed by monitoring the CD formation of the aforementioned samples (Figure IV-9). These results were consistent with what was

observed and discussed in section 3.1, where Coil enriched with only PL at 5, 10, 15 and 20 mg/g exhibited the same protective effect.

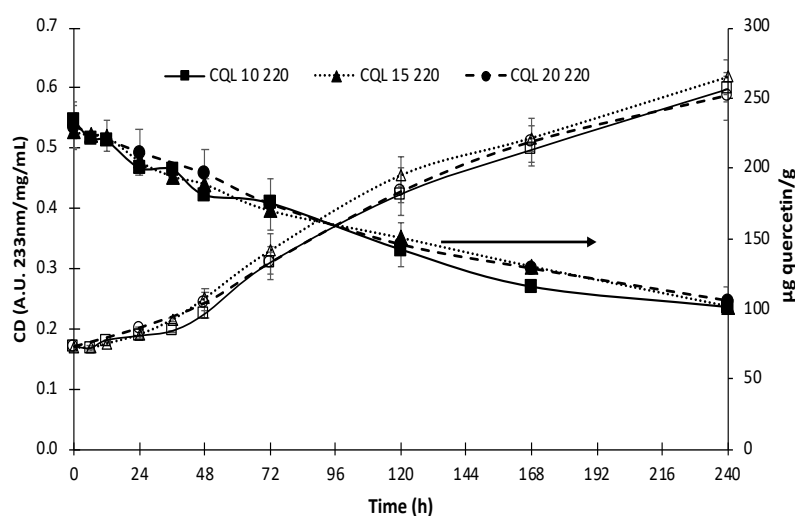


Figure IV-9. Time course formation of CD and remaining quercetin in Coil enriched with 220 µg quercetin/g and 10, 15, 20 mg PL/g

On the other hand, a different behavior was obtained when the effect of quercetin concentration was evaluated in enriched Coil at a fixed PL concentration (either 5, 10, 15 or 20 mg/g oil). Figure IV-14 shows clearly that the increase in quercetin concentration led to higher oxidation lag times whatever the concentration of PL (5, 10, 15 or 20 mg/g oil). This behavior is linked to what can be observed in Figure IV-10 which showed that by increasing the quercetin concentration in oils enriched at a fix PL concentration, the formation of CD was lowered. In general, combination of phospholipids and quercetin led to an enhanced antioxidant activity against lipid oxidation. This result is consistent with Ramadan, (2012) who reported that increases in concentration of quercetin in phenolipid formulations resulted in an increase of its antioxidative potential. According to Ghanbarzadeh et al. (2016) phospholipids may act as carriers of polyphenols and thus improve the antioxidant activity of these compounds. In this sense, in presence of PL, quercetin exhibited a higher ability to interact with free radicals by means of the hydroxyl groups. The hydroxyl groups of quercetin become donors of H-atom to peroxy radicals to stabilize them and delay lipid oxidation. The incorporation of PL to Coil allowed to provide quercetin-phospholipid interactions so that it increased not only the quercetin solubility but also the oxidative stability of Coil.

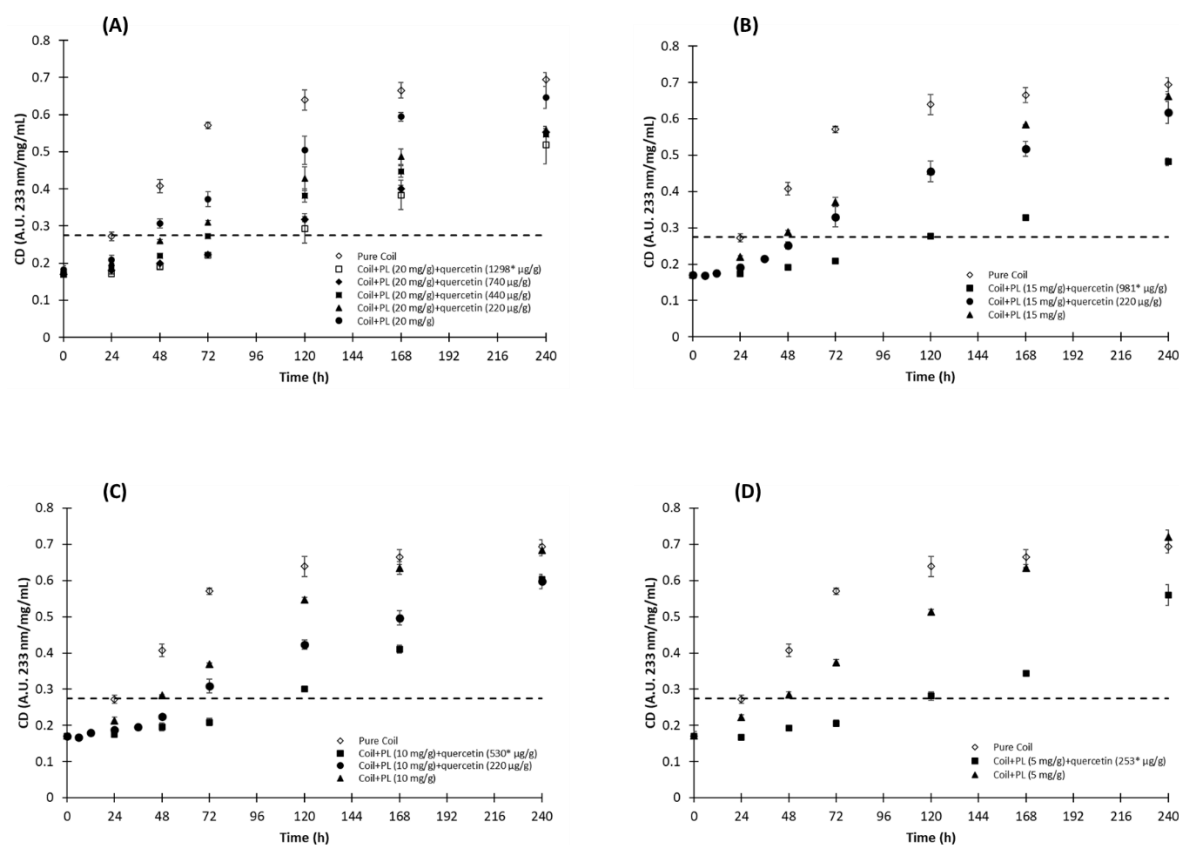


Figure IV-10. Time course formation of CD in Coil enriched with PL at 20 mg/g (A), 15 mg/g (B), 10 mg/g (C) and 5 mg/g (D) mg/g and quercetin at various concentrations.

*Saturated sample

Figure IV-11 also evidenced that the combination of both PL and quercetin led to an enhanced antioxidant activity against lipid oxidation when compared to the individual components. The addition of quercetin along with PL enhanced the protective effect against lipid oxidation exhibited by PL alone, since for instance, oil enriched with both quercetin (at 220 µg/g) and PL (20 mg/g) exhibited a shelf life of 58 ± 4 h, whereas the oxidation lag time of oil enriched with only PL (20 mg/g) was 39 ± 3 h. Similarly, Ramadan, (2012), who used lecithin-quercetin formulations to enrich sunflower oil and triolein, observed that the oxidative stability of oils enriched with phospholipids and quercetin, both at 200 ppm, was greater compared with oils enriched only with phospholipids or quercetin. The author (Ramadan, 2012) also referred this behavior as a consequence of synergisms between polar lipids and quercetin as well as tocopherols.

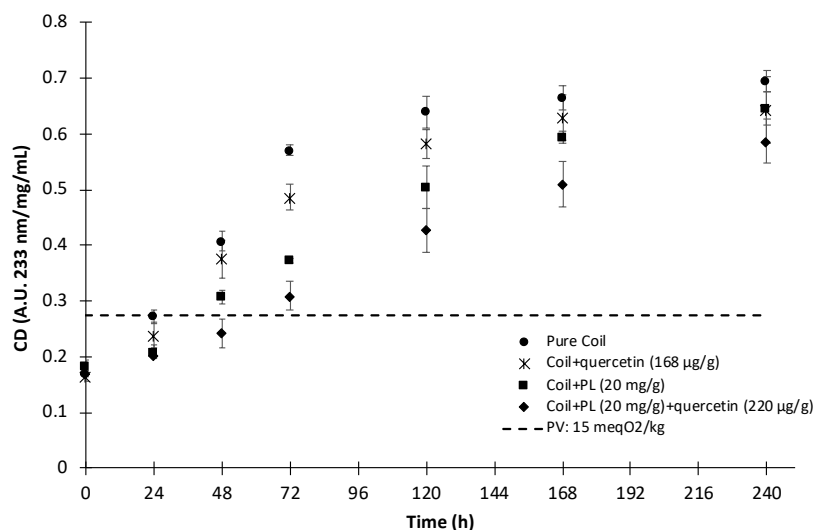


Figure IV-11. Monitoring of CD formation of Coil enriched with individual components (PL or quercetin) and a combination of them (PL+quercetin)

On the other hand, the highest phospholipid and quercetin concentration (20 mg/g and 1 298 µg/g, respectively) in Coil provided the maximum protective effect corresponded to a lag time of 115 ± 7 h, a value similar to that observed simultaneously for other samples enriched also with a mass ratio of $\sim 1:20$ (i.e. 1 molecule of quercetin for 8 molecules of phosphatidylcholine) (5 mg PL/g and 253 µg quercetin/g, 10 mg PL/g and 530 µg quercetin/g, and 15 mg PL/g and 981 µg quercetin/g) (Table IV-1). All these samples correspond to oils saturated with quercetin at various concentrations of PL. The oxidation curves of these samples are shown in Figure IV-12.

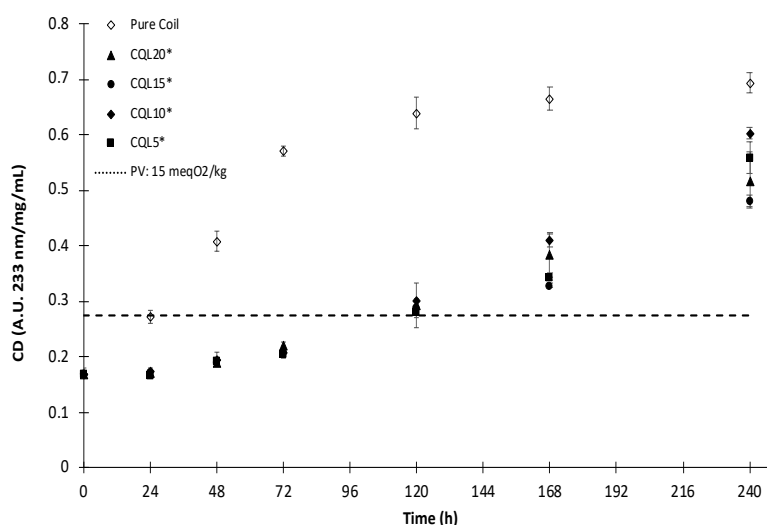


Figure IV-12. Monitoring of CD formation of Coil saturated with quercetin at 5, 10, 15 and, 20 mg of PL/g

For the preparation of the samples saturated with quercetin at different concentration of PL (Figure IV-12), Coil was enriched with PL, oversaturated with quercetin and then, filtered using a PTFE 0.45 μm syringe filter. In this context, the highest protective effect against lipid oxidation exhibited by the saturated samples may come either from the method of their preparation or from the potentiation of the antioxidant activity exhibited by the enrichment with quercetin and PL at a mass ratio of $\sim 1:20$. Our initial hypothesis was that by means of the filtration, some impurities (prooxidants) may be removed from the enriched Coil, thus leaving the oil more stable. It is well known that filtration, among other steps, are carried out in the industrial refining of virgin oils in order to remove substances which can promote the lipid oxidation. Although the filtration during refining of oils is performed along with absorbents that improve the removal of undesirable compounds, we validated if lab syringe filter was able to remove any prooxidant compound.

From that perspective, an aliquot of pure Coil was submitted to all the solvent-free steps including filtration with a 0.45 μm PTFE syringe filter and then oxidized at 60 $^{\circ}\text{C}$ for several hours. CD monitoring (Figure IV-3) of this sample showed a slight increase (no significant, statistically) in the lag time of the filtered pure Coil (29 ± 2 h) compared with the unfiltered pure Coil (24 ± 1 h).

