



# Nanoformulations pour la protection de flavonoïdes instables : exemple de la quercétine

Tri Truong Công Truong Công

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## THÈSE DE DOCTORAT

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par

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# Nanoformulations pour la protection de flavonoïdes instables: exemple de la quercétine

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## SIGLES ET ABRÉVIATIONS

### En anglais

|                  |                                                                     |
|------------------|---------------------------------------------------------------------|
| A <sub>a</sub>   | Average area                                                        |
| A <sub>c</sub>   | Collapse area                                                       |
| API              | Active pharmaceutical ingredient                                    |
| BNP              | Blank nanodispersions                                               |
| Cryo-TEM         | Cryogenic transmission electron microscopy                          |
| d                | Repeat distance between two reticular plans                         |
| DPPC             | Dipalmitoylphosphatidylcholine                                      |
| DSC              | Differential scanning calorimetry                                   |
| EPR              | Enhanced permeability and retention                                 |
| G/P              | Gelucire <sup>®</sup> 50/13 – Phospholipon <sup>®</sup> 90G mixture |
| HLB              | Hydrophilic – lipophilic balance                                    |
| HPH              | High pressure homogenization                                        |
| HPLC             | High performance liquid chromatography                              |
| IC <sub>50</sub> | Half maximal inhibitory concentration                               |
| NLC              | Nanostructured lipid carriers                                       |
| o/w              | Oil-in-water                                                        |
| PdI              | Polydispersity index                                                |
| PEG              | Polyethylene glycol                                                 |
| PXRD             | Powder X-ray diffraction                                            |
| q                | Scattering vector                                                   |
| QAD              | Quercetin anhydrous after drying                                    |
| QAR              | Quercetin anhydrous recrystallized from ethanol                     |
| QHD              | Quercetin hydrate after drying                                      |
| QHR              | Quercetin hydrate recrystallized from ethanol                       |
| RES              | Reticulo-endothelial system                                         |
| SAXS             | Small-angle X-ray scattering patterns                               |
| SGF              | Simulated gastric fluid                                             |
| SIF              | Simulated intestinal fluids                                         |
| SLN              | Solid lipid nanoparticles                                           |
| t <sub>1/2</sub> | Half-life of degradation                                            |

|           |                                      |
|-----------|--------------------------------------|
| UV        | Ultraviolet                          |
| w/o       | Water-in-oil                         |
| WAXS      | Wide-angle X-ray scattering patterns |
| XRD       | X-ray diffraction                    |
| $\theta$  | Scattering angle                     |
| $\lambda$ | Wavelength                           |
| $\pi$ -A  | Surface pressure – area              |

### En français

|                    |                                                 |
|--------------------|-------------------------------------------------|
| $\lambda$          | Longueur d'onde                                 |
| Cryo-MET           | Cryo - microscopie électronique en transmission |
| d                  | Distance réticulaire                            |
| DRX                | Diffraction des rayons X                        |
| H/E                | Huile dans l'eau                                |
| HHP                | Homogénéisation haute pression                  |
| MET                | Microscopie électronique en transmission        |
| q                  | Vecteur de diffusion                            |
| $T_{\text{onset}}$ | Température de début de transition              |
| $\Delta H$         | Enthalpie de transition                         |
| $\theta$           | Angle de réflexion                              |
| IV                 | Intraveineuse                                   |

## SOMMAIRE

|                                                                                      |           |
|--------------------------------------------------------------------------------------|-----------|
| <b>INTRODUCTION GÉNÉRALE .....</b>                                                   | <b>12</b> |
| <b>PARTIE BIBLIOGRAPHIQUE.....</b>                                                   | <b>16</b> |
| <b>FLAVONOÏDES ET QUERCÉTINE .....</b>                                               | <b>17</b> |
| I. Flavonoïdes : structure, propriétés physico-chimiques et activités biologiques... | 17        |
| 1. Introduction.....                                                                 | 17        |
| 2. Structure .....                                                                   | 18        |
| 3. Propriétés physico-chimiques.....                                                 | 20        |
| 3.1. Solubilité .....                                                                | 20        |
| 3.2. Instabilité.....                                                                | 21        |
| 4. Activités biologiques.....                                                        | 21        |
| 4.1. Propriétés antioxydantes .....                                                  | 21        |
| 4.2. Effets protecteurs vasculaires.....                                             | 24        |
| 4.3. Activité anticancéreuse .....                                                   | 25        |
| 4.4. Activité anti-inflammatoire .....                                               | 28        |
| 4.5. Autres effets biologiques.....                                                  | 29        |
| 5. Biodisponibilité et métabolisme .....                                             | 30        |
| II. Quercétine : le chef de file des flavonoïdes .....                               | 33        |
| 1. Généralités .....                                                                 | 33        |
| 2. Propriétés physico-chimiques.....                                                 | 36        |
| 2.1. Cristallinité et pseudo-polymorphisme .....                                     | 36        |
| 2.2. Stabilité et solubilité.....                                                    | 41        |
| 3. Nanoformulations encapsulant la quercétine.....                                   | 43        |
| Références .....                                                                     | 47        |
| <b>REVUE</b>                                                                         |           |
| Chemical and physical stability of labile APIs in nanodispersions .....              | 54        |
| Abstract.....                                                                        | 55        |
| 1. Introduction.....                                                                 | 57        |
| 2. General aspects .....                                                             | 58        |
| 2.1. Chemical and physical stabilities.....                                          | 58        |
| 2.2. Overview of the main nanocarriers.....                                          | 60        |

|                                                                                       |    |
|---------------------------------------------------------------------------------------|----|
| 3. General factors in optimizing the stability of APIs entrapped in nanocarriers..... | 64 |
| 3.1. Drug location .....                                                              | 64 |
| 3.2. Drug-loading capacity .....                                                      | 65 |
| 3.3. Stability of colloidal carriers.....                                             | 66 |
| 3.4. Dispersion medium and storage conditions .....                                   | 67 |
| 4. Chemical stability of labile APIs in nanodispersions .....                         | 68 |
| 4.1. Nanocarriers for improvement of shelf life.....                                  | 68 |
| 4.1.1. Hydrolysis .....                                                               | 68 |
| 4.1.2. Oxidation .....                                                                | 69 |
| 4.1.3. Photodegradation.....                                                          | 70 |
| 4.1.4. Thermal degradation.....                                                       | 71 |
| 4.2. Nanocarriers for increase of half life.....                                      | 72 |
| 4.2.1. pH sensibility .....                                                           | 73 |
| 4.2.2. Enzymatic degradation .....                                                    | 75 |
| 5. Additional stability issues relate to macromolecules.....                          | 76 |
| 6. Chemical and physical stabilities of labile APIs in nanocrystals.....              | 79 |
| 7. Conclusion.....                                                                    | 81 |
| References .....                                                                      | 82 |

## **PARTIE EXPÉRIMENTALE ..... 90**

### **PUBLICATION N°1**

|                                                                                                               |    |
|---------------------------------------------------------------------------------------------------------------|----|
| Nanodispersions stabilized by PEG diesters and phospholipids: towards compartmented lipid nanoparticles ..... | 91 |
| Abstract.....                                                                                                 | 92 |
| 1. Introduction.....                                                                                          | 94 |
| 2. Materials and Methods.....                                                                                 | 95 |
| 2.1. Materials .....                                                                                          | 95 |
| 2.2. Methods .....                                                                                            | 96 |
| 2.2.1. Preparation of nanodispersions by HPH process.....                                                     | 96 |
| 2.2.2. Particle size and zeta potential measurements .....                                                    | 96 |
| 2.2.3. X-ray diffraction .....                                                                                | 96 |
| 2.2.4. MTT assay.....                                                                                         | 97 |
| 2.2.5. HPLC analysis.....                                                                                     | 98 |

|                                                         |     |
|---------------------------------------------------------|-----|
| 2.2.6. Drug loading and encapsulation efficiency .....  | 98  |
| 2.2.7. Cryogenic transmission electron microscopy ..... | 99  |
| 2.2.8. Differential scanning calorimetry .....          | 99  |
| 3. Results and discussion .....                         | 100 |
| 3.1. Formulation studies.....                           | 100 |
| 3.2. SAXS-WAXS studies .....                            | 103 |
| 3.3. Cytotoxicity .....                                 | 105 |
| 3.4. Encapsulation studies.....                         | 106 |
| 3.5. Cryo-TEM .....                                     | 107 |
| 3.6. Gelucire® 50/13 – Phospholipon® 90G mixture .....  | 109 |
| 3.6.1. Anhydrous mixtures .....                         | 109 |
| 3.6.2. Hydrated mixtures.....                           | 114 |
| 4. Conclusion.....                                      | 118 |
| References .....                                        | 119 |

## PUBLICATION N°2

|                                                                                                                                 |     |
|---------------------------------------------------------------------------------------------------------------------------------|-----|
| Quercetin solubility and chemical stability: enhancement with polyoxylglyceride-based multi-compartmented nanodispersions ..... | 128 |
| Abstract.....                                                                                                                   | 129 |
| 1. Introduction.....                                                                                                            | 131 |
| 2. Materials and methods.....                                                                                                   | 132 |
| 2.1. Materials .....                                                                                                            | 132 |
| 2.2. HPLC analysis.....                                                                                                         | 133 |
| 2.3. Solubilisation capacity in solvents and aqueous media.....                                                                 | 133 |
| 2.4. Solubilisation capacity in excipients .....                                                                                | 134 |
| 2.5. Stability of quercetin in different media.....                                                                             | 134 |
| 2.6. Surface pressure measurements .....                                                                                        | 134 |
| 2.7. Preparation of nanodispersions by HPH process .....                                                                        | 135 |
| 2.8. Particle size and zeta potential analysis.....                                                                             | 135 |
| 2.9. Drug loading and encapsulation efficiency.....                                                                             | 135 |
| 2.10. Transmission electron microscopy and cryogenic transmission electron microscopy.....                                      | 136 |
| 2.11. <i>In vitro</i> release studies .....                                                                                     | 137 |

|                                                                                      |     |
|--------------------------------------------------------------------------------------|-----|
| 2.12. Long-term stability.....                                                       | 137 |
| 3. Results and discussion .....                                                      | 137 |
| 3.1. Solubility and stability of quercetin dissolved in different aqueous media .... | 137 |
| 3.2. Solubility of quercetin in excipients and organic solvents.....                 | 139 |
| 3.3. Physico-chemical properties of nanodispersions.....                             | 140 |
| 3.3.1. Size analysis, zeta potential measurements and physical stability .....       | 140 |
| 3.3.2. Drug payload .....                                                            | 141 |
| 3.3.3. Morphological studies .....                                                   | 142 |
| 3.4. <i>In vitro</i> release .....                                                   | 147 |
| 3.5. Long-term stability of quercetin-loaded nanodispersions .....                   | 149 |
| 4. Conclusion.....                                                                   | 150 |
| References .....                                                                     | 151 |

## PUBLICATION N°3

|                                                                                                                          |     |
|--------------------------------------------------------------------------------------------------------------------------|-----|
| Enrichment of quercetin-loaded nanodispersions with nanocrystals: preparation and physico-chemical characterization..... | 160 |
| Abstract.....                                                                                                            | 161 |
| 1. Introduction.....                                                                                                     | 163 |
| 2. Materials and methods.....                                                                                            | 165 |
| 2.1. Materials .....                                                                                                     | 165 |
| 2.2. HPLC analysis.....                                                                                                  | 165 |
| 2.3. Solubility in solvent and excipients .....                                                                          | 166 |
| 2.4. Preparation of quercetin nanocrystals by HPH process .....                                                          | 166 |
| 2.5. Lyophilisation.....                                                                                                 | 166 |
| 2.6. Differential scanning calorimetry.....                                                                              | 167 |
| 2.7. Powder X-ray diffraction.....                                                                                       | 167 |
| 2.8. X-ray diffraction of the nanocrystal suspensions .....                                                              | 167 |
| 2.9. Particle size and zeta potential measurements .....                                                                 | 168 |
| 2.10. Drug loading and encapsulation efficiency.....                                                                     | 168 |
| 2.11. Polarized light microscopy .....                                                                                   | 169 |
| 2.12. Dark field microscopy.....                                                                                         | 169 |
| 2.13. Transmission electron microscopy and Cryogenic transmission electron microscopy.....                               | 169 |

|                                                                    |            |
|--------------------------------------------------------------------|------------|
| 2.14. <i>In vitro</i> release studies .....                        | 169        |
| 2.15. Long-term stability .....                                    | 170        |
| 3. Results and discussion .....                                    | 170        |
| 3.1. Particle size, zeta potential and drug payload analysis ..... | 170        |
| 3.2. Particle morphology (TEM and Cryo-TEM) .....                  | 174        |
| 3.3. Crystallinity and polymorphism of quercetin .....             | 175        |
| 3.3.1. Shape observation by polarized-light microscopy .....       | 175        |
| 3.3.2. DSC analysis .....                                          | 176        |
| 3.3.3. XRD characterization .....                                  | 178        |
| 3.4. <i>In vitro</i> release of nanodispersions .....              | 182        |
| 3.5. Long-term stability of quercetin in nanodispersions .....     | 183        |
| 4. Conclusion .....                                                | 184        |
| References .....                                                   | 185        |
| <b>DISCUSSION GÉNÉRALE .....</b>                                   | <b>191</b> |
| <b>CONCLUSION GÉNÉRALE .....</b>                                   | <b>200</b> |
| <b>ANNEXE .....</b>                                                | <b>203</b> |
| 1. Homogénéisation haute pression .....                            | 204        |
| 2. Calorimétrie différentielle à balayage .....                    | 206        |
| 3. Diffractométrie des rayons X .....                              | 209        |
| 4. Cryo - microscopie électronique en transmission .....           | 215        |
| 5. Balance de Langmuir .....                                       | 221        |
| 6. Dosage de la quercétine par HPLC .....                          | 224        |
| 7. Excipients : les lipides et les tensioactifs .....              | 226        |
| 7.1. Triglycérides .....                                           | 226        |
| 7.2. Polyoxylglycérides .....                                      | 229        |
| 7.3. Phospholipides .....                                          | 231        |

# INTRODUCTION GÉNÉRALE

La recherche et le développement de nouvelles formes médicamenteuses issus des composés phyto-chimiques (substances actives naturelles d'origine végétale) suscitent un grand intérêt dans le domaine pharmaceutique en raison de leurs avantages potentiels pour la santé humaine (Li et al. 2009; Ajazuddin & Saraf 2010). Parmi ces composés intéressants, les antioxydants, tels que les flavonoïdes, ont été particulièrement étudiés à cause de leurs implications et effets bénéfiques dans la prévention et le traitement des maladies cardiovasculaires, des cancers, des maladies neurodégénératives (Parkinson, Alzheimer), ou d'autres pathologies liées au vieillissement (Harborne & Williams 2000; Valko et al. 2007).

Les flavonoïdes ont été découverts en 1938 par un scientifique hongrois, le Dr Albert Szent-Györgyi, impliqué également dans la découverte de la vitamine C (lauréat du prix Nobel de physiologie ou médecine en 1937). À l'origine, il donna aux flavonoïdes le nom de « vitamine P ». Cette dénomination fut abandonnée dans les années 1950 faute de preuves substantielles quant à leur caractère indispensable au bon fonctionnement de l'organisme, corollaire de la notion de « vitamine » (Kavuru 2008). A ce jour, les flavonoïdes sont encore en phase d'exploration. Par exemple, l'Union Européenne a financé à hauteur de 3.3 millions d'euros un projet intitulé FLORA en 2008 sur les bienfaits de ces molécules sur organisme humain. Le projet FLORA vise à mieux comprendre le lien entre régime alimentaire et santé, ainsi que les effets bénéfiques des flavonoïdes chez les humains (<http://cordis.europa.eu>). Ces molécules font actuellement l'objet de nombreuses études, du fondamental à l'appliqué, dans de multiples champs disciplinaires. Les thèmes concernés sont essentiellement (1) leur biosynthèse et synthèse chimique; (2) leur extraction, isolement et purification; (3) leur identification; (4) leur fonctionnalisation; (5) l'étude de leurs propriétés physico-chimiques; (6) des essais biologiques *in vitro* et *in vivo*; et (7) le développement de formes galéniques adaptées.

En pharmacie galénique, les flavonoïdes sont reconnus comme étant des molécules très fragiles *in vitro* et *in vivo*. Ainsi, afin d'améliorer leur efficacité *in vivo*, il apparaît nécessaire de développer des systèmes galéniques agissant comme des réservoirs de médicaments afin de protéger ces molécules contre la dégradation dans le temps de conservation, de prolonger le temps de résidence de la molécule active au site d'administration, et de contrôler leur libération dans le temps. Parmi les différentes stratégies existantes, nous avons choisi la vectorisation particulière, et plus précisément la mise au point de nanoparticules lipidiques permettant d'encapsuler des principes

actifs hydrophobes et fragiles. Les intérêts de la vectorisation d'un médicament sont nombreux. Il est possible de citer notamment (1) la protection du principe actif contre la dégradation; (2) l'amélioration de sa biodisponibilité; (3) la réduction des effets secondaires; et (4) le ciblage actif pour les vecteurs fonctionnalisés en surface (Couvreur 2012).

Parmi plus de 4000 flavonoïdes, les multiples activités biologiques de la quercétine en font l'un des « *leads* » dans le domaine comme le confirment les nombreuses publications référencées dans les différents moteurs de recherche : plus de 9000 citations dans PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>), plus de 21000 articles dans Sciencedirect (<http://www.sciencedirect.com>) et plus de 6000 articles dans Springerlink (<http://www.springerlink.com>) (chiffres de juillet 2012). Au-delà de ces propres propriétés biologiques, la quercétine représente un très bon modèle de principe actif peu soluble et instable chimiquement d'où le travail de thèse effectué autour de cette molécule.

La thèse se divisera en deux parties :

- Dans une première partie (bibliographique), nous allons présenter les flavonoïdes et la quercétine depuis leurs propriétés physico-chimiques jusqu'à leurs applications biologiques. Parallèlement, nous envisagerons la protection des principes actifs instables chimiquement et physiquement par l'encapsulation dans les vecteurs de médicaments (en anglais sous la forme d'un article de revue).
- La partie expérimentale de ce manuscrit sera découpée en trois grands chapitres, rédigés sous forme d'articles mais non soumis, un brevet étant en cours de dépôt. Le premier chapitre s'intéressera à la préparation et à la caractérisation de nanoparticules originales à base de polyoxylglycérides (Publication n°1). Le second chapitre décrira l'encapsulation de la quercétine dans ces nanoparticules (Publication n°2). Enfin, le dernier chapitre concernera la préparation et la caractérisation de nanocristaux permettant d'augmenter la teneur en molécule active au sein des suspensions colloïdales (Publication n°3). Entre chacun de ces chapitres, des transitions en français résumeront les principaux résultats tout en incorporant quelques données complémentaires. Les méthodes expérimentales et les matières premières principalement utilisées dans ce travail seront décrites en annexe, après la discussion générale et la conclusion.

## Références

1. Ajazuddin & Saraf, S., 2010. Applications of novel drug delivery system for herbal formulations. *Fitoterapia*, 81(7), pp.680–689.
2. Couvreur, P., 2012. Nanoparticles in drug delivery: Past, present and future. *Advanced drug delivery reviews*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22580334>.
3. Harborne, J.B. & Williams, C.A., 2000. Advances in flavonoid research since 1992. *Phytochemistry*, 55(6), pp.481–504.
4. Kavuru, P., 2008. Crystal engineering of flavonoids. *Graduate School Theses and Dissertations*. Available at: <http://scholarcommons.usf.edu/etd/325>.
5. Li, D.-C. et al., 2009. Application of targeted drug delivery system in Chinese medicine. *Journal of controlled release: official journal of the Controlled Release Society*, 138(2), pp.103–112.
6. Valko, M. et al., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*, 39(1), pp.44–84.
7. <http://cordis.europa.eu>
8. <http://www.ncbi.nlm.nih.gov/pubmed>
9. <http://www.sciencedirect.com>
10. <http://www.springerlink.com>

# PARTIE BIBLIOGRAPHIQUE

|                                                                                      | Page |
|--------------------------------------------------------------------------------------|------|
| 1 Flavonoïdes et quercetine                                                          | 17   |
| I. Flavonoïdes : structure, propriétés physico-chimiques<br>et activités biologiques | 17   |
| II. Quercétine : le chef de file des flavonoïdes                                     | 33   |
| 2 Chemical and physical stability of labile APIs in nanodispersions                  | 54   |

## FLAVONOÏDES ET QUERCETINE

### I. Flavonoïdes : structure, propriétés physico-chimiques et activités biologiques

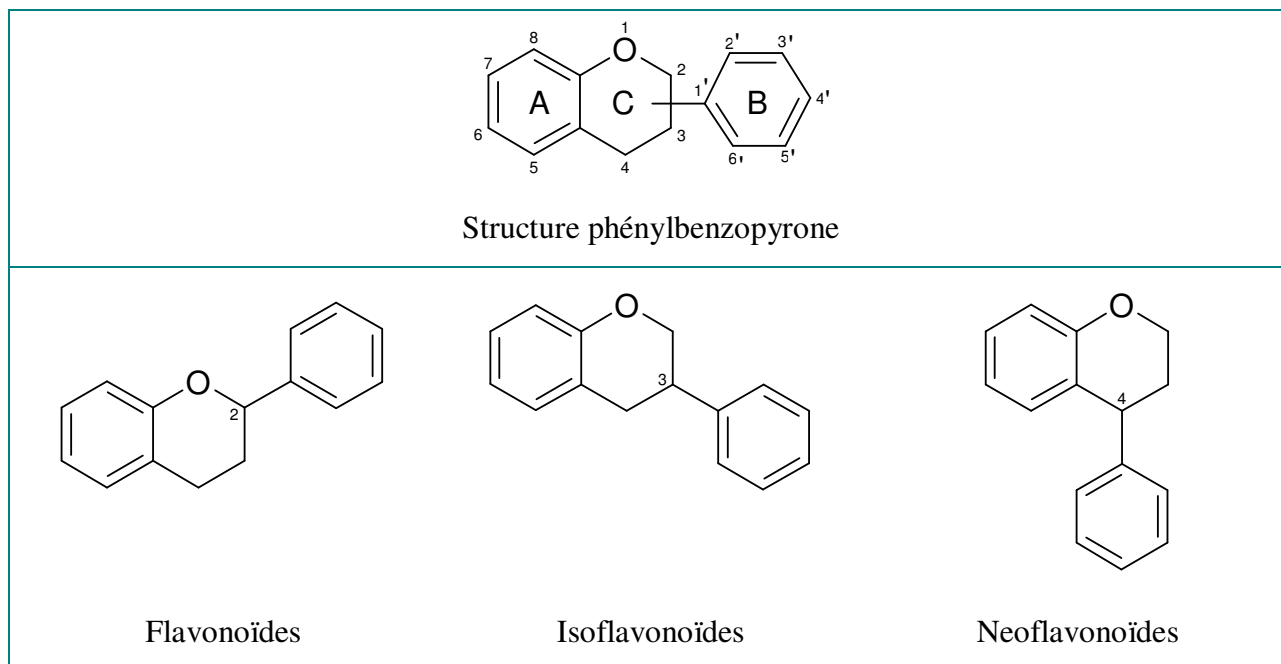
#### 1. Introduction

Les flavonoïdes sont un groupe de composés polyphénoliques largement distribués dans le règne végétal et sont consommés quotidiennement sous forme de fruits, légumes, noix, graines et boissons telles que le vin, le cacao et le thé ([Bouayed et al. 2008](#)). Ils sont capables de moduler l'activité de certaines enzymes et de modifier le comportement de plusieurs systèmes cellulaires, suggérant qu'ils pourraient exercer une multitude d'activités biologiques. De part ces effets potentiellement thérapeutiques, les flavonoïdes sont reconnus comme candidats intéressants pour être utilisés dans le traitement de plusieurs pathologies comme les cancers, les maladies cardiovasculaires, les maladies neurodégénératives (Parkinson, Alzheimer), ou d'autres affections liées au vieillissement. En outre, ils auraient aussi des effets antibactériens, antiviraux, anti-inflammatoires et anti-allergiques. Toutes ces activités biologiques sont associées aux propriétés antioxydantes, piègeurs de radicaux libres, et chélateurs de cations divalents des flavonoïdes ([Cook & Samman 1996](#); [Mendes et al. 2012](#))

Les flavonoïdes sont donc de plus en plus utilisés dans les domaines pharmaceutique ([Harborne & Williams 2000](#)), cosmétique ([Arct & Pytkowska 2008](#)) et agroalimentaire ([Marín et al. 2002](#)). De nombreuses stratégies thérapeutiques ont été testées chez l'animal et l'homme en vue de prévenir les maladies oxydatives ([Peterson & Dwyer 1998](#); [Harborne & Williams 2000](#)). Toutefois, leur utilisation se heurte à leur faible solubilité dans les milieux aqueux et à leur grande instabilité chimique ([Chebil 2006](#); [Anthoni 2007](#)). Deux voies sont actuellement principalement envisagées pour surmonter ces limitations: la fonctionnalisation des flavonoïdes et leur incorporation dans des formulations adaptées. Pour la fonctionnalisation, il est notamment possible de citer l'acylation, la glycosylation, la polymérisation ou l'hydroxylation ([Chebil 2006](#)). Au cours des dernières années, de nombreux nouveaux systèmes de délivrance de médicaments contenant des flavonoïdes purs ou en mélange ont été développés comme les microparticules, les micelles, les liposomes, les nanoparticules polymériques et les nanoparticules lipidiques ([Li et al. 2009](#); [Ajazuddin & Saraf 2010](#)).

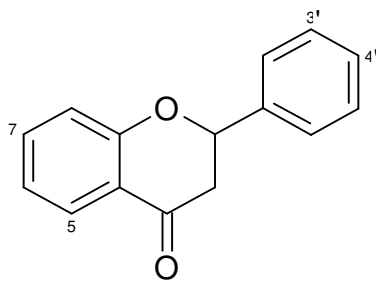
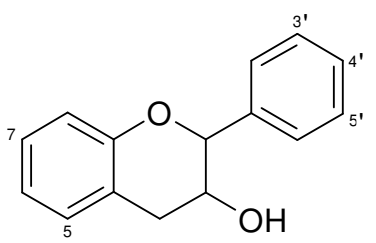
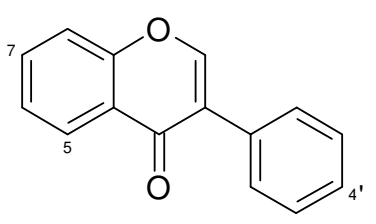
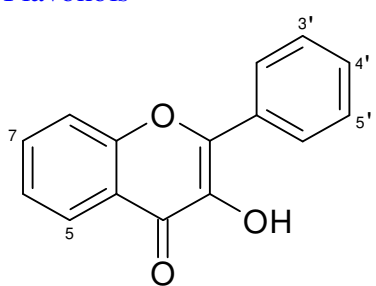
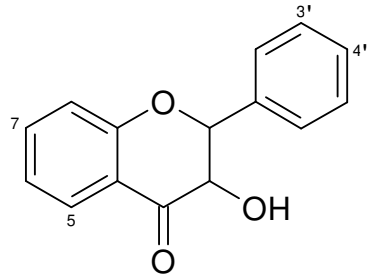
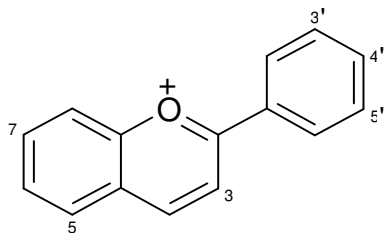
## 2. Structure

Le terme «flavonoïde» est généralement utilisé pour décrire une large collection de produits naturels (plus de 4000 composés identifiés) qui comprennent une même structure de base formée par deux cycles aromatiques en C<sub>6</sub> (A et B) reliés par une chaîne en C<sub>3</sub> souvent fermée en un hétérocycle oxygéné hexagonal (cycle C). Selon la position de la liaison du cycle aromatique sur le benzopyrane, ces substances peuvent être divisées en trois groupes: (1) flavonoïdes, (2) isoflavonoïdes et (3) néoflavonoïdes (Figure 1).



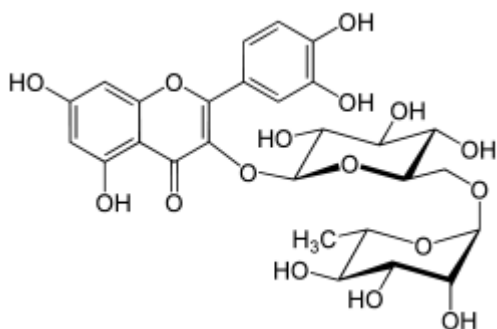
**Figure 1.** Squelettes des phénylbenzopyrones et des trois classes principales de flavonoïdes (d'après Marrais et al. 2006)

En fonction du degré d'oxydation et d'insaturation du cycle C, les flavonoïdes sont subdivisés en différentes classes : flavonols, flavones, flavanones, isoflavones, flavanols, anthocyanidines et dihydroflavonols (Marais et al. 2006; Mendes et al. 2012). Pour chaque classe, les molécules diffèrent entre elles par le nombre et la position des groupements – OH. Les flavonoïdes sont essentiellement hydroxylés en positions 5, 7, 4' et éventuellement en positions 3 et 3' (Figure 2). Ces groupes hydroxyles peuvent être alkylés.

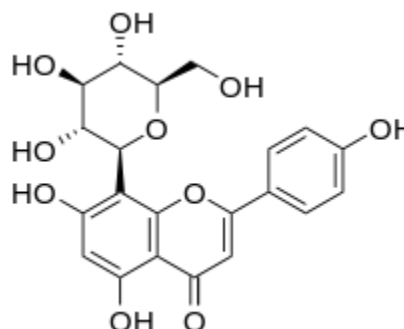
|                                                                                                                                                                                                                                                                                                                                                                                                                                             |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|----|----|-----|----|----|--------------|--|--|--|--|--|---|---|----|----|----|-------------|---------------|----|----|----|--|----------------|----|-----------|----|-----|----|------------|----|----|--------------|----|----|----|----|----|----|
| Flavanones                                                                                                                                                                                                                                                                                                                                                                                                                                  |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                            |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Citron                                                                                                                                                                                                                                                                                                                                                                                                                                      |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| <table><tr><td></td><td colspan="5">Substitution</td></tr><tr><td></td><td>5</td><td>7</td><td>3'</td><td>4'</td><td></td></tr><tr><td>Nagingénine</td><td>OH</td><td>OH</td><td></td><td>OH</td><td></td></tr><tr><td>Hesperitine</td><td>OH</td><td>OH</td><td>OH</td><td>OMe</td><td></td></tr></table>                                                                                                                                  |              |    |    |     |    |    | Substitution |  |  |  |  |  | 5 | 7 | 3' | 4' |    | Nagingénine | OH            | OH |    | OH |  | Hesperitine    | OH | OH        | OH | OMe |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                                                                                                             | Substitution |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                                                                                                             | 5            | 7  | 3' | 4'  |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Nagingénine                                                                                                                                                                                                                                                                                                                                                                                                                                 | OH           | OH |    | OH  |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Hesperitine                                                                                                                                                                                                                                                                                                                                                                                                                                 | OH           | OH | OH | OMe |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Flavanols                                                                                                                                                                                                                                                                                                                                                                                                                                   |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                           |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Thé<br>Pommes<br>Pêches                                                                                                                                                                                                                                                                                                                                                                                                                     |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
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|                                                                                                                                                                                                                                                                                                                                                                                                                                             | Substitution |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                                                                                                             | 5            | 7  | 3' | 4'  | 5' |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Catéchine                                                                                                                                                                                                                                                                                                                                                                                                                                   | OH           | OH | OH | OH  |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Gallocatechine                                                                                                                                                                                                                                                                                                                                                                                                                              | OH           | OH | OH | OH  | OH |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Isoflavones                                                                                                                                                                                                                                                                                                                                                                                                                                 |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                           |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Tofou<br>Lait de soja                                                                                                                                                                                                                                                                                                                                                                                                                       |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| <table><tr><td></td><td colspan="4">Substitution</td><td></td></tr><tr><td></td><td>5</td><td>7</td><td>4'</td><td></td><td></td></tr><tr><td>Daidzeine</td><td></td><td>OH</td><td>OH</td><td></td><td></td></tr><tr><td>Génisteine</td><td>OH</td><td>OH</td><td>OH</td><td></td><td></td></tr></table>                                                                                                                                   |              |    |    |     |    |    | Substitution |  |  |  |  |  | 5 | 7 | 4' |    |    | Daidzeine   |               | OH | OH |    |  | Génisteine     | OH | OH        | OH |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                                                                                                             | Substitution |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                                                                                                             | 5            | 7  | 4' |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Daidzeine                                                                                                                                                                                                                                                                                                                                                                                                                                   |              | OH | OH |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Génisteine                                                                                                                                                                                                                                                                                                                                                                                                                                  | OH           | OH | OH |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Flavonols                                                                                                                                                                                                                                                                                                                                                                                                                                   |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                          |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Brocoli<br>Ail<br>Oignon                                                                                                                                                                                                                                                                                                                                                                                                                    |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
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|                                                                                                                                                                                                                                                                                                                                                                                                                                             | Substitution |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                                                                                                             | 5            | 7  | 3' | 4'  | 5' |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Kaempférol                                                                                                                                                                                                                                                                                                                                                                                                                                  | OH           | OH |    | OH  |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Quercétine                                                                                                                                                                                                                                                                                                                                                                                                                                  | OH           | OH | OH | OH  |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Myricétine                                                                                                                                                                                                                                                                                                                                                                                                                                  | OH           | OH | OH | OH  | OH |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Dihydroflavonols                                                                                                                                                                                                                                                                                                                                                                                                                            |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                         |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Fusétine<br>Taxifoline                                                                                                                                                                                                                                                                                                                                                                                                                      |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
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|                                                                                                                                                                                                                                                                                                                                                                                                                                             | Substitution |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                                                                                                             | 5            | 7  | 3' | 4'  |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Fusétine                                                                                                                                                                                                                                                                                                                                                                                                                                    |              | OH | OH | OH  |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Taxifoline                                                                                                                                                                                                                                                                                                                                                                                                                                  | OH           | OH | OH | OH  |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Anthocyanes                                                                                                                                                                                                                                                                                                                                                                                                                                 |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                          |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Vin rouge<br>Myrtilles<br>Cerises                                                                                                                                                                                                                                                                                                                                                                                                           |              |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| <table><tr><td></td><td colspan="5">Substitution</td></tr><tr><td></td><td>3</td><td>5</td><td>7</td><td>3'</td><td>4'</td><td>5'</td></tr><tr><td>Pelargonidine</td><td>OH</td><td>OH</td><td>OH</td><td></td><td>OH</td><td></td></tr><tr><td>Cyanidine</td><td>OH</td><td>OH</td><td>OH</td><td>OH</td><td>OH</td><td></td></tr><tr><td>Delphinidine</td><td>OH</td><td>OH</td><td>OH</td><td>OH</td><td>OH</td><td>OH</td></tr></table> |              |    |    |     |    |    | Substitution |  |  |  |  |  | 3 | 5 | 7  | 3' | 4' | 5'          | Pelargonidine | OH | OH | OH |  | OH             |    | Cyanidine | OH | OH  | OH | OH         | OH |    | Delphinidine | OH | OH | OH | OH | OH | OH |
|                                                                                                                                                                                                                                                                                                                                                                                                                                             | Substitution |    |    |     |    |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
|                                                                                                                                                                                                                                                                                                                                                                                                                                             | 3            | 5  | 7  | 3'  | 4' | 5' |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Pelargonidine                                                                                                                                                                                                                                                                                                                                                                                                                               | OH           | OH | OH |     | OH |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Cyanidine                                                                                                                                                                                                                                                                                                                                                                                                                                   | OH           | OH | OH | OH  | OH |    |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |
| Delphinidine                                                                                                                                                                                                                                                                                                                                                                                                                                | OH           | OH | OH | OH  | OH | OH |              |  |  |  |  |  |   |   |    |    |    |             |               |    |    |    |  |                |    |           |    |     |    |            |    |    |              |    |    |    |    |    |    |

**Figure 2.** Structures générales des flavonoïdes les plus abondants  
(d'après Cook & Samman 1996; Peterson & Dwyer 1998; Hodek et al. 2002; Ramos 2007)

Dans la nature, les flavonoïdes existent fréquemment sous forme d'hétérosides, par exemple, la rutine, la diosmine, l'hespéridine, la naringine, la piine, la génistine, la daidzine ou la vitexine. Ils sont constitués par un ou plusieurs oses et une partie non glucidique appelée « génine » ou « aglycone ». Le glucose, le rhamnose, le glucorhamnose, le galactose et l'arabinose sont les oses les plus couramment rencontrés. Selon les modes de liaisons entre l'aglycone et l'ose, on distingue les O-glycosides (la liaison entre l'ose et l'aglycone est du type C–O–C) et les C-glycosides (la liaison s'établit entre un carbone de l'ose et un carbone de l'aglycone) (Figure 3) (Kavuru 2008).



Rutine (quercétine-3-O-rutinoside)



Vitexine (apigénine-8-C-glucoside)

**Figure 3.** Exemples de flavonoïdes de type O-glycoside et C-glycoside

### 3. Propriétés physico-chimiques

#### 3.1. Solubilité

En fonction de la structure du flavonoïde, différentes interactions favorables à la solubilisation peuvent se développer en présence d'un solvant: (1) des interactions hydrophobes avec les solvants apolaires concernant les cycles aromatiques (A et B) et les substituants carbonés aliphatiques; (2) des interactions dipolaires entre les solvants polaires et les groupes fonctionnels des flavonoïdes (carbonyle, éther, ester, hydroxyle); (3) des liaisons hydrogènes entre le solvant (eau, alcool, amine) et les divers groupes donneurs ou accepteurs de ce type de liaison présent sur le flavonoïde (Chebil 2006; Anthoni 2007). Toutefois, il peut exister des interactions intramoléculaires parmi les groupes hydroxyles et carboxyliques qui limitent la solubilité dans l'eau.

En général, les flavonoïdes envisagés dans le domaine pharmaceutique sont très peu solubles dans l'eau. Grâce à la partie osidique hydrophile, les hétérosides sont plus

solubles que leurs génines. La solubilité d'un flavonoïde est fréquemment dépendante du pH, de la température, de la nature du solvant. Par exemple, la solubilité de la rutine en fonction du pH est respectivement de l'ordre de 26 µg/ml, 45 µg/ml et 128 µg/ml dans le suc gastrique artificiel (pH 1,2), l'eau (pH 5,6) et le suc intestinal artificiel (pH 6,8) à la température ambiante (Lauro et al. 2002). Elle est peu soluble dans l'eau mais facilement soluble dans le propanol, l'éthanol et le méthanol. Sa solubilité dans ces solvants augmente fortement lorsque la température augmente (Zi et al. 2007).

### 3.2. Instabilité

Les flavonoïdes sont des molécules globalement très réactives. Tant durant leur extraction ou leur purification que pendant leur stockage, ils sont sensibles à de nombreux facteurs environnants. Il est notamment possible de citer l'oxygène, l'humidité, la température, la lumière, la nature du solvant, le pH et la force ionique de la solution, la présence d'ion métallique. Ces facteurs peuvent affecter fortement l'autoxydation des flavonoïdes. Par exemple, si le gallate d'épicatéchine, la (+)-catéchine, l'épicatéchine, le gallate d'épigallocatechine et l'épigallocatechine sont très instable en solution aqueuse, la vitesse d'oxydation de ces cinq flavonoïdes à pH 4,5 sera significativement plus rapide qu'à pH 3,5 (Lin et al. 2008). Dans un autre travail, la stabilité de 11 flavonoïdes comprenant des flavonols (le kaempférol, la myricétine, la rhamnetine, la quercétine et la rutine), des flavanones (la naringénine, la naringine, l'hespéredine), des flavones (l'apigénine, la lutéoline) et isoflavones (la génistéine) a été étudiée lors de leur extraction par quatre procédés différents. Les résultats montrent que la stabilité des flavonoïdes dépend essentiellement de leur structure chimique. En effet, parmi les grandes tendances, un grand nombre de groupements hydroxyles favorise la dégradation oxydative, tandis que la partie osidique et les groupements alkyles protègent contre cette dégradation. En outre, la présence d'un groupement hydroxyle libre en position 3 est particulièrement défavorable pour la stabilité d'un flavonoïde (Biesaga 2011).

## 4. Activités biologiques

### 4.1. Propriétés antioxydantes

Depuis plus d'une décennie, le concept de «stress oxydant», c'est-à-dire une situation où la cellule ne contrôle plus la présence excessive de radicaux oxygénés toxiques,

s'est largement développé et semble impliqué dans de nombreuses maladies humaines. En cas de déséquilibre entre les sources de pro-oxydants (radicaux libres, [Tableau 1](#)) et des systèmes antioxydants dans le corps se produit le stress oxydant qui peut endommager les macromolécules intracellulaires, oxydant les lipides, les protéines ou les acides nucléiques ([Heim et al. 2002](#)). Les dysfonctionnements cellulaires couplés à ces dommages biochimiques entraînent différents niveaux de stress: l'excès dans la prolifération cellulaire, la mort cellulaire par apoptose, la mutagenèse et le dépôt de lipides oxydés. En créant ces troubles, le stress oxydant est impliqué dans de nombreuses pathologies liées au vieillissement comme les cancers, les troubles cardio-vasculaires ou les maladies neurodégénératives ([Favier 2003](#)). Pour lutter contre les radicaux libres en excès, hors des systèmes endogènes de défense (les enzymes antioxydants, le glutathion, l'acide urique, l'acide lipoïque, le coenzyme Q10, la mélatonine, la bilirubine et l'albumine), l'organisme a besoin d'antioxydants exogènes comme les vitamines C et E, les caroténoïdes et les polyphénols, en particulier les flavonoïdes ([Favier 2003](#); [Valko et al. 2007](#)).