In addition, since CQL5* (Coil with 5 mg PL/g + 253 μg quercetin/g, filtered sample) exhibited higher oxidative stability than non-filtered samples with similar quercetin concentrations, i.e. 10 mg PL/g + 220 μg quercetin/g (CQL10-220), 15 mg PL/g + 220 μg quercetin/g (CQL15-220) and 20 mg PL/g + 220 μg quercetin/g (CQL20-220) (Table IV-1), a new sample with 5 mg PL/g oil + 220 μg quercetin/g (called CQL5-220) was prepared without filtration step. This latter sample was then submitted to accelerated oxidation at 60 $^{\circ}\text{C}$ in order to verify if the filtration step was responsible for the high oxidative stability of CQL5*.

The monitoring of CD formation of the sample CQL5-220 was similar of the CQL5* as shown in Figure IV-13. Hence, oxidation lag time of CQL5-220 (103 ± 5 h) was close of CQL5* (117 ± 7 h) and higher than of CQL10-220, CQL15-220 and CQL20-220 (60 ± 3 h). This confirms that the filtration was not responsible for the enhanced oxidative stability of CQL5*. Moreover, as the quercetin concentration of CQL5-220 sample (220 ± 16 $\mu\text{g}/\text{g}$) was close to the solubility of quercetin in Coil enriched at 5 mg PL/g oil, i.e. 253 ± 4 $\mu\text{g}/\text{g}$, it could be stated that the highest protective effect against lipid oxidation is exhibited when Coil is

enriched with quercetin and PL at a mass ratio of ~1:20, i.e. quercetin saturated samples CQL5*, CQL10*, CQL15*, CQL20*.

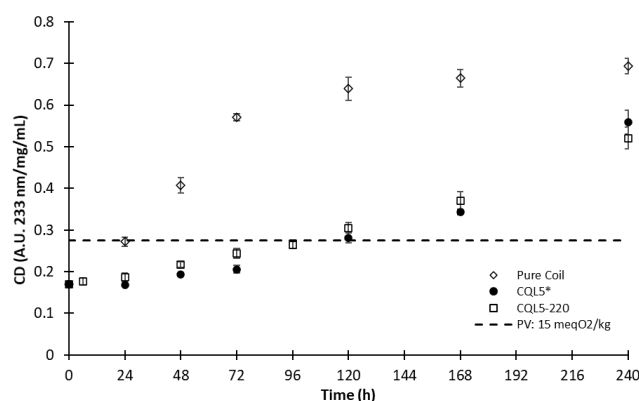


Figure IV-13. Monitoring of CD formation of CQL5* (Coil with 5 mg PL/g + 253 μg quercetin/g, filtered sample) and CQL5-220 (Coil with 5 mg PL/g oil + 220 μg quercetin/g oil, not filtered sample)

In general, when phospholipids are used as carriers of polyphenols for food fortification, they are able to improve the antioxidant activity of these compounds (Ghanbarzadeh et al., 2016; Bhattacharyya et al., 2014). Globally, the effectiveness of antioxidants in oils is influenced by numerous parameters: the structure of the bioactive molecules, the solubilization site, the concentration, mechanism of action as well as by the composition of the oil phase. The microenvironment formed by surface-active compounds such as phospholipids is recognized as the active site of lipid oxidation, therefore oxidation cannot be explained merely by chemical reactions but also by considering molecular positions in space (Budilarto & Kamal-Eldin, 2015). In the schematic representation of the hydrophilic and hydrophobic antioxidant distribution in bulk oil (Budilarto & Kamal-Eldin, 2015; Chaiyasit et al., 2007; Wanasundara & Shahidi, 2005), the hydrophobic antioxidants are diluted in the bulk oil whereas surfactant and hydrophilic antioxidants are located at the oil-water interface of the micelles/association colloids.

Quercetin is usually described as a hydrophobic molecule because of its poor solubility in water (< 1 g/L). Although there is a considerable disagreement about the quercetin solubility data that ranged from 3.8 to 970 $\mu\text{g}/\text{mL}$ water (Abraham & Acree, 2014), it stands in the same range than the solubility of quercetin in Coil measured in this work (168 $\mu\text{g}/\text{g}$). Its predicted octanol-water partition coefficient ($\log P$) of 1.5 (PubChem) indicated some hydrophobicity

and, Van Dijk et al. (2000) estimated the ratio of quercetin partitioning between water and olive oil to 53%. Therefore, quercetin can be considered as neither fully lipophilic nor hydrophilic (Mignet et al., 2013). On the other hand, although the existence of two aromatic rings with highly unsaturated carbon-carbon bonds reinforce the hydrophobicity of quercetin, its log P value is one of the lowest comparing with other flavonoids, indicating that its partition into hydrophobic phases is limited (Buchweitz et al., 2016). In this context, quercetin added at its solubility level to pure Coil is probably dispersed in the oil or associated to endogenous surfactants like free fatty acids, phospholipids, among others. We have shown that the addition of phospholipids influenced the quercetin solubility in oil. Hence, our hypothesis is that the solubilizing effect of phospholipids drives the excess of quercetin molecules close to the water-oil interface generated by the PL micelles. The hypothesis is based on the assumption that in bulk oils, quercetin is interacting with the polar part of phospholipids as it does in aqueous liposome suspension (Ghanbarzadeh et al., 2016; Maiti et al., 2007; Kidd, 2009). The hypothesis is also sustained by the fact that the planar configuration of quercetin favors its intercalation into organized structures of phosphatidylcholine (Van Dijk et al., 2000).

Consequently, quercetin molecules are located at the proximity of the polar heads of phospholipids, in close contact with the aqueous microenvironment recognized as being the active site of lipid oxidation. According to Frankel et al. (1994) the antioxidant potential is strongly associated to the effective concentration of the compound at the location where oil oxidation begins. Therefore, the improved accessibility of the antioxidant to the aqueous microenvironment present in the oil, due to the surface-active PL, was expected to enhance the antioxidant activity of the bioactive compound, which was actually observed.

The oxidation lag times of all enriched Coil has been schematically represented in Figure IV-14 in order to facilitate comparison among different data.

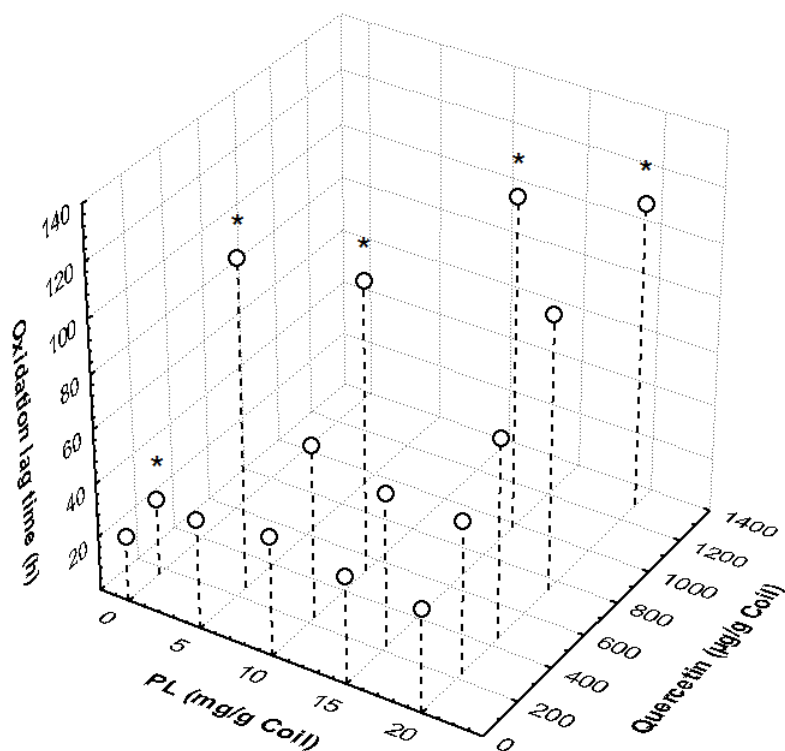


Figure IV-14. 3D-plot of the oxidation lag times of enriched and non-enriched Coil.

*Samples saturated with quercetin

3.3. Monitoring of quercetin concentration during oxidation

The concentration of quercetin was monitored by HPLC for Coil saturated with quercetin at different PL concentrations (0, 5, 10, 15 and 20 mg/g oil) (samples labeled with * in Figure IV-14 and Table IV-1) and also in the samples enriched with PL (10-20 mg/g oil) and 220 µg quercetin/g oil. The concentration of remaining quercetin in the oxidized samples was expressed in µg of quercetin per gram of oil.

The evolution of the remaining quercetin during oxidation is shown in Figure IV-15(A) for Coil enriched with only quercetin at 168 µg/g. As can be observed, the remaining quercetin in Coil without PL showed a deep decrease during the first 120 h of oxidation, with 70% of quercetin consumed at this point. The consumption of quercetin offered only a slight protection to the oil against oxidation, contrary to authors such as Palma et al. (2017) who reported that the consumption of quercetin in bulk methyl linoleate was correlated with the ability of the flavonoid to protect the model oil. Nonetheless, and as mentioned before, the effectiveness of an antioxidant for preventing oil oxidation depends on the solubilization site, the composition of the lipid system and the interaction with other endogenous antioxidants.

Goupy et al. (2007) for instance observed that quercetin was partially spared by tocopherols, based on the experimental evidence that residual quercetin concentration in presence of tocopherols was generally higher than that found in its absence.

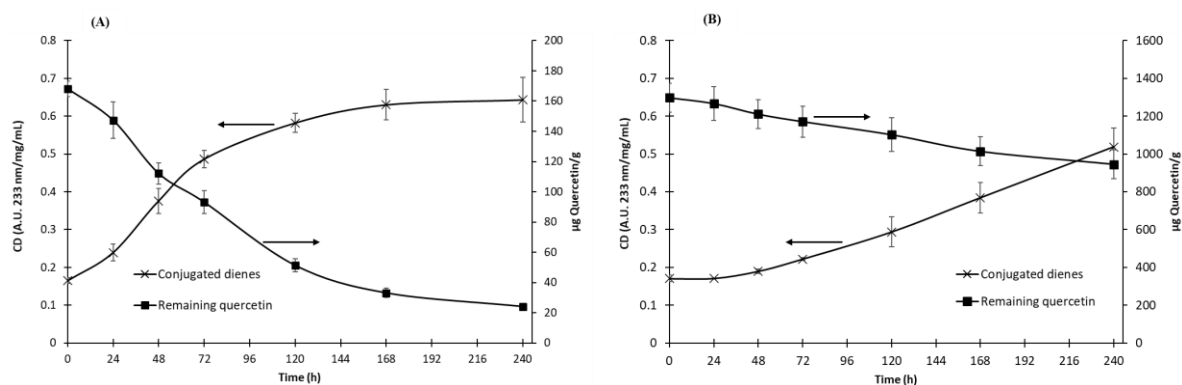


Figure IV-15. Time course formation of CD and remaining quercetin in Coil saturated with quercetin at (A) 168 µg/g and 0 mg of PL/g oil and at (B) 1298 µg/g and 20 mg of PL/g oil

In Coil saturated with quercetin at various PL concentrations (5, 10, 15 and 20 mg/g oil), higher quercetin concentrations remained during oil oxidation in comparison with CQ* sample (Figure IV-15(B), and Figure IV-16). The addition of PL to Coil from 5 to 20 mg/g allowed to introduce increasing amounts of quercetin in the oil and therefore, maintained a reservoir of quercetin during accelerated oxidation. This may have favored a higher Coil oxidative stability along with the already discussed protection effect exhibited by PL against lipid oxidation.

In Figure IV-9 were shown the results of the monitoring of CD formation and remaining quercetin of enriched Coil at different concentration of PL (from 10 to 20 mg/g) and quercetin at a fixed concentration (220 µg/g). As previously discussed, it can be observed that all enriched samples in Figure IV-9 exhibited the same protective effect, regardless of the phospholipid concentration. This behavior was consistent with the monitoring of quercetin degradation since also equal kinetic curves were observed for the different enriched oils (CQL 10-220, CQL 15-220, CQL 20-220).

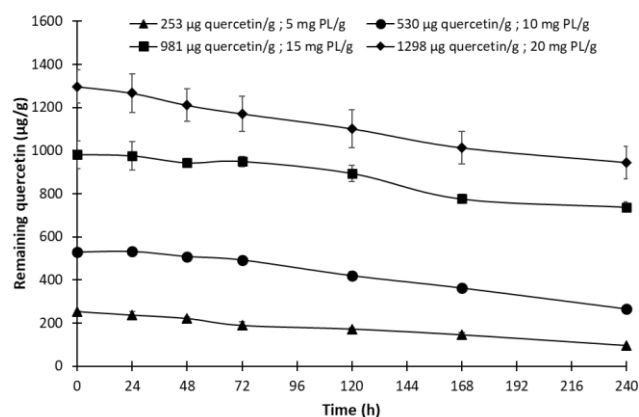


Figure IV-16. Monitoring of remaining quercetin in Coil saturated with quercetin at various PL concentrations

Koga & Terao, (1995) also observed that phospholipids, in presence of exogenous vitamin E antioxidant, could retard the oxidation of bulk oil comprising methyl linoleate and methyl laurate exposed to a water-soluble radical initiator and simultaneously, delayed the consumption of vitamin E in the oil. In this sense, it can be stated that consumption of an antioxidant compound in a bulk oil and its protective effect depend on several factors and, the system investigated in this work was quite complex since the non-stripped Camelina oil already contained endogenous antioxidants as tocopherols and prooxidants like metals. Because of the addition of several formulations of PL and quercetin, the studied system contained several species active on the lipids oxidation either by acting on molecular organizations and/or by chemical effects through radical scavenging that can moreover interact with each other.

3.4. Oxidation at room temperature of pure Coil and Coil saturated with quercetin

Nowadays, the oxidative stability of oils has been widely studied by manufacturers in order to determine their shelf life and maintain their quality by retarding the lipid oxidation. As already stated, the rate of lipid oxidation depends on intrinsic factors such as fatty acid composition, minor compounds, presence of prooxidants, etc. and on external factors related to the storage conditions, such as temperature, air (oxygen) concentration, and light (Das, 1991). In this context, this work studied the oxidative stability of pure Coil and Coil saturated with quercetin (CQ*) (168 µg quercetin/g) simulating home storage conditions in order to estimate a global correlation with the accelerated oxidation at 60 °C. To mimic those conditions, both oil samples were storage at room conditions (temperature ranging 25 °C-28 °C), protected from the light for several days.

The time course formations of conjugated dienes of both pure Coil and CQ* at room temperature are displayed in Figure IV-17. The oxidation lag time of Coil at room temperature, considering as limit a CD value of 0.2747, was 28 ± 2 days. From this result it can be concluded that the oxidation of Coil at 60 °C decreased the oil shelf life 28-fold, since at this temperature the lag time of Coil was only 24 h. Elevated temperatures can increase the instability of lipids by promoting hydroperoxide breakdown in a process that generates free radicals. For instance, Conte et al. (2020) found that increasing the storage temperature of virgin olive oil from 25 °C to 60 °C decreased its shelf life about 12-fold. In this sense, it has been widely reported that decreasing the storage temperature of oils is an effective way to control lipid oxidation that triggers harmful effects (Johnson & Decker, 2015).

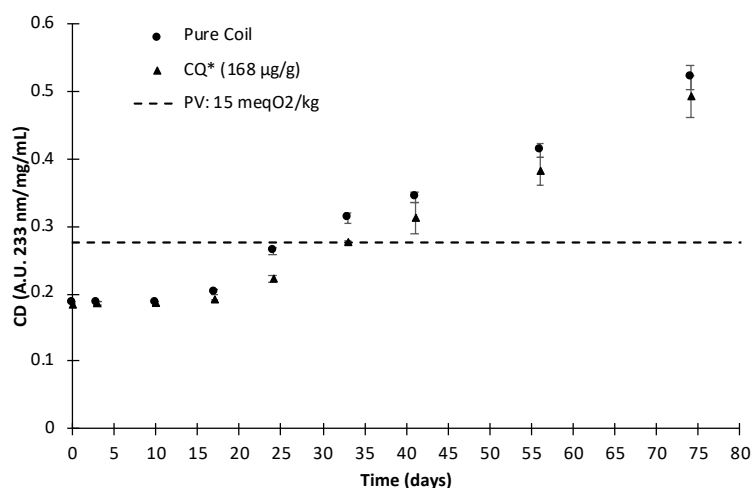


Figure IV-17. Time course formation of conjugated dienes of pure Coil and Coil saturated with quercetin (168 µg/g) simulating the home storage conditions

On the other hand, a slight protective effect of quercetin (168 µg/g) was also observed when Coil was maintained at room temperature (Figure IV-17) since the oxidation lag time observed for CQ* at room temperature was 35 ± 2 days. Therefore, it can be calculated that at room temperature, the protective effect of quercetin in Coil could increase the oxidation lag time only 1.25-fold. This result was consistent with that observed under accelerated conditions of oxidation (60 °C), since at those conditions, the incorporation of quercetin at 168 µg/g to Coil, also showed slight protective effect against lipid oxidation (oxidation lag time increased about 1.2-fold at 60 °C by quercetin incorporation).

Considering that the oxidation lag times of pure Coil exposed at 60 °C and at room temperature were estimated in 24 h and 28 days, respectively, it can be stated that 1 hour at

60 °C is equivalent to 1.17 days at room temperature. Although we have not developed a predicted model for estimating the shelf life of Coil at room temperature, the aforementioned correlation can be helpful to globally estimate the oxidation lag time at room temperature of enriched oils already oxidized at 60 °C. For instance, considering that the sample CQL20*, i.e. Coil enriched with 20 mg PL/g and 1298 µg quercetin/g, exhibited an oxidation lag time of 115 h at 60 °C, it would have a shelf life of 134.2 days under home storage conditions, which is 106 days more than non-enriched Coil.

4. Conclusion and perspectives

From this chapter, a linear correlation between the formation of conjugated dienes and the peroxide value of camelina and sunflower oil submitted to accelerated oxidation at 60 °C was obtained. From the correlation between these primary oxidation products, it was possible to estimate the peroxide value of any oil sample only by the conjugated diene measurement which is a simpler analytical method. The oxidative stability assessment of enriched oils focused on primary oxidation products as indicators for determining the maximum oxidation level of an edible oil. The influence of various quercetin-phospholipids formulations on oxidation lag time, defined as the time needed for the different oils submitted to accelerated oxidation to reach the maximum peroxide value permitted for edible oils, was successfully studied. By means of the calculation of the oxidation lag times it was observed that phospholipids in camelina oil exerted an antioxidant effect, probably attributed to the ability of chelating metals, to their amine function which could directly interact with free radicals and to the synergism with endogenous antioxidants such as tocopherols. Nonetheless, the antioxidant activity of phospholipids was not dose-dependent in a concentration range from 5 to 20 mg/g. On contrary, the oil enrichment with only quercetin exerted a poor protective effect against lipid oxidation attributed to the lack of proximity between the site of solubilization of quercetin and the site where oxidation takes place. Same behavior was observed when the oxidation of oil samples was monitored at room temperature. Furthermore, the degradation of quercetin in absence of phospholipids in bulk oils submitted to accelerated oxidation was not correlated with a protective effect against lipid oxidation.

On the other hand, the oil enrichment with quercetin-phospholipid formulations by the solvent free route, developed in previous chapter, besides improving the solubility of quercetin in oil, also enhanced the oxidative stability of oil. The protective effect of

phospholipids and quercetin was probably determined by a more specific synergism than the additivity (concentration) of both components since several combinations provided the same retarded oxidation. In this sense, samples saturated with quercetin at the different phospholipid concentrations provided the highest protective effect against oxidation of camelina oil. The aforementioned condition corresponded to an oil enrichment with quercetin and PL at a mass ratio ~1:20. Moreover, the presence of phospholipids in bulk oils allowed to incorporate higher amounts of quercetin which may act as reservoir during storage or later oil processing.

It has been suggested that quercetin, by interacting with the polar head of phosphatidylcholine, participated to the micellar water-oil microenvironment where the oil oxidation takes place and therefore, an enhanced protective effect of quercetin formulated along with phospholipid could be observed.

For future works, it is recommended the verification of colloidal structuration in bulk oils enriched with phospholipids and various concentrations of quercetin (above 168 $\mu\text{g/g}$), in order to evidence the correlation between these associations and the enhanced antioxidant effect of quercetin. In this context, some analysis such as small-angle X-ray scattering (SAXS), surface tension measurement (interfacial tensiometer) or nuclear magnetic resonance (NMR) may be highly useful.

On the other hand, the monitoring of secondary oxidation products during accelerated oxidation of enriched oils can also provide additional information related to the effect of quercetin-phospholipid formulations.

In general, this chapter opens up potential new applications for phospholipid-polyphenol formulations in the food, cosmetic and pharmaceutical industries, since it was demonstrated that the oxidative stability of edible oils can be also significantly improved by means of the incorporation of quercetin with commercial phospholipid.

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Chapter V: Enrichment of camelina oil by polyphenol-rich extracts from walnut cake

Introduction

In this chapter we have aimed at the enrichment of camelina oil by polyphenol-rich extracts from walnut cake in order to enhance its oxidative stability. In the previous chapters, the enrichment of camelina oil has been carried out by quercetin-phosphatidylcholine formulation, but in this chapter more complex extracts (mix of polyphenols) from walnut cake have been considered.

Walnut, the seed of *Juglans regia* is a highly nutritious food due to its high content of unsaturated fatty acids, such as linoleic and oleic acid, and tocopherols which reduces the risk of many heart diseases. Moreover, walnuts are an important source of various polyphenols, such as phenolics acids and condensed tannins, which exhibit favorable effects on human health owing to their antioxidant and antiatherogenic properties (Labuckas et al., 2008). Walnut press-cake is the by-product that results after the oil pressing extraction from walnut seeds therefore, large amounts of this undervalued residue which are often thrown away, are produced. However, walnut press-cake still constitutes a rich and cheap source of proteins, fibers, bioactive molecules as phenolics compounds, etc.

Phenolic compounds may be extracted from plant matrices by different conventional methods such as maceration and Soxhlet, or by more intensive process as ultrasound assisted extraction, pressurized liquids, microwave assisted extraction, supercritical fluids, etc. Thus, the first issue approached in this chapter is the study of polyphenol extraction from walnut press-cake by maceration. For that, a fractional factorial design was conducted in order to optimize the phenolic extraction yield and the antioxidant activity of walnut extracts. We have evaluated the influence of time, temperature, solvent ratio and the presence or not of phospholipid in extraction solvent on the total phenolic content and on the scavenging activity of walnut extracts. A mixture ethanol-water was selected as extraction solvent and, Folin-Ciocalteu assay and the scavenging activity of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical were used for total phenolic quantification and antioxidant activity measurement, respectively. Furthermore, a semi-continuous extraction of polyphenols from walnut cake was also carried for comparison purposes. The two-site kinetic desorption model was adjusted to

the experimental kinetic extraction of polyphenols from walnut cake by the semi-continuous method.

Chapter IV evaluated methods for improving oxidative stability of camelina oil by enriching with quercetin-phosphatidylcholine formulation. The above implied the use of quercetin as purified flavonoid molecule (purity >95 %). However, in this chapter we have been concerned to assess the effect of enriching camelina oil by polyphenol-rich walnut extracts. In this context, the second part of *Chapter V* consisted of developing a method for enriching camelina oil by walnut extracts and then, evaluate the oxidative stability of enriched camelina oil. For that, polyphenol-rich extracts, previously extracted from walnut cake by maceration, were added to camelina oil, containing or not phosphatidylcholine, followed by solvent evaporation. After the enriched oils, which increased its phenolic content, were submitted to accelerated oxidation in order to evaluate the effect of polyphenols from walnut on the protective effect against lipid oxidation. For that, we also have assessed the enrichment of camelina oil at different concentration of polyphenol extracts and the influence of also incorporating phospholipids to camelina oil.

In addition, some methods for enriching directly camelina oil with phenolics from walnut cake without using solvents were assessed. These methods basically consisted of maceration and ultrasound assisted extraction of walnut cake in camelina oil. Finally, the oxidation course of camelina oil provided with walnut particles during accelerated oxidation was also evaluated.

This chapter intended to evaluate the enrichment of edible oils with several formulations of natural polyphenols from agro-residues. However, the disruptions and delays caused by the Covid-19 pandemic forced to shorten the experimental plan and prevented the development of some tests and analyzes that are recommended for future works.