**Tableau 1.** Les radicaux libres : symbole, nom, concentration et temps de demi-vie dans les tissus (d'après [Pryor 1986](#); [Fontaine 2007](#))

| Symbole                     | Nom                  | Concentration             | Demi-vie (37°C) |
|-----------------------------|----------------------|---------------------------|-----------------|
| $\cdot\text{O}_2^-$         | Radical superoxyde   | $10^{-12}$ à $10^{-11}$ M | Enzymatique*    |
| $\cdot\text{OH}$            | Radical hydroxyle    | -                         | $10^{-9}$ s     |
| $\cdot\text{NO}$            | Monoxyde d'azote     | -                         | 1 à 10 s        |
| $\cdot\text{H}_2\text{O}_2$ | Peroxyde d'hydrogène | $10^{-9}$ à $10^{-7}$ M   | Enzymatique*    |
| $\cdot\text{ONOO}^-$        | Peroxynitrite        | $10^{-9}$ à $10^{-7}$ M   | 0,05 à 1 s      |

\* Les demi-vies du radical superoxyde et du peroxyde d'hydrogène varient en fonction de l'activité enzymatique des enzymes assurant leur métabolisme.

Les flavonoïdes sont des antioxydants et des piègeurs de radicaux libres parmi les plus efficaces ([Tableau 2](#)). La capacité d'un flavonoïde pour inhiber les radicaux libres est gouvernée par sa structure chimique. Les éléments structuraux responsables de l'activité antioxydante sont: (1) le groupement 3',4'-catéchol dans le cycle B, (2) la double liaison C2-3, et (3) le groupement hydroxyle en C3 avec (4) le groupement 4-oxo ([Cook & Samman 1996](#); [Heim et al. 2002](#); [Seyoum et al. 2006](#)). La glycosylation ou la méthylation de l'hydroxyle en position 3 des flavonols conduit à une diminution

importante de l'activité antioxydante. Les flavonoïdes qui ont un groupe 3-OH tels que la fisétine, la (+)-catéchine, la quercétine, la myricétine, et la morine sont des inhibiteurs efficaces de la peroxydation lipidique par rapport à ceux qui n'ont pas de substitution 3-OH tels que la diosmétine, l'apigénine, l'hespéridine, et la naringénine (Das & Ratty 1986). En raison de l'absence d'un carbonyle en C-4, le piégeage des radicaux libres par la catéchine est plus faible que celui de la quercétine. Les flavonoïdes sous forme d'aglycones ont fréquemment une activité antioxydante plus forte que celle des flavonoïdes sous forme d'hétérosides. En outre, l'activité antioxydante de nombreux flavonoïdes est plus élevée que celle des vitamines C et E (Tableau 2) (Ishige et al. 2001; Marín et al. 2002).

**Tableau 2.** Activité antioxydante, exprimée en équivalent Trolox (TEAC: Trolox equivalent antioxidant capacity), de quelques flavonoïdes comparativement aux vitamines C et E (d'après Ishige et al. 2001; Marín et al. 2002). Plus la concentration indiquée est importante, plus l'activité antioxydante est forte.

| Composé                          | Activité antioxydante (mM) | Composé     | Activité antioxydante (mM) |
|----------------------------------|----------------------------|-------------|----------------------------|
| Vitamine C                       | 1,0                        | Cyanidine   | 3,6 – 4,4                  |
| Vitamine E                       | 1,0 – 1,1                  | Delphidine  | 4,4                        |
| Quercétine                       | 4,7 – 4,8                  | Apigénine   | 1,5 – 2,8                  |
| Kaempférol                       | 1,3 – 1,5                  | Diosmine    | 1,2                        |
| Rutine                           | 2,4 – 2,7                  | Lutéoline   | 2,1 – 2,5                  |
| Catéchine                        | 3,4 – 3,8                  | Naringénine | 1,5 – 2,5                  |
| Epigallocatechine gallate (EGCG) | 4,8                        | Hespérétine | 1,4                        |

Plusieurs mécanismes d'action de l'activité antioxydante des flavonoïdes ont été proposés:

- (1) le piégeage direct des radicaux libres : flavonoïde (OH) + R<sup>•</sup> → flavonoïde (O<sup>•</sup>) + RH;
- (2) la chélation des ions métalliques (notamment Fe<sup>3+</sup> et Cu<sup>+</sup>);
- (3) l'inhibition d'oxydases;
- (4) l'activation des enzymes antioxydantes;
- (5) la régénération des antioxydants liés aux membranes (réduction des radicaux de l'alpha-tocophérol par exemple) (Heim et al. 2002).

## 4.2. Effets protecteurs vasculaires

Les espèces réactives de l'oxygène (ROS : reactive oxygen species) et de l'azote (RNS : reactive nitrogen species) sont bien connues pour jouer un double rôle, à la fois nuisible et bénéfique, dans l'organisme. Les ROS et RNS sont normalement générées par des enzymes finement régulées, tels que la NO synthase et les isoformes des NADPH oxydases. Toutefois, la surproduction des ROS et RNS peut entraîner la peroxydation lipidique et la production de peroxynitrite qui vont contribuer aux maladies cardio-vasculaires. La peroxydation lipidique entraîne l'oxydation des acides gras polyinsaturés et l'oxydation des lipoprotéines de faible densité (LDL: low-density lipoprotein) ainsi qu'une déstabilisation des membranes. Le peroxynitrite, un oxydant généré par la réaction de l'oxyde nitrique (NO) avec le superoxyde dans l'endothélium vasculaire, induit l'oxydation des LDL et la dysfonction myocardique liée aux cytokines pro-inflammatoires. De plus, la nitrotyrosine, un produit de nitration des protéines par les RNS, est présente dans les lésions athérosclérotiques humaines ([Heim et al. 2002](#); [Valko et al. 2007](#)).

Grâce à leurs propriétés antioxydantes et les capacités de piégeage des ROS et RNS, les flavonoïdes ont démontré des effets cardioprotecteurs particuliers. L'ingestion de flavonoïdes d'origine alimentaire a été associée à une réduction très importante de la mortalité liée aux maladies cardio-vasculaires comme les maladies coronariennes, l'infarctus du myocarde et la maladie ischémique cardiaque ([Hertog et al. 1993](#)). Les flavonoïdes comme la rutine ou la quercétine agissent sur les vaisseaux sanguins en maintenant une perméabilité vasculaire normale. En outre, ils sont aussi responsables d'une augmentation de la résistance des capillaires en rapport avec leurs effets sur les plaquettes, les leucocytes et sur les enzymes intervenant dans la coagulation sanguine ([Gryglewski et al. 1987](#)). Les interactions entre les vaisseaux sanguins et les plaquettes sont impliquées dans le développement de la thrombose et de l'athérosclérose. Les flavonoïdes inhibent particulièrement l'agrégation et l'adhésion plaquettaire réduisant les tendances thrombotiques ([Gryglewski et al. 1987](#); [Tzeng et al. 1991](#)). La relation entre la consommation de flavonoïdes alimentaires et le risque de maladie coronarienne a été étudiée chez 805 hommes âgés de 65 à 84 ans. Ces études épidémiologiques montrent une corrélation inverse entre la consommation de flavonoïdes alimentaires et la mortalité par la maladie coronarienne qui est expliquée par l'inhibition de l'oxydation des LDL et la réduction de l'agrégation plaquettaire ([Hertog et al. 1993](#)). Le vin rouge et le thé sont de

riches sources de flavonoïdes. Ainsi, la consommation régulière de vin rouge à dose raisonnable (un ou deux verres par jour) et de thé préviendrait le développement des maladies cardio-vasculaires expliquant le “paradoxe français”. En outre, les préparations à base de flavonoïdes ont été largement utilisées dans la pratique médicale pour traiter les troubles de la circulation périphérique ([Jaeger et al. 1988](#)).

### 4.3. Activité anticancéreuse

A des concentrations élevées, les ROS peuvent être des médiateurs importants de dommages aux constituants élémentaires des structures cellulaires : les acides nucléiques, les lipides et les protéines. Le radical hydroxyle est connu pour réagir avec la molécule d'ADN, en endommageant le squelette du désoxyribose, des bases puriques et pyrimidiques. La modification permanente du matériel génétique pouvant résulter de stress oxydant représente la première étape impliquée dans la mutagenèse, la cancérogenèse, et le vieillissement ([Valko et al. 2007](#)). En piégeant les ROS, les flavonoïdes ont montré un intérêt dans la chimioprévention du cancer chez l'animal.

Par ailleurs, ils peuvent interagir avec le processus fondamental de carcinogenèse à plusieurs niveaux (initiation, promotion et progression) ([Tableau 3](#)) ([Galati & O'Brien 2004](#); [Ramos 2007](#)). De façon intéressante, la catéchine a montré une activité antiproliférative significative sur différents types de cancers comme certaines leucémies, ou des cancers gastriques, prostatiques ou pulmonaires ([Ramos 2007](#)). De plus, une certaine spécificité de ce même composé a été mise en évidence par Chung et al ([Chung et al. 2003](#)). En effet, dans les kératinocytes normaux, un effet prolifératif a été observé à 0,5 µmol/L alors qu'une même concentration conduit à l'apoptose des cellules de carcinomes squameux. Enfin, un fort effet dose a été fréquemment constaté pour les flavonoïdes avec des concentrations cytoprotectrices 5 à 10 fois inférieures aux concentrations pro-apoptotiques ou susceptibles de générer des dommages à l'ADN cellulaire ([Wätjen et al. 2005](#)). Plusieurs études épidémiologiques ont été menées à grande échelle pour évaluer le potentiel effectif des flavonoïdes alimentaires comme agents anticancéreux *in-vivo*. Le [Tableau 4](#) résume ces études chez l'homme et suggère une grande contradiction dans les bénéfices de l'utilisation de ces polyphénols dans le domaine du cancer.

**Tableau 3.** Effets moléculaires des flavonoïdes sur les différents stades du processus de cancérogenèse (initiation, promotion et progression) (d'après Ramos et al. 2007).

| Stade / Flavonoïde                     | Effets biologiques                                                             | Système d'étude                |
|----------------------------------------|--------------------------------------------------------------------------------|--------------------------------|
| <b>Initiation</b>                      |                                                                                |                                |
| Quercétine                             | Diminution de la 8-oxo-dG                                                      | HepG2                          |
| Génistéine                             | Augmentation de l'activité des CYP                                             | HepG2                          |
| Myricétine                             | Diminution de l'oxydation de l'ADN ;<br>Inhibition de la topoisomérase II      | Hépatocytes de<br>rat          |
| Daidzéine                              | Inhibition de la topoisomérase II                                              | V79 (poumon, le<br>hamster)    |
| <b>Promotion</b>                       |                                                                                |                                |
| Quercétine                             | Arrêt du cycle cellulaire (G <sub>2</sub> /M, G <sub>0</sub> /G <sub>1</sub> ) | CNE2                           |
|                                        | Augmentation des caspases -3, -7 et de Bax                                     | Neurones (culture<br>primaire) |
|                                        | Diminution de l'AKT et ERK1/2                                                  |                                |
|                                        | Arrêt du cycle cellulaire (G <sub>1</sub> )                                    | Thymocytes de<br>souris        |
|                                        | Induction de l'apoptose                                                        |                                |
| Thé                                    | Arrêt du cycle cellulaire (G <sub>2</sub> /M)                                  | LNCaP, DU145                   |
| Épigallocatechine-3-<br>gallate (EGCG) | Arrêt du cycle cellulaire                                                      | YCU-H891,                      |
|                                        | Augmentation de la caspase-9 et de Bax                                         | YCU-H861                       |
|                                        | Diminution de Bcl-2, Bcl-xL, p-EGFR, p-STAT-3 et<br>p-ERK                      |                                |
|                                        | Augmentation de Fas, Bax et p53                                                | HepG2                          |
|                                        | Diminution de PI-3K and AKT                                                    | LNCaP                          |
|                                        | Augmentation de ERK1/2                                                         |                                |
|                                        | Arrêt du cycle cellulaire                                                      | HT-29                          |
|                                        | Induction de l'apoptose                                                        |                                |
|                                        | Diminution de p-EGFR, p-HER2, p-AKT et p-ERK                                   |                                |
|                                        | Augmentation des caspases-3/7, -8/10 et -9                                     | H4IIE                          |
| Lutéoline                              | Augmentation des caspases-3, -8, et-7 et de PARP                               | HLF                            |
|                                        | Diminution de Bcl-xL                                                           |                                |
|                                        | Augmentation de la translocation Bax/Bak et JNK                                | HepG2                          |

| Stade / Flavonoïde    | Effets biologiques                                                          | Système d'étude |
|-----------------------|-----------------------------------------------------------------------------|-----------------|
| Apigénine             | Arrêt du cycle cellulaire (G <sub>2</sub> /M)                               | MCF-7           |
|                       | Augmentation de libération du cytochrome c, et des caspases-3 et -9         | HL-60           |
|                       | Augmentation des caspases-3, -8, -7 et de PARP                              | HLF             |
| Génistéine            | Augmentation de p38; Diminution de ERK1/2                                   | MCF-10F         |
|                       | Diminution de AKT, NF- $\kappa$ B et p-EGFR                                 | MDA-MB-231      |
|                       | Diminution de Bcl-xL, AKT et EGFR                                           | A549            |
| Tectorigénine         | Arrêt du cycle cellulaire, Échelonnement de l'ADN                           | HL-60           |
|                       | Diminution de Bcl-2 et p-EGFR                                               |                 |
| <b>Progression</b>    |                                                                             |                 |
| Quercétine            | Diminution de FAK, MMP-2 et MMP-9                                           | MiaPaCa2        |
|                       | Blocage de la signalisation de l'EGFR                                       |                 |
|                       | Diminution du VEGF et EGF                                                   |                 |
| Pomegranate           | Diminution du VEGF                                                          | MCF-7           |
|                       | Augmentation de MIF                                                         | MCF-10F         |
| Fraise                | Diminution du VEGF et NF- $\kappa$ B                                        | HaCat           |
| Catechin, epicatechin | Augmentation de mRNA, t-PA et u-PA; Diminution de l'activité fibrinolytique | HUVEC           |
| Thé vert (EGCG)       | Diminution du VEGF                                                          | NHK             |
|                       | Diminution de migration, MMP-2 et MMP-9                                     |                 |
|                       | Diminution de migration, MMP-2 et MMP-9                                     | U87             |
| Lutéoline             | Diminution de FAK, MMP-2 et MMP-9                                           | MiaPaCa2        |
|                       | Diminution du VEGF et STAT-3                                                | HLF             |
|                       | Blocage de la signalisation de l'EGFR                                       |                 |

**Tableau 4.** Bilan, contradictoire, des études épidémiologiques sur l'association entre les flavonoïdes (ou les aliments riches en composés phénoliques) et le cancer (d'après Ramos et al. 2007).

| Composants                          | Effet                                                                                        | Panel                        |
|-------------------------------------|----------------------------------------------------------------------------------------------|------------------------------|
| Flavonoïdes                         | Diminution globale du risque de cancer                                                       | 9959 hommes                  |
|                                     | Diminution du risque de cancer dans la cavité buccale, le pharynx, le larynx et l'œsophage   | 540 personnes                |
|                                     | Pas de réduction du risque de cancer de la vessie                                            | 497 personnes                |
|                                     | Pas de réduction du risque de l'incidence du de cancer                                       | 728 hommes                   |
|                                     | Pas de réduction du risque de cancer du poumon                                               | 103 femmes                   |
| Quercétine, oignons, raisins blancs | Diminution de récurrence du cancer du poumon                                                 | 582 personnes                |
| Quercétine                          | Diminution de l'incidence du cancer du poumon                                                | 10054 hommes                 |
| Myricétine                          | Diminution du risque de cancer de la prostate                                                |                              |
| Quercétine, kaempférol              | Diminution du risque de cancer de l'estomac                                                  | 354 personnes                |
| Catéchines                          | Diminution de l'incidence du cancer du rectum                                                | 34651 femmes                 |
| Thé                                 | Diminution du risque de cancer du côlon                                                      | 12170 personnes              |
| Thé vert                            | Réduction des risques de cancer dans les différents organes                                  | 8552 personnes               |
|                                     | Diminution du risque de récurrence du cancer du sein et des métastases                       | 472 personnes                |
| Thé noir                            | Aucune association avec le cancer gastrique                                                  | 11902 hommes et 14409 femmes |
|                                     | Aucune association avec les risques de cancer colorectal, de l'estomac, du poumon et du sein | 58279 hommes et 62573 femmes |
| Soja                                | Diminution du risque de cancer du poumon                                                     | 999 hommes                   |
|                                     | Diminution du risque de cancer du sein                                                       | 34759 femmes                 |

#### 4.4. Activité anti-inflammatoire

Plusieurs flavonoïdes sont capables d'inhiber les enzymes cyclo-oxygénase et/ou 5-lipoxygénase du métabolisme de l'acide arachidonique plaquettaire. Le kaempférol, la myricétine, l'artémétine, la quercétine, la cyanidine, l'apigénine, l'hespéridine et leurs dérivés ont montré une bonne activité anti-inflammatoire (Harborne & Williams 2000;

Ghedira 2005). En fait, la consommation de cerises étant traditionnellement signalée pour soulager la douleur arthritique et la goutte. L'efficacité anti-inflammatoire des anthocyanes de ce fruit a donc été testée. La cyanidine a montré une activité inhibitrice significative vis-à-vis des deux enzymes citées précédemment (Wang et al. 1999). L'héspéridine, administrée par voie sous-cutanée (car inactive *per os*), présente une activité anti-inflammatoire significative chez le rat dont l'œdème a été induit aussi bien par la carragénine que par le dextran (Galati et al. 1994). Le kaempférol, dans l'huile de croton, a également montré une bonne activité dans le traitement des dermatites (Harborne & Williams 2000). Enfin, deux glycosides de flavonols, la quercétine 3-xylosyle (1→2) - rhamnoside et la quercétine 3-rhamnoside, possèdent une activité contre l'inflammation aiguë chez la souris (Harborne & Williams 2000).

#### 4.5. Autres effets biologiques

Des propriétés antibactériennes et antivirales des flavonoïdes vis-à-vis de différentes souches bactériennes ont été mises en évidence (Ghedira 2005), notamment une activité antibactérienne directe, une synergie avec des antibiotiques et/ou une diminution de la virulence bactérienne. Parmi les flavonoïdes les plus puissamment antibactériens, il est possible de mentionner : la panduratine A, l'isobavachalcone, la scandenone, le kaempférol, la licochalcone A, la sepicanine A, l'isolupalbigenine, la flavone, le 3'-O-methyldilacol (Cushnie & Lamb 2011). Les flavonoïdes affectent la réplication intracellulaire des virus tels que le virus respiratoire syncytial (VRS), l'herpès simplex virus (HSV) et les adénovirus (Gonçalves et al. 2001).

Les groupes de flavonoïdes principalement connus pour posséder des activités oestrogéniques sont les isoflavones, comme la génistéine. Pour les femmes ménopausées asymptomatiques qui consomment des légumes tels que le soja et les légumineuses, possédant des teneurs élevées en isoflavonoïdes, les effets oestrogéniques ont été montrés comme significatifs sur la maturation vaginale et la concentration de l'hormone stimulatrice des follicules.

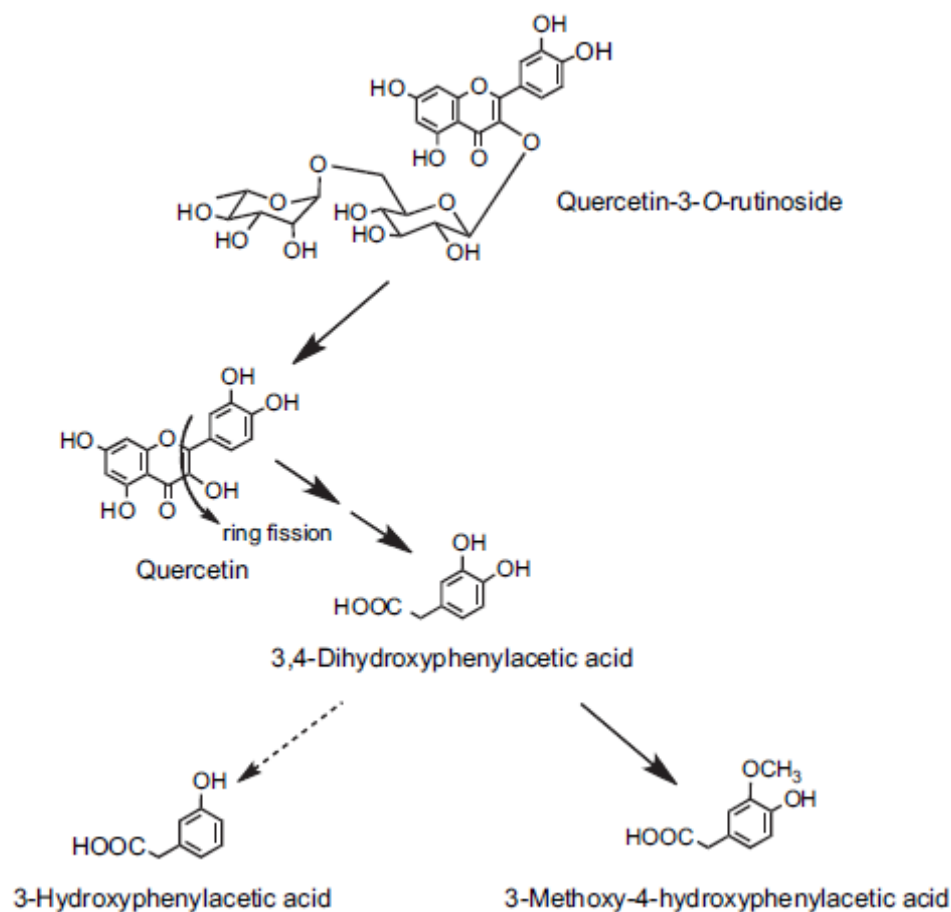
En outre, les flavonoïdes pourraient prévenir la cataracte diabétique par inhibition de l'aldose réductase du cristallin (Chaudhry et al. 1983), intervenir positivement sur certaines maladies auto-immunes (Damre et al. 2003; Kuo et al. 2004; Wang et al. 2004) et neurodégénératives (Valko et al. 2007). Enfin, la myricétine présente des effets hypoglycémisants et hypotriglycéridémisants chez les animaux diabétiques (Ong & Khoo 2000).

## 5. Biodisponibilité et métabolisme

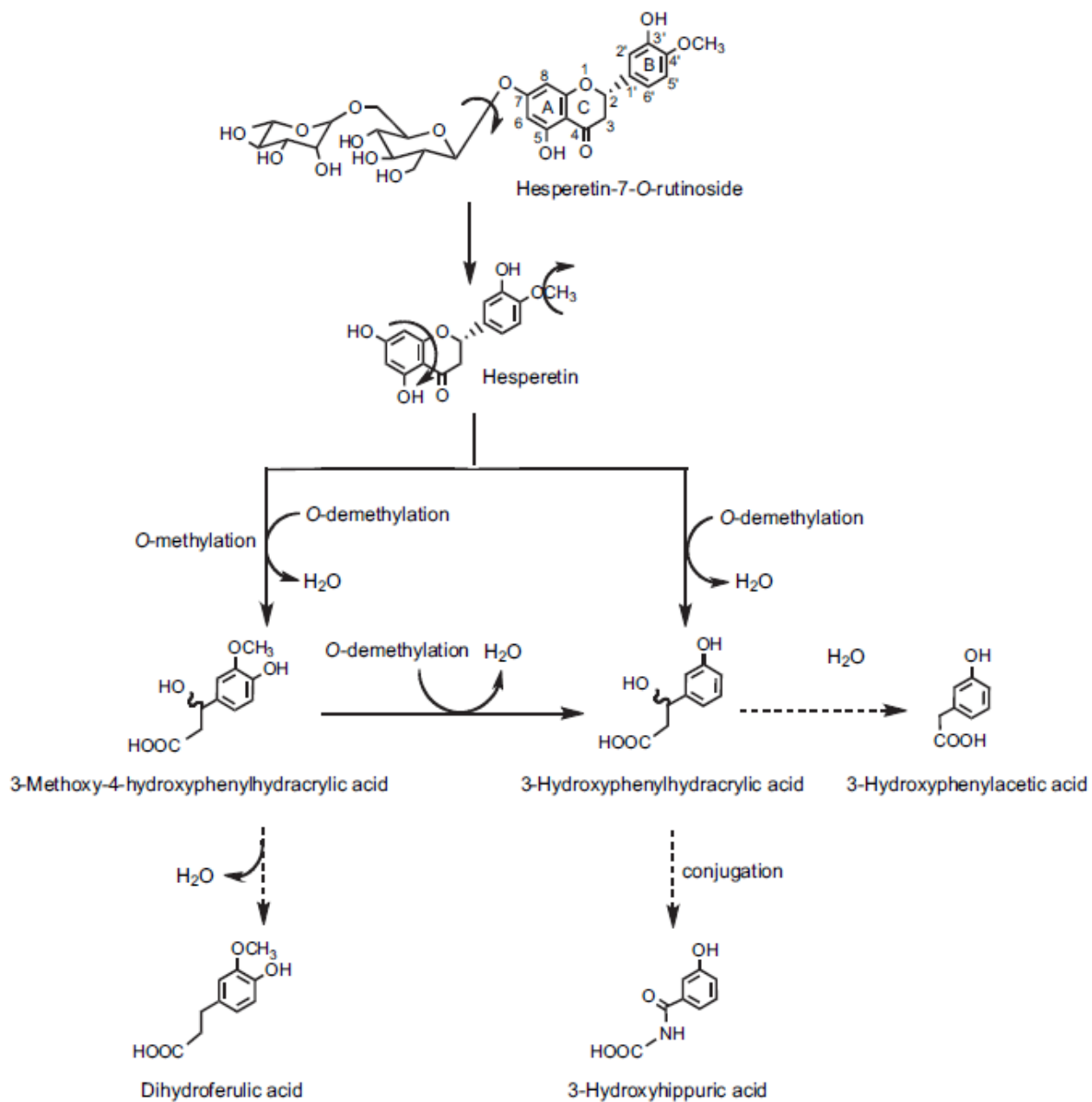
En dépit d'effets potentiellement importants des flavonoïdes dans la thérapie, et malgré une prise quotidienne conséquente par la simple alimentation (de l'ordre de 20 mg à 1 g dans les pays occidentaux), les bénéfices *in vivo* restent parfois contradictoires comme l'ont montré les [Tableaux 3 et 4](#). Ceci peut très certainement s'expliquer par la faible biodisponibilité et la métabolisation/catabolisation importante des flavonoïdes.

Du point de vue de la biodisponibilité, à l'exception de quelques uns de leurs métabolites, les anthocyanines sont très faiblement absorbées et leur champ d'action reste essentiellement intestinal. Les concentrations plasmatiques de flavonols comme la catéchine et l'épicatéchine sont de l'ordre de 0,1–0,7  $\mu\text{mol/L}$  après une prise orale de 90–165 mg. Les flavanones vont donner les meilleurs résultats avec des concentrations plasmatiques pouvant atteindre 5  $\mu\text{mol/L}$  pour des apports de l'ordre de 200 mg ([Manach et al. 2004](#)).

Concernant les études sur la digestion des flavonoïdes alimentaires chez l'homme, il est maintenant bien établi que ces composés subissent un catabolisme considérable ([illustration dans les Figures 4 et 5](#)). Concernant par exemple la catéchine et l'acide tannique, moins de 5% de ces composés polyphénoliques sont excrétés sous forme inchangée dans les fèces après administration orale chez des rats ([Bravo et al. 1994](#)). Dans d'autres études menées chez homme, essentiellement des métabolites de la (+)-catéchine ont été détectés dans le plasma, l'urine et les fèces ([Das & Sothy 1971](#); [Das 1971](#); [Stalmach et al. 2010](#)) après son administration orale. Les sites importants du catabolisme des flavonoïdes sont le lumen de l'intestin (enzymes, microflores intestinales), les cellules de la paroi intestinale et le foie. Ce catabolisme est une question d'intérêt car il affecte directement l'activité biologique d'un composé et sa capacité à pénétrer dans les cellules. Pour ces raisons, il est probablement à l'origine d'une forte variabilité inter-individuelle dans les travaux *in vivo* sur les activités biologiques de ces polyphénols ([Manach et al. 2004](#); [Ramos 2007](#)). En outre, une caractéristique commune de flavonoïdes est leur métabolisation importante au niveau hépatique. Après leur passage dans le foie, ils sont produits sous forme de conjugués glucuronide et/ou sulfate dans la circulation sanguine afin de diminuer leur hydrophobicité et faciliter leur excrétion urinaire et biliaire ([Erlund et al. 2000](#); [Walle 2004](#); [Erlund 2004](#); [Del Rio et al. 2010](#)). Les temps de demi-vie plasmatiques des flavonoïdes sont rarement calculés avec précision mais ils ont été évalués à 2-3h pour des anthocyanines et des flavanols ([Manach et al. 2004](#)).



**Figure 4.** Voie du catabolisme de la quercétine-3-O-rutinoside (rutine) chez l'homme. Les conversions sont principalement faites par la microflore colique. La métabolisation de l'acide 3,4-dihydroxyphénylacétique en acide 3-methoxy-4-hydroxyphénylacétique a lieu dans le foie avant son excrétion dans l'urine. La flèche en pointillés indique une voie mineure (d'après Del Rio 2010).

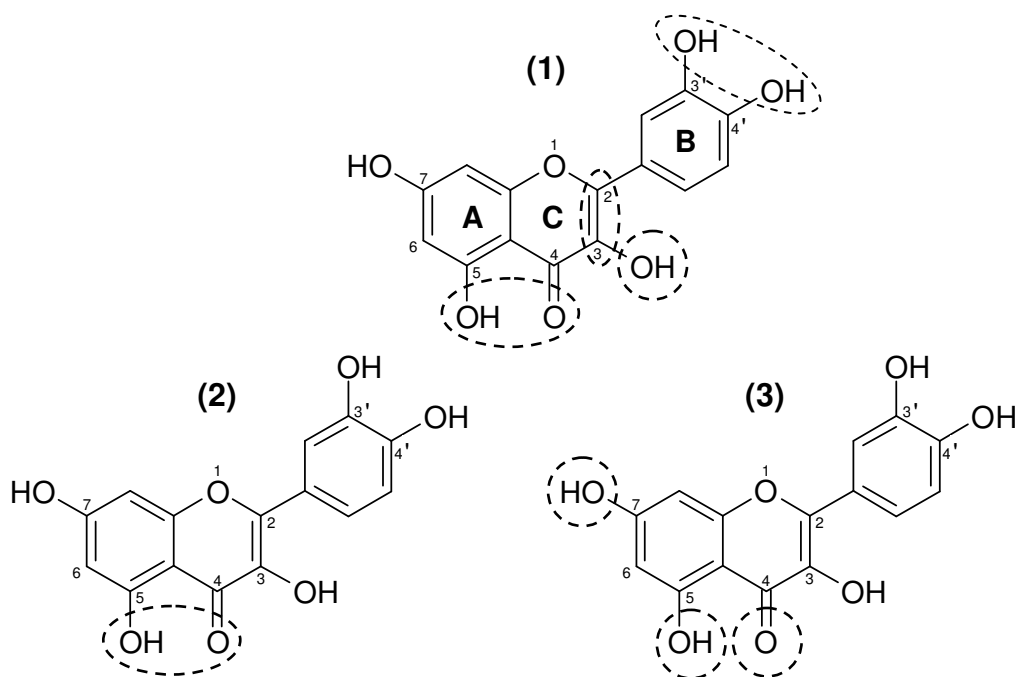


**Figure 5.** Voie du catabolisme de l'hespérétine-7-O-rutinoside (hespéridine) chez l'homme. Les conversions sont principalement faites par la microflore colique mais les étapes de méthylation peuvent avoir lieu dans le foie avant son excrétion dans l'urine. Les flèches en pointillés indiquent des voies mineures (d'après Del Rio 2010).

## II. Quercétine : le chef de file des flavonoïdes

### 1. Généralités

Comme évoqué dans le chapitre précédent, la quercétine (3,3',4',5,7-pentahydroxyflavone) est un flavonoïde parmi les plus intéressants. Ses propriétés antioxydantes et anti-inflammatoires ont été associées à la prévention et au traitement de maladies cardiovasculaires, neurodégénératives et de cancers. Nous l'avons vu ([Tableau 2](#)), son activité antioxydante est 4 à 5 fois plus élevée que celle de la vitamine C ou de la vitamine E (Ishige et al. 2001; Marín et al. 2002). Les éléments structuraux responsables de cette forte activité sont: (1) le groupement 3',4'-catéchol dans le cycle B, (2) une double liaison C2-3, et (3) le groupement hydroxyle en C3 avec (4) le groupement 4-oxo (Heim et al. 2002; Seyoum et al. 2006). En outre, le groupement hydroxyle en C5 avec le groupement 4-oxo est en charge de la chélation du fer. Les groupements hydroxyles en C5, C7 et le groupement 4-oxo sont nécessaires à la neuroprotection ([Figure 6](#)) ([Dajas 2012](#)).



**Figure 6.** Caractéristiques structurales de la quercétine liées à: (1) l'activité antioxydante; (2) la chélation du fer; (3) la neuroprotection ([d'après Dajas 2012](#)).

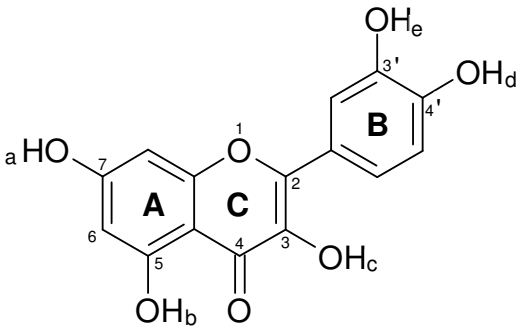
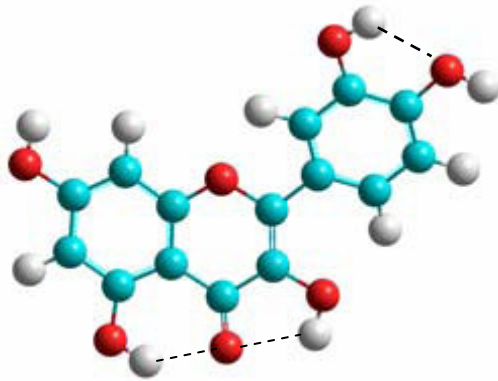
La lecture du [Tableau 3](#) suggère que cette molécule est également capable d'intervenir à plusieurs niveaux du cycle cellulaire et dans le processus de cancérogenèse. En fonction notamment de la dose administrée, il a été montré que la quercétine pouvait avoir un effet soit cytoprotecteur (à des concentrations de quelques  $\mu\text{mol/L}$ ), soit apoptotique (à des concentrations de quelques dizaines de  $\mu\text{mol/L}$ ) ([Ramos 2007](#)). La cytoprotection serait due à ces propriétés antioxydantes, qui diminuent la cytotoxicité induite par l' $\text{H}_2\text{O}_2$ , ainsi qu'à l'activation de la voie des MAP kinases qui aboutit à l'expression de gènes de survie (c-fos, c-jun) et de gènes de défense (Glutathion-S-transférase). La mort cellulaire programmée (apoptose) est caractérisée par des changements morphologiques et biochimiques dans la cellule, notamment une fragmentation de l'ADN. Dans de nombreuses lignées cellulaires (leucémie, cancers du sein, du colon, du foie, des poumons,...), la quercétine a été capable d'induire cette mort cellulaire, probablement par l'activation de la voie des caspases (-3, -7, -9) et le clivage de la poly ADP-ribose polymérase (PARP). A des concentrations de quelques dizaines de  $\mu\text{mol/L}$ , la quercétine modifie également la balance entre les protéines Bcl2 pro-apoptotiques et anti-apoptotiques en faveur des produits favorisant l'apoptose comme Bax. Enfin, des effets synergiques ont été observés entre la quercétine et le cisplatine, le resveratrol, le busulfan, le cyclophosphamide, la gemcitabine ou le topotecan ([Lamson & Brignall 2000](#)).

Compte-tenu de ces effets préventifs et thérapeutiques potentiellement très importants, des suppléments diététiques de quercétine, sous forme de capsules ou de comprimés de 50 mg jusqu'à 500 mg, sont disponibles sur le marché pharmaceutique. En effet, bien que la quercétine soit le flavonoïde alimentaire le plus abondant, il est présent dans un grand nombre de fruits, de légumes, de boissons et de plantes médicinales, comme *Ginkgo biloba*, *Hypericum perforatum*, *Sambucus canadensis* ([Kavuru 2008](#)), son apport journalier par la seule alimentation ne semble pas suffisant pour atteindre ces objectifs thérapeutiques. La consommation quotidienne de quercétine dans un régime alimentaire occidental moyen est estimée à 5-40 mg/jour et jusqu'à 200-500 mg/jour chez les personnes qui consomment de grandes quantités de produits riches en flavonols (les pommes, les oignons et les tomates) ([Russo et al. 2012](#)) alors que la dose quotidienne recommandée est de 400 à 1200 mg/jour ([Kavuru 2008](#)). Les effets secondaires liés à l'utilisation des suppléments de quercétine sont relativement rares,

quelques signalements ponctuels de nausées aux doses élevées ([Kavuru 2008](#)), mais se pose la question de leur réelle efficacité après administration par voie orale ([Tableau 4](#)). Après une telle administration, les études de pharmacocinétiques centrées sur cette molécule sont assez contradictoires. Dans un premier travail, après administration d'une dose unique de 4 g chez l'homme, aucune concentration mesurable du flavonoïde ou de ses dérivés n'a été détectée dans le plasma ou l'urine alors qu'environ 53% de la dose orale ont été retrouvés inchangés dans les selles ([Gugler et al. 1975](#)). Pour Erlund et al., la quercétine peut être mesurable sous forme de conjugués glucuronide et/ou sulfate dans la circulation sanguine chez l'homme, mais elle est difficilement détectable sous forme aglycone libre ([Erlund et al. 2000](#)). Dans une autre étude ([Wätjen et al. 2005](#)), l'apport complémentaire de 1 à 2 g de quercétine permet d'atteindre des concentrations plasmatiques entre 10 et 50  $\mu\text{mol/L}$ , donc du même ordre de grandeur que celles induisant les effets pro-apoptotiques. Comme pour les flavonoïdes en général, les causes de cette faible biodisponibilité sont l'insolubilité de la quercétine dans les milieux aqueux, sa très grande instabilité chimique et sa métabolisation importante ([Azuma et al. 2010](#); [Lauro et al. 2002](#); [Harborne & Williams 2000](#); [Moon et al. 2008](#); [Ramešová et al. 2012](#)). Autant de paramètres qu'il faudra surmonter par l'emploi d'une formulation adaptée.