1. Maceration extraction from walnut cake (WAC)

For maceration extraction from walnut cake (WAC), a 2^{4-1} fractional factorial design was used to determine the influence of two levels (-1, +1) of extraction time (Z1), temperature (Z2), solid-to-solvent ratio (Z3) and percentage of phospholipid in the solvent (Z4) on the extraction of total phenolic compounds (TPC) and free radical scavenging activity (DPPH) of extracts. Z4 factor was aliased with the second order interaction $Z1Z2Z3$ giving an experimental design of resolution IV. Additional center points (level 0) were added to assess reproducibility of experiments, to check the absence of drift and to verify the linearity of the responses. The current design comprised 11 experimental runs including 3 center points (level 0) as shown in Table V-1. The extraction time (Z1) varied between 15-60 minutes, the temperature (Z2) was assessed at 28 °C (room temperature) up to 60 °C in order to evaluate the increased of mass transfer rate at higher temperatures. Solid-to-solvent ratios (Z3) between 1/13 and 1/25 were also studied in order to observe the influence of the modification in the gradient of concentration as the solid-to-solvent ratio increases. Finally, the mixture ethanol-water 60:40 (v/v) (called EtOH60) containing phospholipids (PL) (Z4) (in a concentration range of 0 to 1.5% wt/v) was also assessed as extraction solvent in order to study the effect of phospholipids on the solubilization capacity of the solvent. EtOH60 was used as extraction solvent according to previous works (Bodoira & Maestri, 2020; Drevelegka & Goula, 2020; Yuan et al., 2020; Riciputi et al., 2018; Odabaş & Koca, 2016) as well as to other works carried out in our team (Faber Ariel Espinosa Pardo doctoral thesis, 2020), which showed that the mixture ethanol-water 60:40 (v/v) provided the highest capacity to extract phenolic compounds from walnut. The previous experiments in our team aimed at evaluating the phenolic extraction yield from defatted walnut cake using ethanol-water mixtures at different proportions (v/v) (20:80, 60:40, 80:20) and absolute ethanol as extraction solvents. Those tests consisted of maceration extraction (two successive extractions of 45 min each one at 28 °C, solid-to-solvent ratio of 1:13) and the results showed that the ethanol-water mixtures at 20:80, 60:40, 80:20 and absolute ethanol were able to extract 9.87, 17.35, 10.65 and 0.9 mg GAE/g of cake, respectively. Thus, the 60:40 ethanol-water mixture (EtOH60) exhibited the highest capacity for extracting phenolic compounds from defatted walnut cake.

The influence of the different independent factors on TPC and DPPH was predicted by adjusting the response variable to a first-order polynomial model (Equation V-1) using the

freeware RStudio (RStudio[®], USA). Moreover, in order to identify the significant terms, an analysis of variance (ANOVA) has been performed with a confidence level of 95%.

$$Y = \beta_0 + \sum_{i=1}^4 \beta_i Z_i + \sum_{i=1}^4 \sum_{j=1}^4 \beta_{ij} Z_i Z_j \quad \text{Equation V-1}$$

where Y represents the response variable (TPC, or DPPH); β_0 , β_i and β_{ij} are the intercept, linear and interaction coefficient, respectively; Z_i and Z_j are the independent factors affecting the response. For interpretation purpose the classical hypothesis used for fractional factorial design are used. The three hypotheses are: i) interactions of higher order (second order interactions and higher) are negligible; ii) effects aliased in non-significant contrasts are non-significant; iii) all interactions of a non-significant factor are non-significant.

Table V-1. Coded and natural values of the fractional factorial design with the response variables for maceration extraction from WAC

Run	Independent factors (Z)				Response variables			
	Time (min) (Z ₁)	Temperature (°C) (Z ₂)	Solid-to-solvent ratio (m/v) (Z ₃)*	PL concentration (%) (Z ₄)	TPC (mg GAE/g WAC)	TPC (mg GAE/mL)	DPPH (μmol Trolox Equiv. /g WAC)	DPPH (μmol Trolox Equiv. /mL)
1	37.5 (0)	44 (0)	1/19 (0)	0.75 (0)	8.23	0.46	68.93	3.83
2	15 (-1)	28 (-1)	1/13 (-1)	0 (-1)	10.27	0.86	90.53	7.55
3	60 (+1)	28 (-1)	1/13 (-1)	1.5 (+1)	2.54	0.21	42.72	3.56
4	15 (-1)	28 (-1)	1/25 (+1)	1.5 (+1)	2.22	0.10	12.14	0.52
5	60 (+1)	28 (-1)	1/25 (+1)	0 (-1)	12.04	0.52	99.72	4.27
6	37.5 (0)	44 (0)	1/19 (0)	0.75 (0)	7.85	0.44	70.01	3.89
7	60 (+1)	60 (+1)	1/13 (-1)	0 (-1)	11.83	1.01	104.91	8.99
8	60 (+1)	60 (+1)	1/25 (+1)	1.5 (+1)	13.40	0.56	91.26	3.80
9	15 (-1)	60 (+1)	1/13 (-1)	1.5 (+1)	6.56	0.55	60.50	5.04
10	15 (-1)	60 (+1)	1/25 (+1)	0 (-1)	14.78	0.62	136.92	5.71
11	37.5 (0)	44 (0)	1/19 (0)	0.75 (0)	8.27	0.46	68.75	3.82

Runs 1, 6 and 11 comprised the three replicates at the central point. * The volume of solvent for the solid-to-solvent ratios 1/13, 1/19 and 1/25 was 39 mL, 57 mL, 75 mL, respectively

Responses obtained during fractional factorial experiments are also reported in Table V-1. As can be observed, for TPC, values between 2.2 and 14.8 mg GAE/g WAC were obtained with a mean of 8.9 mg GAE/g WAC. These results are lower than that reported by Bakkalbaşı, (2018) (58.2 mg GAE/g walnut cake), but closer to those of Luo et al. (2017) (19-35 mg GAE/ g walnut flour) and Shahidi & Ambigaipalan, (2015) (15 mg/g walnut). These

discrepancies may be related to the differences among walnut varieties, and extraction method used for recovering the polyphenols.

Statistical analysis through multiple regression and ANOVA highlighted the PL concentration as the most significant factor at 95% level for TPC. A backward-forward method was used for determining the best model fitting the experimental data using AIC criterion as proposed by Akaike, (1974). For TPC, the best model (in coded values) was: $TPC \text{ (mg GAE/g WAC)} = 8.9 + 0.7 \cdot \text{time} + 2.4 \cdot \text{temperature} + 1.4 \cdot \text{solid-to-solvent ratio} - 3 \cdot \text{PL concentration} + 1.0 \cdot \text{temperature} \cdot \text{solid-to-solvent ratio} + 1.3 \cdot \text{temperature} \cdot \text{PL concentration}$.

ANOVA results (Table V-A1, Appendix) highlighted the temperature, solid-to-solvent ratio, PL concentration and the interactions temperature-PL concentration and temperature-ratio as significant terms at 95% level. The fitted model explained 95% of the experimental variance and showed that the effect of PL concentration was negative, so adding PL was deleterious to the extraction of TPC whereas increasing temperature and ratio favored it. In this sense, the TPC can be theoretically maximized at highest levels of time, temperature and solid-to-solvent ratio in absence of PL. Nonetheless, other factors such as energy and solvent consumption should be also considered for optimizing the extraction process.

The polynomial model showed a positive influence of time on TPC which was expected and already known (Santos et al., 2016). Furthermore, the results showed that higher temperatures favored the extraction of phenolic compounds. This behavior has been widely reported for the extraction of phenolic compounds from several plant matrices such as grape seeds (Bucic-Kojic et al., 2013), tea leaves (Santos et al., 2016), medicinal herbs (Marete et al., 2009), citrus peel (Li et al., 2006), *Agave* leaves (Ben Hamissa et al., 2013) and even during winemaking (Sacchi et al., 2005). Higher temperatures is related to increased ease with which solvent diffuses into cells and the enhancement of desorption and solubility, moreover temperature increases vapor pressure of solutes whereas reducing the surface tension, and viscosity of the liquid medium, which facilitates mass transfer (Goula et al., 2016; Palma et al., 2013). Sharma et al. (2015) have reported higher concentration of phenolic in onion by heating it up to 120 °C for 30 minutes, however, these authors also highlighted the key role of the correlation between the temperature and time since the combination of these two parameters determines the breakdown of cellular constituents to release phenolic compounds. Sharma et al. (2015) also reported that at high temperatures the content of specific flavonoids in onions may decrease although the global phenolic content increases. This latter behavior was also

observed by (Garcia-Mendoza et al., 2017). On the other hand other authors such as Odabaş & Koca, (2016) have reported no significant influence of temperature on phenolic extraction from hazelnut skin when ultrasound assisted extraction (temperature from 20 °C to 60 °C) and supercritical fluid extraction (temperature from 40 °C to 60 °C) were studied. In this case, the effect of temperature was not significant when combined with other factors such as ethanol concentration, power of ultrasound, extraction time and pressure of supercritical carbon dioxide. Roselló-Soto et al. (2019) performed a maceration extraction from tiger nuts by-products and observed a slight decrease in the TPC above 43 °C when ethanol 50% was used as extraction solvent. This decrease comes from the thermal degradation of some particular phenolic compounds which is influenced by the phenol structure and heating time (Sharma et al., 2015). These authors (Roselló-Soto et al., 2019) carried out the extraction for 3 hours which is much longer than that of this work.

Regarding the solid-to-solvent-ratio, it was expected that higher values of ratio improve the extraction yield since the gradient of concentration increases as the solid-to-solvent-ratio increases. Thereby, a higher gradient of concentration between solid surface and bulk solvent enhanced the mass transfer. On the other hand, it is worth mentioning that although higher solid-to-solvent ratios led to higher extraction yields, it also provided less concentrated phenolic extracts (Table V-1) because of the use of larger volume of solvent. The incorporation of PL to EtOH60 at concentrations ranging 0.75-1.5% (wt./v) exhibited a deleterious effect on the extraction of phenolic compounds. We could say that this behavior was unexpected since in *Chapter III*, it has been shown the positive influence of PL in the solubility of quercetin in edible oils. However, the extraction of phenolic compounds from plant matrices is a process much more complex than solubilization of pure quercetin in oils. According to Palma et al. (2013) the extraction of solutes from a solid matrix requires five steps: i) transfer the solvent from the fluid phase to the solid surface and pervades it, ii) that the solvent penetrates into the solid matrix by molecular diffusion, iii) solubilize the soluble material by desorption from the matrix and solvation into the extraction solvent. For that, many chemical bonds should be broken, iv) that the solution with the solutes returns to the surface of the solid by molecular diffusion, and v) transfer the solution from the solid surface to the bulk fluid by natural or forced convection. In this context, the presence of PL in EtOH60 could have modified the physical properties of the solvent which hindered the penetration of EtOH60 into the solid matrix. PL could also modify the polarity of EtOH60 which directly impacted the solubility of phenolic compounds in the solvent. It has been reported that phenols of walnut

are mostly polar compounds (Bakkalbaşı, 2018), with a high solubility in ethanol with 30-50% (v/v) of water (Bodoira & Maestri, 2020). Since EtOH60 achieved the optimal polarity for phenolic extraction from walnut, the incorporation of PL led to moving from this optimal condition and thus, the ability of the solvent for solubilizing phenolic compounds significantly decreased.

Another hypothesis that may explain the detrimental effect of PL on the TPC, is based on the fact that the formation of many colloidal structures, in which phenols are contained, could prevent them from reacting with the Folin–Ciocalteu’s reagent, resulting in the underestimation of the TPC of extracts (Li et al., 2015; Suárez et al., 2011).

For DPPH response, values ranged between 12 and 137 $\mu\text{mol Trolox Equiv./g WAC}$, with a mean of 77 $\mu\text{mol Trolox Equiv./g WAC}$. The fitted model was: $\text{DPPH } (\mu\text{mol Trolox Equiv./g WAC}) = 76.9 + 18.5 * \text{temperature} - 28.1 * \text{PL concentration}$ (ANOVA results in Table V-A2, Appendix).

PL was also the most significant factor in DPPH at 95% level. As can be observed in Table V-A2 (Appendix), this model included, temperature and PL and as significant factors, and explained 77% of the variability. The model highlights the positive influence of temperature and the deleterious effect of PL. The extraction conditions maximizing the DPPH are at highest temperature (60 °C) and in absence of PL, having only a slight influence of time (for instance, the model predicts 119 $\mu\text{mol Trolox Equiv./g WAC}$ for 15 min of extraction and 128 $\mu\text{mol Trolox Equiv./g WAC}$ for 60 min). Similar to TPC, the antioxidant activity of walnut extracts was positively influenced by temperature whereas PL exhibited a deleterious effect. This behavior was expected under the consideration that TPC and DPPH were highly correlated (Pearson’s coefficient of 0.94). In this sense, it can be stated that the scavenging activity of walnut extracts obtained by maceration, was mainly due to the action of phenolic compounds. The positive correlation between TPC and antioxidant activity (DPPH) of plant extracts has been widely reported (Piluzza & Bullitta, 2011; Thaipong et al., 2006; Gil et al., 2002). Our range of antioxidant activity (DPPH) was in agreement with that reported by Fregapane et al. (2020) for walnut press-cake (149 $\mu\text{mol Trolox Equiv./g cake}$). According to Bodoira & Maestri, (2020), the free radical scavenging properties of nut extracts are related to the high average number of phenolic OH groups, which represent potential hydrogen atom transfer sites.