## 2. Propriétés physico-chimiques

**Tableau 5.** Structure et propriétés physico-chimiques de la quercétine

| Structure                                                                          | Propriétés                |                                                                                  |
|------------------------------------------------------------------------------------|---------------------------|----------------------------------------------------------------------------------|
|   | Nom scientifique          | 2-(3,4-dihydroxyphényl) -3,5,7-trihydroxy-4H-chromén-4-one                       |
|                                                                                    | (Nom IUPAC)               |                                                                                  |
|                                                                                    | Synonymes                 | 3,3',4',5,7-pentahydroxyflavone; xanthaurine ; quercétol ; quercitine ; quertine |
|                                                                                    | Formule brute             | C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>                                   |
|                                                                                    | Poids moléculaire         | 302,24 (g/mol)                                                                   |
| Liaisons hydrogènes intramoléculaires                                              | LogP                      | 1,82 +/- 0,32 (Rothwell et al. 2005)                                             |
|  | pKa                       | 6,6 et 9,7 (Zenkevich & Guschina 2010)                                           |
|                                                                                    | Point de fusion           | 317 – 327,5 °C (Zhang et al. 2011; Borghetti et al. 2012)                        |
|                                                                                    | Masse volumique           | 1,799 g/cm <sup>3</sup>                                                          |
|                                                                                    | λ <sub>max</sub> (UV-Vis) | 371 nm                                                                           |
|                                                                                    | Apparence                 | poudre jaune                                                                     |
| Doneurs de ponts hydrogènes: 5 (a – e)                                             | Formes physiques :        | anhydre, hydrate ou dihydrate                                                    |
| Accepteurs de ponts hydrogènes: 7                                                  |                           |                                                                                  |

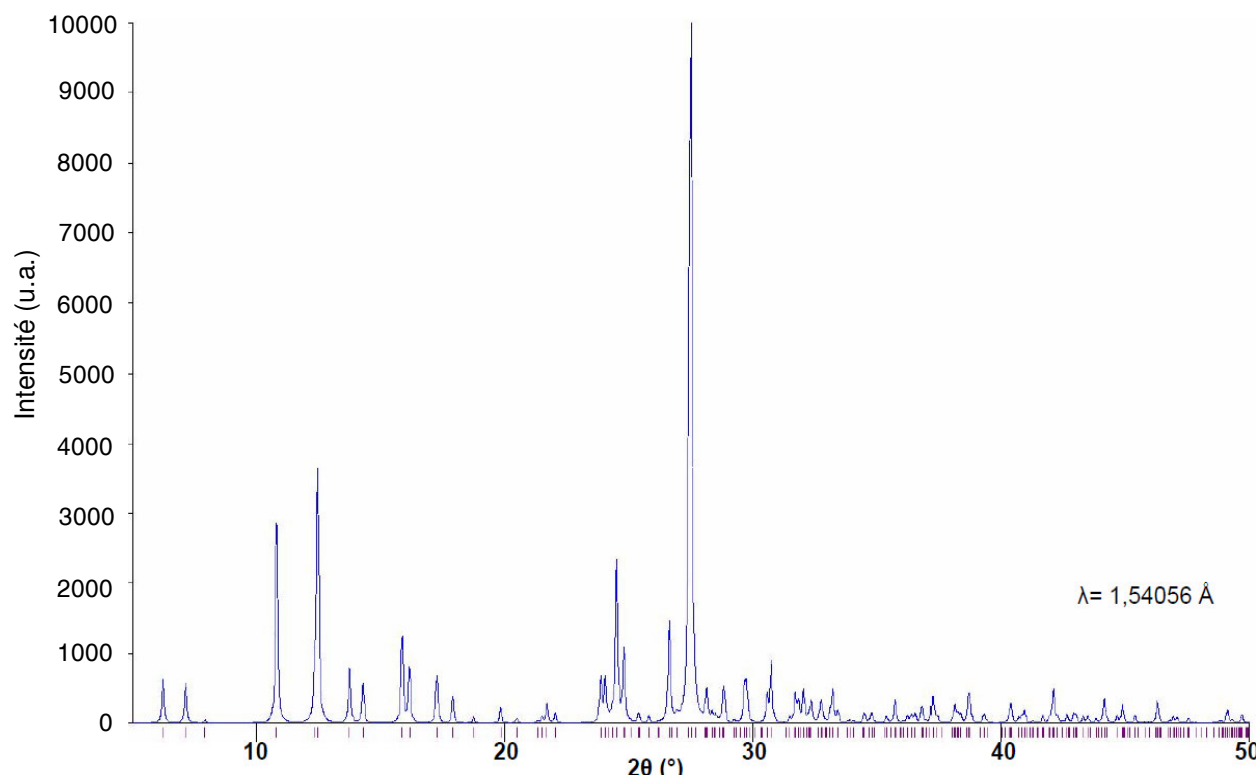
### 2.1. Cristallinité et pseudo-polymorphisme

La structure moléculaire de la quercétine se compose de trois cycles désignés A, B et C, respectivement (Tableau 5). Les cinq groupements hydroxyle (a – e), peuvent agir comme accepteurs et/ou donneurs de liaisons hydrogène et les groupements carbonyle et éther sont capables de servir comme accepteurs de liaisons hydrogènes.

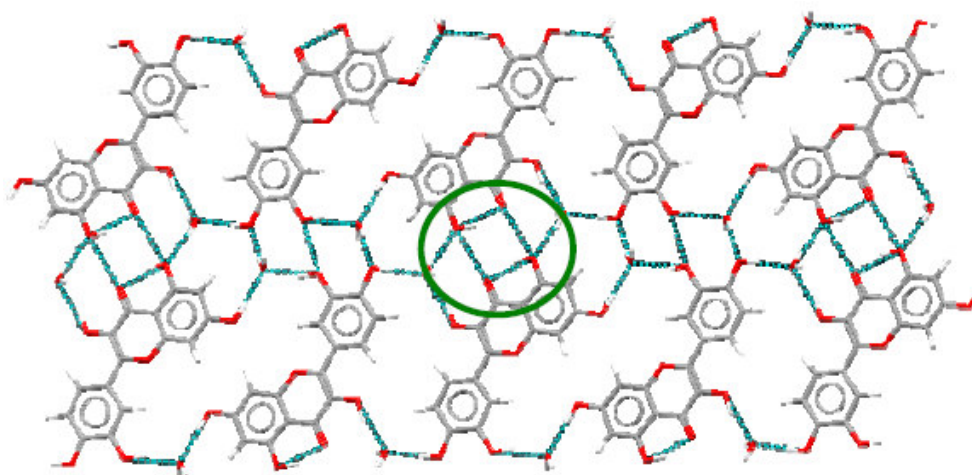
A l'état solide, la quercétine peut exister sous différentes formes, notamment une forme anhydre, deux hydrates (mono- et dihydrate) et d'autres solvates (pyridine, tétrahydrofurane,...) (Kavuru 2008). La forme la plus fréquemment rencontrée dans la

littérature, la forme dihydrate, est décrite dans la base de données cristallographiques *Cambridge Structural Database* (CSD) sous les dénominations FEFBEX et FEFBEX01. Le diagramme de poudre reconstitué à partir de FEFBEX01, qui possède une meilleure adéquation entre les données expérimentales et la structure proposée (Facteur-R), est décrit [Figure 7](#).

La [Figure 8](#) représente l'organisation cristalline de la quercétine dihydrate. Dans le réseau cristallin, tous les groupements hydroxyles et le carbonyle de la quercétine ainsi que deux molécules d'eau participent aux liaisons hydrogènes. On note des liaisons hydrogènes intramoléculaires formées par le groupement hydroxyle ( $-OH_b$ ) et le groupement carbonyle adjacent. De plus, les trois cycles de la molécule ne sont pas dans le même plan. Les cycles A et C se situent dans un plan commun faisant un angle dièdre d'environ  $8^\circ$  avec le plan contenant le noyau B ([Kavuru 2008](#)).

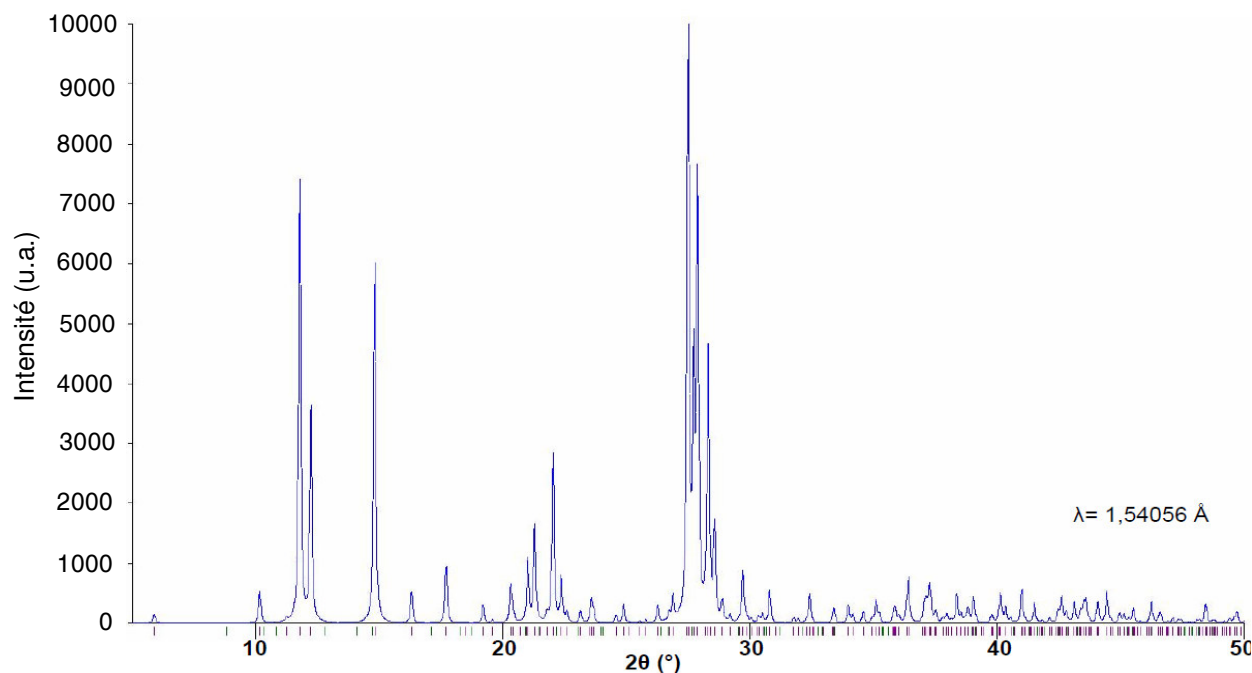


**Figure 7.** Diagramme de poudre de la quercétine dihydrate reconstitué à partir des données de la [Cambridge Structural Database](#) (données FEFBEX 01) à l'aide du logiciel Mercury 3.0. Facteur-R : 4,8%. Dernière consultation le 4 septembre 2012.



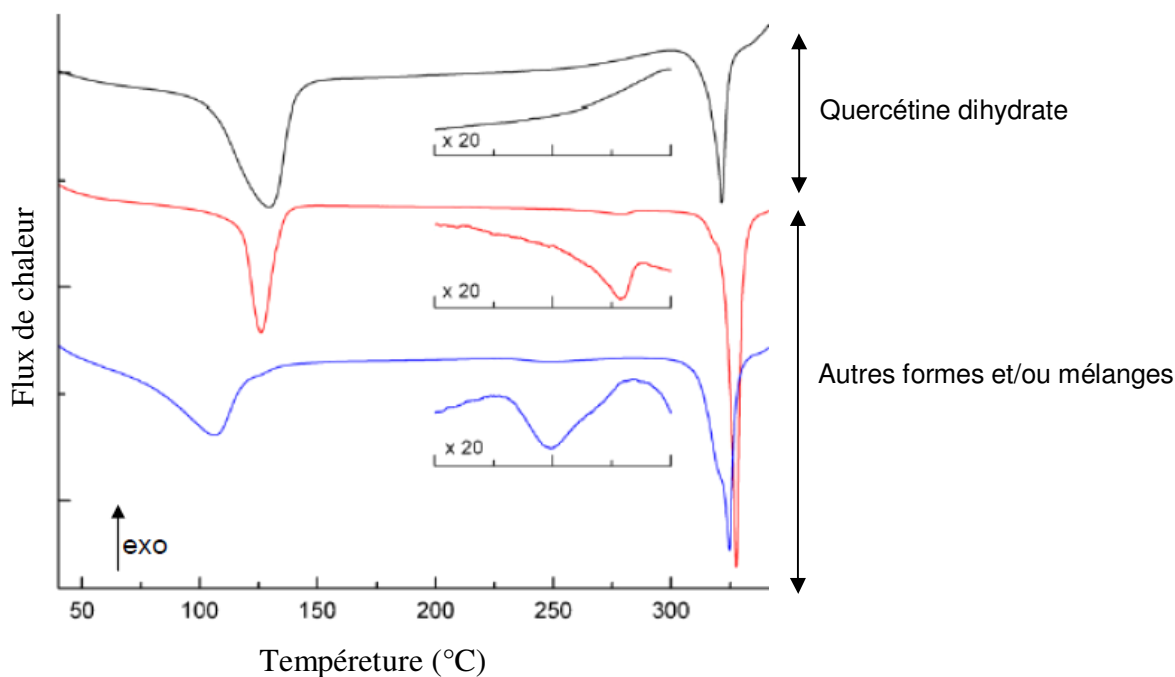
**Figure 8.** Liaisons hydrogènes dans la structure cristalline de la quercétine dihydrate. Le cycle vert montre les liaisons intramoléculaires, entre le groupement hydroxyle ( $-OH_b$ ) et le groupement carboxyle adjacent, ainsi que les liaisons intermoléculaires (d'après Kavuru 2008).

Plus récemment (2011), la structure de la quercétine monohydrate, dont le diagramme de poudre reconstitué est décrit dans la Figure 9, a été intégrée dans la base CSD (AKIJEK 01/02). D'autres solvates de quercétine sont décrits dans cette base de données, par exemple avec la pyridine (NIXLUC) ou avec le formamide (EVIJUO), ainsi que des co-cristaux (avec la théobromine, MUPPOD) alors que la forme anhydre n'est pas référencée.

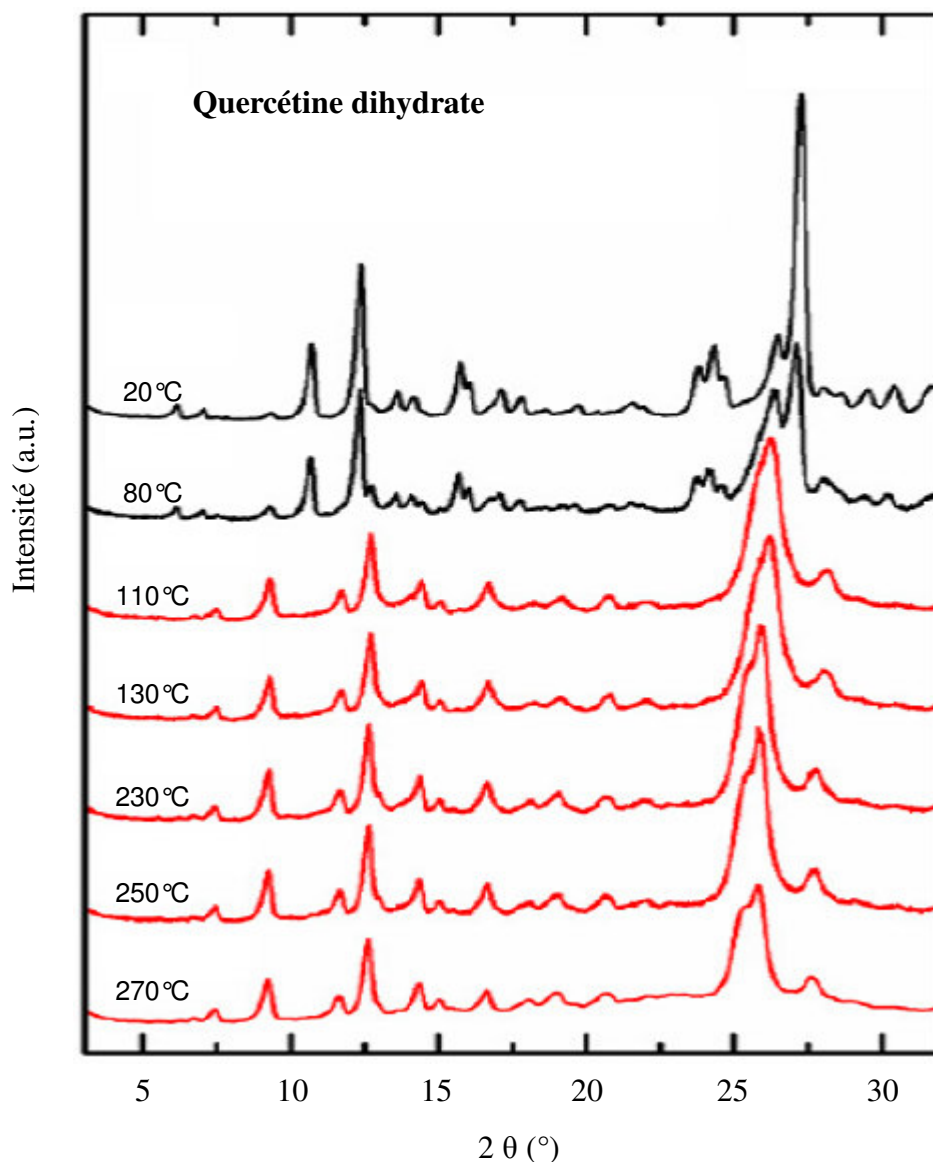


**Figure 9.** Diagramme de poudre de la quercétine monohydrate reconstitué à partir des données de la Cambridge Structural Database (données AKIJEK 02) à l'aide du logiciel Mercury 3.0. Facteur-R : 2.0%. Dernière consultation le 4 septembre 2012.

D'un point de vue thermique, la [Figure 10](#) représente un thermogramme typique de la quercétine dihydrate. A un premier évènement thermique autour de 105-120°C et correspondant à la déshydratation de l'échantillon, succède sa fusion aux environs de 320°C ([Borghetti et al. 2012](#)). L'enthalpie de cette fusion est de l'ordre de 137,4 - 153,5 J/g ([Chebil 2006](#); [Sahoo et al. 2010](#)). Les changements structuraux accompagnant la déshydratation sont décrits dans la [Figure 11](#).

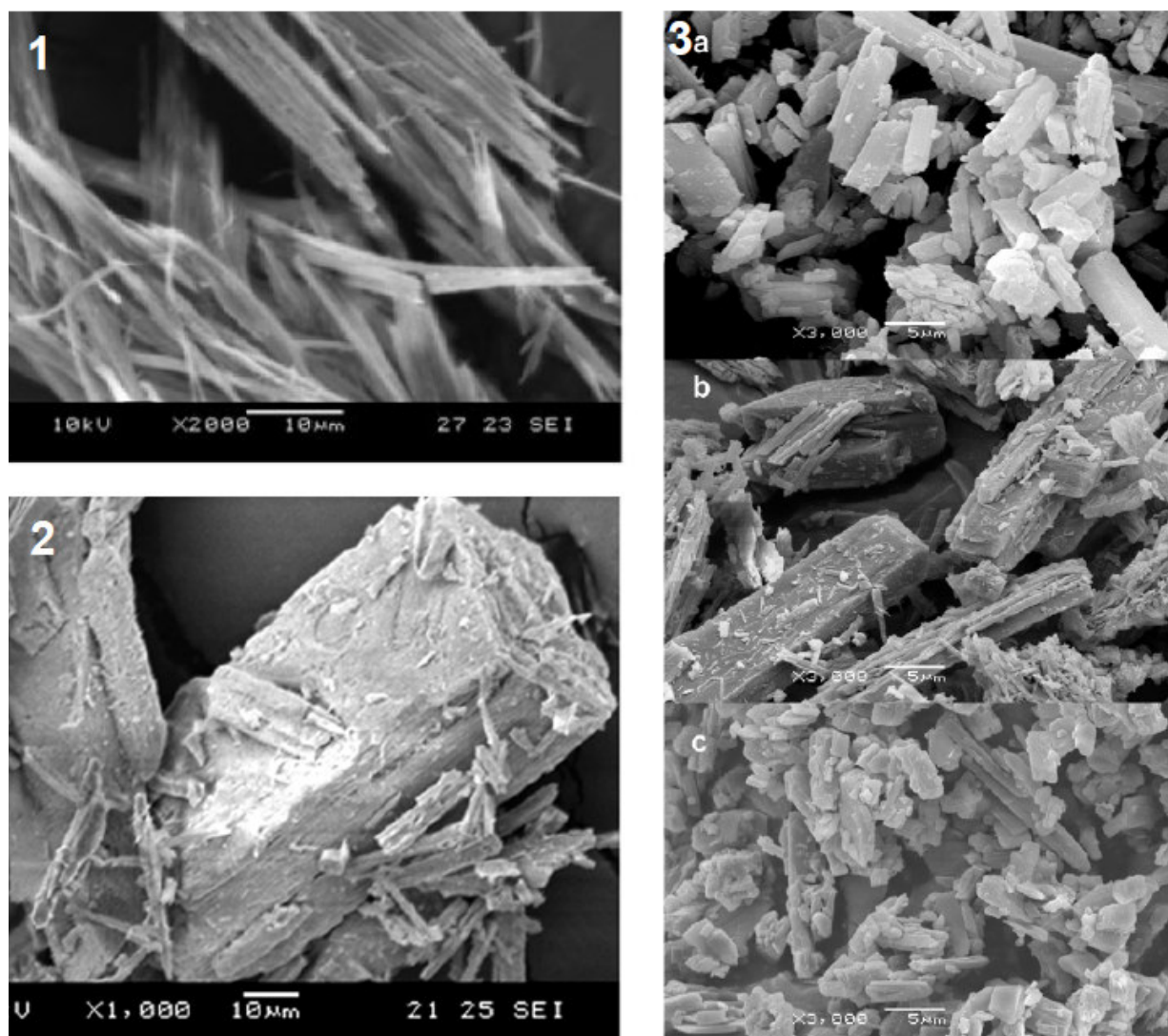


**Figure 10.** Thermogrammes de différents échantillons commerciaux de quercétine ([d'après Borghetti et al. 2012](#)). Par diffraction des rayons X, l'échantillon en noire a été identifié comme étant de la quercétine dihydrate. Les autres échantillons ne sont pas typiques d'une forme précise et sont probablement des mélanges.



**Figure 11.** Diffractogrammes de la quercétine dihydrate en fonction de la température (d'après Borghetti et al. 2012). En noir : avant la déshydratation, en rouge : après la déshydratation.

Outre l'exemple de la quercétine dihydrate, la Figure 10 montre une certaine hétérogénéité dans les thermogrammes de la quercétine selon l'origine de l'échantillon avec, par exemple l'apparition d'un petit événement thermique supplémentaire autour de 250°C pour deux des échantillons. Cette grande variabilité a été retrouvée dans la littérature, tant sur les profils thermiques que sur les diffractogrammes, rendant parfois délicates l'analyse et la comparaison de différentes études expérimentales (da Costa et al. 2002; Wu et al. 2008; Sahoo et al. 2011; Zhang et al. 2011; Borghetti et al. 2012). Le faciès des cristaux représenté dans la Figure 12 illustre parfaitement cette diversité.

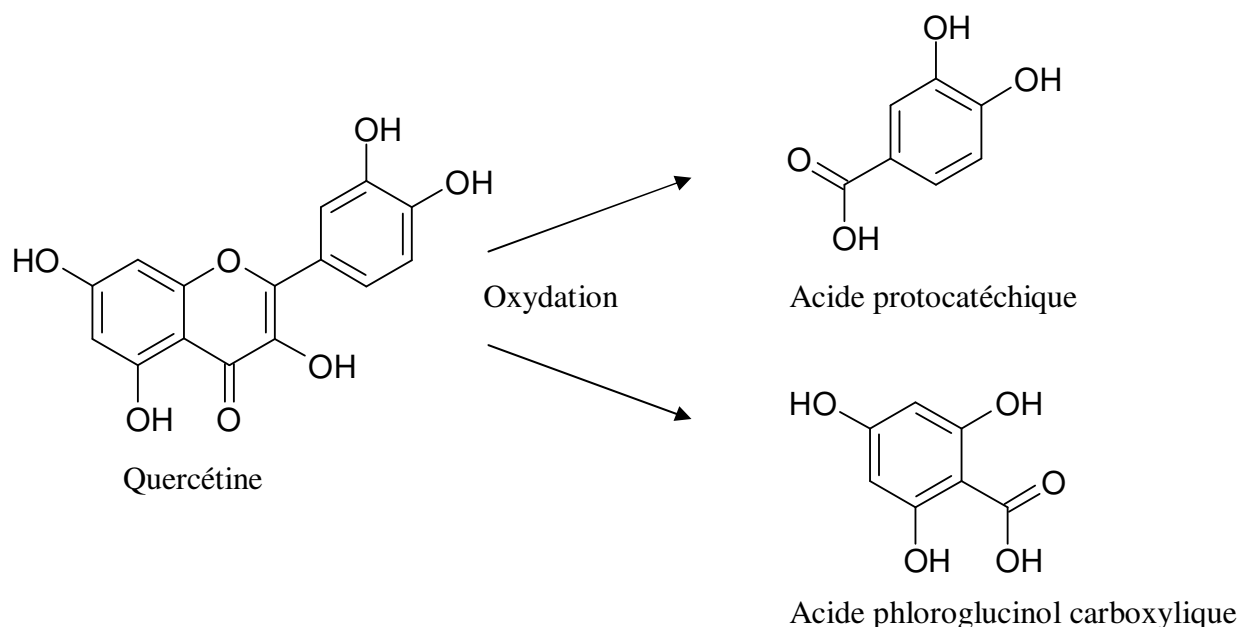


**Figure 12.** Images de microscopie de particules de quercétines extraites de différents travaux (1) d'après Sahoo et al, 2010 ; (2) d'après Kakran et al, 2011 ; et (3) d'après Borghetti et al, 2012.

## 2.2. Stabilité et solubilité

La quercétine est instable dans les solutions à pH neutre ou basique comme l'eau, le plasma sanguin (Moon et al. 2008) et elle est également sensible à l'oxygène atmosphérique (Ramešová et al. 2012) et à la lumière (Fahlman & Krol 2009; Scalia & Mezzena 2009; Scalia & Mezzena 2010; Gao et al. 2011). L'oxydation de la quercétine peut produire au moins 18 produits de dégradation (Zhou et al. 2007) mais les deux principaux sont l'acide protocatéchique (acide 3,4-dihydroxybenzoïque) et l'acide phloroglucinol carboxylique (acide 2,4,6-trihydroxybenzoïque) (Figure 13) (Pinelo et al. 2004; Ramešová et al. 2012). Cependant, la quercétine est plus stable en milieu acide

(pH 2,7) à température ambiante et dans l'eau à des températures inférieures ou égale à 4 °C (Moon et al. 2008).



**Figure 13.** Produits de dégradation les plus stables de la quercétine  
(d'après Pinelo et al. 2004; Ramešová et al. 2012)

A température ambiante, sa solubilité est de l'ordre de 0,17 à 7,7 µg/ml dans l'eau, de 5,5 µg/ml dans le suc gastrique artificiel pH 1,2 et de 28,9 µg/ml dans le suc intestinal artificiel à pH 6,8 (Lauro et al. 2002; Gao et al. 2009). Dans le tampon phosphate pH 7,4, sa solubilité est de 11 µg/ml à température ambiante et de 44 µg/ml à 37 °C (Priprem et al. 2008). Dans l'eau, la solubilité de la quercétine anhydre varie de 2,15 µg/ml à 25 °C à 33 µg/ml à 82 °C et celle de la quercétine dihydrate de 2,63 µg/ml à 25 °C à 45 µg/ml à 82 °C (Srinivas et al. 2010), suggérant une meilleure solubilité de la forme hydrate, à température ambiante comme à chaud. Ce résultat est toutefois contradictoire avec la loi générale considérant qu'une forme anhydre est toujours plus soluble qu'une forme hydratée car l'hydrate interagissant déjà intimement avec l'eau, l'énergie libre libérée pendant la dissolution du cristal sera moins importante (Khankari & Grant 1995). Par des études en résonance magnétique nucléaire (RMN), Olejniczak et Potrzebowski ont montré des liaisons hydrogènes entre l'eau d'hydratation et les hydroxyles en C3', C4' et C7 de la quercétine dans la forme dihydrate. Dans la forme anhydre, le groupement hydroxyle en C3' est impliqué dans une liaison hydrogène avec le groupement C4' ce qui réduirait la solubilité de

la forme anhydre pour ces mêmes auteurs ([Olejniczak & Potrzebowski 2004](#)). A l'inverse, pour Borghetti et al, l'eau d'hydratation contribue à la cohérence de la structure cristalline et diminue l'énergie du cristal, faisant de la forme dihydrate la forme la plus stable (donc implicitement la moins soluble) et la plus intéressante pour un développement pharmaceutique ([Borghetti et al. 2012](#)). Probablement à cause de l'instabilité chimique de la quercétine, les différentes valeurs de solubilité aqueuse obtenues dans la littérature dépendent beaucoup des conditions opératoires (pH, température) et de la méthode utilisée ce qui pourraient entretenir l'incertitude autour de la comparaison hydrate / anhydre. Enfin, l'emploi de différentes familles d'excipients pharmaceutiques permet d'améliorer cette solubilité aqueuse. Ainsi, des co-solvants comme le propylène glycol (50%), le polyéthylène glycol (50%) ou l'éthanol (50%) ont effectivement amélioré la solubilité aqueuse de la quercétine d'environ 400 à 15000 fois à 25°C ([Priprem et al. 2008](#)). De nombreux tensioactifs, e.g. polysorbate 80, lécithine, taurocholate de sodium,, laurylsulfate de sodium, bromure de cétyltriméthylammonium, ont aussi accru significativement la solubilité de cette molécule ([Azuma et al. 2002](#); [Liu & Guo 2006](#); [Priprem et al. 2008](#)).

### 3. Nanoformulations encapsulant la quercétine

Pour augmenter l'absorption et la biodisponibilité de la quercétine après administration chez l'homme, plusieurs approches de formulations galéniques innovantes ont été proposées. Il est possible de citer l'association de la quercétine avec des lipides et des tensioactifs ([Azuma et al. 2002](#)), les complexes de quercétine – cyclodextrine ([Borghetti 2009](#)), la dispersion quercétine – PVP K25 ([Mello-Costa et al. 2010](#)) et notamment les micro- et nanoparticules encapsulant cette molécule en vue d'améliorer sa solubilité, sa stabilité *in vitro* et *in vivo* et sa biodisponibilité. En effet, comme résumé dans le [Tableau 6](#), de nombreuses publications récentes ont montré l'intérêt de l'encapsulation de la quercétine dans des nanovecteurs.

**Tableau 6.** Exemples des micro- et nanoformulations de quercétine et synthèse des résultats les plus marquants.

| Formulation                              | Excipients                                                                                                      | Effets marquants                                                                                                                                                           | Référence               |
|------------------------------------------|-----------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|
| Microparticules polymériques<br>1 – 3 µm | Polyméthacrylate de méthyle (PMMA),<br>1,3-butylène glycol et glycérine<br>Arlacel <sup>®</sup> 83 (surfactant) | Aucune différence significative de stabilité entre la quercétine libre et la quercétine encapsulée durant le stockage à 25°C. Amélioration de la stabilité décrite à 42°C. | (Lee et al. 2007)       |
| Microparticules lipidiques<br>10 – 45 µm | Tristéarine (matrice lipidique)<br>et phosphatidylcholine (émulsifiant)                                         | La teneur en quercétine dans les microparticules est stable après 3 mois à température ambiante et à l'abri de la lumière                                                  | (Scalia & Mezzena 2009) |
| Nanosuspensions<br>~200 nm (PdI = 0,2)   | Pluronic <sup>®</sup> F68<br>et lécithine                                                                       | La teneur en quercétine dans les nanosuspensions est stable pendant 10 jours sous lampes fluorescentes et 1 mois à 25°C à l'abri de la lumière                             | (Gao et al. 2011)       |
| Micelles polymériques<br>14 – 19 nm      | PEG-OCL (poly (éthylène glycol)-b-oligo (ε-capro-lactones))<br>avec des groupes terminaux naphtyle ou benzyle   | Augmentation de la solubilité de la quercétine jusqu'à 1 mg/ml, soit environ 110 fois sa solubilité aqueuse                                                                | (Khonkarn et al. 2011)  |
| Microémulsions<br>39 nm                  | Oléate d'éthyle (huile, 7%), Tween 80 (surfactant, 48%),<br>et éthanol (cosurfactant, 45%)                      | Amélioration de la solubilité (4,1 mg/ml) et de l'absorption orale de la quercétine                                                                                        | (Gao et al. 2009)       |
| Liposomes<br>110 – 150 nm                | Lécithine – cholestérol<br>– PEG 4000 (13:4:1)                                                                  | Augmentation de l'accumulation de la quercétine dans les tissus tumoraux                                                                                                   | (Yuan et al. 2006)      |

| Formulation                                     | Excipients                                                                                              | Effets marquants                                                                                                                     | Référence            |
|-------------------------------------------------|---------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|----------------------|
| Nanoparticules polymériques<br>~ 150 nm,        | Acide polylactique (PLA) et alcool polyvinylique (PVA)                                                  | Amélioration de la solubilité et de la vitesse de dissolution de la quercétine                                                       | (Kumari et al. 2010) |
| Nanoparticules polymériques                     | Eudragit <sup>®</sup> E (copolymères de méthacrylate d' aminoalkyle) – alcool polyvinylique (PVA) (1:1) | Augmentation de la vitesse de dissolution du principe actif environ 70 fois plus élevée par rapport aux suspensions de la quercétine | (Wu et al. 2008)     |
| Nanoparticules lipidiques solide (SLN), ~150 nm | Lécithine, PEG 400, Tween 80                                                                            | Amélioration de l'absorption gastro-intestinale de la quercétine                                                                     | (Li et al. 2009)     |
| Nanocapsules lipidiques (LNC)<br>27 – 54 nm     | Labrafac <sup>TM</sup> Lipophile WL 1349, Phospholipon <sup>®</sup> 90G, et Solutol <sup>®</sup> HS15   | La charge élevée de quercétine dans les particules augmente sa solubilité apparente d'un facteur 100                                 | (Barras et al. 2009) |
| Nanocristaux<br>483 nm                          | Tween 80 (0,5 – 2,5%)                                                                                   | Amélioration de la vitesse de dissolution de la quercétine                                                                           | (Sahoo et al. 2011)  |
| Nanocristaux<br>276 –787 nm                     | Tween 80 (1 – 2%)                                                                                       | Amélioration de la vitesse de dissolution de la quercétine                                                                           | (Kakran et al. 2012) |

Comme le montre le [Tableau 6](#), les bénéfices décrits après encapsulation de la quercétine sont très souvent en relation avec sa solubilité ou sa vitesse de dissolution mais peu d'études se sont focalisées sur la stabilité chimique. De même, les problématiques de stabilité physique et de polymorphisme ont été très peu abordées dans les travaux sur les nanovecteurs incorporant de la quercétine. Pourtant, tant le procédé de préparation que la nature des excipients, peuvent avoir une incidence importante sur ces aspects. Par exemple, sa transformation polymorphique a lieu dans les complexes de quercétine - amidon ([Zhang et al. 2011](#)) comme l'ont montré des

études de diffraction des rayons X mais sans que ces formes ne soient clairement identifiées. L'amorphisation de la quercétine a également été constatée dans des microsphères de cellulose ([Scarfato et al. 2008](#)), des nanoparticules polymériques d'Eudragit® E – alcool polyvinylique (1:1) ([Wu et al. 2008](#)) ou des nanosuspensions préparées par précipitation – évaporation de solvant d'une solution éthanolique de Pluronic® F68 et de lécithine ([Gao et al. 2011](#)).

Nous avons vu dans cette présentation des flavonoïdes en général et de la quercétine en particulier, que l'utilisation thérapeutique de ces produits va nécessiter le développement de formulations adaptées, des nanovecteurs dans le travail de thèse présent, tant pour augmenter leur solubilité apparente que pour améliorer leur stabilité chimique et biologique. Les problèmes de solubilité étant largement abordés dans la littérature, le prochain chapitre, rédigé sous la forme d'un article de revue, se focalisera sur les aspects de protection de principes actifs chimiquement et physiquement instables par l'encapsulation dans des vecteurs de médicaments.

## Références

1. Ajazuddin & Saraf, S., 2010. Applications of novel drug delivery system for herbal formulations. *Fitoterapia*, 81(7), pp.680–689.
2. Anthoni, J., 2007. *Synthèse enzymatique, modélisation moléculaire et caractérisation d'oligomères de flavonoïdes*. Thèse de doctorat. Available at: <http://www.sudoc.fr/13021695X>.
3. Arct, J. & Pytkowska, K., 2008. Flavonoids as components of biologically active cosmeceuticals. *Clinics in dermatology*, 26(4), pp.347–357.
4. Azuma, K. et al., 2002. Combination of lipids and emulsifiers enhances the absorption of orally administered quercetin in rats. *Journal of Agricultural and Food Chemistry*, 50(6), pp.1706–1712.
5. Azuma, K., Ippoushi, K. & Terao, J., 2010. Evaluation of tolerable levels of dietary quercetin for exerting its antioxidative effect in high cholesterol-fed rats. *Food and Chemical Toxicology*, 48(4), pp.1117–1122.
6. Barras, A. et al., 2009. Formulation and characterization of polyphenol-loaded lipid nanocapsules. *International Journal of Pharmaceutics*, 379(2), pp.270–277.
7. Biesaga, M., 2011. Influence of extraction methods on stability of flavonoids. *Journal of chromatography. A*, 1218(18), pp.2505–2512.
8. Borghetti, G.S. et al., 2012. Physicochemical properties and thermal stability of quercetin hydrates in the solid state. *Thermochimica Acta*, 539(0), pp.109–114.
9. Bouayed, J. et al., 2008. Caractérisation et bioévaluation des polyphénols: nouveaux domaines d'application en santé et nutrition. *Phytothérapie*, 6(2), pp.71–74.
10. Bravo, L. et al., 1994. Degradation of polyphenols (catechin and tannic acid) in the rat intestinal tract. Effect on colonic fermentation and faecal output. *The British journal of nutrition*, 71(6), pp.933–946.
11. Chaudhry, P.S. et al., 1983. Inhibition of human lens aldose reductase by flavonoids, sulindac and indomethacin. *Biochemical pharmacology*, 32(13), pp.1995–1998.
12. Chebil, L., 2006. *Acylation des flavonoïdes par les lipases de Candida antarctica et de Pseudomonas cepacia: études cinétique, structurale et conformationnelle*. les-Nancy.
13. Chung, J.H. et al., 2003. Dual mechanisms of green tea extract (EGCG)-induced cell survival in human epidermal keratinocytes. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*, 17(13), pp.1913–1915.
14. Cook, N.C. & Samman, S., 1996. Flavonoids—Chemistry, metabolism, cardioprotective effects, and dietary sources. *Journal of Nutritional Biochemistry*, 7(2), pp.66–76.

15. da Costa, E.M. et al., 2002. Thermal characterization of the quercetin and rutin flavonoids. *Thermochimica Acta*, 392-393, pp.79–84.
16. Cushnie, T.P.T. & Lamb, A.J., 2011. Recent advances in understanding the antibacterial properties of flavonoids. *International journal of antimicrobial agents*, 38(2), pp.99–107.
17. Dajas, F., 2012. Life or death: Neuroprotective and anticancer effects of quercetin. *Journal of ethnopharmacology*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22820241>.
18. Damre, A.S. et al., 2003. Studies on the immunomodulatory activity of flavonoidal fraction of *Tephrosia purpurea*. *Fitoterapia*, 74(3), pp.257–261.
19. Das, N.P., 1971. Studies on flavonoid metabolism. Absorption and metabolism of (+)-catechin in man. *Biochemical pharmacology*, 20(12), pp.3435–3445.
20. Das, N.P. & Ratty, A.K., 1986. Effects of flavonoids on induced non-enzymic lipid peroxidation. *Progress in clinical and biological research*, 213, pp.243–247.
21. Das, N.P. & Sothy, S.P., 1971. Studies on flavonoid metabolism. Biliary and urinary excretion of metabolites of (+)-(U- <sup>14</sup> C) catechin. *The Biochemical journal*, 125(2), pp.417–423.
22. Erlund, I. et al., 2000. Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. *European Journal of Clinical Pharmacology*, 56(8), pp.545–553.
23. Erlund, Iris, 2004. Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. *Nutrition Research*, 24(10), pp.851–874.
24. Fahlman, B.M. & Krol, E.S., 2009. UVA and UVB radiation-induced oxidation products of quercetin. *Journal of photochemistry and photobiology. B, Biology*, 97(3), pp.123–131.
25. Favier, A., 2003. Le stress oxydant : Intérêt conceptuel et expérimental dans la compréhension des mécanismes des maladies et potentiel thérapeutique. *L'actualité chimique*, (11-12), pp.108–115.
26. Fontaine, E., 2007. Radicaux libres. In *Traité de nutrition artificielle de l'adulte*. Paris: Springer Paris, pp. 251–257. Available at: [http://www.springerlink.com/index/10.1007/978-2-287-33475-7\\_19](http://www.springerlink.com/index/10.1007/978-2-287-33475-7_19).
27. Galati, E.M. et al., 1994. Biological effects of hesperidin, a citrus flavonoid. (Note I): antiinflammatory and analgesic activity. *Farmaco (Società chimica italiana: 1989)*, 40(11), pp.709–712.

28. Galati, G. & O'Brien, P.J., 2004. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free radical biology & medicine*, 37(3), pp.287–303.
29. Gao, L. et al., 2011. Preparation of a chemically stable quercetin formulation using nanosuspension technology. *International Journal of Pharmaceutics*, 404(1-2), pp.231–237.
30. Gao, Y. et al., 2009. Formulation optimization and in situ absorption in rat intestinal tract of quercetin-loaded microemulsion. *Colloids and Surfaces B: Biointerfaces*, 71(2), pp.306–314.
31. Ghedira, K., 2005. Les flavonoïdes: structure, propriétés biologiques, rôle prophylactique et emplois en thérapeutique. *Phytothérapie*, 3(4), pp.162–169.
32. Gonçalves, J.L. et al., 2001. In vitro antiviral effect of flavonoid-rich extracts of *Vitex polygama* (Verbenaceae) against acyclovir-resistant herpes simplex virus type 1. *Phytomedicine: international journal of phytotherapy and phytopharmacology*, 8(6), pp.477–480.
33. Gryglewski, R.J. et al., 1987. On the mechanism of antithrombotic action of flavonoids. *Biochemical pharmacology*, 36(3), pp.317–322.
34. Gugler, R., Leschik, M. & Dengler, H.J., 1975. Disposition of quercetin in man after single oral and intravenous doses. *European Journal of Clinical Pharmacology*, 9(2-3), pp.229–234.
35. Harborne, J.B. & Williams, C.A., 2000. Advances in flavonoid research since 1992. *Phytochemistry*, 55(6), pp.481–504.
36. Heim, K.E., Tagliaferro, A.R. & Bobilya, D.J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *The Journal of nutritional biochemistry*, 13(10), pp.572–584.
37. Hertog, M.G. et al., 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet*, 342(8878), pp.1007–1011.
38. Hodek, P., Trefil, P. & Stiborová, M., 2002. Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. *Chemico-biological interactions*, 139(1), pp.1–21.
39. Ishige, K., Schubert, D. & Sagara, Y., 2001. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free radical biology & medicine*, 30(4), pp.433–446.
40. Jaeger, A., Wälti, M. & Neftel, K., 1988. Side effects of flavonoids in medical practice. *Progress in clinical and biological research*, 280, pp.379–394.

41. Kakran, M. et al., 2012. Fabrication of quercetin nanocrystals: comparison of different methods. *European journal of pharmaceuticals and biopharmaceutics: official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V*, 80(1), pp.113–121.
42. Kavuru, P., 2008. Crystal engineering of flavonoids. *Graduate School Theses and Dissertations*. Available at: <http://scholarcommons.usf.edu/etd/325>.
43. Khankari, R.K. & Grant, D.J.W., 1995. Pharmaceutical hydrates. *Thermochimica Acta*, 248(0), pp.61–79.
44. Khonkarn, R. et al., 2011. PEG-OCL micelles for quercetin solubilization and inhibition of cancer cell growth. *European journal of pharmaceuticals and biopharmaceutics: official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V*, 79(2), pp.268–275.
45. Kumari, A., Yadav, S.K. & Yadav, S.C., 2010. Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids and Surfaces B: Biointerfaces*, 75(1), pp.1–18.
46. Kuo, Y.C., Yang, L.-M. & Lin, L.-C., 2004. Isolation and immunomodulatory effect of flavonoids from *Syzygium samarangense*. *Planta medica*, 70(12), pp.1237–1239.
47. Lamson, D.W. & Brignall, M.S., 2000. Antioxidants and cancer, part 3: quercetin. *Alternative medicine review: a journal of clinical therapeutic*, 5(3), pp.196–208.
48. Lauro, M.R. et al., 2002. Fast- and slow-release tablets for oral administration of flavonoids: rutin and quercetin. *Drug Development and Industrial Pharmacy*, 28(4), pp.371–379.
49. Lee, D.H. et al., 2007. Preparation and characterization of quercetin-loaded polymethyl methacrylate microcapsules using a polyol-in-oil-in-polyol emulsion solvent evaporation method. *The Journal of Pharmacy and Pharmacology*, 59(12), pp.1611–1620.
50. Li, D.C. et al., 2009. Application of targeted drug delivery system in Chinese medicine. *Journal of Controlled Release*, 138(2), pp.103–112.
51. Li, H. et al., 2009. Enhancement of gastrointestinal absorption of quercetin by solid lipid nanoparticles. *Journal of Controlled Release*, 133(3), pp.238–244.
52. Lin, Q.L., Akesson, B. & Bergensthl, B., 2008. Effect of colloidal structures on the stability of five flavonoids with different hydrophilicity. *Food hydrocolloids*, 22(4), pp.700–705.
53. Liu, W. & Guo, R., 2006. Interaction between flavonoid, quercetin and surfactant aggregates with different charges. *Journal of Colloid and Interface Science*, 302(2), pp.625–632.
54. Manach, C. et al., 2004. Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, 79(5), pp.727–747.

55. Marais, J.P.J. et al., 2006. The Stereochemistry of Flavonoids. In E. Grotewold, ed. *The Science of Flavonoids*. Springer New York, pp. 1–46. Available at: <http://www.springerlink.com/content/k61175724527x8p1/abstract>.
56. Marín, F.R. et al., 2002. Flavonoids as nutraceuticals: Structural related antioxidant properties and their role on ascorbic acid preservation. In *Bioactive Natural Products*. Elsevier, pp. 741–778.
57. Mello Costa, A.R. et al., 2010. Quercetin-PVP K25 solid dispersions. *Journal of Thermal Analysis and Calorimetry*, 104(1), pp.273–278.
58. Mendes, A.P.S. et al., 2012. The basic antioxidant structure for flavonoid derivatives. *Journal of molecular modeling*, 18(9), pp.4073–4080.
59. Moon, Y.J. et al., 2008. Quercetin pharmacokinetics in humans. *Biopharmaceutics & Drug Disposition*, 29(4), pp.205–217.
60. Olejniczak, S. & Potrzebowski, M.J., 2004. Solid state NMR studies and density functional theory (DFT) calculations of conformers of quercetin. *Organic & biomolecular chemistry*, 2(16), pp.2315–2322.
61. Ong, K.C. & Khoo, H.E., 2000. Effects of myricetin on glycemia and glycogen metabolism in diabetic rats. *Life sciences*, 67(14), pp.1695–1705.
62. Peterson, J. & Dwyer, J., 1998. Flavonoids: Dietary occurrence and biochemical activity. *Nutrition Research*, 18(12), pp.1995–2018.
63. Pinelo, M. et al., 2004. Solvent effect on quercetin antioxidant capacity. *Food Chemistry*, 88(2), pp.201–207.
64. Priprem, A. et al., 2008. Anxiety and cognitive effects of quercetin liposomes in rats. *Nanomedicine: Nanotechnology, Biology and Medicine*, 4(1), pp.70–78.
65. Pryor, W.A., 1986. Oxy-radicals and related species: their formation, lifetimes, and reactions. *Annual review of physiology*, 48, pp.657–667.
66. Ramešová, S. et al., 2012. On the stability of the bioactive flavonoids quercetin and luteolin under oxygen-free conditions. *Analytical and bioanalytical chemistry*, 402(2), pp.975–982.
67. Ramos, S., 2007. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. *The Journal of nutritional biochemistry*, 18(7), pp.427–442.
68. Del Rio, D. et al., 2010. Polyphenols and health: What compounds are involved? *Nutrition, Metabolism and Cardiovascular Diseases*, 20(1), pp.1–6.
69. Rothwell, J.A., Day, A.J. & Morgan, M.R.A., 2005. Experimental determination of octanol-water partition coefficients of quercetin and related flavonoids. *Journal of Agricultural and Food Chemistry*, 53(11), pp.4355–4360.

70. Russo, M. et al., 2012. The flavonoid quercetin in disease prevention and therapy: facts and fancies. *Biochemical Pharmacology*, 83(1), pp.6–15.
71. Sahoo, N. G et al., 2011. Preparation and characterization of quercetin nanocrystals. *Journal of Pharmaceutical Sciences*, 100(6), pp.2379–2390.
72. Scalia, S. & Mezzena, M., 2009. Incorporation of quercetin in lipid microparticles: Effect on photo- and chemical-stability. *Journal of Pharmaceutical and Biomedical Analysis*, 49(1), pp.90–94.
73. Scalia, S. & Mezzena, M., 2010. Photostabilization effect of quercetin on the UV filter combination, butyl methoxydibenzoylmethane-octyl methoxycinnamate. *Photochemistry and photobiology*, 86(2), pp.273–278.
74. Scarfato, P. et al., 2008. Quercetin microspheres by solvent evaporation: Preparation, characterization and release behavior. *Journal of Applied Polymer Science*, 109(5), pp.2994–3001.
75. Seyoum, A., Asres, K. & El-Fiky, F.K., 2006. Structure-radical scavenging activity relationships of flavonoids. *Phytochemistry*, 67(18), pp.2058–2070.
76. Srinivas, K. et al., 2010. Solubility and solution thermodynamic properties of quercetin and quercetin dihydrate in subcritical water. *Journal of Food Engineering*, 100(2), pp.208–218.
77. Stalmach, A. et al., 2010. Absorption, metabolism, and excretion of green tea flavan-3-ols in humans with an ileostomy. *Molecular nutrition & food research*, 54(3), pp.323–334.
78. Tzeng, S.H. et al., 1991. Inhibition of platelet aggregation by some flavonoids. *Thrombosis research*, 64(1), pp.91–100.
79. Valko, M. et al., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*, 39(1), pp.44–84.
80. Walle, T., 2004. Absorption and metabolism of flavonoids. *Free radical biology & medicine*, 36(7), pp.829–837.
81. Wang et al., 1999. Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *Journal of natural products*, 62(5), p.802.
82. Wang, H.B. et al., 2004. Flavone glucosides with immunomodulatory activity from the leaves of *Pleioblastus amarus*. *Phytochemistry*, 65(7), pp.969–974.
83. Wätjen, W. et al., 2005. Low concentrations of flavonoids are protective in rat H4IIE cells whereas high concentrations cause DNA damage and apoptosis. *The Journal of nutrition*, 135(3), pp.525–531.
84. Wu, T.H. et al., 2008. Preparation, physicochemical characterization, and antioxidant effects of quercetin nanoparticles. *International Journal of Pharmaceutics*, 346(1-2), pp.160–168.

85. Yuan, Z.P., Chen, L. & Fan, L., 2006. Liposomal Quercetin Efficiently Suppresses Growth of Solid Tumors in Murine Models. *Clinical Cancer Research*, 12(10), pp.3193–3199.
86. Zenkevich, I. & Guschina, S., 2010. Determination of dissociation constants of species oxidizable in aqueous solution by air oxygen on an example of quercetin. *Journal of Analytical Chemistry*, 65(4), pp.371–375.
87. Zhang, L. et al., 2011. Preparation, physicochemical characterization and in vitro digestibility on solid complex of maize starches with quercetin. *LWT - Food Science and Technology*, 44(3), pp.787–792.
88. Zhou, A., Kikandi, S. & Sadik, O.A., 2007. Electrochemical degradation of quercetin: Isolation and structural elucidation of the degradation products. *Electrochemistry Communications*, 9(9), pp.2246–2255.
89. Zi, J., Peng, B. & Yan, W., 2007. Solubilities of rutin in eight solvents at T = 283.15, 298.15, 313.15, 323.15, and 333.15 K. *Fluid Phase Equilibria*, 261(1–2), pp.111–114.

## Chemical and physical stability of labile APIs in nanodispersions

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**Abbreviations:** API, active pharmaceutical ingredient; CMC, Critical Micellar Concentration; EPR effect, enhanced permeability and retention effect; NLC, nanostructured lipid carriers; SLN, solid lipid nanoparticles; UV, ultraviolet.

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## Abstract

Stability is a critical aspect in the design, manufacturing, regulatory review and approval of drugs. The review is directed to colloïd systems that might protect labile drugs (both small molecules and large biomolecules) from different degradation pathways including hydrolysis, oxidation, photodegradation, enzymatic degradation, effects of temperature and pH. A better understanding about the protection mechanisms is discussed to develop suitable nanovectors encapsulating sensitive APIs.

**Keywords:** drug degradation, nanoparticulate systems, labile APIs, *in vitro* stability, *in vivo* stability, physical stability, chemical stability, shelf life, half life.

## Contents

1. Introduction
2. General aspects
  - 2.1. Chemical and physical stabilities
  - 2.2. Overview of the nanocarriers
3. General factors in optimizing the stability of labile APIs entrapped in nanocarriers
  - 3.1. Drug location
  - 3.2. Drug-loading capacity
  - 3.3. Stability of colloidal carriers
  - 3.4. Dispersion medium and storage conditions
4. Chemical stability of labile APIs in nanodispersions
  - 4.1. Nanocarriers for improvement of shelf life
    - 4.1.1. Hydrolysis
    - 4.1.2. Oxidation
    - 4.1.3. Photodegradation
    - 4.1.4. Other causes
  - 4.2. Nanocarriers for increase of half life
    - 4.2.1. pH sensibility
    - 4.2.2. Enzymatic degradation
5. Additional stability issues relate to macromolecules
6. Physical stability of labile APIs in nanocrystals
7. Conclusion
- References

## 1. Introduction

The stability is expressed as the capacity of an active pharmaceutical ingredient (API) or drug product to remain within established specifications of identity, strength, quality, purity in a specified period of time (FDA 1998). In general, the active ingredient in a drug product must be stable during manufacturing, shipping, storage, distribution. The expiration date is usual to expect the quality of drug products to be retained for as long as 3–5 years. For *in vitro* instability, various methods have been devised to achieve improvement of the chemical stability such as alteration of the potentially hostile environmental parameters, conversion of the chemical compound to a more stable prodrugs or formulation of novel dosage forms including monolithic/reservoir devices, use of excipients as stabilizers or micro- and nanoparticulate systems (Crowley 1999; Liversidge & Wei 2003). However, APIs can become fragile entities in hostile *in vivo* environments after administration in humans (*in vivo* instability). For instance, new fragile materials derived from biotechnological or other natural sources are a case in point. Therefore, this article will focus on physical and chemical stability of fragile active ingredients entrapped in nanocarriers to increase their shelf life (the amount of time a stored drug retains its activity) and half life (the amount of time it takes for one-half of a drug dose to be lost through biological processes).

Nanocarriers belongs to the drug-targeting paradigm initially theorized by Paul Ehrlich (Nobel Prize, 1908) in the “magic bullet” concept. As API properties significantly differ in chemical structure, molecular weight, hydrophilicity/lipophilicity or metabolism, the essential characteristics that govern efficacy are highly complex. Furthermore, many promising new compounds are compromised by inappropriate physicochemical properties that lead to poor biodistribution. An answer to these limits is to substitute API properties by the properties of nanometric carriers which could be designed to improve API pharmacokinetics and delivery. This concept implies a strong interaction between the API and the whole formulation before and after administration allowing to consider the potential stabilizing effect of the excipients. Based on the structure of nanocarriers, the nature of sensitive molecules (solubility, labile groups) as well as environmental factors, the problems, solutions and stability mechanisms have been devised to develop suitable nanovectors for sensitive drug substances.

## 2. General aspects

### 2.1. Chemical and physical stabilities

APIs, whether in solid, liquid, gas, or semisolid states, decompose or degrade at various rates. Such decomposition or degradation may be due to hydrolysis, oxidation, photodegradation, enzymatic degradation, effects of temperature and pH. The rate of degradation or decomposition varies considerably depending on the structural, physical and chemical nature of APIs. This rate is also often significantly affected by numerous environmental factors, including temperature, light, radiation, enzyme or other catalysts, pH and ionic strength of the solution, solvent type, and buffer species ([Liversidge & Wei 2003](#); [Baertschi & Reynolds 2005](#)).

Chemical instability due to degradation or decomposition is highly undesirable for several reasons. (1) Extensive drug degradation decreases its potency and shortens its effective shelf life. (2) Instability may alter dissolution/bioavailability and physical appearance of the dosage form causing product failures. (3) Degradation products may result in adverse effects or be unsafe for consumption. (4) Another importance of stability is requirement for approval by regulatory agencies ([Baertschi et al. 2005](#)).

Chemical degradation of molecules preferentially takes place when they are in solution that means being molecularly dispersed in a dissolution medium explaining why it should be interesting to keep API into solid state as long as possible ([Muller & Keck 2004](#)). However, physical instability often occurs in the solid state. In the solid state, it is well known that depending on the compound of interest and the conditions in which it was obtained, the molecules may be arranged in a regular pattern (crystalline material) or with disordered arrangements (amorphous material). Crystalline state is one of the most important parameters affecting solid drug stability, solubility, dissolution and efficacy ([Wu et al. 2011](#)). It is well known that more amorphous substances show higher solubility than the crystalline ones, while crystalline substances are physically more stable compared to the amorphous forms. Polymorphism is the ability of a compound to exist in more than one crystal with different unit cell parameters. Polymorphic forms of a drug substance can have different chemical and physical properties, including particle shape (habit), melting point, chemical reactivity, solubility, dissolution rate, optical and mechanical properties, vapor pressure, and density. Solvates are crystal forms

containing either stoichiometric or nonstoichiometric amounts of a solvent. If the incorporated solvent is water, the solvate is commonly known as a hydrate. This pseudo-polymorphism also generates significant differences in physical properties. A metastable pharmaceutical solid form can change crystalline structure or solvate/desolvate in response to changes in environmental conditions, processing, or over time. Thus, polymorphism can affect the quality, safety, and efficacy of the drug product (FDA 2007; Kawakami 2012). Crystal forms of API are evaluated in developmental studies from three viewpoints: (1) definition of the existing API, (2) determination of the thermodynamic relationship of the existing API, and (3) finding of the most stable form (Kawakami 2012). The most thermodynamically stable polymorphic form of a drug substance is often chosen during development based on the minimal potential for conversion to another polymorphic form and on its greater chemical stability. However, a metastable form can be chosen for various reasons, including bioavailability enhancement. Because drug product stability is affected by a multitude of other factors, including formulation, manufacturing process, and packaging, it is the stability of the drug product and not stability of the drug substance polymorphic form that should be the most relevant measure of drug quality. This scrutiny is essential to ensure that polymorphic differences are addressed via design and control of formulation and process conditions to physical and chemical stability of the product over the intended shelf-life, and bioavailability/bioequivalence (FDA 2007). Indeed, as a large range in equilibrium solubilities, and then dissolution rates, can exist for the various crystal forms of a same compound this can lead to significant and dramatic differences in dissolution kinetics as it is illustrated by the frequently told Norvir capsules story (Bauer et al. 2001).

Mechanisms involved in the stability of small and large biomolecule formulations are different due to their molecular structure differences. A small molecule drug is defined as a low molecular weight non-polymeric organic compound while large biomolecules refer to large bioactive molecules such as proteins, peptides and nucleic acids (DNA, ODN, siRNA). One of the major issues with macromolecule stability is to maintain the native 3D conformational integrity in order to keep their biological activities, whilst there is no such concern for small organic molecules (McNally & Hastedt 2007). Recent advances in the fields of biotechnology and genetic research have resulted in promotion of proteins and

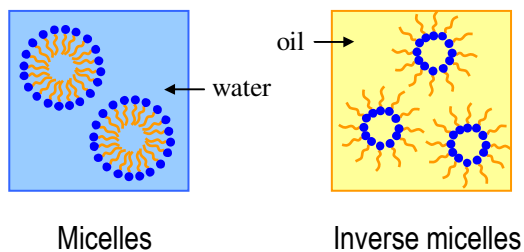
peptides as a major class of therapeutic agents. The U.S. Food and Drug Administration has approved nearly 80 protein drugs, of which 80% are recombinant proteins successfully used for the treatment of a broad range of diseases, and many more are under clinical investigation (Umashankar et al. 2010). However, these large biomolecules often have short half lives *in vivo* mainly due to enzymatic degradation (Fu et al. 2000; Zhu et al. 2000).

## 2.2. Overview of the main nanocarriers

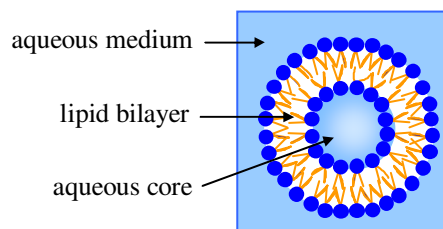
Conceptually, nanotechnologies in general and liposomes or nanoparticles in particular have revolutionized the administration of medicines. Liposomes were discovered by Bangham in 1968 (Bangham & Haydon 1968). The development pipeline of nanoparticles is focused on biodegradable materials and nanoparticle surface functionalization allowing new treatments strategies. The first paper on nanoparticles was published in 1976 by Peter Speiser (Kreuter 2007), a pioneer in the conception of nanoparticles. Today, the development of nanoparticulate systems which contain medicines (nanomedicines) has made it possible to increase the therapeutic index of many active ingredients (improvement of the activity, reduction of toxicity) by selectively directing them towards the diseased tissues and cells (drug targeting) (Couvreur 2012).

This section will present the most frequent nanostructures developed in the pharmaceutical field including micelles, polymeric nanoparticles, liposomes, microemulsions, nanoemulsions, solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), lipid nanocapsules and drug nanocrystals (Figure R1). The understanding of the design of the nanocarriers and the way they incorporate the drugs are very important to understand their putative protection effect against *in vitro* and *in vivo* degradation.

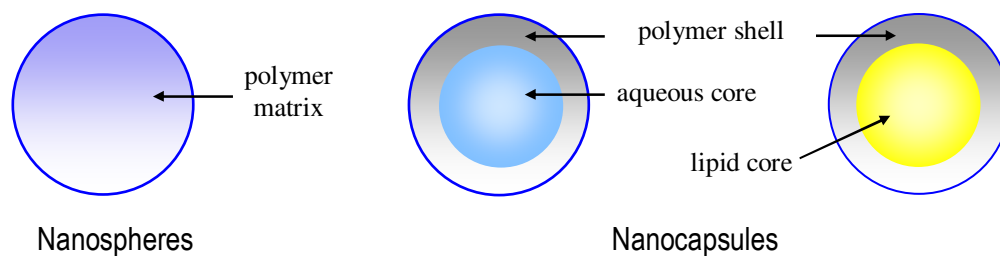
A. Micelles



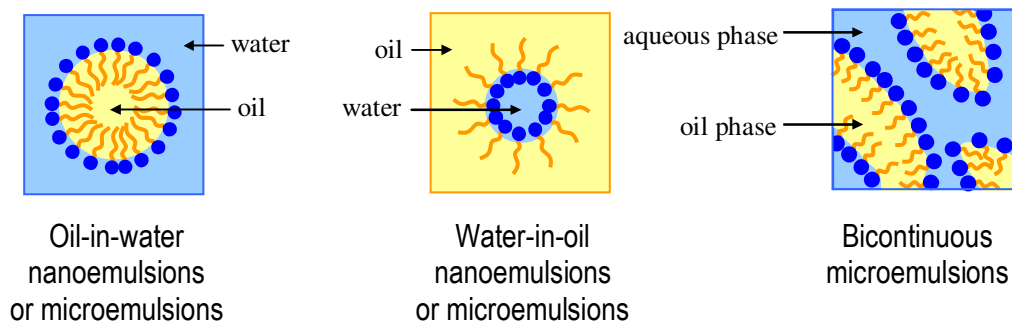
B. Liposomes



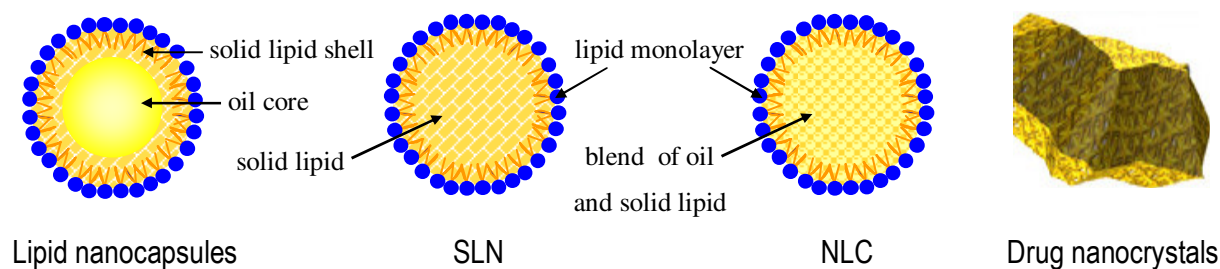
C. Polymeric nanoparticles



D. Nanoemulsions and microemulsions



E. Lipid nanoparticles and drug nanocrystals



**Figure R1.** Basic structure of (A) micelles, (B) liposomes, (C) polymeric nanoparticles, (D) nanoemulsions and microemulsions, (E) lipid nanoparticles and drug nanocrystals.

**Micelles** consist of a core and shell structure. They belong to a group of dynamic colloids, which form spontaneously under certain concentration (Critical Micellar Concentration, CMC) and temperature from amphiphilic agents such as surfactants or amphiphilic polymers, molecules of which consist of two clearly distinct regions with opposite affinities towards a given solvent. In aqueous media, hydrophobic fragments of amphiphilic agents form the core of a micelle, while hydrophilic fragments form the micelle's shell. When used as drug nanocarriers, micelles solubilize poorly soluble APIs within the micelle core, while more hydrophilic APIs could be distributed along the amphiphilic agents in intermediate positions (mixed micelles) or eventually adsorbed on the micelle ([Torchilin 2007](#); [Kedar et al. 2010](#); [Xiong et al. 2011](#)).

**Liposomes** are spherical, self-closed structures formed by one or several concentric lipid bilayers with an aqueous phase inside and between the lipid bilayers. Liposomes can entrap water-soluble substances in their internal water compartment and water-insoluble actives into the membrane ([Torchilin 2005](#)).

**Nanoemulsions** (mini-emulsions) are kinetically stable systems obtained by shearing a mixture of two immiscible liquids with surfactant and mostly covering the droplet size range from 50 to 500 nm. **Microemulsions** are optically transparent and thermodynamically stable dispersion systems of at least three components: a polar and a non-polar liquid phase (water and oil, respectively) and a surfactant. Microemulsions form spontaneously, without the need of high energy processes. Three basic types of microemulsion have been defined: direct (oil-in-water) droplets, reversed (water-in-oil) droplets and bicontinuous systems. In the case of direct microemulsion, the droplet size is typically between 10 and 100 nm ([Tadros et al. 2004](#); [Mason et al. 2006](#); [Burguera & Burguera 2012](#)).

**Nanoparticles** for pharmaceutical purposes are defined as solid colloidal particles ranging in size from 1 nm to 1000 nm. They consist of macromolecular materials, such as lipids and polymers, and can be used therapeutically as drug carriers, in which therapeutic entities, such as small-molecule drugs, peptides, proteins and nucleic acids, entrapped or encapsulated, or to which the active principle is adsorbed or attached ([Davis et al. 2008](#); [Petros & DeSimone 2010](#)).

**Polymer nanoparticles** are a collective term given for any type of polymer nanoparticle. **Nanospheres** are matrix particles, i.e., particles whose entire mass is solid and active molecules may be adsorbed at the sphere surface or encapsulated within the particle. **Nanocapsules** are vesicular systems, acting as a kind of reservoir, in which the entrapped substances are confined to a cavity consisting of a liquid core (either oil or water) surrounded by a solid material shell ([Rao & Geckeler 2011](#)).

**Lipid nanoparticles** can simply be derived from oil-in-water nanoemulsions by replacing the liquid lipid (oil) by a solid lipid. The first generation of **solid lipid nanoparticles (SLN)** is solid particle matrix at the physiological temperature. In the second generation technology, the **nanstructured lipid carriers (NLC)**, the particles are produced by using a blend of a solid lipid with a liquid lipid, this blend also being solid at body temperature. The advantage of the second generation technology is the increased loading with actives compared to SLN and firmer inclusion of the active inside the particle matrix during the shelf life. By preparing the particles from a solid lipid, especially highly purified solid lipids, the particle matrix tends to form a relatively perfect crystal lattice leaving limited space to accommodate the API. This limits the loading capacity and can lead to expulsion of API from the lipid matrix during storage. In contrast, the use of a lipid mixture with very differently structured (acyl chain lengths for example) molecules distorts the formation of a perfect crystal. The particle matrix contains many imperfections providing space to accommodate the active in molecular form or as amorphous clusters. One could state that “the perfectness” of the NLC system is its “imperfectness” in its crystalline structure ([Müller et al. 2002](#); [Müller et al. 2011](#)). Besides, **lipid nanocapsules**, vesicular structures containing an oil core surrounded by a lipid wall based on triglycerides and surfactant molecules, has been developed using the properties of the phase inversion process of an emulsion ([Heurtault et al. 2003](#)). Due to the lipophilic nature of the particle matrix, lipid nanoparticles are predestined for incorporation of poorly water-soluble, lipophilic compounds ([Müller et al. 2002](#)).

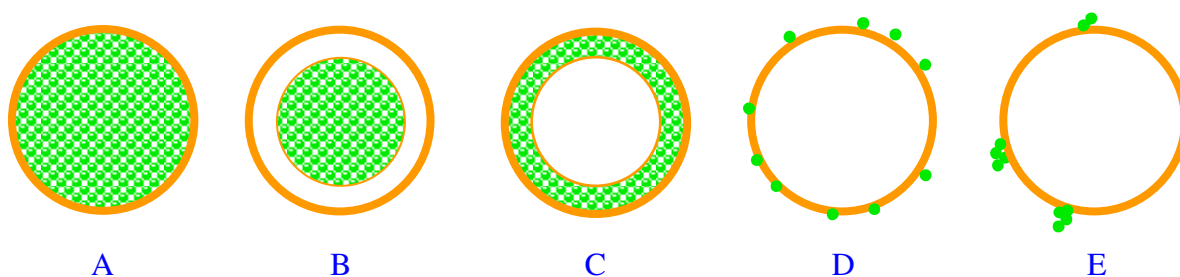
**Drug nanocrystals** are particles made from almost 100% of API and are stabilized by surfactants or polymeric steric stabilizers. This approach generally produces dispersions of drug nanocrystals in a liquid medium (typically water), which are called

“nanosuspensions”. Nanosuspensions can be used for compounds that are water insoluble but which are soluble in oil (high  $\log P$ ), although other lipidic systems, such as liposomes and emulsions, can be used to formulate these compounds as well. In contrast with lipidic systems, nanosuspensions can also be used to successfully formulate compounds that are insoluble in both water and oil ([Rabinow 2004](#); [Müller & Keck 2011](#)).

### 3. General factors in optimizing the stability of APIs entrapped in nanocarriers

#### 3.1. Drug location

Strategies to address chemical stability issues of labile APIs are usually designed according to incorporation of the drugs into a nanovector. Methods for incorporating a fragile API into engineered nanoparticles, with regard to the ultimate delivery of drugs to the desired location *in vivo*, can be classified into two broad categories. In one category, API is physically entrapped in or absorbed onto the nanoparticle through non-covalent interactions. The second category includes examples in which API has been directly attached to the nanoparticle matrix by degradable or non-degradable covalent bonds and frequently coupled with activated release ([Petros & DeSimone 2010](#)). For incorporation of drugs in the nanocarriers through non-covalent interactions, API can be dissolved and/or dispersed homogeneously in the matrix. They can be entrapped only in the core or located in the shell of particle. Besides, active molecules and/or aggregates/agglomerates can be adsorbed on the surface ([Figure R2](#)).



**Figure R2.** Incorporation of drugs in the nanocarriers through non-covalent interactions. **(A)** API dissolved and/or dispersed homogeneously in the matrix; **(B)** API entrapped in the core of particle; **(C)** API located in the shell of particle; **(D)** API adsorbed on the surface; **(E)** Aggregates/agglomerates of API adsorbed on the surface.

The drug adsorption on the surface contributes significantly to initial burst effect defined as the initial release of a large bolus of API ([Huang & Brazel 2001](#)), and makes it easily

accessible to the degradation conditions *in vitro* et *in vivo*. Overall, the long-term stability of chemical compounds depends on drug distribution in the nanosystems studied. In particular, the localization of the sensitive groups in a nanovector is crucial for its stability. Fragile functional groups must be carefully hidden inside nanocarriers. Besides, the solubility of sensible drug substance should be considered in nanovector selection improving stability of labile drugs. It is possible that hydrophilic molecules might be entrapped into the aqueous core of nanocarriers such as inverse micelles, liposomes, polymer nanocapsules, water-in-oil nanoemulsions or microemulsions. In contrast, amphiphilic or lipophilic molecules are usually encapsulated into micelles, lipid nanoparticles, nanocrystals, polymer nanospheres, polymer oil-core nanocapsules, oil-in-water nanoemulsions or microemulsions and lipid bilayer of liposomes.

### 3.2. Drug-loading capacity

Nanocarriers can carry a large payload of drug entity and protect it from degradation. For example, a 70 nm nanoparticle can contain approximately 2000 small interfering RNA (siRNA) molecules, while a liposome of Ambisome<sup>®</sup> having the same diameter will carry ~5000 molecules of amphotericin B and a liposome of Myocet<sup>®</sup> (180 nm) contains ~90000 molecules of doxorubicin. These high payload amounts can also be achieved with other drug types such as small-molecule or peptide drugs (Davis et al. 2008). Ideally, a successful nanoparticulate system should have a high drug-loading capacity and a crystalline structure of API in the nanoparticulate system results in higher drug stability (Liversidge & Wei 2003). Generally, the more the active ingredient is soluble in the components constituting the nanocarriers, the more the drug payload increases. Design and development of specific nanoformulations and/or preparation methods remain an important role to improve drug entrapment capacity in nanovectors. As an interesting example of formulation applied to solve the encapsulation of labile and hydrophilic compounds in lipid nanoparticles, the polypeptide insulin was successfully encapsulated in SLN by incorporating the drug in reverse micelles loaded in the lipid matrix using a double emulsion method (Liu et al. 2008). In brief, based on the nature of molecule, especially labile moieties and its solubility, suitable nanoformulations will be performed to avoid interaction with the catalytic agents for the drug degradation process.

### 3.3. Stability of colloidal carriers

Drug-loaded nanoparticulate systems have to satisfy chemical and physical stability criteria of both nanocarriers and active ingredients. Stability of nanosystems is one of the critical aspects in ensuring safety, efficacy of drug products and notably the protection of sensitive APIs. The stability issues of nanocarriers could arise during manufacturing, storage, shipping (*in vitro* stability) (Wu et al. 2011) as well as after administration (*in vivo* stability) (Muller & Keck 2004).

As regards the *in vitro* stability, the extensive available literature reviews are given on physical and chemical instability of nanodispersions including flocculation, creaming, sedimentation, coalescence, gelling phenomena, inversion phase, Ostwald ripening, separation, crystallization - polymorphism (change of crystalline state), crystal growth, drug expulsion, fusion, degradation of lipid and polymer. There are two main mechanisms through which colloidal dispersions can be physically stabilized in both aqueous and non-aqueous medium: electrostatic repulsion and steric stabilization. These two mechanisms can be achieved by adding ionic and non-ionic stabilizers into the medium, respectively (Heurtault et al. 2003; Tadros et al. 2004; Muller & Keck 2004; Wu et al. 2011; Thorat & Dalvi 2012).

Concerning the *in vivo* stability, the fate of nanocarriers in the body, long or short circulation times depend on size, surface and shape properties of nanovectors (Yoo et al. 2011). A lot of studies have been done on the optimal size of nanocarriers. In the extremities, particles less than 5 nm are rapidly cleared from the circulation through extravasation or renal clearance. Particulate matter larger than ~15 µm is removed from the circulation by mechanical filtration in capillaries and can be lethal depending on the dose. For rigid, spherical particles, ones that are 10 – 200 nm in size have the highest potential for prolonged circulation because they are large enough to avoid uptake in the liver, but small enough to avoid filtration in the spleen (Moghimi et al. 2001; W. Li & Szoka 2007; Davis et al. 2008; Petros & DeSimone 2010; Yoo et al. 2011). With the recent expand in research on particle morphology, it is now clear that shape plays a significant role in drug delivery. For example, a spherical particle moving through a vessel does not deviate from its streamlined motion unless it experiences an external force. On the other hand, nonspherical particles are susceptible to tumbling and torque, resulting in higher

tendency to move towards the vessel wall (Decuzzi et al. 2010; Yoo et al. 2011). Concerning the properties of the surface, the particle surface attribute has three vital roles in the function of engineered nanoparticles with regard to the ultimate delivery of the drugs to the desired location *in vivo*. First, surface chemistry is known to heavily influence the process of opsonization, which ultimately dictates the RES response. Second, to achieve cellular targeting, ligands known to bind cell surface receptors of selected cells should be included in the design of engineered nanoparticles. Third, if organelle targeting is also required, those ligands must be incorporated into surface design (Petros & DeSimone 2010). Particularly, the effect of pegylation (the decoration of nanoparticles with polyethylene glycol (PEG), a hydrophilic and flexible polymer) on particle surface to prolong their blood circulation time plays an important role in function of nanoparticles (Harris & Chess 2003). For example, Doxil® (Ortho Biotech), a PEG-liposome containing doxorubicin, circulates in the body as a nanoparticle and has a half-life ~100-times longer than free doxorubicin (Sutton et al. 2007).

### 3.4. Dispersion medium and storage conditions

Drug nanoformulations are usually prepared in aqueous media. Hydrolysis and/or oxydation in aqueous media are considered to play a significant role in the degradation of both labile drugs and excipients. Thus, the various nanodispersions should be transformed in a dry powder form. To transform aqueous nanodispersions into dry powder, one can use lyophilization and spray drying procedures. Spray drying is an efficient technology for solid dispersion manufacturing since it allows extreme rapid solvent evaporation leading to fast transformation of an API-carrier solution to solid API-carrier powder (Paudel et al. 2012). However, spray drying usually requires high temperature in the process which is not suitable for thermolabile drugs. In such cases, lyophilization is the most convenient procedure. Lyophilization of nanoparticulate systems can be performed to overcome either physical or chemical incompatibility, permitting recovery of the original particle size after reconstitution (Rabinow 2004). In the lyophilization process, classical cryoprotective agents such as mannitol, trehalose, maltodextrin or sucrose are added to the nanoparticulate systems to protect them from freezing damage due to ice formation, to minimise the particle size growth during lyophilisation (Lai et al. 2011), and to avoid particle aggregation after reconstitution of the system (Teeranachaideekul et al. 2008).

In addition, light and heat are known to initiate the reaction leading to the formation of free radical. This free radical combining with the oxygen molecule causes the propagation step and finally the oxidation progresses to the termination step (chain reactions). Elimination of oxygen (flushing with nitrogen gas or inert gas) and storage at the low temperature in the dark can therefore minimize the chain reactions and the oxidation reaction can then be retarded. The combined antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) and vitamin E were also used to enhance the chemical stability of labile drugs ([Teeranachaideekul et al. 2007](#)).

#### 4. Chemical stability of labile APIs in nanodispersions

The potential nanosystems to prevent or retard degradation will be determined by exposure of representative samples of labile drug-loaded nanoformulations to relevant hostile environments including water, oxygen, light and temperature for *in vitro* instability; pH, enzyme and other causes for *in vivo* instability. This part will present typical examples of nanodispersions as promising delivery systems improving shelf life and half life of sensitive APIs. In addition, protection mechanisms of nanoparticulate systems based on the structure of nanocarriers, the nature of sensitive molecules are also discussed.

##### 4.1. Nanocarriers for improvement of shelf life

Drug substances degrade to different extents on separate exposure to water, oxygen, light and heat. A combination of these stresses causes very complex behaviour ([Glass et al. 2004](#)). Thus, effect of nanoparticulate systems on the inherent stabilities of the sensitive active ingredients has been discussed for the following stress conditions: (1) hydrolysis, (2) oxidation, (3) photolysis and (4) thermal degradation.

###### 4.1.1. Hydrolysis

Hydrolysis is the cleavage of a chemical species by water. Since water, either as a solvent or as moisture in the air, contacts most pharmaceutical dosage forms to some degree, the potential for this degradation pathway exists for most drugs and excipients ([Waterman et al. 2002](#)). In general, hydrolysis is one of the most common reactions seen in the APIs having ester and amide functional groups within their structure ([Sehrawat et al. 2010](#)).

Curcumin, a natural diphenolic compound insoluble in water, is a prominent candidate treating anticancer and several other diseases. It is well known that curcumin undergoes rapid hydrolytic degradation in physiological pH (Wang et al. 1997). For improving desirable effects in solubilizing and stabilizing curcumin, various types of nanoparticles, such as polymeric micelles, polymer nanoparticles, liposome, nano-microemulsions, nanogels, SNL, polymer conjugates were established for the delivery of curcumin active form to tumors (Yallapu et al. 2012). The aggregation of surfactant molecules, including sodium dodecyl sulfate, cetyltrimethyl ammonium bromide, Tween 80, Triton X-100 and pluronic polymers, form micelles above CMC and can provide the required influence to stabilize curcumin molecules (Wang et al. 2010). However, cationic micelles provide greater stability to curcumin even at pH 13 and are preferred for their medicinal implications (Leung et al. 2008). Similarly, plasma proteins (human serum albumin and fibrinogen) have also been recognized as carriers for curcumin because of their ability to stabilize curcumin molecules (Leung & Kee 2009). In another study, curcuminoid-loaded SLN consisted of stearic acid and glyceryl monostearate with surfactants were found to be stable during 6 months at room temperature and gave prolonged *in vitro* release of curcuminoids up to 12 h. Furthermore, the light and oxygen sensitivity of curcuminoids was strongly reduced by incorporating curcuminoids into this unique type of formulation (Tiyaboonchai et al. 2007). Concerning polymeric nanoparticles, the amount of curcuminoids loaded in poly(butyl) cyanoacrylate nanoparticles showed no significant changes compared to the freshly nanoparticles after storage for 6 months at 40 °C / 75% RH in the presence and absence of sunlight (Mulik et al. 2009). For liposomal systems, fluorescence quenching experiments revealed that curcumin locates in the hydrophobic region of egg yolk phosphatidylcholine liposomal bilayer and was efficiently protected against hydrolysis (Karewicz et al. 2011). Generally, thanks to curcumin location in lipophilic region of nanosystems, alkaline hydrolysis of curcumin is suppressed even in fully hydrated systems like liposomes or micelles.

#### 4.1.2. Oxidation

Oxidation is a well established degradation pathway for APIs. Oxygen which is required in most oxidation reactions and is abundant in the environment to which pharmaceuticals are exposed, during either processing or long term storage (Sehrawat et al. 2010). Oxidation is probably second only to hydrolytic breakdown as a cause of loss of quality. Oxidation

can be catalysed by exposure to air or light, the presence of trace residues (for example, heavy metals), or by other components (e.g., from excipients) in the formulation. It can also result from a combination of all of these (Crowley 1999).

Ascorbyl palmitate, an ascorbic acid derivative, is as a model drug for nanosized lipid carriers. As an antioxidant, ascorbyl palmitate has been also used in the cosmetics, food, and pharmaceutical industries. However, its practical application is restricted because of its oxidation-induced poor solubility and instability (Moribe et al. 2011). The chemical stability of ascorbyl palmitate could be achieved by carrier systems using microemulsions, liposomes, and SLN. Ascorbyl palmitate was resistant against oxidation in the order of non-hydrogenated soybean lecithin liposomes > SLN > w/o and o/w microemulsions > hydrogenated soybean lecithin liposomes. The hidden location of the nitroxide group of ascorbyl palmitate inside nanocarriers is crucial to its stability (Kristl et al. 2003). In an another study, nondegraded ascorbyl palmitate content in NLC, SLN, nanoemulsions after 3 months was found to be 71.1%, 67.6% and 55.2% respectively (Uner et al. 2005) while the stability of the same API seems not adequate in microemulsions (Spiclin et al. 2001). All these results suggested that the nanocarrier structure is important to the maintenance of ascorbyl palmitate integrity. An improvement of long-term chemical stability of ascorbyl palmitate in NLC was also achieved by addition of combined antioxidants (butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) and vitamin E) with nitrogen gas flushing as compared to those without antioxidant. The percentage of ascorbyl palmitate remaining at both 4°C and room temperature was higher than 85% during 90 days of storage (Teeranachaideekul et al. 2007).