2. Semi-continuous extraction from WAC: TPC recovery and kinetic modeling

The semi-continuous extractions basically consisted of flowing (1 mL/min) EtOH60 or EtOH90 through a bed of walnut cake particles (5 g) for 140 min and collecting periodically the effluent in graduated tubes. The temperature for extraction was set at 60 °C and walnut cake was mixed with glass beads (diameter of 0.5-0.75 mm or 2 mm) prior being loaded in a stainless-steel column.

In order to model the extraction of phenolic compounds from walnut cake (WAC) by semi-continuous extraction at 60 °C, the two-site kinetic desorption model has been used. This empirical model has been described by Kubatova et al. (2002). The model considers that a fraction of the extractable compounds is near the particle surface, being easily accessible to the solvent, and hence desorbed at a fast rate (called washing process). On the other hand, the remaining fraction of extractable compounds is located inside the particles of the plant matrix therefore, it is hardly accessible and desorbed at a slower rate (called slow diffusion). Equation V-2 describes the two-site kinetic model.

$$y_t = y_\infty * [1 - f * e^{-k_1 t} - (1 - f) * e^{-k_2 t}] \quad \text{Equation V-2}$$

where y_t (response variable) is the TPC (mg GAE/g WAC) at time t , y_∞ is the TPC at saturation; f is the fraction of extractable material of easy access, t is the extraction time, k_1 is the first-order rate constant describing the quickly released fraction (min^{-1}), and k_2 is the first-order rate constant describing the slowly released fraction (min^{-1}). This model has been fitted to the experimental data using Microsoft Excel Solver, minimizing the mean relative percent deviation.

As can be observed in Figure V-1 the empirical two-site kinetic desorption model successfully described the TPC kinetics behavior of semi-continuous extraction. The two-site model was quite close to the experimental data of phenolic extraction under the different conditions of extraction; i) EtOH60, beads of 0.5-0.75 mm, ii) EtOH60, beads of 2 mm and, iii) EtOH90, beads of 2 mm. In Table V-2 are presented the estimated parameters of the model. In this sense, it is important to highlight the differences among the different conditions of extraction related to the fraction of extractable material of easy access (f), which varied in the following manner: EtOH60-beads of 0.5-0.75 mm > EtOH60-beads of 2 mm > EtOH90-beads of 2 mm. This indicated a higher amount of phenolic compound than can be easily extracted by EtOH60 when the extraction bed is formed using beads of 0.5-0.75 mm instead of 2 mm

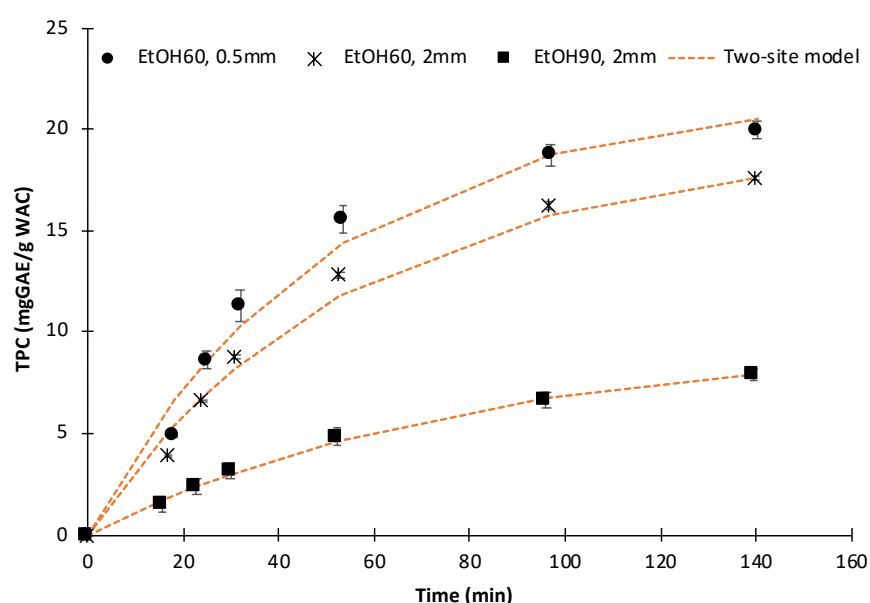
beads. Small beads could promote a greater contact of EtOH60 with the solid matrix into the extraction cell (for instance, by avoiding preferential routes of the solvent through the extraction bed), which enhanced the solubilization of phenolic compounds in the bulk solvent. Regarding EtOH90 (beads of 2 mm), this solvent significantly decreased the yield of phenolic compounds in comparison with EtOH60 (beads of 2 mm); after 140 minutes of extraction the yield decreased from 17 to 8 mg GAE/g WAC. This behavior was consistent with the findings of Odabaş & Koca, (2016) which reported that ethanol-water mixtures about 55% to 70% (v/v) are the optimal conditions for extracting polar phenolics from hazelnut skin. The values of y_{∞} also varied, in particular between EtOH60 (19.1-21.8 mg GAE/g WAC) and EtOH90 (9.7 mg GAE/g WAC) proving the high selectivity of EtOH60 for phenolic compounds.

On the other hand, the values of k_1 were close to those of k_2 for all extractions, i.e. EtOH60-beads of 0.5-0.75 mm, EtOH60-beads of 2 mm and EtOH90-beads of 2 mm (Table V-2). This behavior means that the desorption rate of the easy access material was similar to that of the less accessible material that describes the slow diffusion. In this context, it can be observed in Figure V-1 that none of the curves reached a plateau, indicating that extractions were not finished after 140 min and that longer extraction periods should be carried out for observing the slow diffusion stage. Similarly, Kubatova et al. (2002) have reported that for instance, low solvent flow-rates may not be enough to see both “fast” and “slow” parts of kinetic curves and therefore, k_1 is close to k_2 . A faster flow-rate will give a somewhat faster extraction rate, particularly in the early part of the extraction where the analyte concentration is the highest and then a $k_1 \gg k_2$ can be achieved. Barrales et al. (2018) and Pereira et al. (2019) also found $k_1 \gg k_2$ in kinetics of phenolic extraction using pressurized liquid solvents.

For EtOH60, the value of 17-18 mg GAE/g WAC was higher than the 14.78 mg GAE/g WAC obtained by batch maceration. This might come from a greater contact between the solvent and the matrix in semi-continuous trials where the solvent flow through the particulate bed, and therefore led to a more efficient mass transfer. In addition, semi-continuous extraction allows a higher gradient of concentration in the system, which also enhance the mass transfer.

Table V-2. Estimated parameters of the two-site kinetic desorption model for TPC from WAC by semi-continuous extraction at 60 °C

Two-site model parameters	EtOH60, beads 0.5 mm	EtOH60, beads 2 mm	EtOH90, beads 2 mm
y_{∞} (mg GAE/g WAC)	21.8	19.1	9.7
f	0.087	0.020	0.005
k_1 (min ⁻¹)	0.023	0.021	0.016
k_2 (min ⁻¹)	0.020	0.018	0.012
R^2	0.983	0.991	0.997

**Figure V-1.** Experimental and modeled TPC kinetic curves of semi-continuous extraction at 60 °C from WAC

Regarding the total extracted amount, a similar behavior than that observed for TPC was found since EtOH60-beads of 0.5-0.75 mm allowed to obtain more extract than EtOH60-beads of 2 mm (Figure V-A1, Appendix). Although EtOH90 yielded lower mas of extract than EtOH60 during the first stage of extraction, both ethanolic mixtures yielded similar amounts of extract (about 1.1 g of extract from 5 g of WAC,) at the end of the extraction. Differences in the kinetic of extraction by using EtOH60 or EtOH90 may come from the class of compounds extracted by each solvent, since the concentration of water in ethanol mixture significantly modify its polarity and consequently the type of molecules extracted. Although, the accurate identification of individual compounds extracted under the different extraction conditions was out of scope in this study, we verified that EtOH90 exhibited higher affinity for

neutral lipids in comparison with EtOH60, since this latter extracted about 30% of total oil in WAC, whereas EtOH90 recovered 40% of total oil; after semi-continuous extraction using EtOH60 and EtOH90, the mass of oil in the walnut residue was quantified by Soxhlet extraction giving the following values: 1.33 g and 1.13 g, respectively.

These findings are in agreement with several studies which showed that the presence of water in the alcoholic solvents negatively affect the oil extraction (Capellini et al., 2019, 2017; Navarro et al., 2016; Sawada et al., 2014). This fact is also consistent with the aforementioned statements that argued about the optimal polarity achieved by EtOH60 for extracting polar compounds (like phenolics among others), whereas a lower proportion of water in ethanol mixture led to decrease its polarity and therefore EtOH90 modified its affinity more towards oil (apolar compound) than towards phenolics. In this context the polarity (dielectric constant) of ethanol-water mixtures was estimated as described by Tir et al., 2012 and considering a dielectric constant (at 60 °C) of 21.4 for ethanol (Navarro et al., 2016) and of 66.8 for water (Malmberg & Maryott, 1956). The dielectric constant of EtOH60 was 39.56 whereas of EtOH90 was 25.94, which confirms our hypothesis related to differences in the type of extracted compounds due to the different polarity of EtOH60 and EtOH90.

3. Enrichment of Oil with polyphenol from WAC

3.1. Enrichment with WAC extracts from maceration

As previously described, the purpose of enriching Oil with polyphenols extracts from WAC is both to enhance the oxidative stability of Oil and to provide an added-value to walnut residue as a rich and abundant source of antioxidant compounds. The first oil enrichment consisted of adding a rich-polyphenol extract, obtained previously by maceration, to Oil. In this sense, the extract of run No. 10 of Table V-1 was used to enrich Oil. For that, two levels of enrichment were assessed: 150 and 1 500 mg extract/kg oil. Hence, 1.4 mL or 14 mL of extract were directly added to 70 g of oil (containing or not, 20 mg of PL/g oil) which provide concentrations of 150 and 1 500 mg extract/kg oil, respectively. For enriching the oil containing PL, the 14 mL walnut extract was previously concentrated to 1.5 mL under nitrogen flux before adding to the oil, in order to avoid PL precipitation.

After solvent evaporation, the TPC of enriched oils was measured and the results are shown in Table V-3.

Table V-3. Total phenolic content (TPC) and oxidation lag time of oils enriched with hydroalcoholic extracts from WAC

Sample	TPC (mg GAE/kg oil)		Oxidation Lag time (h)
	Without PL	With PL	
150 mg ext/kg Coil	Without PL	41.2 ^a ± 2.5	41 ^b ± 2
	With PL	42.2 ^a ± 2.8	57 ^c ± 3
1 500 mg ext/kg Coil	Without PL	129.6 ^b ± 7	66 ^c ± 3
	With PL	103.1 ^c ± 6.6	80 ^d ± 5
Pure Coil		30 ^a ± 2.6	24 ^a ± 1

Data expressed as mean values and standard deviations. Equal letters in the same column indicate no significant difference at level of 5% ($p < 0.05$)

The theoretical TPC of oils enriched at 150 and 1 500 mg extract/kg Coil is 42.43 mg GAE/kg oil and 154.3 mg GAE/kg oil, respectively. These values were calculated considering the TPC of pure Coil (30 ± 2.6 mg GAE/kg oil) plus the phenolics incorporated by the addition of the different amount of walnut extract, i.e. 12.43 mg GAE/kg oil and 124.3 mg GAE/kg oil, for 1.4 mL and 14 mL of extract, respectively. In this context, it can be observed that the measured TPC of most of the enriched oils was similar to the theoretical one, which means that during the enrichment process no significant degradation of polyphenols took place. Only the Coil containing PL and enriched at 1 500 mg extract/kg Coil exhibited a lower measured phenolic content (103.1 mg GAE/kg oil) than the theoretically expected (154.3 mg GAE/kg oil). This behavior can be explained by the fact that prior to oil enrichment, and in order to avoid PL precipitation, the 14 mL of walnut extract had to be concentrated under nitrogen flux for several hours. This long concentration step could lead to a partial degradation of polyphenol in the hydroalcoholic extract. On the other hand, it has been also previously mentioned that the formation of many colloidal structures due to PL concentration, may prevent the phenolics from reacting with the Folin–Ciocalteu's reagent which results in the underestimation of oil phenolic content.