#### 4.1.3. Photodegradation

Exposure to light can precipitate a plethora of degradation reactions. These can include addition reactions in unsaturated systems, substitution reactions, polymerization, isomerization and photo-oxidation (Crowley 1999). Interactions between the API and optical radiation can result in the formation of free radicals and/or reactive oxygen species leading to the degradation and/or decomposition of the drug substance itself (Glass et al. 2004). Suitable light-resistant packaging can, in many cases, prevent or reduce degradation. However, degradation during product use is also a possibility and it might be necessary to stabilize the formulation itself (Crowley 1999).

Tretinoin (all-trans retinoic acid) is employed in the topical treatment of various skin diseases including acne and psoriasis. However, its use is strongly limited by side effects and high chemical instability by photodegradation ([Ridolfi et al. 2012](#)). Tretinoin-loaded poly-caprolactone nanocapsules improved tretinoin photostability, independently on the type of oily phase used. The better protection presented by the nanocapsules against ultraviolet (UV)-induced photodegradation of tretinoin could be attributed due to the crystallinity of the polymer, which has the ability of reflecting and scattering UV radiation ([Ourique et al. 2008](#)). Hydrogels containing nanoencapsulated tretinoin demonstrated a lower photodegradation (24.2%) than the formulation containing the non-encapsulated drug (68.6%) after 8 h of UVA irradiation (315-400 nm). The half-life of the former (26.6h) was seven times higher than the latter (3.8h) ([Ourique et al. 2011](#)). In another example, the photostability testing for different nanostructured systems containing clobetasol propionate (a corticosteroid used for the treatment of skin disorders) demonstrate that photostability of this molecule under UVA radiation was improved by its incorporation into nanosystems (polymeric nanocapsules > nanoemulsions > polymeric nanospheres) ([Fontana et al. 2009](#)). The results suggested that both the carrier structures and the excipients used have significant role to the enhancement of photostability.

#### 4.1.4. Thermal degradation

Thermolytic degradation is usually thought of as degradation caused by exposure to temperatures high enough to induce bond breakage. The following list of degradation pathways can be considered as thermolytic pathways: hydrolysis/dehydration, isomerization/epimerization, decarboxylation, rearrangements and some kinds of polymerization reactions. Note that hydrolytic reactions are a subset of thermolytic pathways, but oxidative and photolytic reactions are not primarily driven by temperature ([Jansen & Baertschi 2005](#)). Encapsulating labile drug substances in nanosystems can inhibit synergistic effect of temperature on degradation rate of drugs. Moreover, thermal stability-related issues can affect many areas, including the following: selection of optimal formulation, appropriate storage conditions, shelf-life determination of product and processing parameters in manufacturing.

Coenzyme Q10, a well known antioxidant, is a highly lipophilic compound sensitive to light and temperature. Thermal and photostability of coenzyme Q10 encapsulated within

poly(methyl methacrylate) nanoparticles were examined. An aqueous dispersion of coenzyme Q10 with 0.5% (w/v) of sodium dodecylsulfate (SDS) in deionized distilled water and a solution in caprylic and capric triglycerides (CCT) oil were used as a control. Stress testing of thermal stability of coenzyme Q10 was performed as a function of incubation time at 45°C for 25 days. The hydrophobic polymer matrix of nanoparticles improved thermal stability of coenzyme Q10 from aqueous environment. While degradation to a large extent was observed in the SDS suspension and the CCT oil solution, the stability of coenzyme Q10 encapsulated within the polymer nanoparticles was much better, resulting in a range of 84–88% of residual intact coenzyme Q10 after 25 days. Furthermore, when coenzyme Q10 was exposed to UVA illumination, it gradually decomposed and, after 144 min, the residual percentage of intact coenzyme Q10 was about 48% in the SDS dispersion and 35% in CCT solution. However, the stability of coenzyme Q10 encapsulated within poly(methyl methacrylate) nanoparticles dramatically maintained at 68–78%. This enhanced photo-resistance might be attributed to the physical interference of UV light with polymer nanoparticles ([Kwon et al. 2002](#)).

#### 4.2. Nanocarriers for increase of half life

*In vivo* instability of drug substances after administration can be relevant for LADMER system (schematic of liberation, absorption, distribution, metabolism, excretion, and response of a drug product). For the fate of API in human, drug degradation causing loss of its therapeutic potency almost occurs via nonenzymatic breakdown under effect of pH or enzymatic breakdown. Many biologically active compounds, including various large molecules, need to be delivered intracellularly to exert their therapeutic action inside cytoplasm or onto nucleus or other specific organelles, such as mitochondria. However, the lipophilic nature of the biological membranes restricts the direct intracellular delivery of such compounds. Moreover, large molecules such as DNA, SiRNA, oligonucleotides which are internalized via endocytosis and transferred within endosomes, end in lysosomes resulting in their degradation by lysosomal enzymes. So although many compounds show a promising potential *in vitro*, they cannot be used *in vivo* due to poor bioavailability problems ([Gupta et al. 2005](#)). Nowadays, encapsulating a pH-sensitive API or a drug-degrading enzyme into a nanovector in order to increase its half-life and enhance its deposition in targets seems potentially interesting to formulators.

#### 4.2.1. pH sensibility

Different parts of the body have different optimal pH levels, such as the pH of gastric acid varies from 1.3 to 3.5; the pH of intestinal fluids is in range from 6 to 8, whilst human blood stays in a very narrow pH range 7.35 - 7.45. The active ingredient can be sensitive to pH at release site or site of action. For example, a number of substances usable in the treatment of *Helicobacter pylori* and stomach ulcers are unstable under acid condition in the human stomach lumen, e.g. amoxicillin, clarithromycin, omeprazole (Ramteke et al. 2009), erythromycin (Padovan et al. 2012); many substances are not stable under neutral to moderately basic conditions, e.g. camptothecins (Hao et al. 2005), quercetin (Moon et al. 2008), curcumin (Yallapu et al. 2012). It is believed that the formulation of drugs as nanoparticles could represent today a good solution to protect pH-sensitive drugs against degradation in the drug development pipeline.

Camptothecins, anticancers as parent camptothecin, 9-nitrocamptothecin, Db-67, Sn-38 and topotecan, are chemically labile APIs demonstrating a reversible hydrolysis of the lactone ring to an inactive carboxylate at neutral pH (Hao et al. 2005). Highly stable formulations are required to take full advantage of the EPR effect in treating solid tumors (acidic pH), in maximizing the duration of exposure in blood, and for molecular targeting using ligands to direct the nanovector-encapsulated drug specifically to target cancer cells where the drug can then be released locally (Drummond et al. 2010). In case of Sn-38 lipid nanocapsules for oral administration, the active lactone ring was encapsulated into the lipid core and was protected by the shell composed of Solutol<sup>®</sup>, a non-ionic surfactant (polyethyleneglycol 660-12-hydroxystearate) *In vitro* stability in gastrointestinal fluids (simulated gastric fluid, simulated intestinal fluid in both fasted and fed states), Sn38 remains encapsulated in lipid nanocapsules in both media (less than 10% are release) after 6 h. Therefore, Sn38-loaded lipid nanocapsules with a mean particle size of  $38 \pm 2$  nm was interesting in order to increase transport by endocytosis across the intestinal barrier as the size is inferior to 100 nm (Roger et al. 2011). In another study, the liposomal encapsulation improved the stability of topotecan and changed its distribution pattern *in vivo*. However, the liposome membrane fluidity has effect on the *in vitro* and *in vivo* behavior of topotecan liposomes. Liposomes were prepared with unsaturated soybean phosphatidylcholine which has a phase transition temperature lower than body temperature, and hydrogenated soybean phosphatidylcholine

which has a higher phase transition temperature than body temperature. Compared with 'fluid' liposomes composed of soybean phosphatidylcholine, the hydrogenated soybean phosphatidylcholine liposomes containing topotecan were more stable *in vitro* and *in vivo*, increasing the plasma concentration and the accumulation of topotecan in tumor. One of the possible reasons is that the higher stability of hydrogenated soybean phosphatidylcholine liposome in blood, and then its lower membrane permeability, decreases the leakage of drug from the liposome. The other reason is probably that the rate of clearance of hydrogenated soybean phosphatidylcholine liposome from the blood is lower than that of soybean phosphatidylcholine liposome. Furthermore, topotecan was well encapsulated into liposomes. The acidic intraliposomal environment formed during drug loading by ammonium sulfate gradient method provides another important advantage on increasing the lactone stability of topotecan. In addition, PEG-modified hydrogenated soybean phosphatidylcholine liposome of topotecan prolonged the circulation time, decreased the uptake of liposome by RES, decreased the distribution of topotecan in bone marrow, and increased the tumor accumulation compared with the unmodified hydrogenated soybean phosphatidylcholine liposome (Hao et al. 2005). In another study, anti-EGFR and anti-HER2-immunoliposomal formulations prolonged API circulation lifetimes *in vivo*. They dramatically increased uptake of topotecan, compared to nontargeted liposomal topotecan and poorly permeable free topotecan, in receptor-overexpressing cancer cell lines with a corresponding increase in cytotoxicity in multiple breast cancer cell lines and improved antitumor activity against HER2-overexpressing human breast cancer (BT474) xenografts (Drummond et al. 2010). Other studies have also reported that lactone stability and antitumor efficacy of topotecan-loaded lipid nanoparticles (SLN and NLC) (Souza et al. 2011) or camptothecin-loaded SLN (Huang et al. 2008) were improved in comparison to camptothecin solutions.

Another example, the stability of amoxicillin, clarithromycin, and omeprazole in simulated gastric fluid (pH 1.2) was also improved by encapsulation of these molecules into chitosan–glutamate nanoparticles which reduced acid degradation and helped to maintain effective concentration for longer time at the site of action, whereas all the three drugs were degraded in acidic pH within 3h (Ramteke et al. 2009). Liposomal membranes are also able to maintain a proton concentration gradient sufficient to protect these acid sensitive antimicrobial agent (Bardonnnet et al. 2009).

#### 4.2.2. Enzymatic degradation

Enzymes in the body have two main types: digestive enzymes (Ulleberg et al. 2011) and metabolic enzymes (Choi et al. 2011; Xiong & Guan 2012). They play a very important role in the drug degradation processes. After *in vivo* administration, a lot of active substances are quickly destroyed by enzymatic pathway (Evans 2004). For the protection of drugs from enzymatic degradation / metabolism, nanoparticles may be considered as an effective solution.

Insulin, a peptide used in the treatment of diabetes mellitus, has low stability and poor absorption by oral administration. The ability of liposomes to prevent the enzymatic degradation of insulin in gastrointestinal tract was investigated by using the intestinal fluid obtained in rats. Free insulin degraded rapidly according to first order kinetics in the intestinal fluid whereas PEG-liposomes composed of dipalmitoylphosphatidylcholine (DPPC), cholesterol and distearoylphosphatidylethanolamine polyethyleneglycol 2000 (DSPE PEG) completely suppressed the degradation of insulin during 60 min and uncoated liposomes suppressed it only partially. For hypoglycemic effects of insulin after oral administration, PEG-liposomes showed the longer period of hypoglycemic action. From PEG-liposomes, essentially no leakage of insulin was observed, suggesting the strong resistivity against the digestion by bile salts. The PEG surface coating of liposomes was effective to avoid the direct interaction of bile salts with lipid membrane. Since PEG molecules are well hydrated in an aqueous solution, a thick water layer was formed on the surface of PEG-liposomes which can prevent the direct interaction of bile salts with lipid membrane (Iwanaga et al. 1997). Similarly, chitosan nanoparticles have been commonly regarded as potential carriers for the mucosal delivery of therapeutic peptides because of their biocompatibility, bioadhesion, permeation enhancing properties and ability to protect the entrapped peptides against degradation. Following oral administration in rat, insulin-loaded chitosan nanoparticles formulated by ionic cross-linking with hydroxypropyl methylcellulose phthalate increased the hypoglycemic effect of insulin by more than 9.8-folds as compared to oral insulin solution (Makhlof et al. 2011).

Concerning the development of colloids for siRNA delivery, nanovectors can entrapped these large hydrophilic molecules bypassing the hydrophobic plasma membrane barrier to reach their target mRNA in the cytoplasm and avoiding the enzymatic breakdown

(Ezzat et al. 2012). SiRNA–glycol chitosan–polyethylenimine nanoparticles (siRNA–GC–PEI nanoparticles) serve as a good example to illustrate this. In order to test the stability of siRNA–GC–PEI nanoparticles against nuclease, free siRNA and siRNA–GC–PEI nanoparticles were incubated with 0.3 U of RNase A up to 6 h at 37°C. In case of free nucleic acid, siRNAs were fully degraded in the enzymatic environment within 30 min, indicating their great instability in presence of nuclease. However, the siRNA–GC–PEI nanoparticles presented a distinguishable protecting effect of encapsulated siRNAs against RNase A. To evaluate whether the siRNA–GC–PEI nanoparticles can indeed specifically target the SCC7 tumors, the *in vivo* biodistribution in SCC7 tumor-bearing mice was studied. After intravenous injection, free siRNA was rapidly excreted from the kidney within 1 h post-injection, due to the renal clearance of hydrophilic molecules. Contrary, with siRNA–GC–PEI nanoparticles-treated mice, a substantial NIR fluorescence intensity persisted in the whole body up to 2 days, indicating a sustained blood circulation profile of siRNA–GC–PEI NPs (Huh et al. 2010).

## 5. Additional stability issues relate to macromolecules

For proteins, peptides and gene delivery, the large molecular structures of the API of interest are distinctly different and more complicated compared to that of small molecules. The structures of these macromolecules are generally differentiated into four structures based on weak non-covalent interactions, i.e. primary, secondary, tertiary and quaternary structures. To maintain their therapeutic efficacy, it is essential to preserve the native structure these large biomolecules. Using “naked”, proteins/peptides/genes have various instability problems such as deamidation, oxidation, acylation, aggregation and unfolding. Moreover, the macromolecule delivery at a cellular or subcellular targeting level can be hindered by their three-dimensional structure, spatial occupation, hydrophilic / lipophilic nature and rapid enzymatic degradation. Currently, a possible strategy to prevent protein/peptide/gene denaturation is to encapsulate the biomolecules into nanocarriers. In addition to improve the stability, macromolecule encapsulation can enhance bioavailability and provide controlled therapeutic release (Tan et al. 2010). Because of the high biochemical and structural complexity of macromolecules, several new delivery nanosystems emerges for therapeutic peptides, proteins and genes, e.g. polyplex, lipoplex (Huang et al. 1998), aquasomes, archaeosomes, cubosomes, hexosomes,

isasomes, enzymosomes, ethosomes, emulsomes, erythrosomes (proteoliposomes), cryptosomes, discomes, genosomes, novasomes, photosomes, transferosomes (elastic liposomes), proteosomes, vesosomes, virosomes (Umashankar et al. 2010). These systems join other more or less mature technology such as polymeric or lipid based microparticles (Decapeptyl and Nutropin depot) or hydrogels (Stolnik & Shakesheff 2009).

Among the “some” families, liposomes were probably the most investigated systems. Substantial improvements are being made with liposomes for small-drug delivery, until marketed pharmaceutical products, and lessons learnt from such studies may be useful for development of liposomes in case of macromolecule delivery. Unfortunately, even it is generally considered that liposomes have an important carrying capacity, it is not important enough to carry large molecules such as proteins. For that reason, it needs simple ways to improve encapsulation capacity and protein-loaded liposome stability (Dass & Choong 2006). DepoFoam, large and multivesicular liposomes give some very promising results with enkephalin or insulin (Ye et al. 2000). However, as their sizes range between few micrometers to hundreds micrometers, they don't belong to the scope of the present review. Aquasomes and isasomes are good examples of nanosize systems that are finding a niche as peptide and protein carriers. Aquasomes are three-layered self-assembled structures that comprise a central solid nanocrystalline core whose surface is noncovalently modified with carbohydrates to obtain a sugar ball, e.g. sucrose and trehalose, which is then exposed to adsorption of a therapeutic agent. The solid core provides the structural stability, while the carbohydrate coating protects against dehydration and stabilizes the large biomolecules. The surface modification with carbohydrates creates a glassy molecular stabilization film that adsorbs therapeutic proteins. Thus, these particles provide complete protection of an aqueous nature to the adsorbed drugs against the denaturing effects of external pH and temperature, because there are no swelling and porosity changes with change in pH or temperature. Aquasomes have been successfully utilized for the delivery of insulin, hemoglobin, enzymes and various antigens (Umashankar et al. 2010). Insulin-bearing aquasomes were prepared using a calcium phosphate ceramic core for parenteral delivery. This core was coated with various disaccharides such as cellobiose, pyridoxal-5-phosphate, or trehalose under sonication and was further loaded with the drug at low temperature by a partial adsorption mechanism. The *in vivo* performance of aquasome formulations

of insulin compared with standard porcine insulin solution was evaluated using albino rats. Prolonged reduction of blood glucose was observed with all formulations except cellobiose-coated particles. The blood glucose reduction and duration of action for pyridoxal-5-phosphate or trehalose-coated particles are significantly higher compared to porcine insulin solution. Pyridoxal-5-phosphate-coated particles were found to be more effective in reducing blood glucose levels than aquasomes coated with trehalose or cellobiose. This could be attributed to the high degree of molecular preservation by pyridoxal-5-phosphate. The prolonged activity was attributed to slow release of drug from the carrier and structural integrity of the peptide. The polyhydroxy oligomers act like water since they are enriched with hydroxyl groups and give a waterlike atmosphere. They may even replace the water around the polar residues in proteins, thereby protecting the drug molecule against dehydration and maintaining their integrity in the absence of water ([Cherian et al. 2000](#)).

Isasomes, internally self-assembled particles, are a relatively new family of colloidal particles containing nano-objects like cubosomes, hexosomes, micellar cubosomes, and emulsified microemulsions ([Yaghmur et al. 2006](#)). These nanostructured aqueous dispersions are spontaneously self-assembled multicompartiment objects, candidates for delivering API and especially peptides and proteins ([Angelova et al. 2011](#)). The fully hydrated bilayers of the amphiphile of interest create a thermodynamically stable network that separates water channels having pore diameters in the range of few nanometers. The driving force of these supra-molecular organizations is the curvature elasticity and the packing frustration of the constitutive compounds. The most important phases in this field are the inverted hexagonal phase ( $H_2$  space group) and the reversed bicontinuous cubic phases ( $Pn3m/Q^{224}$  or  $Im3m/Q^{229}$ ) leading to nanostructured aqueous dispersions named hexosome and cubosome, respectively. The main characteristics of these dispersions are the presence of water-filled nanochannels and their consequent interfacial area, few hundreds of  $m^2/g$ , allowing the solubilization of large amounts of amphiphilic components as well as hydrophilic or hydrophobic active molecules. The lipid matrix is generally formed of glyceryl monoleate, phytantriol derivatives or some phospholipids, stabilized by PEO-PPO-PEO triblock copolymer or polysorbate 80. Among the proteins entrapped in such kind of particles, it is possible to cite apo-feritin (474 kDa), transferring (75 kDa) or fibrinogen (420 kDa) ([Angelova et al. 2011](#)). Several

papers deal with the possible denaturation and unfolding of the entrapped protein. With circular dichroism, a technique which is sensitive to the secondary structure of macromolecules, Clogston and Caffrey clearly show the lack of significant denaturation of cytochrome c, lysozyme, myoglobin, ovalbumin, conalbumin and apo-ferritin following their entrapment into monoolein cubic phase (Clogston & Caffrey 2005). Into phytantriol-based cubic phase, resonance Raman spectroscopic investigations demonstrated that both cytochrome c active center, especially the central iron ion, and  $\alpha$ -helix do not undergo structural changes (Misiūnas et al. 2012) confirming the potential of the self-assembled particles to stabilize sensitive macromolecules.

## 6. Chemical and physical stabilities of labile APIs in nanocrystals

Over the past two decades, drug nanocrystal technology has been undoubtedly one of the highlighted approaches in the pharmaceutical delivery field. Its major contribution is the benefits that can be gained by formulating poorly soluble drugs. Drug nanocrystals are reported as a safe and well tolerated formulation in many different routes of administration (Gao et al. 2012). A lot of experiments have shown that nanocrystal systems improve chemical stability *in vitro* and *in vivo* of labile APIs due to role of surfactants or polymer stabilizers and crystalline nature of drugs. For instance, the chemical stability of ascorbyl palmitate could be achieved by formulating the active as nanosuspension by selecting the suitable surfactant composition. In fact, the percentage of ascorbyl palmitate remaining in nanosuspensions stabilized with Tween 80 was higher than 90% after 3 months storage at 40°C; whereas chemical instability of ascorbyl palmitate was significantly higher in the nanosuspensions stabilized with sodium dodecyl sulfate stored in the same conditions. Tween 80 is a non-ionic surfactant composed of polyethylene glycol chains. When nanocrystals are stabilized with this polymer, their surface will be surrounded by a thickened layer. This might reduce the diffusion of oxygen molecules in the aqueous phase around the crystal and further exposure of the active nanocrystals to oxidative processes (Teeranachaideekul et al. 2008). With the immunosuppressant rapamycin, another interesting example, the main degradation scheme is the cleavage of the macrocyclic lactone ring by hydrolysis. However, when rapamycin is formulated as nanocrystals including a mixture of 5% rapamycin and 2.5% Flurionic F68<sup>TM</sup> in an aqueous medium, minimal or no rapamycin degradation is observed after 3 weeks of

storage at room temperature, even following prolonged exposure to an aqueous medium (Liversidge & Wei 2003). Similar results were found on other fragile molecules such as omeprazol (Möschwitzer et al. 2004), paclitaxel (Liversidge & Wei 2003; Muller & Keck 2004), quercetin (Gao et al. 2011), camptothecin (Zhang et al. 2011), etoposide and pifosulfan (Merisko-Liversidge et al. 1996). The results demonstrated that the crystalline nature of drug substances and the surface coverage of nanocrystals by stabilizers as barriers to diffusion of oxygen and/or water into particles improving the chemical stability of interest, and degradation took place only at the outermost surface of the particle protecting inner core of drug nanocrystals (Möschwitzer et al. 2004; Muller & Keck 2004). Physical instability, due to crystal growth and change of crystalline state (polymorphism), is a common concern for drug nanocrystals. Crystal growth in colloidal suspensions is generally known as Ostwald ripening, and is responsible for changes in particle size and size distribution (Wu et al. 2011). Ostwald ripening is the tendency for a particle dispersion to grow in diameter over time, by a process in which the smaller particles dissolve preferentially, because of their higher solubility, with subsequent crystallization onto larger particles, making them even larger (Rabinow 2004). Van Eerdenbrugh et al. demonstrated that Ostwald ripening occurred in nanosuspensions for 9 model drug compounds after 3 months of storage at room temperature (Van Eerdenbrugh et al. 2008).

Few works investigated the polymorphism of nanocrystals. The calcium-channel blocker nifedipine is known to have three polymorphic forms,  $\alpha$ ,  $\beta$  and  $\gamma$  (or I, II, and III respectively), the  $\alpha$ -form being the most stable with a melting temperature of 172°C against 163°C and 140°C for the two other polymorphs. This compound also exhibits several solvated structures. Interestingly, nanocrystals obtained with a high-pressure homogenization process described a x-ray diffraction pattern typical of the nifedipine stable  $\alpha$ -form confirming that the process did not interfere with the crystal form in that case (Hecq et al. 2005). Opposite trends have been observed by Müller et al with a poorly-water soluble pteridine derivative, RMKP22, which is very sensitive to the process involved in the preparation of the nanocrystals. Thus, the proportion of amorphous material in the nanosuspensions directly increases with the applied pressure (duration, intensity) and only low energy milling leads to totally crystalline nanoparticles. As with bulk crystals, these data confirm that solid state of nanosuspensions should be analyzed with care as it can significantly modify drug dissolution profiles (Müller et al. 2001).

## 7. Conclusion

Chemical and physical stabilities of labile APIs are an important consideration in discovery and development of drugs products. The knowledge gained from *in vitro* stability is useful for formulation and packaging development. However, *in vivo* instability is essential in explaining why some compounds efficient *in vitro*, in pharmacological models, show no or poor *in vivo* activity. This remains a challenging issue during pharmaceutical product development. Incorporation of a labile active ingredient in multifunctional nanoparticles to get *in vitro* and *in vivo* stability has emerged during the last decades as an optimal solution. Stability of a fragile API in a nanoparticulate system is affected by various factors such as physico-chemical properties of nanocarrier (size, shape, surface and charge), stability of system, drug location, drug-loading capacity, dispersion medium, storage conditions. Further understanding of the degradation pathways of active ingredient and protection mechanisms of nanovectors is essential to facilitate efficient nanocarrier selection. Development of nanoparticulate systems, although currently seen as very complicated and challenging in pharmaceutical industry, is expected to improve *in vitro* and *in vivo* stability of labile APIs.

## References

1. Angelova, A. et al., 2011. Self-assembled multicompartment liquid crystalline lipid carriers for protein, peptide, and nucleic acid drug delivery. *Accounts of chemical research*, 44(2), pp.147–156.
2. Baertschi, S., Alsante, K. & Olsen, B., 2005. Stress Testing: Relation to the Development Timeline. In S. Baertschi, ed. *Pharmaceutical Stress Testing*. Informa Healthcare, pp. 173–179.
3. Baertschi, S. & Reynolds, D., 2005. Introduction. In S. Baertschi, ed. *Pharmaceutical Stress Testing*. Informa Healthcare, pp. 1–12.
4. Bangham, A.D. & Haydon, D.A., 1968. Ultrastructure of membranes: biomolecular organization. *British medical bulletin*, 24(2), pp.124–126.
5. Bardonnnet, P.L. et al., 2009. Glycosylated liposomes against *Helicobacter pylori*: behavior in acidic conditions. *Biochemical and biophysical research communications*, 383(1), pp.48–53.
6. Bauer, J. et al., 2001. Ritonavir: an extraordinary example of conformational polymorphism. *Pharmaceutical research*, 18(6), pp.859–866.
7. Burguera, J.L. & Burguera, M., 2012. Analytical applications of emulsions and microemulsions. *Talanta*, 96, pp.11–20.
8. Cherian, A.K., Rana, A.C. & Jain, S.K., 2000. Self-assembled carbohydrate-stabilized ceramic nanoparticles for the parenteral delivery of insulin. *Drug development and industrial pharmacy*, 26(4), pp.459–463.
9. Choi, Y.H., Chin, Y.W. & Kim, Y.G., 2011. Herb-drug interactions: focus on metabolic enzymes and transporters. *Archives of pharmacal research*, 34(11), pp.1843–1863.
10. Clogston, J. & Caffrey, M., 2005. Controlling release from the lipidic cubic phase. Amino acids, peptides, proteins and nucleic acids. *Journal of controlled release: official journal of the Controlled Release Society*, 107(1), pp.97–111.
11. Couvreur, P., 2012. Nanoparticles in drug delivery: Past, present and future. *Advanced drug delivery reviews*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22580334>.
12. Crowley, 1999. Excipients as stabilizers. *Pharmaceutical science & technology today*, 2(6), pp.237–243.
13. Dass, Crispin R & Choong, Peter F M, 2006. Carrier-mediated delivery of peptidic drugs for cancer therapy. *Peptides*, 27(11), pp.3020–3028.
14. Davis, M.E., Chen, Z. (Georgia) & Shin, D.M., 2008. Nanoparticle therapeutics: an emerging treatment modality for cancer. *Nature Reviews Drug Discovery*, 7(9), pp.771–782.

15. Decuzzi, P. et al., 2010. Size and shape effects in the biodistribution of intravascularly injected particles. *Journal of controlled release: official journal of the Controlled Release Society*, 141(3), pp.320–327.
16. Drummond, D.C. et al., 2010. Development of a highly stable and targetable nanoliposomal formulation of topotecan. *Journal of controlled release: official journal of the Controlled Release Society*, 141(1), pp.13–21.
17. Van Eerdenbrugh, B. et al., 2008. Drying of crystalline drug nanosuspensions-the importance of surface hydrophobicity on dissolution behavior upon redispersion. *European Journal of Pharmaceutical Sciences: Official Journal of the European Federation for Pharmaceutical Sciences*, 35(1-2), pp.127–135.
18. Evans, G. ed., 2004. *A Handbook of Bioanalysis and Drug Metabolism* 1st ed., CRC Press.
19. Ezzat, K. et al., 2012. Solid formulation of cell-penetrating peptide nanocomplexes with siRNA and their stability in simulated gastric conditions. *Journal of controlled release: official journal of the Controlled Release Society*, 162(1), pp.1–8.
20. FDA, 2007. Guidance for Industry. ANDAs: Pharmaceutical Solid Polymorphism. Chemistry, Manufacturing, and Controls Information.
21. FDA, 1998. Guidance for Industry. Manufacturing, Processing, or Holding Active Pharmaceutical Ingredients.
22. Fontana, M.C. et al., 2011. Improved efficacy in the treatment of contact dermatitis in rats by a dermatological nanomedicine containing clobetasol propionate. *European journal of pharmaceutics and biopharmaceutics: official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V*, 79(2), pp.241–249.
23. Fontana, M.C. et al., 2009. Nanoencapsulation as a way to control the release and to increase the photostability of clobetasol propionate: influence of the nanostructured system. *Journal of biomedical nanotechnology*, 5(3), pp.254–263.
24. Fu, K., Klibanov, A.M. & Langer, R., 2000. Protein stability in controlled-release systems. *Nat Biotech*, 18(1), pp.24–25.
25. Gao, L. et al., 2012. Drug nanocrystals: In vivo performances. *Journal of controlled release: official journal of the Controlled Release Society*, 160(3), pp.418–430.
26. Gao, L. et al., 2011. Preparation of a chemically stable quercetin formulation using nanosuspension technology. *International Journal of Pharmaceutics*, 404(1-2), pp.231–237.
27. Glass, B.D., Novak, C. & Brown, M.E., 2004. The thermal and photostability of solid pharmaceuticals: A review. *Journal of thermal analysis and calorimetry*, 77(3), pp.1013–1036.

28. Gupta, B., Levchenko, T.S. & Torchilin, V.P., 2005. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. *Advanced drug delivery reviews*, 57(4), pp.637–651.
29. Hao, Y.L. et al., 2005. In vitro and in vivo studies of different liposomes containing topotecan. *Archives of Pharmacal Research*, 28(5), pp.626–635.
30. Harris, J.M. & Chess, R.B., 2003. Effect of pegylation on pharmaceuticals. *Nature reviews. Drug discovery*, 2(3), pp.214–221.
31. Hecq, J. et al., 2005. Preparation and characterization of nanocrystals for solubility and dissolution rate enhancement of nifedipine. *International journal of pharmaceutics*, 299(1-2), pp.167–177.
32. Heurtault, B. et al., 2003. Interfacial stability of lipid nanocapsules. *Colloids and Surfaces B: Biointerfaces*, 30(3), pp.225–235.
33. Heurtault, B. et al., 2003. Physico-chemical stability of colloidal lipid particles. *Biomaterials*, 24(23), pp.4283–4300.
34. Huang, C.Y. et al., 1998. Lipitoids-novel cationic lipids for cellular delivery of plasmid DNA in vitro. *Chemistry & biology*, 5(6), pp.345–354.
35. Huang, X. & Brazel, C.S., 2001. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. *Journal of controlled release: official journal of the Controlled Release Society*, 73(2-3), pp.121–136.
36. Huang, Z. et al., 2008. Development and evaluation of lipid nanoparticles for camptothecin delivery: a comparison of solid lipid nanoparticles, nanostructured lipid carriers, and lipid emulsion. *Acta Pharmacologica Sinica*, 29(9), pp.1094–1102.
37. Huh, M.S. et al., 2010. Tumor-homing glycol chitosan/polyethylenimine nanoparticles for the systemic delivery of siRNA in tumor-bearing mice. *Journal of controlled release: official journal of the Controlled Release Society*, 144(2), pp.134–143.
38. Iwanaga, K. et al., 1997. Oral delivery of insulin by using surface coating liposomes: Improvement of stability of insulin in GI tract. *International Journal of Pharmaceutics*, 157(1), pp.73–80.
39. Jansen, P. & Baertschi, S., 2005. Stress Testing: A Predictive Tool. In S. Baertschi, ed. *Pharmaceutical Stress Testing*. Informa Healthcare, pp. 13–49.
40. Karewicz, A. et al., 2011. Interaction of curcumin with lipid monolayers and liposomal bilayers. *Colloids and surfaces. B, Biointerfaces*, 88(1), pp.231–239.
41. Kawakami, K., 2012. Modification of physicochemical characteristics of active pharmaceutical ingredients and application of supersaturatable dosage forms for improving bioavailability of poorly absorbed drugs. *Advanced drug delivery reviews*, 64(6), pp.480–495.

42. Kedar, U. et al., 2010. Advances in polymeric micelles for drug delivery and tumor targeting. *Nanomedicine: Nanotechnology, Biology and Medicine*, 6(6), pp.714–729.
43. Kreuter, J., 2007. Nanoparticles-a historical perspective. *International journal of pharmaceutics*, 331(1), pp.1–10.
44. Kristl, J. et al., 2003. Effect of colloidal carriers on ascorbyl palmitate stability. *European journal of pharmaceutical sciences: official journal of the European Federation for Pharmaceutical Sciences*, 19(4), pp.181–189.
45. Kwon, S.S. et al., 2002. Preparation and characterization of coenzyme Q10-loaded PMMA nanoparticles by a new emulsification process based on microfluidization. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 210(1), pp.95–104.
46. Lai, F. et al., 2011. Nanocrystals as tool to improve piroxicam dissolution rate in novel orally disintegrating tablets. *European journal of pharmaceutics and biopharmaceutics: official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V*, 79(3), pp.552–558.
47. Leung, M.H.M., Colangelo, H. & Kee, T.W., 2008. Encapsulation of curcumin in cationic micelles suppresses alkaline hydrolysis. *Langmuir: the ACS journal of surfaces and colloids*, 24(11), pp.5672–5675.
48. Leung, M.H.M. & Kee, T.W., 2009. Effective stabilization of curcumin by association to plasma proteins: human serum albumin and fibrinogen. *Langmuir: the ACS journal of surfaces and colloids*, 25(10), pp.5773–5777.
49. Li, W. & Szoka, F., 2007. Lipid-based Nanoparticles for Nucleic Acid Delivery. *Pharmaceutical Research*, 24(3), pp.438–449.
50. Liu, J. et al., 2008. Solid lipid nanoparticles for pulmonary delivery of insulin. *International Journal of Pharmaceutics*, 356(1–2), pp.333–344.
51. Liversidge, E. & Wei, L., 2003. Stabilization of chemical compounds using nanoparticulate formulations. Available at: <http://www.freepatentsonline.com/y2003/0054042.html>.
52. Makhlof, A., Tozuka, Y. & Takeuchi, H., 2011. Design and evaluation of novel pH-sensitive chitosan nanoparticles for oral insulin delivery. *European journal of pharmaceutical sciences: official journal of the European Federation for Pharmaceutical Sciences*, 42(5), pp.445–451.
53. Mason, T.G. et al., 2006. Nanoemulsions: formation, structure, and physical properties. *Journal of Physics: Condensed Matter*, 18(41), pp.R635–R666.
54. McNally, E.J. & Hastedt, J.E., 2007. *Protein Formulation and Delivery, Second Edition*, Taylor & Francis.
55. Merisko-Liversidge, E. et al., 1996. Formulation and antitumor activity evaluation of nanocrystalline suspensions of poorly soluble anticancer drugs. *Pharmaceutical research*, 13(2), pp.272–278.

56. Misiūnas, A. et al., 2012. Horse heart cytochrome c entrapped into the hydrated liquid-crystalline phases of phytantriol: X-ray diffraction and Raman spectroscopic characterization. *Journal of colloid and interface science*, 378(1), pp.232–240.
57. Moghimi, S.M., Hunter, A.C. & Murray, J.C., 2001. Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacological reviews*, 53(2), pp.283–318.
58. Moon, Y.J. et al., 2008. Quercetin pharmacokinetics in humans. *Biopharmaceutics & Drug Disposition*, 29(4), pp.205–217.
59. Moribe, K. et al., 2011. Drug Nanoparticle Formulation Using Ascorbic Acid Derivatives. *Journal of Drug Delivery*, 2011, pp.1–9.
60. Möschwitzer, J. et al., 2004. Development of an intravenously injectable chemically stable aqueous omeprazole formulation using nanosuspension technology. *European Journal of Pharmaceutics and Biopharmaceutics: Official Journal of Arbeitsgemeinschaft Für Pharmazeutische Verfahrenstechnik e.V.*, 58(3), pp.615–619.
61. Müller, R.H., Jacobs, C. & Kayser, O., 2001. Nanosuspensions as particulate drug formulations in therapy. Rationale for development and what we can expect for the future. *Advanced Drug Delivery Reviews*, 47(1), pp.3–19.
62. Müller, R.H. & Keck, C.M., 2011. Twenty years of drug nanocrystals: Where are we, and where do we go? *European Journal of Pharmaceutics and Biopharmaceutics: Official Journal of Arbeitsgemeinschaft Für Pharmazeutische Verfahrenstechnik e.V.* Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21971369>.
63. Müller, R.H., Radtke, M. & Wissing, S.A., 2002. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. *Advanced drug delivery reviews*, 54 Suppl 1, pp.S131–155.
64. Müller, R.H., Shegokar, R. & Keck, C.M., 2011. 20 years of lipid nanoparticles (SLN and NLC): present state of development and industrial applications. *Current Drug Discovery Technologies*, 8(3), pp.207–227.
65. Mulik, R., Mahadik, K. & Paradkar, A., 2009. Development of curcuminoids loaded poly(butyl) cyanoacrylate nanoparticles: Physicochemical characterization and stability study. *European journal of pharmaceutical sciences: official journal of the European Federation for Pharmaceutical Sciences*, 37(3-4), pp.395–404.
66. Muller, R.H. & Keck, C.M., 2004. Challenges and solutions for the delivery of biotech drugs--a review of drug nanocrystal technology and lipid nanoparticles. *Journal of Biotechnology*, 113(1-3), pp.151–170.
67. Ourique, A.F. et al., 2008. Tretinoin-loaded nanocapsules: Preparation, physicochemical characterization, and photostability study. *International journal of pharmaceutics*, 352(1-2), pp.1–4.
68. Ourique, Aline Ferreira et al., 2011. Improved photostability and reduced skin permeation of tretinoin: development of a semisolid nanomedicine. *European journal of pharmaceutics and biopharmaceutics: official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V.*, 79(1), pp.95–101.

69. Padovan, J. et al., 2012. Investigating the barriers to bioavailability of macrolide antibiotics in the rat. *European journal of drug metabolism and pharmacokinetics*, 37(3), pp.163–171.
70. Paudel, A. et al., 2012. Manufacturing of solid dispersions of poorly water soluble drugs by spray drying: Formulation and process considerations. *International journal of pharmaceutics*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22820134>.
71. Petros, R.A. & DeSimone, J.M., 2010. Strategies in the design of nanoparticles for therapeutic applications. *Nat Rev Drug Discov*, 9(8), pp.615–627.
72. Rabinow, B.E., 2004. Nanosuspensions in drug delivery. *Nature Reviews Drug Discovery*, 3(9), pp.785–796.
73. Ramteke, S. et al., 2009. Amoxicillin, clarithromycin, and omeprazole based targeted nanoparticles for the treatment of H. pylori. *Journal of drug targeting*, 17(3), pp.225–234.
74. Rao, J.P. & Geckeler, K.E., 2011. Polymer nanoparticles: Preparation techniques and size-control parameters. *Progress in Polymer Science*, 36(7), pp.887–913.
75. Ridolfi, D.M. et al., 2012. Chitosan-solid lipid nanoparticles as carriers for topical delivery of tretinoin. *Colloids and surfaces. B, Biointerfaces*, 93, pp.36–40.
76. Roger, E., Lagarce, F. & Benoit, J.-P., 2011. Development and characterization of a novel lipid nanocapsule formulation of Sn38 for oral administration. *European journal of pharmaceutics and biopharmaceutics: official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V*, 79(1), pp.181–188.
77. Sehwat, R., Maithani, M. & Singh, R., 2010. Regulatory Aspects in Development of Stability-Indicating Methods: A Review. *Chromatographia*, 72(1), pp.1–6.
78. Souza, L.G. et al., 2011. Development of topotecan loaded lipid nanoparticles for chemical stabilization and prolonged release. *European Journal of Pharmaceutics and Biopharmaceutics: Official Journal of Arbeitsgemeinschaft Für Pharmazeutische Verfahrenstechnik e.V*, 79(1), pp.189–196.
79. Spiclin, P., Gasperlin, M. & Kmetec, V., 2001. Stability of ascorbyl palmitate in topical microemulsions. *International journal of pharmaceutics*, 222(2), pp.271–279.
80. Stolnik, S. & Shakesheff, K., 2009. Formulations for delivery of therapeutic proteins. *Biotechnology letters*, 31(1), pp.1–11.
81. Sutton, D. et al., 2007. Functionalized micellar systems for cancer targeted drug delivery. *Pharmaceutical research*, 24(6), pp.1029–1046.
82. Tadros, T. et al., 2004. Formation and stability of nano-emulsions. *Advances in Colloid and Interface Science*, 108-109, pp.303–318.
83. Tan, M.L., Choong, P.F.M. & Dass, C.R., 2010. Recent developments in liposomes, microparticles and nanoparticles for protein and peptide drug delivery. *Peptides*, 31(1), pp.184–193.