After oxidation of these enriched samples, the results of CD monitoring showed that both levels of enrichment (150 and 1 500 mg extract/kg Coil) improved significantly the oxidative stability of Coil (containing or not PL) (Table V-3 and Figure V-2). The results also showed a higher protective effect when oil was enriched at 1 500 mg extract/kg Coil than at 150 mg extract/kg Coil. For Coil enriched with 150 and 1 500 mg extract/kg Coil in absence

of PL, the oxidative stability (41 h and 66 h, respectively) increased about 1.7-fold and 2.7-fold, whereas in presence of PL, the oxidation lag time (57 and 84 h, respectively) increased about 2.4-fold and 3.5-fold, respectively. From that perspective, it can be stated: i) the oxidative stability of Coil increased at higher TPC of oil but not in the same proportion. It means for instance, that TPC of Coil enriched at 1 500 mg extract/kg oil is 3.1-fold higher than that enriched at 150 mg extract/kg oil, but the oxidative stability of Coil enriched at 1 500 mg extract/kg oil was only 1.7-fold greater than that Coil enriched at 150 mg extract/kg oil. We have observed in *Chapter IV* that although higher quercetin concentration in Coil enriched at a fix PL proportion, provided a greater oxidative stability, the protective effect of quercetin is also significantly influenced by its synergism with other antioxidants, physical location and not only by its concentration. ii) the presence of PL in oils enriched with walnut extracts further improved the oxidative stability of Coil. This observation is consistent with that already discussed in *Chapter IV*; PL exert a protective effect against lipid oxidation due to its capacity to chelate metals and scavenge free radicals. The negatively charged phosphoryl group of PL is able to chelate metals especially iron and copper, thus limiting their reactions with PUFAs (Cui & Decker, 2016; Koga & Terao, 1995). Moreover, the amine function of phosphatidylcholine can directly interact with free radicals and block the propagation reactions (Henna Lu et al., 2011).

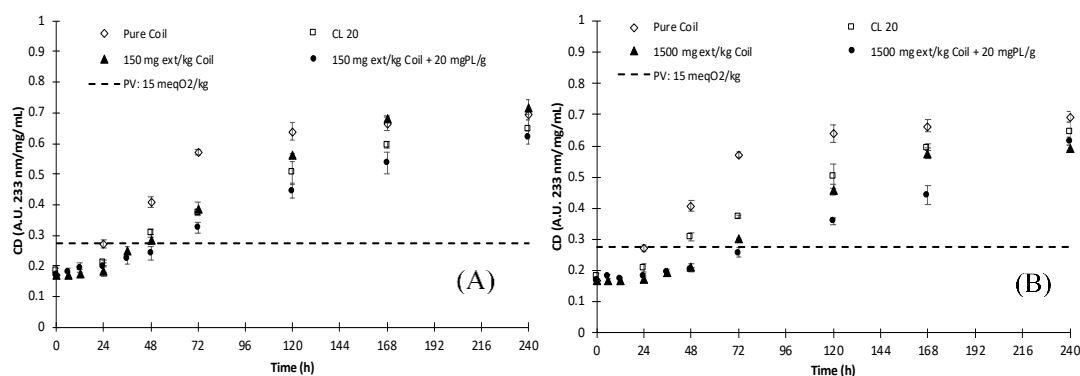


Figure V-2. Time course formation of CD in Coil (containing or not, 20 mg PL/g oil) enriched with walnut extract at (A) 150 mg/kg oil, and at (B) 1 500 mg/kg oil

The protective effect of phenolic extracts against lipid oxidation of bulk oils has been widely reported in agreement with our results. For instance, Bakkalbasi et al. (2018) observed an increasing in the oxidative stability of walnut oil by the enrichment with phenolic extracts from walnut press-cake. A significant decrease of the formation of peroxides, conjugated dienes and trienes, hexanal is observed in walnut oil enriched with 50, 100 and 200 mg phenolic

extract/kg oil, in comparison with non-enriched oil. The enriched oils were submitted to accelerated oxidation at 65 °C, and the protective effect was dependent on the extract concentration (Bakkalbasi et al., 2018). In particular, the non-enriched walnut oil reached a PV of 12 m_{eq}O₂/kg after about 6.5 days of oxidation, whereas the enriched oil with 200 mg phenolic extract/kg oil needed 9 days for reaching the same PV, which means 1.4-fold greater oxidative stability of enriched oil. In the same way, Fregapane et al. (2020) observed a significant increase in the antioxidant capacity (evaluated both by DPPH and ORAC assays) of olive, pistachio and walnut oils when they were enriched with either pistachio or walnut extracts obtained by maceration with ethanol 80% (v/v). An increase of 2.4-fold the oxidative stability, measured by Rancimat method, of virgin walnut oil by enriching it with walnut cake extracts up to a total phenolic content of 518 mg/kg oil (the TPC of non-enriched walnut oil was 50 mg/kg) (Fregapane et al., 2020). Moreover, these authors reported that phenolic extracts from walnut exhibit a higher protective effect against lipid oxidation than those from pistachio.

Other authors such as Zribi et al. (2013) significantly increased (about 1.3-fold) the induction time of pan-frying refined olive and soybean oil enriched with hydrolysate olive leaf extracts (incorporated at 500 mg/kg), and Poiana, (2012) retarded the lipid oxidation of sunflower oil enriched with grape seed extracts submitted to convection and microwave heating. The authors studied various concentrations (from 600 to 1 000 mg/kg) of grape seed extract in oil and reported that the protective effect against lipid oxidation was dose-dependent. Oil enrichment at a level of 1 000 mg/kg was able to reduce the formation of hydroperoxides, conjugated dienes, conjugated trienes in a range of 30-40%, and it was comparable to the effect of synthetic antioxidants like butylated hydroxytoluene (BHT) (Poiana, 2012). Delgado-Adamez et al. (2014) observed that the enrichment of virgin olive oil (up to a TPC higher than 1 000 mg/mL oil) with extracts from olive leaf/cake increased the oxidative stability, measured by Rancimat method, 1.5-fold in comparison with the control oil. In addition, the oxidative stability of this oil increased approximately 40% by incorporating lecithin (6 mg/mL oil). Suárez et al. (2011) enriched olive oil with phenolic compound (7 mg extract/mL oil) from dried olive cake and lecithin (0.3%), and reported that enriched oils had an average enhancement of oxidative stability of 65% compared with the value of the control oil. These authors (Delgado-Adamez et al., 2014; Suárez et al., 2011) also stated that the stabilization of the phenolic compound added to bulk oils is attributed to the amphiphilic character of lecithin that allow the formation of reverse micelles in the oil matrix. This latter allowed to observe an

enhanced oxidative stability of bulk oils enriched with both polar phenolic extracts and phospholipids.

In general, the significant protective effect against lipid oxidation of bulk oils exhibited by polar polyphenols can be explained by the polar paradox theory (Bakkalbasi et al., 2018; Li et al., 2015). From that perspective, it is worth providing the most important concepts around this theory.

Porter, (1993) argued that primary antioxidants that are polar or amphiphiles of high hydrophilic-lipophilic balance (HLB) tend to be more effective in nonpolar media such as bulk oils, while non-polar or amphiphilic antioxidants with low HLB are more effective in polar media or systems with relatively higher polarity as emulsions. This theory emerged as a way of interpreting the action of antioxidants in different lipid systems such as bulk oils, emulsion, micelles, liposomes, etc., which are complex systems of variable composition that influence directly the mechanisms of action of antioxidant molecules.

The polar paradox behavior of antioxidants in bulk oils was an empirical observation until considering the interfacial phenomenon. This latter consideration established that the interfacial properties of antioxidants, as well as their partition in the medium, are determinant in the effectiveness of antioxidants (Shahidi & Zhong, 2011). Early studies on oil oxidation argued that the oxidation reactions take place in homogenous medium, in which the oil-air interface was considered to be the site where oxidation is initiated and propagated. In this sense, polar (hydrophilic) antioxidants oriented themselves at the oil-air interface (Figure V-3A) and therefore protected the system from oxidation.

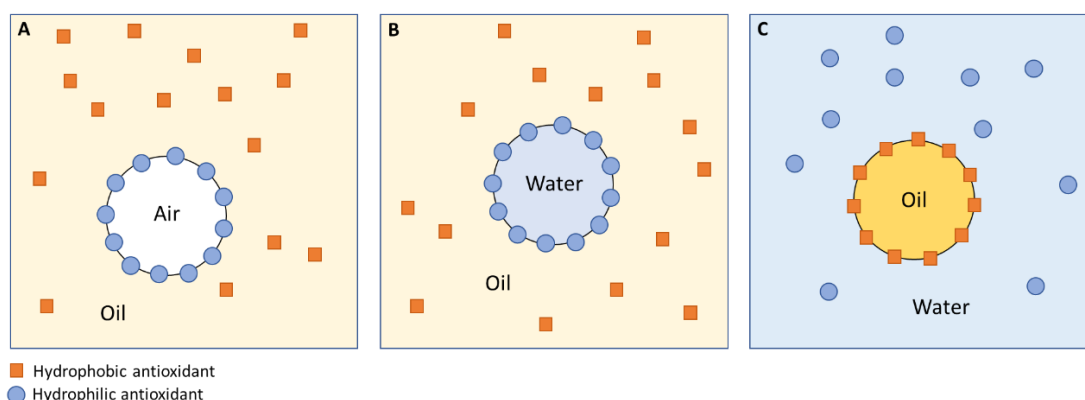


Figure V-3. Distribution of antioxidants in bulk oils considering the interfacial phenomena and polar paradox (Adapted from Shahidi & Zhong, 2011)

Nevertheless, since air is less polar than oil, the distribution of polar antioxidants at the oil-air interface was questioned. In this context, it was later proved that various microenvironments existing in edible oils are responsible for altering the physical location of both antioxidants and lipid substrates. In addition, these microenvironments (association colloids) are currently known as the site of lipid oxidation in bulk oils (Shahidi & Zhong, 2011).

As it was discussed previously in *Chapter IV*, different types of association of colloids such as reverse micelles could be formed in those microenvironments due to the presence of traces of water. In the case of amphiphilic molecules such as phospholipids, their self-assembly in the aqueous microenvironment are well evidenced. Moreover, oxidation products such as aldehydes, hydroperoxides and ketones have also the ability to self-assemble in oils. In this context, the formation of colloidal structures stated a different location of the polar antioxidants in bulk oils; as shown in Figure V-3B, polar antioxidant are distributed at the interface of colloids (oil-water interface) instead of being located at the oil-air interface. Hence, polar antioxidants are physically close to the oxidation site, being more effective to inhibit oxidation reactions than the nonpolar ones that are dissolved in the lipid phase (Li et al., 2015; Shahidi & Zhong, 2011). This theory was later supported by the observations of Frankel et al. (1994) who reported higher efficacy of polar antioxidants like eriodictyol and caffeic acid, in bulk oils, than lipophilic BHT. Figure V-3C corresponds to emulsion systems, where non-polar antioxidants are preferably at located the oil-water interface (around the lipid droplet), whereas polar antioxidants are predominantly dissolved in the aqueous phase.

3.2. Direct enrichment with WAC by maceration and UAE

In order to enrich Coil with polyphenols or polyphenol-phospholipid formulations, the direct extraction of phenolic compounds from WAC was attempted. The purpose of this enrichment was to simplify the extraction process, by the absence of the evaporation step or solvent separation. For that, WAC was directly added to camelina oil and then both maceration and ultrasound assisted extraction (UAE) were carried out. Nonetheless, after maceration and UAE, the total phenolic content of Coil exhibited only a slight increase, even in the Coil previously enriched with PL (20 mg/g) as shown in Table V-4.