84. Teeranachaideekul, V. et al., 2008. Development of ascorbyl palmitate nanocrystals applying the nanosuspension technology. *International Journal of Pharmaceutics*, 354(1-2), pp.227–234.
85. Teeranachaideekul, V., Müller, R.H. & Junyaprasert, V.B., 2007. Encapsulation of ascorbyl palmitate in nanostructured lipid carriers (NLC)--effects of formulation parameters on physicochemical stability. *International journal of pharmaceutics*, 340(1-2), pp.198–206.
86. Thorat, A.A. & Dalvi, S.V., 2012. Liquid antisolvent precipitation and stabilization of nanoparticles of poorly water soluble drugs in aqueous suspensions: Recent developments and future perspective. *Chemical Engineering Journal*, 181–182(0), pp.1–34.
87. Tiayaboonchai, W., Tungpradit, W. & Plianbangchang, P., 2007. Formulation and characterization of curcuminoids loaded solid lipid nanoparticles. *International Journal of Pharmaceutics*, 337(1–2), pp.299–306.
88. Torchilin, V.P., 2007. Micellar nanocarriers: pharmaceutical perspectives. *Pharmaceutical research*, 24(1), pp.1–16.
89. Torchilin, V.P., 2005. Recent advances with liposomes as pharmaceutical carriers. *Nature reviews. Drug discovery*, 4(2), pp.145–160.
90. Ulleberg, E.K. et al., 2011. Human Gastrointestinal Juices Intended for Use in In Vitro Digestion Models. *Food digestion*, 2(1-3), pp.52–61.
91. Umashankar, M.S., Sachdeva, R.K. & Gulati, M., 2010. Aquasomes: a promising carrier for peptides and protein delivery. *Nanomedicine: nanotechnology, biology, and medicine*, 6(3), pp.419–426.
92. Uner, M. et al., 2005. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) for application of ascorbyl palmitate. *Die Pharmazie*, 60(8), pp.577–582.
93. Wang, Y.J. et al., 1997. Stability of curcumin in buffer solutions and characterization of its degradation products. *Journal of pharmaceutical and biomedical analysis*, 15(12), pp.1867–1876.
94. Wang, Z. et al., 2010. The role of charge in the surfactant-assisted stabilization of the natural product curcumin. *Langmuir: the ACS journal of surfaces and colloids*, 26(8), pp.5520–5526.
95. Waterman, K.C. et al., 2002. Hydrolysis in pharmaceutical formulations. *Pharmaceutical development and technology*, 7(2), pp.113–146.
96. Wu, L., Zhang, J. & Watanabe, W., 2011. Physical and chemical stability of drug nanoparticles. *Advanced Drug Delivery Reviews*, 63(6), pp.456–469.
97. Xiong, X.B. et al., 2011. Engineering of amphiphilic block copolymers for polymeric micellar drug and gene delivery. *Journal of controlled release: official journal of the Controlled Release Society*, 155(2), pp.248–261.

98. Xiong, Y. & Guan, K.L., 2012. Mechanistic insights into the regulation of metabolic enzymes by acetylation. *The Journal of cell biology*, 198(2), pp.155–164.
99. Yaghmur, A. et al., 2006. Control of the internal structure of MLO-based isosomes by the addition of diglycerol monooleate and soybean phosphatidylcholine. *Langmuir: the ACS journal of surfaces and colloids*, 22(24), pp.9919–9927.
100. Yallapu, M.M., Jaggi, M. & Chauhan, S.C., 2012. Curcumin nanoformulations: a future nanomedicine for cancer. *Drug discovery today*, 17(1-2), pp.71–80.
101. Ye, Q. et al., 2000. DepoFoam technology: a vehicle for controlled delivery of protein and peptide drugs. *Journal of controlled release: official journal of the Controlled Release Society*, 64(1-3), pp.155–166.
102. Yoo, J.W., Doshi, N. & Mitragotri, S., 2011. Adaptive micro and nanoparticles: temporal control over carrier properties to facilitate drug delivery. *Advanced drug delivery reviews*, 63(14-15), pp.1247–1256.
103. Zhang, H. et al., 2011. Preparation and antitumor study of camptothecin nanocrystals. *International Journal of Pharmaceutics*, 415(1-2), pp.293–300.
104. Zhu, G., Mallery, S.R. & Schwendeman, S.P., 2000. Stabilization of proteins encapsulated in injectable poly (lactide- co-glycolide). *Nat Biotech*, 18(1), pp.52–57.

# PARTIE EXPÉRIMENTALE

| <b>N°</b> | <b>Titre des publications</b>                                                                                                | <b>Page</b> |
|-----------|------------------------------------------------------------------------------------------------------------------------------|-------------|
| 1         | Nanodispersions stabilized by PEG diesters and phospholipids:<br>towards compartmented lipid nanoparticles                   | 91          |
| 2         | Quercetin solubility and chemical stability: enhancement with<br>polyoxylglyceride-based multi-compartmented nanodispersions | 128         |
| 3         | Enrichment of quercetin-loaded nanodispersions with nanocrystals:<br>preparation and physico-chemical characterization       | 160         |

## **Nanodispersions stabilized by PEG diesters and phospholipids: towards compartmented lipid nanoparticles**

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**Abbreviations:** APIs, active pharmaceutical ingredients; BNP, blank nanodispersions; Cryo-TEM, cryogenic transmission electron microscopy; DSC, differential scanning calorimetry; HLB, hydrophilic – lipophilic balance; HPLC, high performance liquid chromatography; HPH, high pressure homogenization; IC<sub>50</sub>, half maximal inhibitory concentration; NLC, nanostructured lipid carriers; Pdl, polydispersity index; PEG, polyethylene glycol; RES, reticulo-endothelial system; SAXS, small-angle X-ray scattering patterns; SLN, solid lipid nanoparticles; XRD, X-Ray diffraction; WAXS, wide-angle X-ray scattering patterns.

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**Abstract**

Lipid nanoparticles stabilized by Gelucire® 50/13 – Phospholipon® 90G mixture were successfully prepared using high pressure homogenization process. They were made from four lipid phases (Labrafac® CC, cocoa butter, their 25/75 (w/w) mixture and Labrafil® M2125 CS) with different physical states at room temperature (liquid and/or solid) and different hydrophilic-lipophilic balance (HLB) from 1 to 4. The particle sizes were in range of 100 - 200 nm with polydispersity indices about 0.1 and unimodal size distribution. All dispersions were physically stable at least 3 months at room temperature. These particles were able to highly encapsulate hydrophobic molecules. Particularly, analysis of microscopic structure and supramolecular organization demonstrated the formation of compartmented lipid nanoparticles for lipid phase of Labrafil® M2125 CS, which could be very interesting to co-administrate both lipophilic and hydrophilic molecules.

**Keywords:** compartmented lipid nanoparticles, lipid nanoparticles, Gelucire® 50/13, high pressure homogenization, nanodispersions, Phospholipon® 90G, transmission electron microscopy, X-Ray diffraction.

## Contents

### 1. Introduction

### 2. Materials and Methods

#### 2.1. Materials

#### 2.2. Methods

2.2.1. Preparation of nanodispersions by high-pressure homogenization process

2.2.2. Particle size and zeta potential measurements

2.2.3. X-ray diffraction

2.2.4. MTT assay

2.2.5. HPLC analysis

2.2.6. Drug loading and encapsulation efficiency

2.2.7. Cryogenic Transmission Electron Microscopy

2.2.8. Differential scanning calorimetry

### 3. Results and discussion

#### 3.1. Formulation studies

#### 3.2. SAXS-WAXS studies

#### 3.3. Cytotoxicity

#### 3.4. Encapsulation studies

#### 3.5. Cryo-TEM

#### 3.6. Gelucire<sup>®</sup> 50/13 – Phospholipon<sup>®</sup> 90G mixture

3.6.1. Anhydrous mixtures

3.6.2. Hydrated mixtures

### 4. Conclusion

### Acknowledgments

### References

## 1. Introduction

More than 35% of active pharmaceutical molecules in development are classified as poorly water-soluble drugs according to the biopharmaceutics classification system. In addition, Lipinski et al. pointed out that leads obtained with high-throughput screening tend to have even higher molecular weights causing greater lipophilicity than the older ones. In the case where such a lead is transformed into a medicine, conventional formulation strategies are no longer adequate to achieve acceptable bioavailability (Lipinski 2000; Shah & Agnihotri 2011). Among alternative technologies, lipid-based colloidal formulations have shown promise in enhancing the bioavailability of lipophilic drugs. Their small particle size makes them interesting as delivery systems for different modes of administration, including the parenteral route.

As regards lipid-based colloids, classical formulations are liposomes which primarily consist of phospholipid bilayers, nanoemulsions which are dispersion of nanoscale lipid droplets and solid lipid nanoparticles (SLN) based on solid triglycerides, waxes or fatty acids as matrix lipids. More recently, nanostructured lipid carriers (NLC) containing a mixture of solid and liquid lipids at room temperature have been developed. After intravenous administration, first-generation colloids have two major limitations including sequestration into organs like liver or spleen and recognition by the reticulo-endothelial system (RES). For these reasons, surface-modified nanocarriers have been developed in which hydrophilic polymer, as polyethylene glycol (PEG), covered the drug delivery device. This results in low recognition by the opsonins, decrease of the immune system uptake and long circulation time. Furthermore, the hydrophilic external layer increases the physical stability of particles due to steric hindrance when repulsive electrostatic forces are weak compared to attractive van der Waals forces (Doane et al. 2012). Thus, phospholipid-PEG derivatives such as DSPE-mPEG (1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine) or DMPE-mPEG (1,2-Dimyristoyl-sn-Glycero-3-Phospho-ethanolamine) have been extensively used to stabilize SLN, NLC or liposomes, even at the clinical level (Puri et al. 2009). More recently, lipoamino acid residues have been successfully grafted to PEG (Basile et al. 2012). In the field of copolymers, many types of compounds have been tested for their ability to achieve the stability to nanoparticles. Among them, it is possible to cite poloxamers, PEG-PLGA (poly(lactic-co-glycolic acid)), or PEG-PIBCA (poly(isobutyl 2-cyanoacrylate)) (Owens & Peppas 2006). The present work focused on polyoxylglycerides.

Polyoxylglycerides are a family of pharmaceutical materials composed of mixtures of mono-, di- and triglycerides and of mono- and di-fatty acid esters of polyethylene glycols. The nature and proportion of each component, which are specific to a given product, govern some properties of interest of these excipients such as hydrophilic – lipophilic balance (HLB) or their physical state at room temperature (liquid, solid). Gelucire® 50/13, approved by international instances ([European Pharmacopeia](#), [US Food Additive](#), [US Pharmacopeia National Formulary](#), and [FDA Inactive Ingredient Guide](#)) with broad applications in cosmetic and pharmaceutical formulations has been investigated. It consists in a mixture of mono-, di- and triacyl glycerides (20%) of palmitic and stearic acids as well as mono- and diesters of PEG 1500 (72% with a majority of diesters). The 8% remaining is free PEG 1500 ([Brubach et al. 2004](#)).

The aim of the present work was to investigate the possibility to produce lipid particles incorporating phospholipids and polyoxylglycerides as stabilizers. Several lipid phases have been combined into the formulations. The nanoparticles have been characterized from their long-term physical stability such as size, zeta potential, microscopic structure and supramolecular organization, and from their ability to encapsulate a lipophilic model molecule. Besides, preliminary cytotoxicity studies have been performed.

## 2. Materials and Methods

### 2.1. Materials

Gelucire® 50/13 (stearoyl macrogolglycerides), Labrafil® M 2125 CS (linoleoyl polyoxylglycerides), and Labrafac® CC (capric and caprylic triglycerides) were given by Gattefosse S.A.S. (Saint-Priest, France). Phospholipon® 90G (soybean lecithin at 94 – 102% of phosphatidylcholine) was provided by Phospholipid GmbH, Lipoid Group (Köln, Germany). Cholesterol ( $\geq 99\%$  purity) was purchased from Sigma-Aldrich (Saint Quentin-Fallavier, France). Cocoa butter, originally from Ivory Coast, was supplied by Barry Callebaut (Meulan, France). Methanol and acetonitrile for HPLC were obtained from Carlo Erba (Italy). Water was purified through a Milli-Q water system (Millipore, France).

## 2.2. Methods

### 2.2.1. Preparation of nanodispersions by high-pressure homogenization process

Gelucire<sup>®</sup> 50/13 (0 to 4%) and/or Phospholipon<sup>®</sup> 90G (0 to 3%) were dispersed in Milli-Q water as aqueous phase thanks to an Ultra-Turrax T25 (Janke & Kunkel GMBH & Co.KG, IKA<sup>®</sup>-Labortechnik, Germany) at 9500 rpm during 5 min at 70°C. The lipid phase was also heated at 70°C allowing a complete melt of the solid lipids. Four lipid phases with different physical states at room temperature (liquid and/or solid) and different HLB (1 to 4) have been used. There were Labrafac<sup>®</sup> CC, cocoa butter, their 25/75 (w/w) mixture and Labrafil<sup>®</sup> M2125 CS. The lipid phase was added slowly into the aqueous phase under continuous stirring at 13500 rpm by Ultra-Turrax for 5 min at the same temperature. In some formulations, cholesterol was used as a lipophilic compound to test the drug payload. Thus, 2, 5 or 8% (w/w in lipid) of cholesterol were solubilized in the lipid phase during the experimental protocol as described above. Containing or not containing cholesterol, the coarse emulsions were then quickly introduced into a two-stage homogenizer (APV 2000, Invensys, Albertslund, Denmark) previously heated to 70°C during 5 min to form hot nanodispersions. The pressure of the first floor was 600 bars and that of the 2nd floor was 200 bars. Finally, nanodispersions were kept at room temperature.

### 2.2.2. Particle size and zeta potential measurements

The particle size and zeta potential of nanodispersions were measured using a Nano-ZS90 apparatus (Malvern Instruments, Orsay, France). The average hydrodynamic diameter and polydispersity index (Pdl) were determined by *quasi*-elastic light scattering. The zeta potential determinations were performed using electrophoretic laser Doppler velocimetry. The samples were diluted to 1/300 in Milli-Q water for size analysis and in 1 mM NaCl solution for zeta potential measurements.

### 2.2.3. X-ray diffraction (XRD)

X-ray scattering experiments were performed on the 5.2L beamline at ELETTRA Synchrotron Light Laboratory (Trieste, Italy). The energy and wavelength of the incident X-ray beam were 8 KeV and 1.54 Å respectively. The samples were thermostated in the laboratory made sample holder, Microcalix ([Ollivon et al. 2006](#)) allowing both sample

temperature control and simultaneous differential scanning calorimetry analysis. Small-angle (SAXS) and wide-angle (WAXS) X-ray scattering patterns were recorded simultaneously using a 2D single photon counting detector (Pilatus 100K) based on hybrid pixel technology (Dectris, Swiss) and a position-sensitive linear gas detectors filled with an argon-ethane mixture respectively. After 2D-image treatment in the SAXS experiments, the scattered intensity was reported as a function of the scattering vector  $q = 4\pi\sin\theta/\lambda = 2\pi/d$ , where  $2\theta$  is the scattering angle,  $\lambda$  is the wavelength and  $d$  is the repeat distance between two reticular plans. The calibration of the WAXS and SAXS detectors were achieved using pure tristearine ( $2L\beta$  form), characterized by short spacings of  $4.59, 3.85$  and  $3.70 \text{ \AA} \pm 0.01 \text{ \AA}$  and silver behenate characterized by a long spacing of  $58.380 \text{ \AA} \pm 0.001 \text{ \AA}$  respectively. The samples were loaded into quartz capillaries (Quarzkapillaren, Germany) with external diameter of  $1.4 \pm 0.1 \text{ mm}$  and a wall thickness of  $0.01 \text{ mm}$ . All data were analyzed with the IGOR Pro software (WaveMetrics, Inc. USA).

#### 2.2.4. MTT assay

The cytotoxicity of the nanoparticles on the P388S Leukemia cells was assessed by MTT assay. 5mg/ml of MTT solution was prepared in 0.9% normal saline. The MTT solution was then filtered through  $0.22 \text{ }\mu\text{m}$  filter under the laminar flow. The extraction solution was prepared by mixing 50 ml of 20% sodium dodecyl sulphate and 50 ml dimethyl formamide. Four stock suspensions containing various concentrations of nanoparticles, 100mg/ml (stock-1), 2mg/ml (stock-2), 100 $\mu\text{g/ml}$  (stock-3), and 2 $\mu\text{g/ml}$  (stock-4) were prepared. A series of concentrations, 0.02 – 10000  $\mu\text{g/well}$  (or 0.1 – 50 mg/ml) were obtained from these stock suspensions.

The test procedure was the following: A sterile 96-well plate (12 columns x 8 rows) was taken and all border wells were filled with sterile water under the laminar flow. The remaining wells (10 columns x 6 rows) were filled with the cell suspension up to 100 $\mu\text{l}$ . Next, the well plate was closed with its lid and shaken on a plate shaker for 2 min, and kept for pre-incubation during 24 h. After 24 h, different concentrations of nanoparticles were added to the well plates and further incubated for 24 h (cells/well  $3 \times 10^3$ ) and 48h (cells/well  $1.2 \times 10^3$ ). After the incubation time, the well plate was removed from the incubator, and 20  $\mu\text{l}$  of sterile MTT solution were added to each well containing the cells.

Subsequently, the well plate was shaken on the plate shaker for about 2 min, then incubated for 2.5 h and centrifuged at 2000 rpm and 4°C for 5 min. The supernatant was carefully aspirated to avoid the loss of formazan crystals formed in the wells. To the sediment in the wells was added 200 µl extraction solution and shaken for 5 min on the plate shaker. Afterwards, the well plate was kept in the incubator for 15 min, and then shaken until the crystals dissolved completely and clear solutions were achieved. Finally, the well plate was then read using the plate reader for absorbance at 570 nm. The absorbance of the wells was averaged and converted to the cell survival (%) and plotted against time.

### 2.2.5. HPLC analysis

HPLC analysis for cholesterol was performed on a Waters™ HPLC system (Waters™ 2996 Photodiode Array Detector, Waters™ 717 plus Autosampler, Waters™ 600 Controller – Millipore pump). Chromatographic separation was carried out with a Modulo-cart QS Nucléosil 5C18 column (4.6 x 250 mm, 5 µm, Interchrom). The mobile phase was acetonitrile – methanol (30:70, v/v). The detection wavelength was set at 208 nm. The flow rate was 1.4 ml/min. The sample volume was 100 µl. The calibration curve was linear in the range of 5 to 100 µg/ml ( $y = 26477x - 2750.7$ ,  $r^2 = 0.9999$ ). Before HPLC analysis, all samples were dissolved in methanol at concentrations in the linear range, and filtered through a membrane of 0.45 µm (PVDF Millex-HV, Germany). The cholesterol peak was identified on the chromatograms at the retention time of 13.6 min.

### 2.2.6. Drug loading and encapsulation efficiency

After the preparation, the cholesterol-loaded nanodispersions were purified by ultracentrifugation (LE-80K Ultracentrifuge Optima™, Rotor 701Ti - 93E502, Coulter™ Beckman, Villepinte, France) for 35000 rpm during one hour at 25°C. After ultracentrifugation, the lipid colloidal dispersions could be separated into three parts: lipid nanoparticles (top layer), aqueous phase eventually containing micelles or phospholipidic vesicles (middle layer) and cholesterol precipitate (bottom layer). The top layer was carefully taken off and separated into two parts. The first part was used to quantify the water content (after drying and weighing). The second part was used for cholesterol quantification by HPLC analysis as mentioned above. Amount of entrapped cholesterol in lipid nanodispersions was calculated according to the regression line

(section 2.2.5) and the humidity of the sample. The cholesterol loading and encapsulation efficiency were calculated using the formula:

$$\text{Drug loading (\%)} = \frac{\text{Amount of entrapped cholesterol}}{\text{Initial amount of lipid in the formulation}} \times 100$$

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Amount of entrapped cholesterol}}{\text{Initial amount of drug in the formulation}} \times 100$$

### 2.2.7. Cryogenic transmission electron microscopy (Cryo-TEM)

The morphology of nanodispersions was observed using a cryo-TEM system (JEM 2100, JEOL, Japan). All nanodispersions were diluted to 1/50 (v/v) in Milli-Q water. 10 µl of samples were placed on a copper grid, and the solution excess was carefully removed by a filter paper. The vitrification of the film was achieved by rapidly plunging the grid into liquid ethane. The vitrified sample was then transferred to the microscope. The temperature was kept about -180°C during both the transfer and the viewing procedures in order to prevent sample perturbation and the formation of ice crystals.

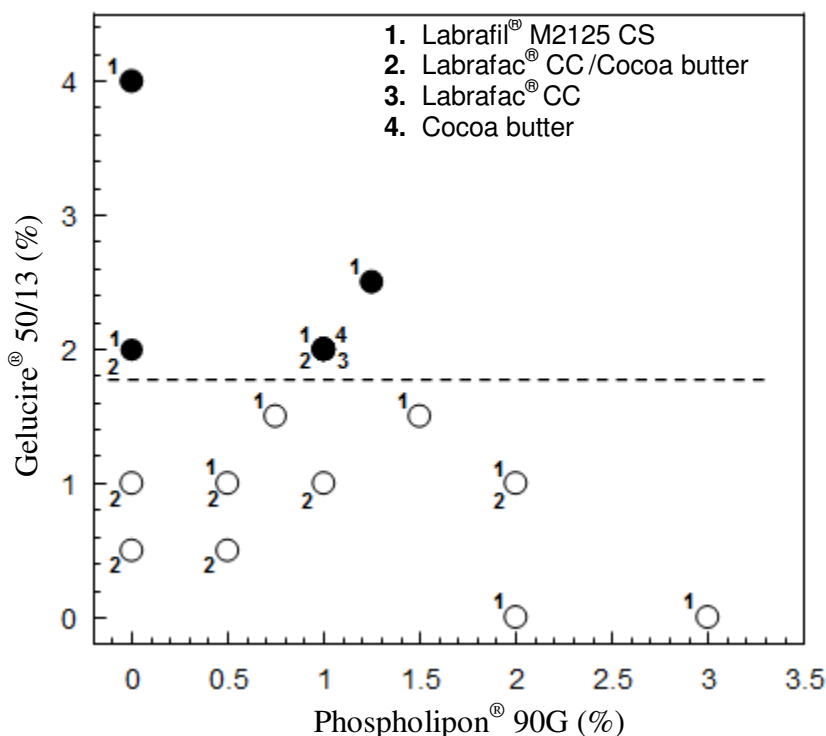
### 2.2.8. Differential scanning calorimetry (DSC)

Thermal analysis has been carried out using a Diamond DSC system (Perkin-Elmer, USA). It was associated to Pyris software version 9 and equipped with a cooling device (Intracooler 2P) in dry air atmosphere. The instrument was standardized using the enthalpy and melting point of lauric acid (99.5% purity,  $T_m = 43.6^\circ\text{C}$ ,  $\Delta H_m = 167.75 \text{ J/g}$ ). To favor their homogeneity, all the samples were dissolved in suitable solvents (chloroform and/or ethanol), then evaporated under reduced pressure, and lyophilized during 24 h to completely remove traces of solvents. The samples (about 2 to 10 mg) were loaded into hermetically sealed aluminium pans (50 µl, Perkin-Elmer, USA) for analysis. An empty pan was used as reference. Concerning temperature cycles, the samples were heated from 10°C to 70°C, cooled to 10°C and heated again to 70°C with a scan rate of 5°C/min. Between heating and cooling, isothermal steps of 5 min were inserted. To compare enthalpies, all DSC curves were normalized by sample weight. For better visualization, the DSC curves were shifted along the ordinate.

### 3. Results and discussion

#### 3.1. Formulation studies

Two fundamental parameters have been changed during the formulation studies, the nature of the lipophilic phase (Labrafac® CC, cocoa butter, Labrafil® M2125 CS) and the Gelucire® 50/13 to Phospholipon® 90G ratio. The parameters concerning the preparation process (temperature, mixing and homogenization times, applied pressures...) remained constant. The [Figure 1.1](#) summarized the results obtained during this part of the work and expressed for each formulation the success of the preparation. In the present case, successful criteria have been defined as a formulation with mean diameter comprised between 100 and 200 nm at the end of the preparation process and size distributions stable during at least three months at room temperature.



**Figure 1.1.** Summary of the formulation studies depending on the proportion of stabilizers and the lipid phase composition. *Filled symbol*: successful formulations; *open symbol*: unsuccessful formulations. Successful attributes have been defined as mean diameter comprised between 100 and 200 nm and stable during at least three months. The concentration of the lipid phase was 20% of the total suspension (w/w).

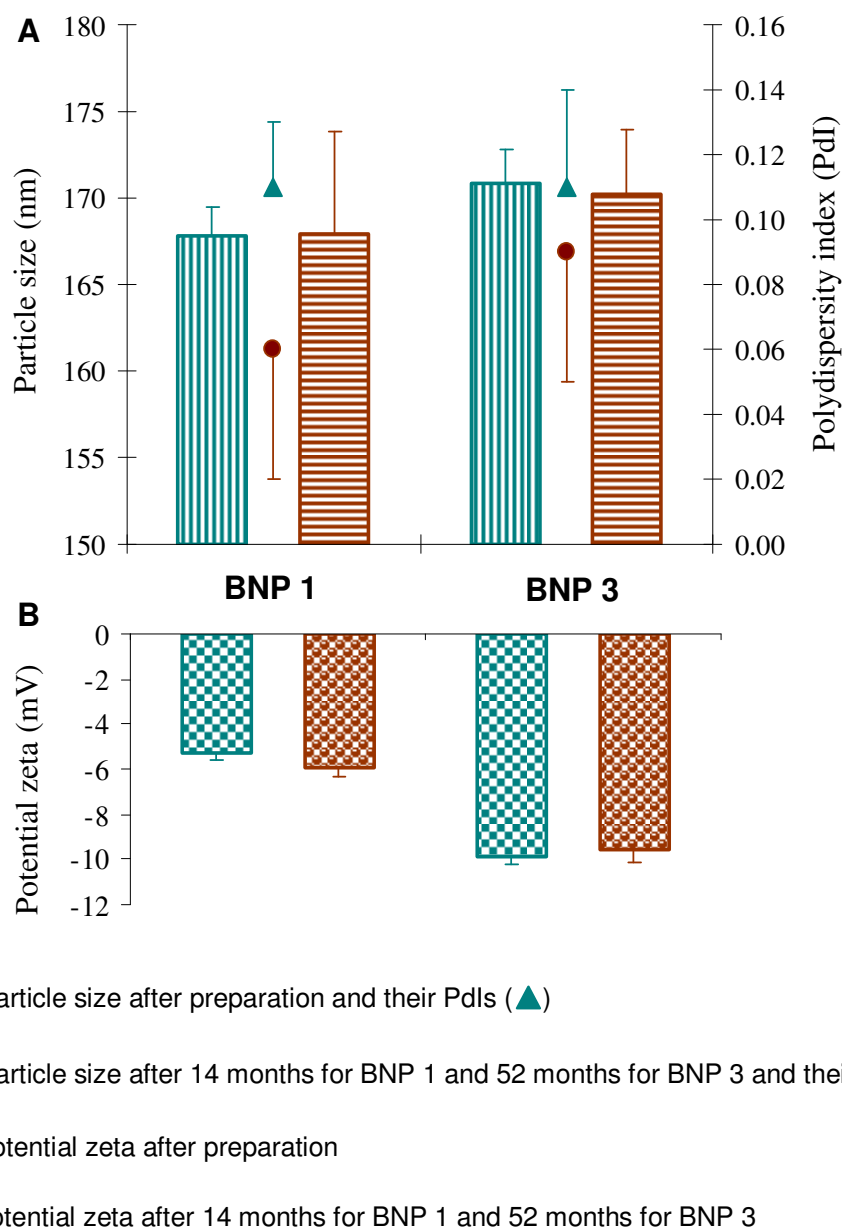
The interests of this target size range have been extensively discussed in the literature depending on the pharmaceutical applications ([Puri et al. 2009](#)). To overview the main ideas, this size range for anti-cancer therapy involves a passive targeting of the drug-loaded

nanocarriers by an accumulation in the tumor area due to its leaky vasculature and the enhanced permeability and retention effect. In the case of administration routes implying mucous layers, these sizes allow carrier passive diffusion into these layers (Ponchel et al. 1997; Dawson et al. 2004; Li et al. 2011). Furthermore, particle endocytosis is possible with these particle sizes (Iversen et al. 2011; Zhao et al. 2011; Ruthardt et al. 2011). The results showed that the composition of the lipid phase did not significantly affect the size distribution and stability of the lipid nanoparticles, while a threshold amount of surface active agents was required, especially concerning Gelucire® 50/13. This limit could be fixed around 2% when the amount of total lipid was 20%. At the concentration used, pure phospholipid mixture was not sufficient to efficiently stabilize the nanoparticles.

The main characteristics of the selected nanoparticles are summarized in the Table 1.1. As defined as critical attribute, the size of the particle was comprised in the range 100 to 200 nm and remained in this range during at least three months at room temperature. The polydispersity indexes were around 0.1 for each formulation, suggesting narrow size distributions. Determined in a 1 mM NaCl solution, the zeta potential of these nanoparticles was quite low, from -10 to -7 mV.

**Table 1.1.** Composition of blank nanodispersions (BNP) stabilized by the Gelucire® 50/13 – Phospholipon® 90G (2:1) mixture – their particle size (D: diameter) and polydispersity index (PdI); n = 3 – 9 measurements.

| Composition (g)                     | BNP 1       | BNP 2       | BNP 3       | NPB 4       |
|-------------------------------------|-------------|-------------|-------------|-------------|
| Gelucire® 50/13                     | 2           | 2           | 2           | 2           |
| Phospholipon® 90G                   | 1           | 1           | 1           | 1           |
| Labrafac® CC                        |             | 20          | 5           |             |
| Labrafil® M2125 CS                  | 20          |             |             |             |
| Beurre de cacao                     |             |             | 15          | 20          |
| Eau MilliQ ( <i>quantum satis</i> ) | 100         | 100         | 100         | 100         |
| Particle size (nm)                  | BNP 1       | BNP 2       | BNP 3       | NPB 4       |
| D <sub>0</sub>                      | 167.8 ± 1.7 | 167.7 ± 2.5 | 170.8 ± 2.0 | 197.6 ± 2.7 |
| PdI <sub>0</sub>                    | 0.11 ± 0.02 | 0.09 ± 0.03 | 0.11 ± 0.03 | 0.10 ± 0.03 |
| D <sub>3 months</sub>               | 166.6 ± 0.5 | 176.7 ± 3.5 | 165.7 ± 2.5 | 206 ± 8.0   |
| PdI <sub>3 months</sub>             | 0.10 ± 0.02 | 0.11 ± 0.03 | 0.10 ± 0.04 | 0.11 ± 0.04 |



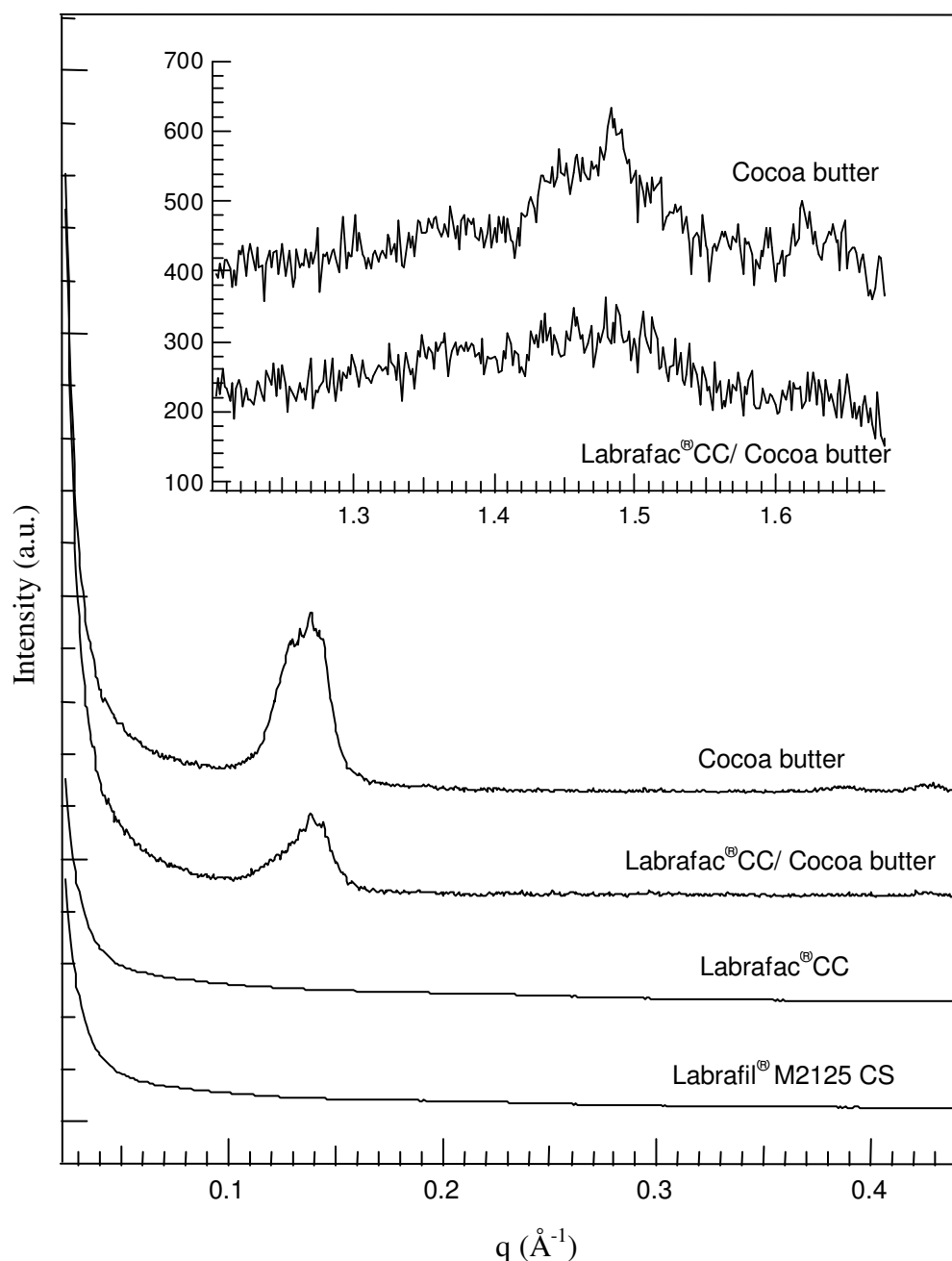
**Figure 1.2.** Long-time size stability of the nanoparticles prepared with 3% of the Gelucire® 50/13 – Phospholipon® 90G (2:1) mixture. **(A):** mean size  $\pm$  SD (nm) and polydispersity index  $\pm$  SD; **(B):** zeta potential  $\pm$  SD; n = 3 (three different batches).

Among the investigated stabilizer mixtures, the Gelucire® 50/13 – Phospholipon® 90G (2:1) composition was very interesting as stable nanoparticles had been obtained with all lipophilic phases. A particular focus has been done on this composition, and the long-term stability of these formulations was described in Figure 1.2. Surprisingly, stored in aqueous medium at room temperature, i.e. without any further treatment such as lyophilization step, the nanoparticles remained stable both from mean size and polydispersity point of views. For the oldest particles containing the cocoa butter –Labrafac® CC (75:25) mixture,

the size and polydispersity remained constant for more than 4 years (Figure 1.2A). After the same period of storage, the zeta potential of this colloidal suspension was also stable around -10 mV (Figure 1.2B). These zeta potential values gave us some information on chemical and physical stability of the system. Indeed, it is known that phospholipids and glycerides could be hydrolyzed with time, resulting in the release fatty acids which could destabilize the dispersion (Ickenstein et al. 2006) and decrease the zeta potential (more negative values) (Werling et al. 2008). Phospholipid hydrolysis was limited in these nanodispersions. Because of the low zeta potential values, electrostatic stabilization should be reduced (Heurtault et al. 2003), confirming the crucial role of the steric repulsion, due to PEG layer, on long-time physical stability.

### 3.2. SAXS-WAXS studies

X-ray diffraction experiments have been done on these nanodispersions (Figure 1.3). As expected, diffraction peaks have been observed in two samples containing cocoa butter while the samples containing Labrafil® M2125 CS and Labrafac® CC did not diffract. In the SAXS domain (low  $q$ ), a large and strong peak, at  $q = 0.137 \text{ \AA}^{-1}$  ( $d = 45.9 \text{ \AA}$ ) was observed on both samples and two small peaks at  $q = 0.388 \text{ \AA}^{-1}$  ( $d = 16.2 \text{ \AA}$ ) and  $q = 0.428 \text{ \AA}^{-1}$  ( $d = 14.7 \text{ \AA}$ ) were detected in the pure cocoa butter sample. In the WAXS region (high  $q$ ), in spite of a noisy signal due to the dispersion, a peak at  $q = 1.48 \text{ \AA}^{-1}$  ( $d = 4.25 \text{ \AA}$ ) and two shoulders at  $q = 1.45 \text{ \AA}^{-1}$  ( $d = 4.33 \text{ \AA}$ ) and  $q = 1.62 \text{ \AA}^{-1}$  ( $d = 3.88 \text{ \AA}$ ) respectively should be noticed. It is well known that cocoa butter could crystallize into six polymorphic forms, depending on their 2L or 3L lamellar arrangement (long spacing – SAXS) and the lateral packing of the fatty acid chains into hexagonal, orthorhombic or triclinic sub-cells (short spacing – WAXS) in ascending stability (Bresson et al. 2011). The XRD pattern obtained with pure cocoa butter was typical from a mixture of form II and IV. The large peak at  $0.137 \text{ \AA}^{-1}$  corresponded to the first order of two close lamellar phases which could be discriminated by their respective third orders at  $q = 0.388 \text{ \AA}^{-1}$  and  $q = 0.428 \text{ \AA}^{-1}$ . The  $d$ -spacings of  $48.6 \text{ \AA}$  and  $44.1 \text{ \AA}$  totally agreed with literature for such 2L-forms. The wide-angle results confirmed the presence of a mixture between an hexagonal sub-cell ( $d = 4.25 \text{ \AA}$ , form II) and an orthorhombic sub-cell ( $d = 4.33 \text{ \AA}$  and  $3.88 \text{ \AA}$ , form IV). Similar conclusions could be done with the particles containing the cocoa butter – Labrafac® CC (75:25) mixture, in spite of the less resolution of the peaks certainly due to crystallization defaults induced by the liquid Labrafac® CC.



**Figure 1.3.** SAXS profiles at 20°C of the nanoparticle suspensions depending on the lipid phase. Inset: WAXS signals of the sample containing solid lipids. Results acquired on Synchrotron SAXS beamline (Elettra) after 8 days of storage at 20°C.

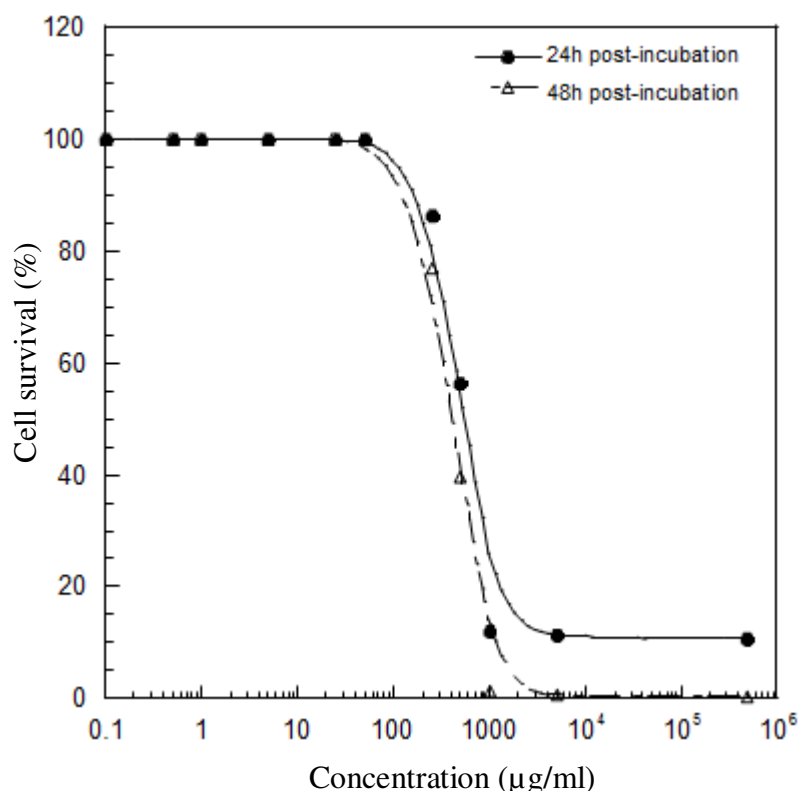
Nanodispersions containing cocoa butter – Miglyol<sup>®</sup> (75:25) mixture have already been studied in the laboratory (Lopez & Ollivon 2009). In spite of bigger diameters ( $\sim 450$  nm) and the use of other surfactants (sodium caseinate and monoglycerides or phospholipids), exactly the same XRD patterns have been obtained, confirming the present results, notably the co-existence of two phases in the nanoparticles. This strongly suggests that the emulsifiers do not greatly affect the structural behavior of this

particular solid/liquid mixture at least from polymorphism point of view, contrary to results observed with other lipid mixtures (Awad & Sato 2002; Bunjes & Koch 2005). The kinetic aspect would not be investigated in this paper.

Concerning the liquid lipid, Labrafil® M2125 CS, synthesized by an alcoholysis / esterification reaction using corn oil (main fatty acid is linoleic acid ~60%) and PEG 300, is clearly more polar than Labrafac® CC which contains pure short chain glycerides. However, even with Synchrotron high-flux beam, and important nanoparticle concentrations (20%), it was not possible to detect liquid-crystalline mesophase organization of Labrafil® M2125 CS, as observed in some polar glycerides (Svensson et al. 2004; Morán et al. 2005; Guo et al. 2010; Angelov et al. 2011). Hence, the long-spacing X-ray pattern demonstrates the lack of structure into these particles.

### 3.3. Cytotoxicity

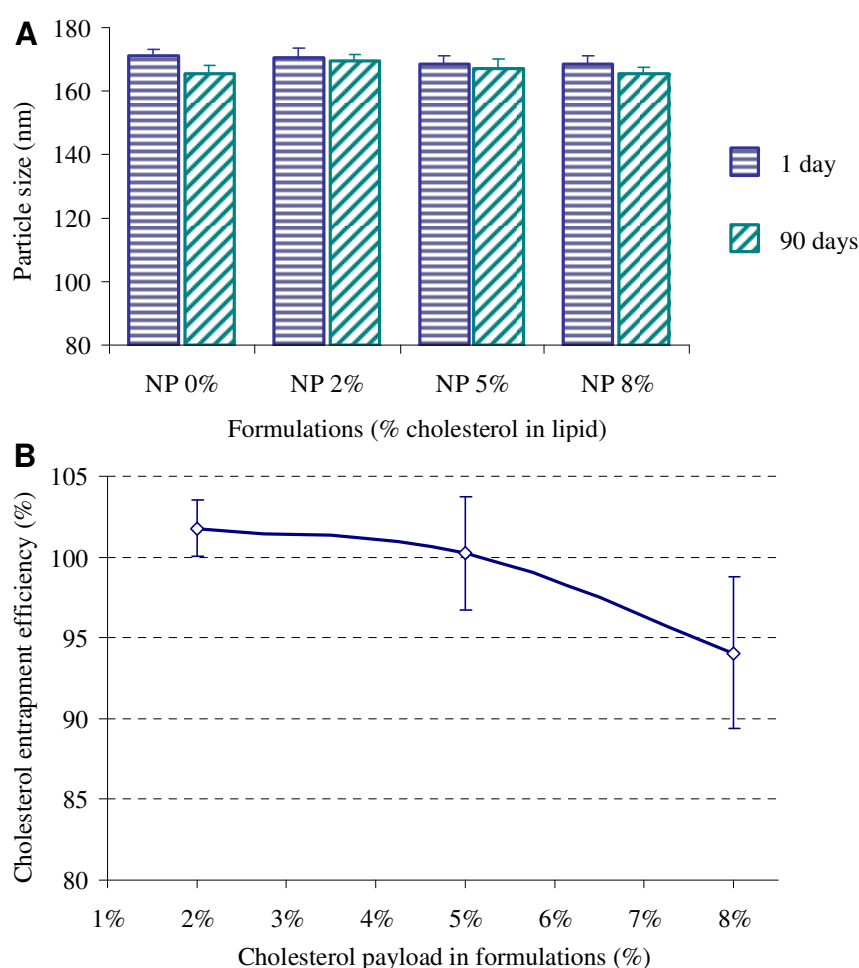
Preliminary cytotoxicity studies have been done with nanodispersions containing the cocoa butter – Labrafac® CC (75:25) mixture. The viability curves of P388S Leukemia cells incubated with the nanodispersions for 24 and 48 h were displayed in Figure 1.4.



**Figure 1.4.** Viability curves of P388S Leukemia cells treated with the cocoa butter – Labrafac® CC (75:25) containing nanodispersions for 24 and 48 h.

The semi-logarithmic relation between particle concentration and cell viability had a classical sigmoidal profile.  $IC_{50}$  values of 550  $\mu\text{g/ml}$  and 410  $\mu\text{g/ml}$  have been calculated after 24 and 48h of incubation respectively. This time evolution seemed logical as shorter incubation duration usually leads to higher  $IC_{50}$  values than a long incubation period. Expressed as lipid concentration,  $IC_{50}$  values were around  $5 \cdot 10^{-2} \%$ . The same range of values had been observed by Petersen et al. during an extensive work on L929 fibroblasts viability, following incubation with various lipid nanoparticles containing trimyristin, tristearin, cholesteryl myristate and several stabilizers such as poloxamer, polysorbate or soybean phospholipids (Petersen et al. 2011).

### 3.4. Encapsulation studies



**Figure 1.5.** Cholesterol encapsulation results by nanodispersions containing the cocoa butter – Labrafac® CC (75:25) mixture. **(A):** mean size  $\pm$  SD of the nanoparticles, for one day and three months after their preparation, depending on the cholesterol amount ( $n=3$ ). **(B):** cholesterol entrapment depending on its initial amount. The concentration of the lipid phase was 20% of the total suspension (w/w).

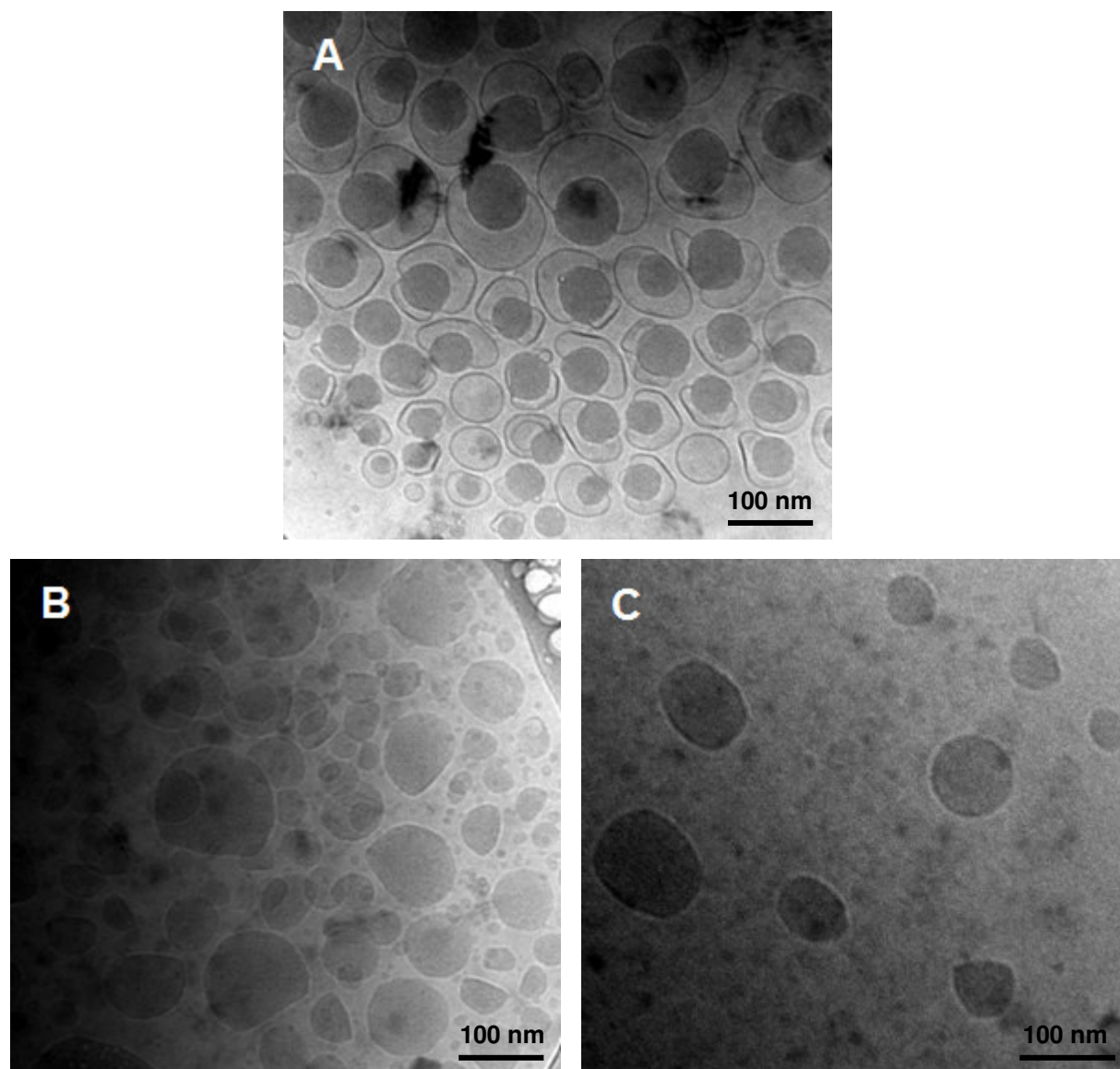
Encapsulation tests have been done with nanodispersions containing the cocoa butter – Labrafac® CC (75:25) mixture. Figure 1.5 summarized the main results obtained with cholesterol which has been chosen as model molecule. Because of the important lipophilicity of cholesterol ( $\log P = 7.62$ ), the entrapment efficiency was quite high, from 94% to 102%. More precisely, such encapsulation efficiency was maximal for cholesterol ratio lower than 5% of the lipid phase, while it reached a value of 94.1% when 8% of cholesterol were added compared to the lipid. As the amount of lipid was high in the whole aqueous dispersion (20% of lipid), the highest cholesterol loading reached to an interesting value of 15 mg/ml. Considering the water solubility of cholesterol around 0.025 µg/ml (Saad & Higuchi 1965), the concentration of cholesterol was  $6 \times 10^5$  higher in the colloidal suspension. Furthermore, the incorporation of cholesterol did not change significantly the size of the nanoparticles (Figure 1.5B) even at the highest payload. The mean diameter of nanoparticles did not change during 3-month storage.

### 3.5. Cryo-TEM

Cryo-TEM after plunge freezing allows direct investigation of colloids very close to their native state due to sample vitrification. Thus, the particle structures of Labrafil® M2125 CS stabilized by the Gelucire® 50/13 – Phospholipon® 90G (2:1) mixture and Gelucire® 50/13 alone have been compared (Figure 1.6A and 1.6B respectively). In Cryo-TEM micrographs, the nanodispersions stabilized only by Gelucire® 50/13 appeared as classical nanoemulsions, i.e. as more or less circular structures with an even distribution of contrast. Because of the low contrast of polyethylene glycol, the dense (dark) structures corresponded to the lipophilic core of the nanoemulsions.

The incorporation of 1% of phospholipids into the formulation led to totally different nanodispersions in which the lipophilic dark core was surrounded by a dense layer. As expected with formulation stabilized by phospholipids, some typical images of liposomes were present on the Figure 1.6A. However, taking into account the observation of tens views from four different samples, the most representative objects of the preparation were compartmented nanoparticles. From these cryo-TEM images, some assumptions could be proposed. The ring-shaped structure strongly suggested the presence of phospholipids bilayers around the lipophilic core. Furthermore, the layer thickness could be estimated between 4 to 5 nm which was totally compatible with that of phospholipid bilayers incorporating the hydrophobic part of Gelucire 50/13. By attentively looking at the cryo-

TEM image, it was possible to notice zone regions where the bilayers were flat or deformed because of the close contact between two particles. The minimal distance among these bilayers was in the range 8 to 10 nm and would probably result from the presence of polyethylene glycol chains at the nanodispersion surfaces. It was totally possible that the lipophilic part of PEGylated glycerides inserted into phospholipid bilayer, while the mobile PEG chains oriented towards the surface. For the nanoparticles made with the cocoa butter – Labrafac® CC (75:25) mixture, the cryo-TEM pictures were not so clear (Figure 1.6C). Indeed, a darker layer seemed to be visible just around the core of the particles but the two compartments (layer and core) were not definitely distinguishable.



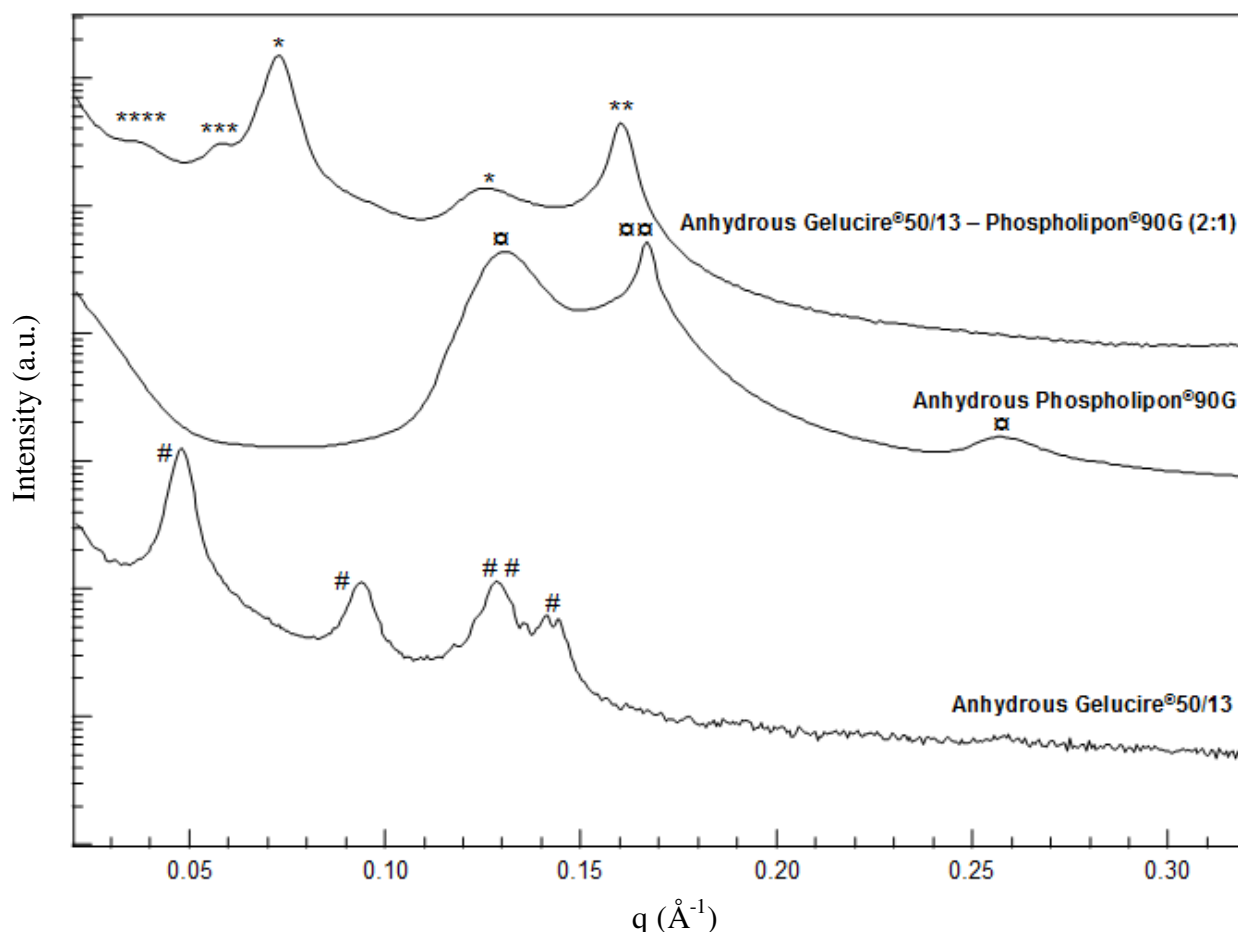
**Figure 1.6.** Cryo-TEM images of Labrafil® M2125 CS nanoparticles stabilized by the Gelucire® 50/13 - Phospholipon® 90G (2:1) mixture (A) or Gelucire® 50/13 alone (B) and cocoa butter - Labrafac® CC (75:25) nanoparticles stabilized by the Gelucire® 50/13 - Phospholipon® 90G (2:1) mixture (C). Scale bar: 100 nm.

### 3.6. Gelucire® 50/13 – Phospholipon® 90G mixture

In order to understand the formation of the compartmented nanoparticles, the mixture between the stabilizers has been carefully investigated in both anhydrous and hydrated states.

#### 3.6.1. Anhydrous mixtures

The structural organization of each stabilizer and their mixture has been observed at 20°C with XRD (Figure 1.7).



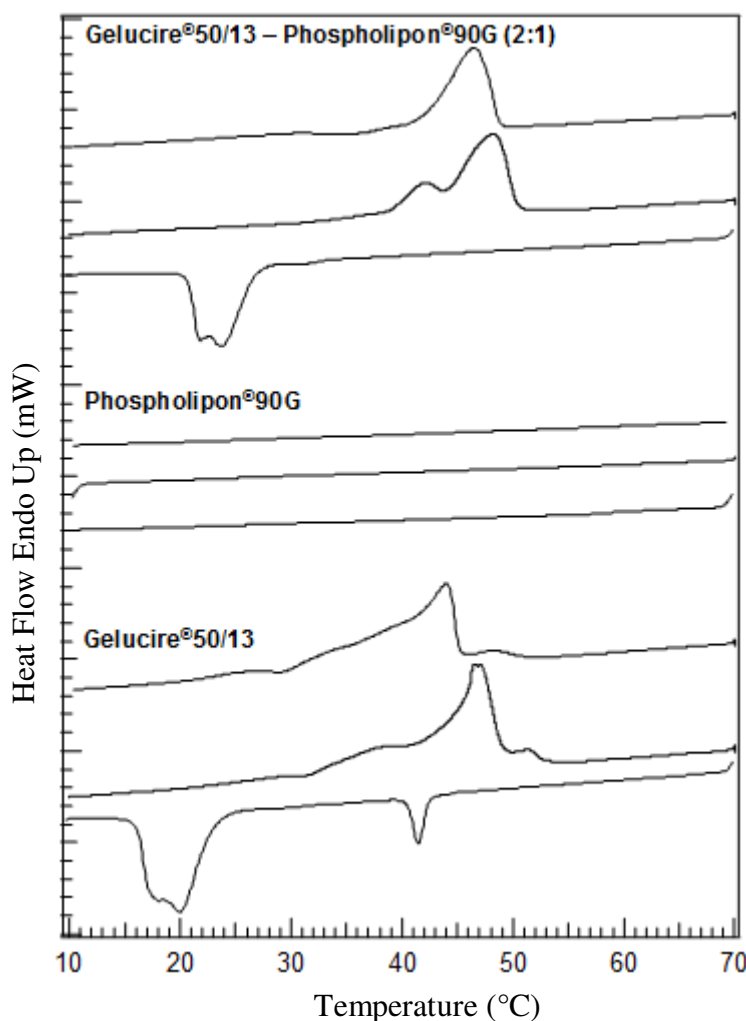
**Figure 1.7.** SAXS profiles at 20°C of the anhydrous stabilizer mixtures. Results acquired on Synchrotron SAXS beamline (Elettra) after 3 days of storage at room temperature. The symbols identify the peak belonging to the same structure.

SAXS pattern of Gelucire® 50/13 showed four intensive peaks at  $0.048 \text{ Å}^{-1}$ ,  $0.094 \text{ Å}^{-1}$ ,  $0.128 \text{ Å}^{-1}$  and  $0.144 \text{ Å}^{-1}$  ( $d = 130.2 \text{ Å}$ ,  $66.8 \text{ Å}$ ,  $48.9 \text{ Å}$  and  $43.8 \text{ Å}$  respectively). Extensive studies have been done in the laboratory on this pharmaceutical excipient (Brubach et al. 2004) and the combination of infrared spectroscopy and X-Ray diffraction at both

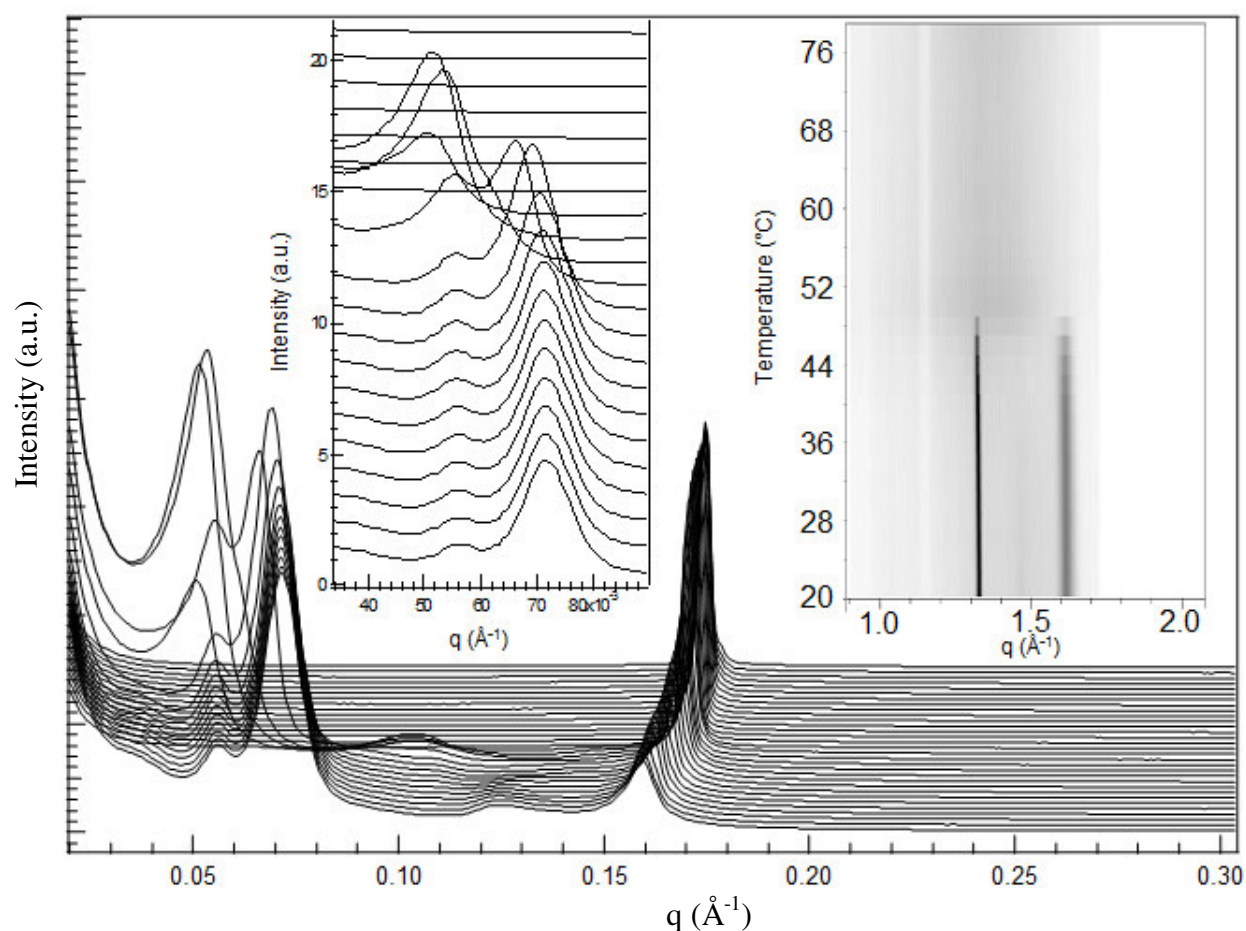
small and wide angle coupled to DSC has enabled to characterize its polymorphic behaviour. Thus, it has been demonstrated that Gelucire<sup>®</sup> 50/13 presents a complex structure which is dependent on its thermal history, i.e. several lamellar phases have been highlighted for the mono- and diacyl polyoxyethylene glycol according to the crystallization conditions. Briefly, slow cooling favors the formation of long lamellar phase without tilt ( $\sim 120\text{-}125\text{ \AA}$ ), while fast cooling generates tilted lamellar phases ( $\sim 90\text{ \AA}$ ). Obtained by rapid cooling, the native pellets (untreated sample analyzed as received) tend to be organized as tilted lamellar phases. A supplementary peak at  $0.127\text{ \AA}^{-1}$  has been attributed to the glyceride part of the sample which forms a lamellar phase in all cases with a long period of  $49.5\text{ \AA}$  and a hexagonal lattice. In the WAXS region, these polyethylene glycol derivatives adopted a helical conformation define by two peaks at  $1.33\text{ \AA}^{-1}$  and  $1.62\text{ \AA}^{-1}$  (Brubach et al. 2004).

In the present work, exactly the same XRD pattern has been obtained in the WAXS region (data not shown) but a strong difference appeared in the long-spacing domain. Indeed, the three Bragg reflections at  $0.048\text{ \AA}^{-1}$ ,  $0.094\text{ \AA}^{-1}$  and  $0.144\text{ \AA}^{-1}$  clearly corresponded to the three first-order peaks of a lamellar phase with a period of  $130\text{ \AA}$ . In order to work on homogenous samples, both individual products and mixtures have been obtained after recrystallization from ethanolic solution. This strongly suggested that this treatment should be compared to a slow cooling allowing the recrystallization into non-tilted phase. The glyceride-based phase was not altered by this crystallization procedure as confirmed by the peak at  $0.128\text{ \AA}^{-1}$ . DSC thermograms of this sample recorded at  $5^{\circ}\text{C}/\text{min}$  (Figure 1.8) showed large endotherms upon heating. It has been demonstrated that these endotherms cover the reorientation of the molecules from tilted to less tilted positions and then the melt of the sample. Before melting point, the higher the temperature is, the more important the long spacing is (Brubach et al. 2004). This perfectly correlated with the thermograms given by Gelucire<sup>®</sup> 50/13 recrystallized from ethanolic solution. Indeed, a significant decrease of the thermal event temperature domain appeared in the second heating run when comparing with the first one, while the third one was totally superimposed to the second (data not shown). Such difference could be easily explained by a crystallization process into non-tilted phase from ethanolic solution and recrystallizations into tilted phases after the first and second fusions.

Mainly composed on phosphatidylcholine, Phospholipon® 90G typically described lamellar phases. More precisely, the SAXS pattern corresponded to the co-existence of two lamellar phases; one characterized by its two first orders at  $0.129 \text{ \AA}^{-1}$  ( $d = 48.4 \text{ \AA}$ ) and  $0.257 \text{ \AA}^{-1}$  ( $d = 24.4 \text{ \AA}$ ) and another one at  $0.167 \text{ \AA}^{-1}$  ( $d = 37.6 \text{ \AA}$ ). The third and second orders of these two lamellar phases were respectively visible at  $0.333 \text{ \AA}^{-1}$  and  $0.384 \text{ \AA}^{-1}$  (data not shown). It was clear from the thermograms that this phospholipid mixture was not sensitive to temperature in the studied range (10 to  $70^\circ\text{C}$ ) (Figure 1.8).



**Figure 1.8.** Differential scanning calorimetry of the stabilizers and their (2:1) mixture. For each sample, heating (middle), cooling (lower) and re-heating (upper) scans performed at  $5^\circ\text{C}/\text{min}$  between 10 and  $70^\circ\text{C}$  were recorded. Isothermal steps of 5 min were inserted at the end of each scan. Curves were shifted along the ordinate to improve visualization.



**Figure 1.9.** Time-resolved SAXS patterns recorded from 20°C to 80°C of the Gelucire® 50/13 – Phospholipon® 90G (2:1) anhydrous mixture at 2°C/min with one XRD diagram every minute. *Left inset:* Focus on an isobestic point in the smallest angle region suggesting equilibrium between two structures. *Right inset:* 2D-matrix of the WAXS domain describing the disappearance of the PEG chains helical conformation ( $q = 1.33 \text{ \AA}^{-1}$  and  $1.62 \text{ \AA}^{-1}$ ) with temperature.

The SAXS pattern of the Gelucire® 50/13 – Phospholipon® 90G (2:1) mixture could not be summarized as the sum of each isolated compound. Indeed, important Bragg reflections have been observed at  $0.073 \text{ \AA}^{-1}$ ,  $0.125 \text{ \AA}^{-1}$  and  $0.160 \text{ \AA}^{-1}$  ( $d = 86.1$ ,  $50.3$  and  $39.3 \text{ \AA}$  respectively), while shoulders appeared at  $0.039 \text{ \AA}^{-1}$  and  $0.059 \text{ \AA}^{-1}$  ( $d = 161.1$  and  $106.5 \text{ \AA}$ ) (Figure 1.7). To correlate Bragg reflections with structures, this mixture has been followed with temperature (Figure 1.9). As the temperature raised, Bragg reflections at  $0.059 \text{ \AA}^{-1}$  and  $0.073 \text{ \AA}^{-1}$  transited to an intense peak at  $0.052 \text{ \AA}^{-1}$ , while a similar phenomenon was observed with the broad and smaller reflection at  $0.125 \text{ \AA}^{-1}$  (to  $0.105 \text{ \AA}^{-1}$ ) which could be assume as the second order of the lamellar phases. As already evoked with Gelucire® 50/13 alone, this corresponded to the progressive stand-up of the molecules with temperature from lamellar long spacings of  $87 \text{ \AA}$  and  $107 \text{ \AA}$  to

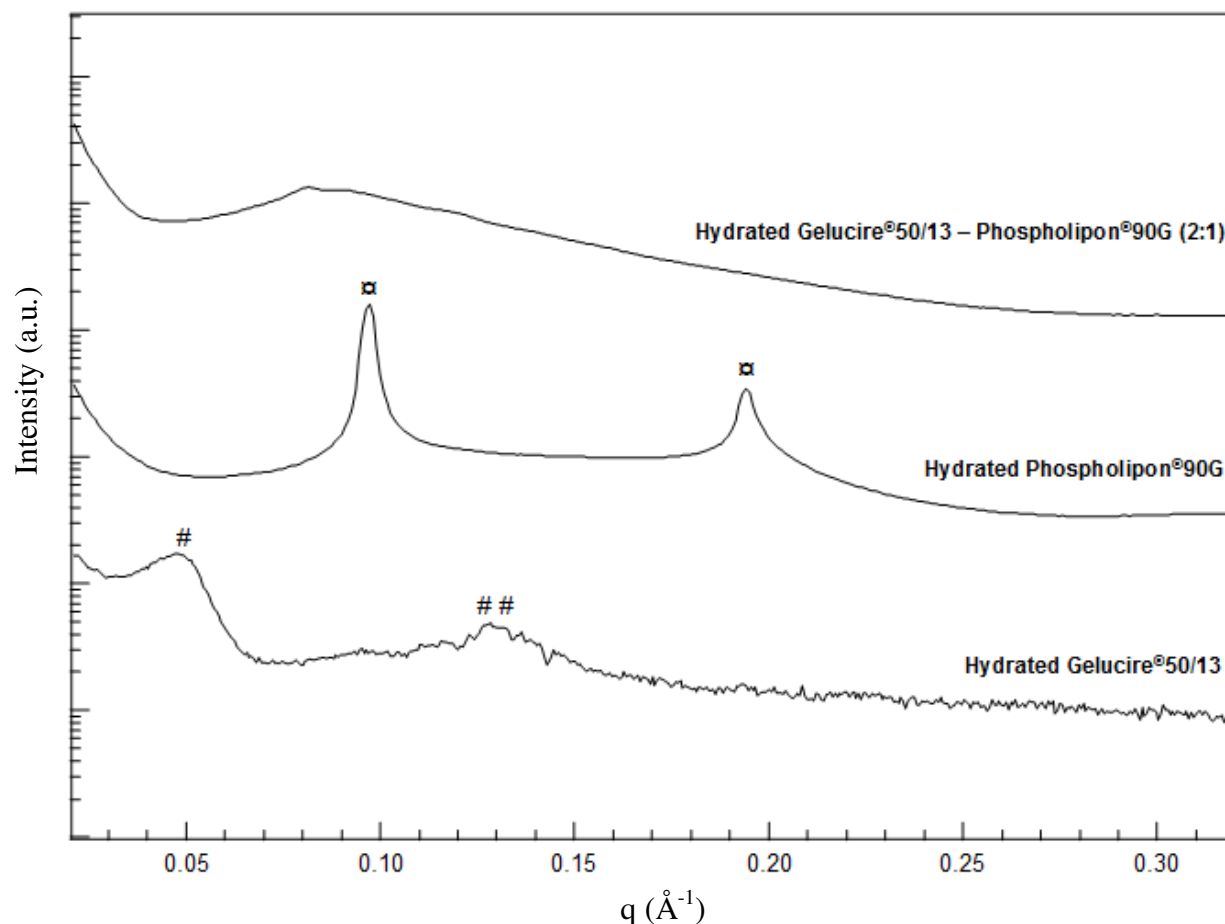
122 Å. The persistence of the Bragg reflection at  $0.160 \text{ Å}^{-1}$  even at high temperature confirmed that it had to be associated to a lamellar phase mainly constituted by phospholipids. With temperature, this reflection presented a gradual shift to  $0.175 \text{ Å}^{-1}$ , a decrease of the peak width at middle height and an important increase in intensity. This suggested a refinement of this lamellar phase. The WAXS pattern showed the typical signature of the helical conformation of PEG chains with an intense reflection at  $1.33 \text{ Å}^{-1}$  and a less intense larger reflection at  $1.62 \text{ Å}^{-1}$  (Brubach et al. 2004). These reflections gradually disappeared between 44 and 48 °C during the melting of Gelucire® 50/13.

Several informations confirming an interaction between Gelucire® 50/13 and Phospholipon® 90G could be extracted from the structural studies. First, whereas Gelucire® 50/13 alone tended to crystallize into non-tilted lamellar structure ( $d = 130 \text{ Å}$ ), the presence of phospholipids strongly oriented the organization to tilted lamellar phases ( $d = 87 \text{ Å}$  and  $107 \text{ Å}$ ). The temperature-dependent transition between tilted and non-tilted phases remained however visible in the mixture. Furthermore, the SAXS imprint of the pure glyceride phase which was present independently of the crystallization process ( $q = 0.128 \text{ Å}^{-1}$ ) seemed to disappear. The structural organization of Phospholipon® 90G was changed by the presence of Gelucire® 50/13. There was only one lamellar period which could be detected in the mixture, while two distinct lamellar phases have been described in the pure product.

The DSC thermograms of the mixture confirmed the interaction between Gelucire® 50/13 and Phospholipon® 90G (Figure 1.8). Thus, a slight shift of 2-3 °C of the thermal events towards higher temperature had to be noticed both heating and cooling cycles. More evident, the first exothermic peak on the cooling cycle of Gelucire® 50/13 alone (42 °C) disappeared in the mixture. This exotherm being attributed to the crystallization of the pure glyceride phase of Gelucire® 50/13 in the literature (Brubach et al. 2004), this confirmed its absence of crystallization. The most probable reason of this is the solubilization of the glycerides into the phospholipids bilayers (Raffournier, Ollivon et al. 2004).

### 3.6.2. Hydrated mixtures

Similar structural characterizations have been done on the hydrated stabilizers and their mixture (Figure 1.10).



**Figure 1.10.** SAXS profiles at 20°C of the hydrated stabilizer mixtures (80 wt % of water). Results acquired on Synchrotron SAXS beamline (Elettra) after 3 days of storage at room temperature. The symbols identify the peak belonging to the same structure.

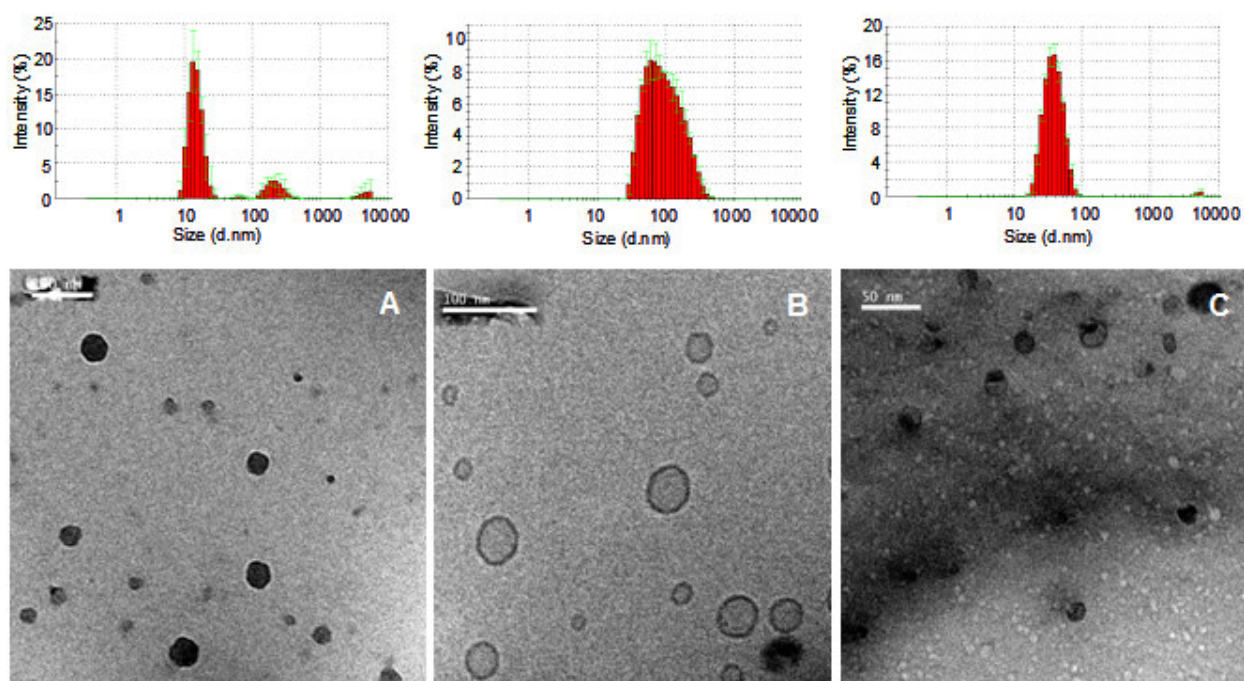
The most informative diffractogram has been obtained with Phospholipon® 90G which described a typical lamellar phase with a d-spacing of 65 Å (two first orders at  $0.097 \text{ Å}^{-1}$  and  $0.194 \text{ Å}^{-1}$ ). As expected, hydration of the phospholipids led to a swelling of the bilayers compared with anhydrous sample.

In the case of Gelucire® 50/13, the results are more difficult to definitely discuss. Indeed, two broad peaks could be distinguished at  $0.049 \text{ Å}^{-1}$  and  $0.129 \text{ Å}^{-1}$ . Unpublished data from the laboratory on hydration of Gelucire® 50/13 or studies in the literature on Gelucire® 44/14 have shown that polyoxyglycerides have a lyotropic behavior and are able to form various mesophases depending on their hydration degrees (Svensson et al.

2004). From our experiments (data not shown), the Bragg reflection at  $0.129 \text{ \AA}^{-1}$  resulted from the presence of glycerides not sensitive to hydration (the position of this reflection remained stable at all studied hydration degrees) and the reflection at  $0.049 \text{ \AA}^{-1}$  resulted from weakly organized mesophase.

Concerning the Gelucire® 50/13 – Phospholipon® 90G (2:1) mixture, Bragg reflections, including the lamellar phases of phospholipids and pure glycerides, clearly disappeared, while a diffusion signal appeared in the sample. This strongly suggested that hydrated Gelucire® 50/13 was able to induce curvature in the phospholipids bilayers and then to create nanoobjects into the sample.

To get more insight onto the stabilizer mixture, oil-free nanodispersions have been prepared as described in the 2.2.1 section with only the surfactants. The dispersions have been then characterized by *quasi*-elastic light scattering and cryo-TEM (Figure 1.11).



**Figure 1.11.** Size distribution and cryo-TEM images of Gelucire® 50/13 (A), Phospholipon® 90G (B) and Gelucire® 50/13 – Phospholipon® 90G (2:1) mixture (C) after dispersion in water and homogenization (see materials and methods section). Scale bars: (A) and (B): 100 nm, (C): 50 nm.

Interestingly, the nanodispersions obtained with the mixture of stabilizers were specific objects which confirmed the interaction between Gelucire® 50/13 and Phospholipon® 90G. In fact, liposome images have been observed with pure Phospholipon® 90G, dark spots have been observed with Gelucire® 50/13, but bicompartmented nanoparticles

resulted from the mixture. As observed in presence of oil, these nanoparticles were hybrid structures containing a bilayer domain and a denser and thicker region. The polyethylene glycol having a low contrast, these dense domains should be due to the lipid part of the PEG esters and to the pure glycerides. This last point is a little bit contradictory with the whole solubilization of the glycerides into the phospholipid bilayers we proposed at the end of the [3.6.1 section](#). However, contrary to the mixtures described previously, the nanoparticles shown here have been obtained without solubilization into ethanolic solution which probably homogenized the mixtures and optimized the interaction between stabilizers.

All these results on anhydrous and hydrated stabilizer mixture demonstrated the strong interaction between Gelucire® 50/13 and Phospholipon® 90G. A key factor concerning the original nanodispersions as described in [Figure 1.6](#) seemed to be the preparation process and notably the incorporation of the stabilizers. In fact, both stabilizers have been mixed into water prior to the incorporation of the oil phase and the results demonstrated that “preformed nanoparticles” could be already present at this step. Then oil phase was gradually incorporated into these preformed nanoparticles. Other procedures have been tested, for example by keeping Gelucire® 50/13 on the water phase and by adding Phospholipon® 90G to the oil phase, but the nanoparticles obtained were unstable.