Table V-4. Total phenolic content (TPC) of Coil after direct enrichment with WAC by several methods

Extraction method	Solvent	TPC (mg GAE/kg oil)
Maceration (solid-to-solvent ratio 1:5 wt, 35 °C, 37.5 min)	Coil without PL	39 ^a ± 3.2
	Coil with PL	36.1 ^a ± 2.9
UAE (solid-to-solvent ratio 1:4 wt, room temperature, 7 min, 275 W, 50% pulse)	Coil without PL	37.5 ^a ± 0.7
	Coil with PL	36 ^a ± 1.9
UAE - Addition of 2% water	Coil without PL	36 ^a ± 0.4
	Coil with PL	35.1 ^a ± 1.6
UAE - Addition of 20% ETOH60	Coil without PL	38 ^a ± 1.7
	Coil with PL	37.2 ^a ± 2.1

Data expressed as mean values and standard deviations. Equal letters in the same column indicate no significant difference at level of 5% ($p < 0.05$)

Throughout this manuscript, it has been largely discussed the poor solubility of phenolic compounds in edible oils due to the polar nature and multi-ring structure of polyphenols. Yara-Varón et al. (2017) have reported the convenience of using edible oils, in particular sunflower oil, for direct extraction of aroma compounds such as linalool, estragole, eucalyptol, trans-anethole and limonene, or of lipophilic pigments such as carotenoids, from plant matrices. Moreover, these authors have suggested the use of vegetable oils as an potential solvent for extracting even polar polyphenols, based on the paradoxical behavior of polar antioxidants in bulk lipids; vegetable oils can not only be the solvents for the lipophilic antioxidants in light of the “like dissolve like” principle (Yara-Varón et al., 2017). However, the extraction of target compounds from plant matrices by vegetable oil is a quite complex systems since it is affected by several factors (both intrinsic and extrinsic). From a macroscopic point of view, a solvent is a continuum characterized by physical constants (boiling point, melting point, vapor pressure, thermal conductivity, surface tension, density, viscosity, refractive index, etc.) (Reichardt, 2007), whereas from a microscopic point of view, it is a discontinuum that consists of individual interacting molecules characterized by molecular properties (dipole moment, electronic polarizability, hydrogen bond donor or acceptor character, electron donor or acceptor character, etc.) (Yara-Varón et al., 2017). All these properties directly influence the solute-solvent interactions and therefore, the efficiency of extraction of target compounds. In addition, when vegetable oils are used as solvent, the

triglycerides nature and the presence of minor components such as mono- and diglycerides, phospholipids, sterols, tocopherols, tocotrienols, free fatty acids, vitamins, pigments, proteins, phenolic compounds, water, etc., are added to the factors that may influence the solute-solvent interactions.

As also discussed in previous chapters, several authors (Yara-Varón et al., 2017; Li et al., 2015; Delgado-Adámez et al., 2014) have highlighted the key role of the formation of colloidal structures (reverse micelle and lamellar structure, for instance), in the capacity of oils for dissolving polar compounds. This is mainly because the colloidal associations provide a large interface between oil and water, which allows the coexistence of both water- and oil-soluble substances (Li et al., 2015). Therefore, a higher capacity of oil rich in amphiphilic molecules for extracting phenolic compounds from plant matrices is theoretically expected.

Globally, our results showed only a slight ability of Coil for extracting phenolic compounds from WAC. Polyphenols of walnut are mainly polar compounds (Bakkalbaşı, 2018), therefore exhibited a poor solubilization in Coil (non-polar medium). Walnut phenolics are preferably extracted by polar solvent such as hydroalcoholic mixtures (Bodoira & Maestri, 2020). On contrary, Bakkalbaşı, 2018 succeed to prepare phenol-enriched walnut oil by ultrasonic maceration of walnut oil with walnut press-cake. This author reported an increase of phenolic content of walnut oil from 9.7 mg GAE/kg oil to 78.8 mg GAE/kg oil, through the direct oil enrichment. Li et al. (2015) also increased the phenolic content of virgin sunflower oil (32% of extraction efficiency), castor oil (considered a polar oil exhibited 73% of extraction efficiency) by direct enrichment with olive leaves, whereas peanut oil exhibited poor extraction efficiency (about 9.9%) of olive leaf phenolics. These discrepancies may come from intrinsic factors such as fatty acid composition of the various oils, the content of both minor compounds and water which significantly influence the extraction process. For instance, the presence of mono- and diglycerides in vegetable oils may facilitate the extraction of polar phenolics, since they have free hydroxyls groups which give them a certain hydrophilic character (Yara-Varón et al., 2017). In this sense, Coil exhibited a low mono- and diglyceride content (< 1%) (Table III-1, *Chapter III*). Moreover, Coil is composed mainly of triglycerides with C18 (~71%) and C20 (~19%) chain lengths (Table III-1, *Chapter III*); and long-chain fatty acids are unfavorable to the extraction efficiency (Li et al., 2015). On the other hand, extrinsic factors such a solid-to-solvent ratio, extraction time and temperature also influence markedly the extraction of phenolics from plant matrices. For instance, Bakkalbaşı, (2018)

performed the ultrasonic extraction for 1 hour at 25 °C and Li et al. (2015) carried out the maceration extraction for 3 hours at 40 °C, whereas in this work the direct extraction by maceration and ultrasound consisted of only 37.5 and 7 minutes, respectively. Longer extraction periods should provide higher phenolic extraction than those observed in Table V-4, however the short extractions carried out in this work aimed at preventing the oxidation of Coil by the combined effect of long time-high temperature. In the same sense, it can be stated that the positive effect of adding water or EtOH60 to Coil, on the extraction efficiency of polar phenolics could be observed when longer extractions are performed. On the other hand, the addition of only 2% of water to oil could be also insufficient to modify the polarity of Coil. In general, oils exhibit a high viscosity and surface tension which makes the diffusion processes and extraction rates slow, hence long extraction periods (several hours) should be considered when vegetable oils are used as solvents.

It is also worth mentioning that the initial content of extractable polyphenols from raw material also governs the level of oil enrichment. For instance, the phenolic content of walnut cake used for Bakkalbaşı, (2018) was 58 mg GAE/g and of olive leaves reported by Li et al. (2015) was 66 mg/g. This values are higher than that found for the WAC used in this work (< 20 mg GAE/g), therefore a lower level of oil enrichment was expected in this work compared with those achieved by both Bakkalbaşı, (2018) and Li et al. (2015).

As already shown in previous section, an alternative for oil enrichment consists of first extracting the phenolic compounds with hydroalcoholic mixtures, which provides a high extraction yield, and then incorporate these polar extract to vegetable oil (Fregapane et al., 2020; Khemakhem et al., 2015; Delgado-Adamez et al., 2014; Zribi et al., 2013; Poiana, 2012; Orozco et al., 2011).

Regarding the phenolic extraction of Coil enriched with PL, it did not vary in relation to pure Coil. This result was unexpected under the hypothesis that the head of PL would provide polar interaction with walnut phenolics, and that the formation of colloidal association in bulk oil, by the presence of this surfactant (and eventually enhanced by the addition of water), allows a larger interface creation with stronger interaction with phenolic compounds. Hence, a greater phenolic extraction of Coil with PL was expected according also to simulations of Yara-Varón et al. (2017). Nonetheless, Li et al. (2015) have reported that the addition of surfactants to sunflower oil for extracting phenolics needs to be optimized since at certain concentration of these additives, the extraction yield may significantly decrease. For instance, by the addition

of monoglycerides to sunflower oil at ratios of 2% and 5%, the oil exhibited lower phenolic content after direct enrichment with olive leaves than when only 1% of monoglycerides were added. In this sense, Li et al. (2015) revealed that a competition may exist between extraction and micellization depending on the addition ratio of surfactants; as the ratio gradually increased above 1%, the generated micelles, with the phenolics inside, became too many to disperse in the oil-continuous phase and thus, are separated from the oil. In that case, the micelles in excess would be separated from the enriched oil when this latter was centrifuged for WAC particles removal. Moreover, the formation of many colloidal structures could prevent them from reacting with the Folin–Ciocalteu’s reagent, which results in the underestimation of the TPC in enriched oils (Li et al., 2015). Similarly, other works have observed a decrease in the TPC directly proportional to the amount of lecithin added to vegetable oils (Delgado-Adamez et al., 2014; Suárez et al., 2011; Koprivnjak et al., 2008). From that perspective, the amount of PL in Coil should be first optimized for enhancing the TPC of enriched oil.

Finally, it is worth remembering that longer extractions than those reported in this work may be required for achieving higher TPC of enriched oils and also for really interpreting the effect of PL in Coil.

3.3. Oxidation stability of Coil enriched with WAC particles

In order to improve the oxidative stability of oils, the incorporation of walnut particles to Coil submitted to accelerated oxidation was evaluated. This method consisted of incorporating WAC particles to oil in a ratio of 1:4 (1.5 g of WAC in 6 g of Coil, containing or not 20 mg of PL/g oil). The enriched oil was stirred overnight (room temperature) and then submitted to accelerated oxidation at 60 °C.

This enrichment method proved to be an efficient technique for delaying lipid oxidation. As shown in Figure V-4, Coil or Coil+PL with walnut particles remained consumable for more than 5 days (120 h), which is significantly higher than non-enriched Coil that after 24 h at 60 °C exceeded a PV of 15 meq.O₂/kg. The incorporation of walnut particles to oil was made in a mass ratio 1:4. Our observations were consistent with those of Joseph, (2018) who also reported an significantly increase about 4-fold the oxidative stability of linseed oil by the incorporation of rapeseed particles in a mass ratio 2:1 (linseed oil: rapeseed particles). However, Joseph, (2018) also observed a greater protective effect against lipid oxidation of defatted rapeseed than non-defatted one.

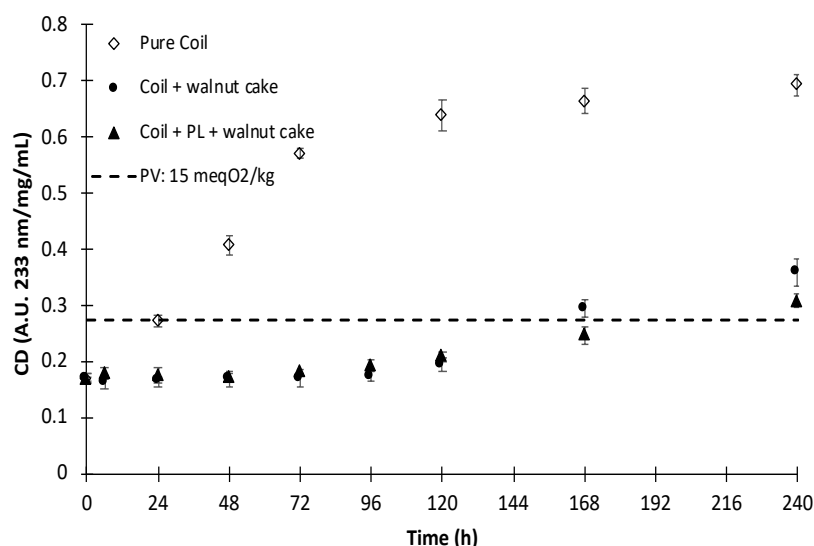


Figure V-4. Time course formation of conjugated dienes of pure Coil (containing or not, 20 mg PL/g oil) enriched with WAC at a ratio 4:1

Our hypothesis for explaining the protective effect provided by walnut particles is related to the solubilization of lipophilic antioxidant compounds during oxidation time. Moreover, high temperature (60 °C) could facilitate the solubilization of these compounds. For instance, theoretically walnut press-cake may contain approximately 200-300 mg tocopherols/kg (Beyhan et al., 2017), which are lipophilic compounds with high antioxidant activity that may act as a hydrogen-donating compounds (Suárez-Jiménez et al., 2016). From that perspective, about 50-75 mg tocopherol/kg oil could be incorporated to Coil by the addition of walnut particles and therefore, the oxidative stability of Coil was enhanced. According to Espín et al. (2000), oil fraction of walnut also exhibits an important capacity for scavenging free radicals due to the presence of tocopherols, which may act in synergism with other compounds such as phospholipids. There is evidence of the protective effect of tocopherol (both α and γ -tocopherol) against lipid oxidation of oils when the antioxidant was added in a concentration of 50 mg tocopherol/kg oil (Yanishlieva et al., 2002). Similarly, Bakkalbaşı, 2018, significantly reduced the formation of peroxides, conjugated dienes and trienes of walnut oil submitted to accelerated oxidation by the incorporation of 100 mg α -tocopherol/kg oil.

In *Chapter IV* it was shown that the addition of exogenous α -tocopherol (36 mg/kg Coil) to Coil did not significantly delay the lipid oxidation. Nonetheless, through the incorporation of walnut particles a higher theoretical value of total tocopherol was added

(50-75 mg tocopherols/kg). Hence, an enhanced oxidative stability of Coil by solubilization of compounds from WAC, such as tocopherols, can be highly considered.

On the other hand, it has been previously discussed that phenolics from walnut are mainly polar compounds and therefore, a poor solubilization of these compounds in oil was expected. A higher temperature (60 °C) of Coil during accelerated oxidation could facilitate the solubilization of some phenolics. Nonetheless, during accelerated oxidation of Coil provided of walnut particles, only a slight increase of TPC was observed over time (Figure V-5). Probably, phenolic compounds were not directly responsible for the protective effect against lipid oxidation since the diffusion of phenolics from walnut particles to Coil seems to be not significant. However, the synergism of some extracted phenolics with other lipophilic compounds (antioxidants) cannot be ruled out.

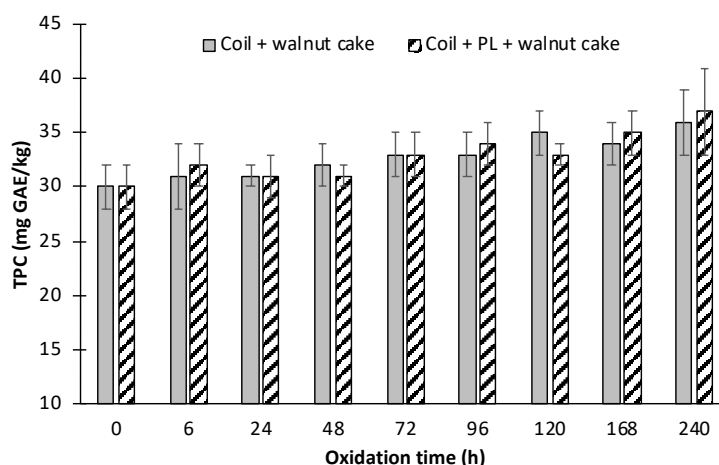


Figure V-5. Monitoring of TPC during accelerated oxidation of Coil (containing or not, 20 mg of PL/g oil) enriched with WAC

Arranz et al. (2008) and Reiter et al. (2005) have also reported that other compounds of walnut such as melatonin and indoleamine which are highly lipophilic, exhibit a high antioxidant activity. In this sense, the solubilization of this type of compounds could be also involved on the enhanced oxidative of Coil enriched with WAC. Nonetheless, the content of tocopherol, melatonin and indoleamine in Coil during accelerated oxidation, was not monitored in this work therefore, their solubilization in Coil and their subsequent action against lipid oxidation is just hypothetical.

On the other hand, it would be worth monitoring the viscosity of Coil enriched with WAC over oxidation time, since author such as Ayadi et al. (2009) have reported an increase

in the viscosity of olive oil after two weeks of infusion with aromatic plants. An increase of oil viscosity may hinder the diffusion of oxygen, necessary for oxidation, in the oil.

4. Conclusion and perspectives

Throughout this chapter, walnut press-cake was presented as cheap, abundant and rich source of phenolics compounds with an important antioxidant capacity which can be useful for several applications in food, pharmaceutical or cosmetic fields. Maceration from walnut cake extraction provided extracts rich in polyphenols and it was mainly benefited by higher temperature of extraction and solid-to-solvent ratio, whereas the incorporation of phospholipids to extraction solvent was deleterious for recovering phenolics. The influence of extraction time, temperature, and solid-to-solvent ratio and phospholipid concentration in extraction solvent on total phenolic extraction and antiradical activity of extracts was successfully studied. In this sense, the total phenolic extraction by maceration fit well to a predicted polynomial model with a coefficient of determination higher than 0.90. Similarly, the free radical scavenging activity (DPPH) of walnut extracts obtained by maceration could be predicted by a polynomial model ($R^2 = 0.77$), which considered only the extraction temperature and the phospholipid concentration as significant factors. Furthermore, the antioxidant activity (measured by means of DPPH assay) of walnut extracts was highly correlated to the total phenolic content (Pearson's coefficient of 0.94), and therefore it was also negatively influenced by the incorporation of phospholipids to hydroalcoholic mixture. The positive effect of increasing extraction temperature is related to higher facility with which solvent diffuses into cells, a higher desorption and solubility, and an increase in the vapor pressure of solutes which facilitates mass transfer.

On the other hand, semi-continuous extraction allowed to slightly increase the phenolic extraction from walnut cake by providing a greater surface area of interaction between solvent and solid particles. Maybe more important than higher extraction yield, the semi-continuous extraction of polyphenols allowed to obtain fractionated extracts that exhibit both different amount of extracted mass and phenolic concentration, thus could be used separately for diverse industrial applications. The two-site kinetic desorption model was successfully adjusted ($R^2 > 0.98$) to the experimental extraction kinetic of polyphenols from walnut cake by the semi-continuous method using EtOH60 and EtOH90. Nonetheless, the low solvent flow-rates probably was not enough to see both "fast" and "slow" parts of kinetic curves and therefore,

k_1 was close to k_2 . In addition, the extraction kinetic of phenolics, by semi-continuous extraction, did not reach the diffusion-controlled stage. From that perspective, the assessment of semi-continuous extraction at both higher flow rates and longer periods is highly suggested for future works. On the other hand the mixture EtOH60 proved to be the best proportion of ethanol-water mixtures for extracting polar phenolics from walnut cake, which was in agreement with literature. Concentrations of ethanol higher than 60%, in the ethanol-water mixtures, was more suitable for oil extraction than phenolic recovery.

Hydroalcoholic extracts rich in walnut polyphenols exhibited a significant capacity for delaying lipid oxidation of camelina oil (bulk oil) in a dose-dependent manner. Although this enrichment method required of a solvent evaporation step, it was efficient for significantly enhancing the oxidative stability of camelina oil. In addition, and in agreement with observations of previous chapter, the incorporation of phospholipid along with polyphenols allowed to obtain and enhanced oxidative stability of enriched oils. Phospholipids exhibited antioxidant activity in bulk camelina oil and also provided colloidal structures in which walnut phenolics are preferably located. The antioxidant activity of polar phenolics of walnut cake in camelina oil, can be explained by polar paradox theory and it was in concordance with that widely reported in literature. Nonetheless, a more complex experimental designs (for future works) of oil enrichment may provide an optimization for incorporating phospholipid-polyphenol extract formulations, in order to obtain a greater oxidative stability. In the same way, the identification of phenolics of walnut extracts may provide additional information related to the type of phenolics responsible for the antiradical activity in bulk oils.

On the other hand, the direct extraction of polyphenol from walnut into the bulk oil by maceration or ultrasound assisted extraction was slightly efficient. Longer extractions should be assessed in order to increase the total phenolic content of oil after direct enrichment. However, the oxidation of camelina oil provided with walnut cake was significantly slower than of oil without walnut. In this sense, the accurate identification of the compounds responsible for the enhanced oxidative stability of oil enriched with walnut is highly recommended for future works.

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Appendix

Table V-A1. Estimated regression coefficients of the predicted model for TPC

Regression coefficient	TPC (Predicted model)			
	Coefficient	Std. Error	t-value	p-value
Intercept β_0	8.9082	0.2653	33.579	4.69×10^{-6} ***
Linear β_i				
Z ₁	0.7475	0.3111	2.403	0.07412•
Z ₂	2.4375	0.3111	7.835	0.00143**
Z ₃	1.4050	0.3111	4.516	0.01068*
Z ₄	-3.0250	0.3111	-9.724	0.00062***
Interactions β_{ij}				
Z ₁₂ + Z ₃₄	0.2250	0.3349	0.672	0.54979
Z ₂₄ + Z ₁₃	1.3625	0.3111	4.380	0.01187*
Z ₂₃ + Z ₁₄	1.0425	0.3111	3.351	0.02853*
Adjusted R²		0.9538		0.001997**

•, *, **, *** significant at 0.1, 0.05, 0.01 and 0.001 level, respectively

Interpretation: Hypothesis ii) Z₁₂ and Z₃₄ non-significative; Hypothesis iii) as Z₁ is a non-significative factor, Z₁₃ and Z₁₄ are non-significative and thus Z₂₄ and Z₂₃ are significant interactions

Table V-A2. Estimated regression coefficients of the predicted model for DPPH

Regression coefficient	DPPH (Predicted model)			
	Coefficient	Std. Error	t-value	p-value
Intercept β_0	76.945	4.852	15.858	9.62×10^{-7} ***
Linear β_i				
Z ₁	4.815	5.690	0.846	0.42539
Z ₂	18.560	5.690	3.262	0.01383*
Z ₄	-28.183	5.690	-4.953	0.00165**
Adjusted R²		0.7669		0.00382**

•, *, **, *** significant at 0.1, 0.05, 0.01 and 0.001 level, respectively

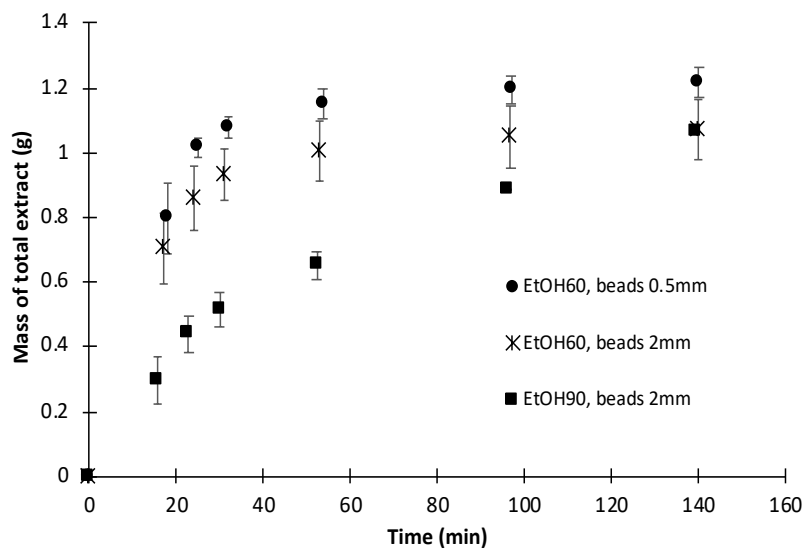


Figure V-A1. Extraction kinetic of global extract by semi-continuous extraction at 60 °C from WAC

General conclusion and perspectives

In this work, the oxidative stability of edible oils, mostly camelina oil, has been successfully enhanced by enriching them with phenolics antioxidants, either as a pure component, quercetin, or as a more complex mixture extracted from a walnut by-product. The solubility of quercetin in oils was influenced by oil composition (fatty acid carbon chain and minor compounds) and from a general point of view, solubilization of quercetin in oils can be limited by multi-ring nature of quercetin. Nonetheless, the solubility of quercetin can be significantly enhanced by the incorporation of phospholipids to edible oils. The level of improvement of quercetin solubility was different among the evaluated oils.

The solvent-free route for the incorporation of phospholipid-quercetin formulations proved to be an efficient method for enhancing quercetin solubility in bulk oils; the enhanced solubility of quercetin was related probably to the interactions formed between the OH group of quercetin and the phosphate group of phosphatidylcholine. On the other hand, it has been also provide several data related to the quercetin solubility in other solvents than oil which are of great interest for food, pharmaceutical, cosmetic and chemical applications. In this sense, amphiphilic solvents such as dimethyl sulfoxide and polyethylene glycol 400 exhibited high capacity to solubilize quercetin whereas water poorly solubilized it.

Regarding oxidative stability assessment, the enrichment of camelina oil with phospholipid-quercetin formulations significantly enhance the shelf life of enriched oil by reducing the lipid oxidation rate. The highest protection effect against lipid oxidation was observed in oils enriched with phospholipids and saturated of quercetin. The addition of only phospholipids to camelina oil also exhibited an antioxidant effect whereas only quercetin poorly protected camelina oil against lipid oxidation. From that perspective, it has been hypothesized that quercetin in presence of phospholipids get closer to the micellar water-oil microenvironment where the oil oxidation takes place and therefore, an enhanced protective effect of quercetin can be observed.

Walnut residue proved to be a rich and abundant source of polar polyphenols with a high antioxidant activity able to delay lipid oxidation of edible oils in a dose-dependent manner. The characteristics of phenolic extracts from walnut cake is influenced by the parameters of extraction process such as temperature, solid-to-solvent ratio and type of solvent.

Globally, it was demonstrated that the oxidative stability of edible oils can be enhanced by the incorporation of phenolic extracts from plant residues and that it is not necessary of using highly pure compounds or synthetic antioxidants for significantly delaying lipid oxidation. This potentiate the use of by-products for obtaining inexpensive antioxidant extracts by GRAS (Generally Recognized As Safe) solvents.

As perspectives for future works, it has been suggested:

- The analysis of the quercetin-PL complex (solvent evaporation method) through infrared spectroscopy, differential scanning calorimetry or x-ray diffraction techniques that allow an accurate characterization of formed complex

- The assessment of the colloidal structuration formed in bulk oils by phospholipids in presence of high concentration of quercetin by means of small-angle X-ray scattering or Nuclear Magnetic Resonance (NMR) analysis

- The monitoring of secondary oxidation products during accelerated oxidation of enriched oils that provide additional information related to the effect of quercetin-phospholipid formulations on lipid oxidation

- The accurate identification of the compounds responsible for the antioxidant activity exhibited by walnut extracts incorporated to edible oils

- The assessment of a solvent-free route to enrich edible oils with phenolics from nuts residues to suppress the use of organic solvent and evaporation steps

- The identification and monitoring of liposoluble compounds of walnut cake able to enhance the oxidative stability of edible oils

- The study of the stability/properties of food, pharmaceutical or cosmetic products fabricated with oils enriched with polyphenols-phospholipids formulations.