At least with Labrafil® M2125 CS, nanoparticles developed here have a very particular structure which could be discussed as the combination of nanoemulsions and liposomes. To make a stable nanoemulsion, a lot of factors must be taken into account as order of incorporation, shear intensities or appropriate composition. It is generally admitted ([Mason et al. 2006](#)) that a significant excess of surfactant is required in the continuous phase in order to rapidly covered new surface area created during coarse emulsion size reduction (homogenization). Depending on its concentration and molecule critical packing parameter, this excess is self-organized into supramolecular objects, such as micelles, in equilibrium with nanodroplets. When phospholipids are used as stabilizers, the process generates liposomes which are often simultaneously observed with nanoparticles or nanodroplets on cryo-TEM pictures ([Klang et al. 2012](#)). Here, nanoemulsion droplets (dark core) and phospholipid bilayers (ring-shape layer) seemed solidarized as the proportion of free liposomes was certainly lower than 2-3% (from cryo-

TEM observations). Such structures are not common but have been described both in natural and synthetic products. Indeed, milk fat globules are natural dispersions corresponding to this unique colloidal assembly ([Lopez 2011](#)). Milk fat globules are constituted by a core rich in liquid and solid triglycerides enveloped by a trilayer of polar lipids and proteins known as the milk fat globule membrane. This membrane could be described as an external phospholipid-based bilayer and a monolayer anchored into the triglyceride core. By considering an equatorial section of the milk fat globules, domains with tight junctions between mono- and bilayers and domains, named cytoplasmic crescents, in which the distance between mono- and bilayer is important, have been observed. The tight junction is insured by the presence glycosylated proteins. We did not use glycoproteins into our formulation but the link between both layers should probably due to the presence of diesters in the Gelucire® 50/13 composition (~40%).

Several works already deal with synthetic nanosystems containing both phospholipid bilayers and lipid core. Teixeira et al. hypothesized that a partial phase separation of the surfactant mixture may be responsible for the formation of “handbag”-like structures with membrane extrusions. This was originally obtained with the addition of positively charged stearylamine to lecithin for the stabilization of triglyceride-based emulsions ([Teixeira et al. 2000](#)). Further studies characterized the mechanism of formation of these objects but also stated on the difficulty to control their preparation and to increase their concentration, less than 20%, compared to other structures such as liposomes and lipid droplets ([Raffournier, Dubernet, et al. 2004](#); [Raffournier et al. 2005](#)). Emulsomes have been also described as emulsion droplets stabilized with phospholipid bilayers ([Paliwal et al. 2009](#)). The proposed preparation process corresponds to the rehydration of phospholipid films with aqueous suspension of lipid nanodispersions (liquid or solid). However, to the author’s knowledge, no evidences on the object structure, as cryo-TEM for example, have been furnished.

Because of their original structure and their long-time stability, the lipid nanoparticles developed here seemed very powerful. The presence of several compartments allowed to envisage the incorporation of lipophilic and hydrophilic API in the same particle. Further studies should be done to deeply investigate their encapsulation potency and their ability to protect unstable API. It could be also informative to study the interaction

between these nanoparticles and serum protein as the presence of mono- and diesters into Gelucire® 50/13 would create a complex interface between the particles and the dispersion medium. Indeed, the PEG shielding and conformation greatly depend on the grafting density which should be high here (2 times more Gelucire® 50/13 than Phospholipon® 90G in the initial mixture). If grafting density is sufficiently low, PEG chains adopt a mushroom conformation, whereas chains minimize their free energy by extending into a brush at higher densities (Alexander 1977; de Gennes 1980). The present nanodispersions should be stabilized by a two-layer PEG coating in which the most external layer corresponds to the end of extended chains (monoesters) and a basal layer composed of PEG chains anchored by both extremities (diesters). At least, their *in vivo* stability and biodistribution could be checked.

#### 4. Conclusion

This paper demonstrated the interest of the Gelucire® 50/13 – Phospholipon® 90G mixture to efficiently stabilize lipid nanoparticles. These particles were able to encapsulate hydrophobic API model molecule. With some lipid phase, especially Labrafil® M2125 CS, we have shown the formation of compartmented lipid particles which could be very interesting to co-administrate lipophilic and hydrophilic APIs.

#### Acknowledgments

The authors gratefully acknowledge Claire GUEUTIN and Ghislaine FRÉBOURG for their assistance with HPLC and cryo-TEM experiments respectively.

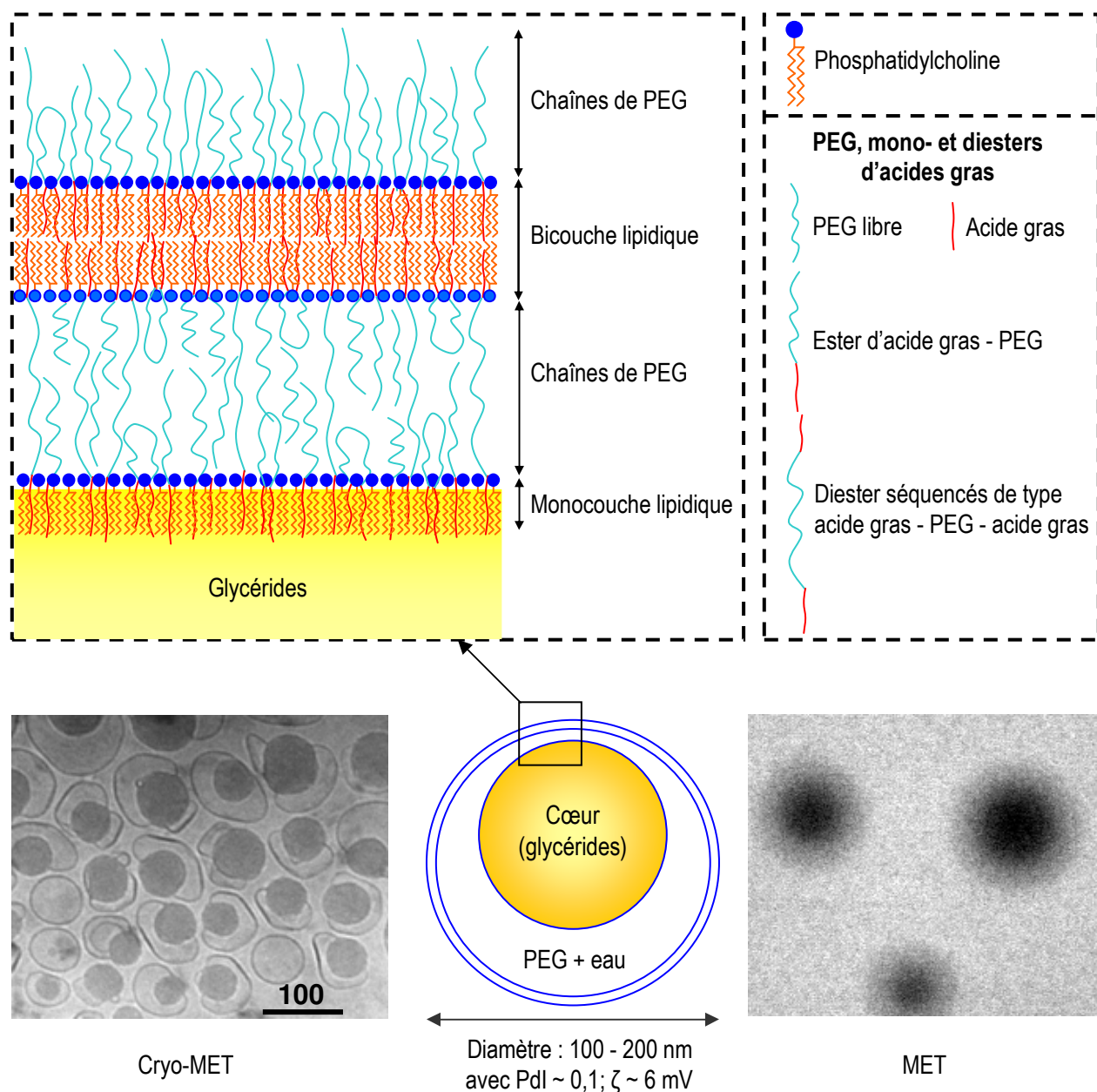
## References

1. Alexander, S., 1977. Adsorption of chain molecules with a polar head a scaling description. *Journal de Physique*, 38(8), pp.983–987.
2. Angelov, B. et al., 2011. SAXS investigation of a cubic to a sponge (L3) phase transition in self-assembled lipid nanocarriers. *Physical chemistry chemical physics: PCCP*, 13(8), pp.3073–3081.
3. Awad, T. & Sato, K., 2002. Acceleration of crystallisation of palm kernel oil in oil-in-water emulsion by hydrophobic emulsifier additives. *Colloids and Surfaces B: Biointerfaces*, 25(1), pp.45–53.
4. Basile, L. et al., 2012. Serum-stable, long-circulating paclitaxel-loaded colloidal carriers decorated with a new amphiphilic PEG derivative. *International journal of pharmaceutics*, 426(1-2), pp.231–238.
5. Bresson, S. et al., 2011. Raman spectroscopy of the polymorphic forms and liquid state of cocoa butter. *European Journal of Lipid Science and Technology*, 113(8), pp.992–1004.
6. Brubach, J.B. et al., 2004. Structural and Thermal Characterization of Mono- and Diacyl Polyoxyethylene Glycol by Infrared Spectroscopy and X-ray Diffraction Coupled to Differential Calorimetry. *The Journal of Physical Chemistry B*, 108(46), pp.17721–17729.
7. Bunjes, H. & Koch, M.H.J., 2005. Saturated phospholipids promote crystallization but slow down polymorphic transitions in triglyceride nanoparticles. *Journal of controlled release: official journal of the Controlled Release Society*, 107(2), pp.229–243.
8. Dawson, M. et al., 2004. Transport of polymeric nanoparticle gene carriers in gastric mucus. *Biotechnology progress*, 20(3), pp.851–857.
9. Doane, T.L. et al., 2012. Nanoparticle  $\zeta$ -potentials. *Accounts of chemical research*, 45(3), pp.317–326.
10. de Gennes, P.G., 1980. Conformations of Polymers Attached to an Interface. *Macromolecules*, 13(5), pp.1069–1075.
11. Guo, C. et al., 2010. Lyotropic liquid crystal systems in drug delivery. *Drug discovery today*, 15(23-24), pp.1032–1040.
12. Heurtault, B. et al., 2003. Physico-chemical stability of colloidal lipid particles. *Biomaterials*, 24(23), pp.4283–4300.
13. Ickenstein, L.M. et al., 2006. Effects of phospholipid hydrolysis on the aggregate structure in DPPC/DSPE-PEG2000 liposome preparations after gel to liquid crystalline phase transition. *Biochimica et biophysica acta*, 1758(2), pp.171–180.
14. Iversen, T.G., Skotland, T. & Sandvig, K., 2011. Endocytosis and intracellular transport of nanoparticles: Present knowledge and need for future studies. *Nano Today*, 6(2), pp.176–185.

15. Klang, V. et al., 2012. Electron microscopy of nanoemulsions: an essential tool for characterisation and stability assessment. *Micron (Oxford, England: 1993)*, 43(2-3), pp.85–103.
16. Li, X. et al., 2011. Novel mucus-penetrating liposomes as a potential oral drug delivery system: preparation, in vitro characterization, and enhanced cellular uptake. *International Journal of Nanomedicine*, 6, pp.3151–3162.
17. Lipinski, C.A., 2000. Drug-like properties and the causes of poor solubility and poor permeability. *Journal of pharmacological and toxicological methods*, 44(1), pp.235–249.
18. Lopez, C. & Ollivon, M., 2009. Crystallisation of triacylglycerols in nanoparticles. *Journal of Thermal Analysis and Calorimetry*, 98(1), pp.29–37.
19. Lopez, Christelle, 2011. Milk fat globules enveloped by their biological membrane: Unique colloidal assemblies with a specific composition and structure. *Current Opinion in Colloid & Interface Science*, 16(5), pp.391–404.
20. Mason, T.G. et al., 2006. Nanoemulsions: formation, structure, and physical properties. *Journal of Physics: Condensed Matter*, 18(41), pp.635–666.
21. Morán, M.C. et al., 2005. The effect of molecular shape on the thermotropic liquid crystal behavior of monolaurylated amino acid glyceride conjugates. *The journal of physical chemistry. B*, 109(48), pp.22899–22908.
22. Ollivon, M. et al., 2006. DSC and high resolution X-ray diffraction coupling. *Journal of Thermal Analysis and Calorimetry*, 85(1), pp.219–224.
23. Owens, D.E. & Peppas, N.A., 2006. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *International journal of pharmaceutics*, 307(1), pp.93–102.
24. Paliwal, R. et al., 2009. Engineered chylomicron mimicking carrier emulsome for lymph targeted oral delivery of methotrexate. *International journal of pharmaceutics*, 380(1-2), pp.181–188.
25. Petersen, S. et al., 2011. The physical state of lipid nanoparticles influences their effect on in vitro cell viability. *European journal of pharmaceutics and biopharmaceutics: official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V*, 79(1), pp.150–161.
26. Ponchel, G. et al., 1997. Mucoadhesion of colloidal particulate systems in the gastrointestinal tract : Bioadhesion : State-of-the-art and perspectives. *European journal of pharmaceutics and biopharmaceutics*, 44, pp.25–31.
27. Puri, A. et al., 2009. Lipid-based nanoparticles as pharmaceutical drug carriers: from concepts to clinic. *Critical Reviews in Therapeutic Drug Carrier Systems*, 26(6), pp.523–580.
28. Raffournier, C. et al., 2005. Oil/water hand-bag like structures: how interfacial rheology can help to understand their formation? *STP pharma sciences*, 15(1), pp.3–9.

29. Raffournier, C., Dubernet, C., et al., 2004. Phospholipid/triglyceride mixtures: analysis and comparison of the solubilisation profiles. In *Surface and Colloid Science*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 139–142.
30. Raffournier, C., Ollivon, M., et al., 2004. Solubility of triacylglycerols or stearylamine in phospholipid vesicles. In *Trends in Colloid and Interface Science XVII*. Progress in Colloid and Polymer Science. Springer Berlin / Heidelberg, pp. 6–13
31. Ruthardt, N., Lamb, D.C. & Bräuchle, C., 2011. Single-particle tracking as a quantitative microscopy-based approach to unravel cell entry mechanisms of viruses and pharmaceutical nanoparticles. *Molecular therapy: the journal of the American Society of Gene Therapy*, 19(7), pp.1199–1211.
32. Saad, H.Y. & Higuchi, W.I., 1965. Water solubility of cholesterol. *Journal of Pharmaceutical Sciences*, 54(8), pp.1205–1206.
33. Shah, A.K. & Agnihotri, S.A., 2011. Recent advances and novel strategies in pre-clinical formulation development: an overview. *Journal of controlled release: official journal of the Controlled Release Society*, 156(3), pp.281–296.
34. Svensson, A., Neves, C. & Cabane, B., 2004. Hydration of an amphiphilic excipient, Gelucire 44/14. *International journal of pharmaceutics*, 281(1-2), pp.107–118.
35. Teixeira, H. et al., 2000. New bicompartmental structures are observed when stearylamine is mixed with triglyceride emulsions. *Pharmaceutical research*, 17(10), pp.1329–1332.
36. Werling, J. et al., 2008. Physicochemical stability of phospholipid-dispersed suspensions of crystalline itraconazole. *European journal of pharmaceutics and biopharmaceutics: official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V*, 69(3), pp.1104–1113.
37. Zhao, F. et al., 2011. Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials. *Small (Weinheim an der Bergstrasse, Germany)*, 7(10), pp.1322–1337.

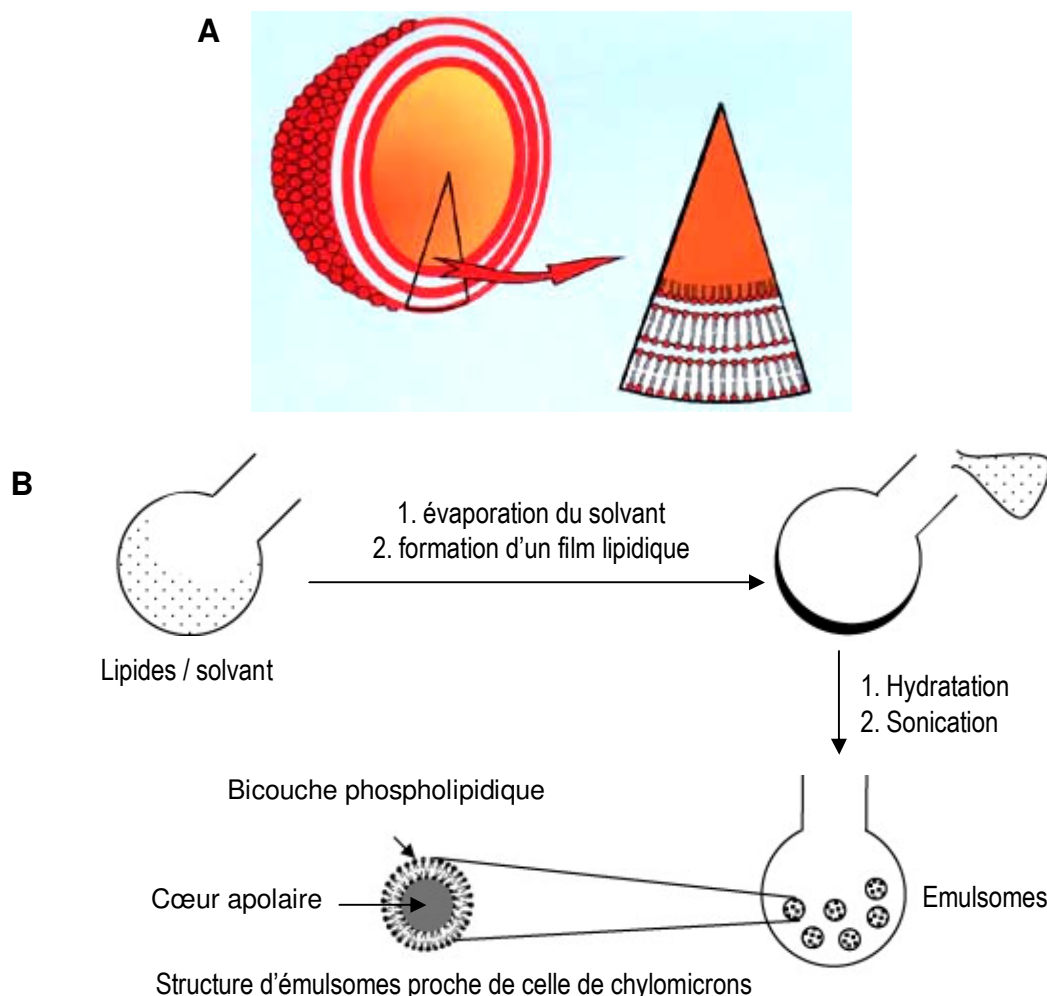
L'originalité du travail présenté dans ce premier chapitre expérimental repose sur la très grande stabilité des nanoparticules formées en présence du couple de tensioactifs utilisé (Gélucire® 50/13 - Phospholipon® 90G), et bien évidemment sur la morphologie de ces particules dans les formulations intégrant du Labrafil® M 2125 CS (Figure 1.12). Ces deux points ont conduit le laboratoire à faire une demande de brevet, dont le dossier est en cours d'instruction au CNRS.



**Figure 1.12.** Représentation schématique de la supra-structure membranaire des nanoparticules lipidiques multicompartimentées revendiquées. La tricouche phospholipidique (une bicouche et une monocouche) est le squelette de la membrane. Dans ce système, les dérivés séquencés de type acide gras - PEG - acide gras sont très certainement le point d'ancrage entre la bicouche externe et la partie lipidique interne. La surface des particules est recouverte par les chaînes de PEG.

Les nano-objets présentés ici s'apparentent principalement à deux familles de particules synthétiques décrites dans la littérature : les émulsomes et les « sacs à main » (particules lipidiques *Janus*). Les émulsomes (Figure 1.13), des objets brevetés, sont représentés comme des particules constituées de bicouches phospholipidiques entourant une gouttelette de lipides (Gupta et al. 2005; Paliwal et al. 2009). Toutefois, ce produit, dont la structure n'a semble-t-il jamais été confirmée expérimentalement, présente deux différences fondamentales avec l'invention proposée :

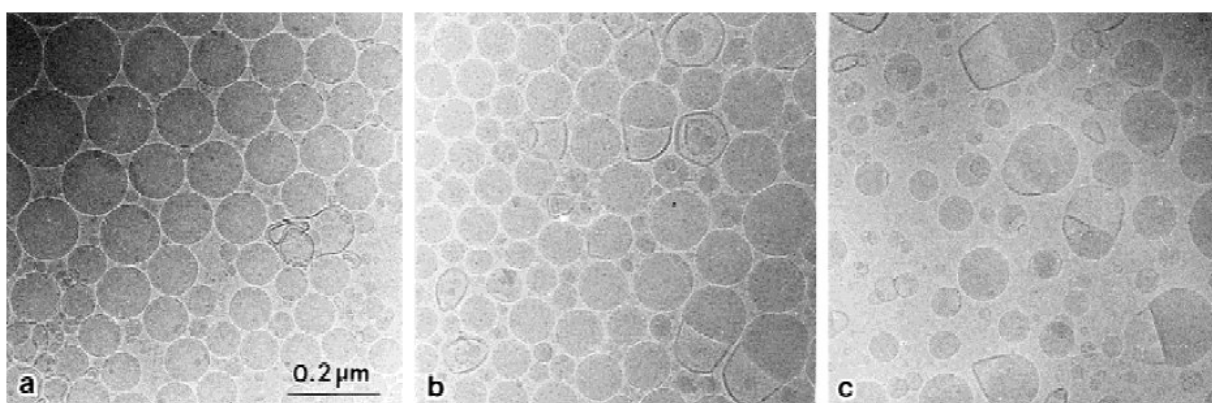
- l'absence de « solidarisation » entre la membrane phospholipidique et le cœur lipophile comme nous l'envisageons avec le Gélucire® 50/13.
- un procédé de préparation a priori (1) difficilement exploitable à très grande échelle avec utilisation de solvant, dépôt et réhydratation d'un film phospholipidique et (2) ne semblant pas garantir la formation de l'objet souhaité.



**Figure 1.13.** (A) Emulsome (d'après [www.pharmoscorp.com](http://www.pharmoscorp.com)),  
(B) Représentation schématique et protocole d'obtention (d'après Paliwal et al. 2009)

D'un point de vue académique, des structures nommées « sacs à main » ou « hand-bags » ont été proposées par l'UMR CNRS 8612 il y a quelques années (Teixeira et al. 2000; Raffournier et al. 2005). La Figure 1.14 schématise ces objets. Le point commun avec les particules revendiquées est la présence de triglycérides et de phospholipides. Toutefois, les grandes différences sont :

- la présence nécessaire de stéarylamine, un lipide cationique monocaténaire
- l'absence de macrogol-glycérides comme le Gélucire® 50/13
- la faible proportion de ces objets par rapport aux gouttelettes de nanoémulsions classiques malgré de nombreuses tentatives d'optimisation de la formulation.

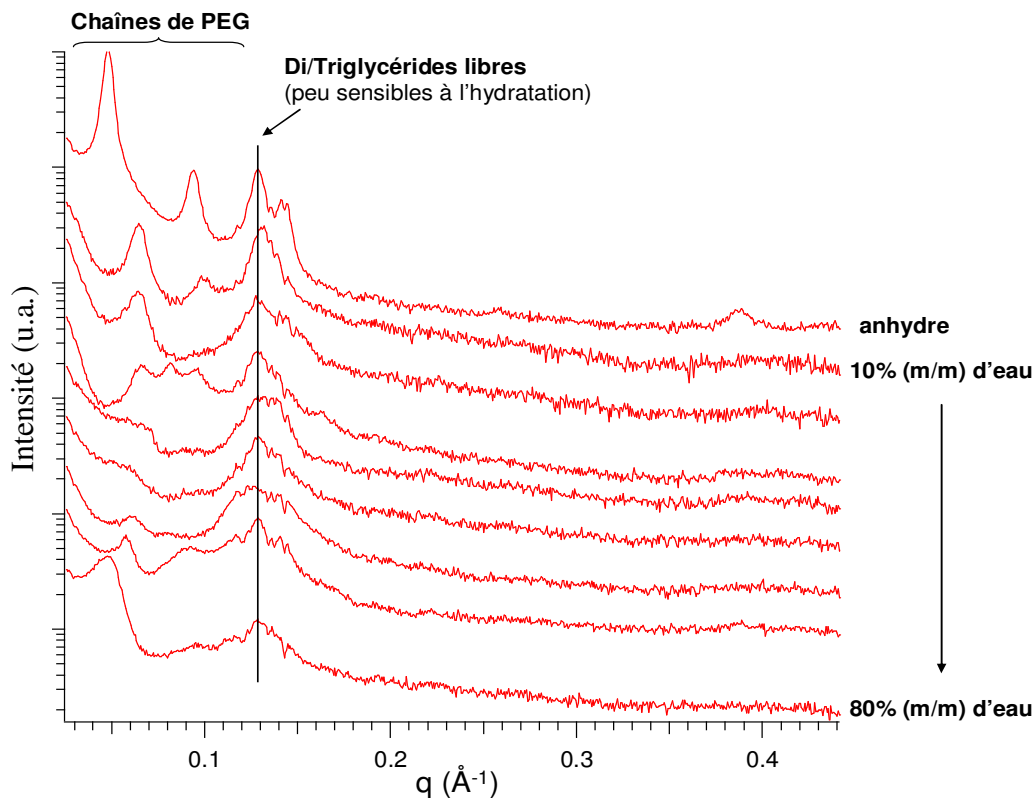


**Figure 1.14.** Images de microscopie électronique décrivant la morphologie et la fréquence des objets de type « sacs à main » pour différentes compositions (d'après Teixeira et al. 2000)

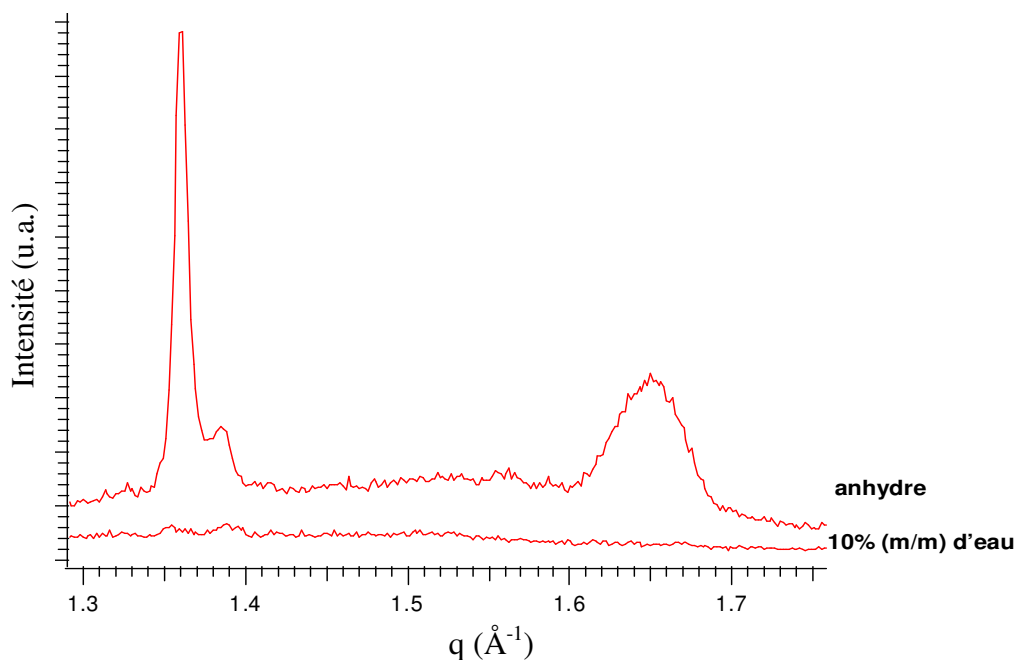
Parmi les excipients utilisés, nous avons vu que le Gélucire® 50/13 avait un rôle important dans la stabilisation des particules. En effet, les phospholipides seuls (le Phospholipon® 90G) n'ont pas permis, en tout cas aux concentrations utilisées, de stabiliser les nanodispersions. Le Gélucire® 50/13 est un mélange complexe (Annexe) qui avait été étudié à l'état anhydre au laboratoire. Pendant cette thèse, des études préliminaires sur son comportement à l'état hydraté ont également été menées, essentiellement par diffraction des rayons X (SAXS, WAXS) et par spectroscopie Raman.

Les Figures 1.15 et 1.16 décrivent les principaux résultats obtenus par diffraction des rayons X. Excepté un pic à  $0.13 \text{ \AA}^{-1}$  qui reste inchangé, les déplacements des pics de Bragg dans le domaine des SAXS correspondant aux structures « pegylées » montrent la complexité du comportement lyotrope du Gélucire® 50/13 et la formation de mésophases diverses (Figure 1.15). Dans la zone des moyens angles (WAXS), la disparition des pics de Bragg entre l'échantillon anhydre et l'échantillon le plus

faiblement hydraté montre la désorganisation totale des hélices formées par les chaînes de PEG (Figure 1.16). Cette déstructuration persiste pour les forts taux d'hydratation.

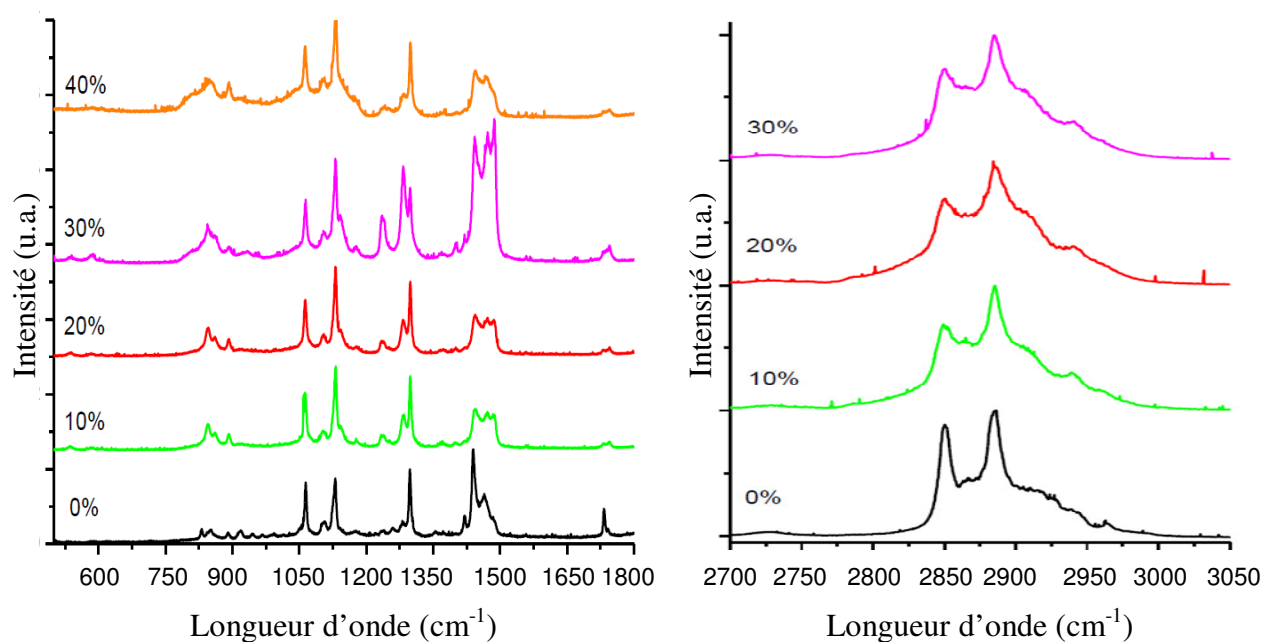


**Figure 1.15.** Diffractogrammes, dans la zone des SAXS, du Gélucire® 50/13 en fonction de son état d'hydratation (1 enregistrement/10 % d'hydratation). Les résultats ont été obtenus au Synchrotron Elettra (Trieste, Italie).



**Figure 1.16.** Diffractogrammes, dans la zone des WAXS, du Gélucire® 50/13 anhydre et hydraté à 10% (m/m). Les résultats ont été obtenus au Synchrotron Elettra (Trieste, Italie).

Pour aller plus loin dans la compréhension de l'interaction entre le Gélucire® 50/13 et l'eau, une collaboration a été initiée avec S. Bresson, du laboratoire de Physique des Systèmes Complexes (Université de Picardie, Amiens), ainsi qu'avec des partenaires marocains dans le cadre d'un projet Egide-Volubilis (2013). L'un des objectifs de cette collaboration est d'étudier le comportement du Gélucire® 50/13 anhydre et hydraté par spectroscopie Raman. Une première étude poussée sur le produit anhydre a été soumise à *Vibrational spectroscopy* (un peu hors du cadre strict de cette thèse, cet article n'a pas été présenté dans le manuscrit) et quelques expériences préliminaires ont été menées sur des échantillons hydratés (Figure 1.17). En fonction de l'état d'hydratation, ces spectres montrent des différences notables qu'il conviendra de fouiller, en corrélation avec les expériences de diffraction des rayons X.



**Figure 1.17.** Spectres Raman d'échantillons de Gélucire® 50/13 anhydre et hydratés. Les résultats ont été obtenus à l'aide d'un spectromètre RAMAN Jobin Yvon T64000 équipé d'un détecteur CCD. Source laser : Ar-ion (514,5 nm,  $\sim 20$  kW/cm<sup>2</sup>). Diamètre du spot : 1  $\mu$ m. Les pourcentages exprimés correspondent au taux d'hydratation (m/m).

Le second chapitre de cette thèse s'intéressera à l'incorporation du principe actif d'intérêt, la quercétine, dans les nanoparticules compartimentées décrites précédemment. Lors de ces travaux, l'accent sera porté principalement sur les problèmes de stabilité ; stabilité du vecteur après incorporation du principe actif et stabilité chimique de ce dernier après son encapsulation.

**Références**

1. Gupta, B., Levchenko, T.S. & Torchilin, V.P., 2005. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. *Advanced drug delivery reviews*, 57(4), pp.637–651.
2. Paliwal, R. et al., 2009. Engineered chylomicron mimicking carrier emulsome for lymph targeted oral delivery of methotrexate. *International journal of pharmaceutics*, 380(1-2), pp.181–188.
3. Raffournier, C. et al., 2005. Oil/water hand-bag like structures: how interfacial rheology can help to understand their formation? *STP pharma sciences*, 15(1), pp.3–9.
4. Teixeira, H. et al., 2000. New bicompartmental structures are observed when stearylamine is mixed with triglyceride emulsions. *Pharmaceutical research*, 17(10), pp.1329–1332.
5. <http://www.pharmoscorp.com/development/nanotechnology.html>

## **Quercetin solubility and chemical stability: enhancement with polyoxylglyceride-based multi-compartmented nanodispersions**

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**Abbreviations:**  $\pi$ -A, surface pressure – area ;  $A_a$ , average area;  $A_c$ , collapse area; API, active pharmaceutical ingredient; Cryo-TEM, cryogenic transmission electron microscopy; G/P, Gelucire® 50/13 – Phospholipon® 90G mixture; HPLC, high performance liquid chromatography; HPH, high pressure homogenization; Pdl, polydispersity index; SGF, simulated gastric fluid; SIF, simulated intestinal fluids;  $t_{1/2}$ , half-life of degradation; TEM, transmission electron microscopy.

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**Abstract**

The flavonoid quercetin is one of the most prominent antioxidants in the disease prevention and therapy. However, its use in the pharmaceutical field is limited by its poor bioavailability. Quercetin is insoluble and unstable in aqueous media. To overcome these drawbacks, polyoxylglyceride-based nanodispersions loading quercetin were successfully prepared by high pressure homogenization (HPH) process. Formulation containing 1% (w/w) quercetin in lipid had particle size of  $207.1 \pm 10.7$  nm with a narrow distribution (Pdl  $\sim 0.1$ ) and an encapsulation efficiency of 96.4 %. The apparent solubility of drug was 4500-fold higher compared with that of the original quercetin ( $0.4 \pm 0.3$   $\mu\text{g/ml}$ ). The TEM and Cryo-TEM images presented the structure of multi-compartmented lipid nanoparticles which were physically stable at least 90 days at room temperature and 4 °C. Meanwhile, the stability studies demonstrated that quercetin was relatively stable in simulated gastric fluid (SGF) at room temperature and 37 °C; it was unstable in Milli-Q water (pH = 5.6,  $t_{1/2}$  = 16.8 h), simulated intestinal fluid (SIF) (pH = 6.5,  $t_{1/2}$  = 13.2 h) and HEPES buffet (pH = 7.4,  $t_{1/2}$  = 9.8 h) at room temperature. The results confirmed that nanodispersion improved the apparent solubility and the chemical stability of quercetin. Furthermore, a controlled release of the API of interest had been observed during 4 h, without any burst effect. The polyoxylglyceride-based multi-compartmented nanoparticle is one of the promising nanocarriers for administration of sensitive active ingredients.

**Keywords:** chemical stability, high pressure homogenization, multi-compartmented lipid nanoparticles, nanodispersions, polyoxylglycerides, quercetin, solubility, surface pressure measurements.

## Contents

### 1. Introduction

### 2. Materials and methods

#### 2.1. Materials

#### 2.2. HPLC analysis

#### 2.3. Solubilisation capacity in solvents and aqueous media

#### 2.4. Solubilisation capacity in excipients

#### 2.5. Stability of quercetin in different media

#### 2.6. Surface pressure measurements

#### 2.7. Preparation of nanodispersions by HPH process

#### 2.8. Particle size and zeta potential analysis

#### 2.9. Drug loading and encapsulation efficiency

#### 2.10. Transmission electron microscopy (TEM) and Cryogenic transmission electron microscopy (Cryo-TEM)

#### 2.11. *In vitro* release studies

#### 2.12. Long-term stability

### 3. Results and discussion

#### 3.1. Solubility and stability of quercetin dissolved in different aqueous media

#### 3.2. Solubility of quercetin in excipients and organic solvents

#### 3.3. Physico-chemical properties of nanodispersions

##### 3.3.1. Size analysis, zeta potential measurements and physical stability

##### 3.3.2. Drug payload

##### 3.3.3. Morphological studies

#### 3.4. *In vitro* release

#### 3.5. Long-term stability of quercetin-loaded nanodispersions

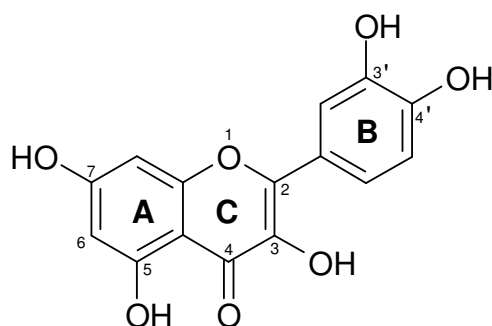
### 4. Conclusion

### Acknowledgement

### References

## 1. Introduction

Quercetin (3,3',4',5,7-pentahydroxyflavone) is one of the most abundant flavonoids in plants. This molecule possesses several structural elements giving anti-oxidant properties: (1) an ortho-dihydroxy or catechol group in ring B; (2) a 2,3-double bond; (3) the 3- and 5-OH groups with the 4-oxo group (Chen et al. 2010) (Figure 2.1). Thus, it is an excellent *in vitro* antioxidant active pharmaceutical ingredient (API) (Boots et al. 2008). It has also been extensively investigated for other pharmacological effects such as anti-tumor (Chen et al. 2010), neuro- and cardioprotective (Russo et al. 2012; Dajas 2012), anti-inflammatory (Kleemann et al. 2011), antibacterial (Cushnie & Lamb 2005) and antiviral (Thapa et al. 2012) activities.



**Figure 2.1.** Molecular structure of quercetin.

In spite of this wide spectrum of potential therapeutic applications, quercetin used in the pharmaceutical field is limited by its very poor bioavailability. Indeed, the absolute bioavailability for the solution is 0.275 and that for suspension is 0.162 after a single administration of 50 mg/kg dose in rats (Khaled et al. 2003), whereas the bioavailability is less than 1% in volunteers after capsule ingestion (Gugler et al. 1975). The low solubility of quercetin is the first reason which explains for this limitation. Indeed, it is a lipophilic aglycon ( $\log P = 1.82 \pm 0.32$ ) (Rothwell et al. 2005) with a solubility in water as low as 0.17 – 7.7  $\mu\text{g/ml}$  (Azuma et al. 2002; Lauro et al. 2002). The second reason is certainly the chemical instability of quercetin in aqueous media. This molecule is unstable in water ( $t_{1/2} = 23.1$  h) at room temperature, but stable at 4°C (Moon et al. 2008). It is decomposed into protocatechuic acid and phloroglucinol carboxylic acid (Pinelo et al. 2004). To overcome these drawbacks, a lot of formulation strategies have been designed. Most of them deal with the API solubility as it has been done with cellulose acetate-based microparticles (Sansone et al. 2011; Scarfato et al. 2008), polymethyl methacrylate microcapsules (Lee et al. 2007), lipid microparticles (Scalia & Mezzena 2009), PLGA

nanocapsules (Ghosh et al. 2009), Eudragit® E and polyvinyl alcohol nanoparticles (Wu et al. 2008), poly-D,L-lactide nanoparticles (Kumari et al. 2010), lipid nanocapsules (Barras et al. 2009), nanocrystals (Sahoo et al. 2011), nanosuspensions (Gao et al. 2011), liposomes (Gordon & Roedig-Penman 1998; Priprem et al. 2008), solid lipid nanoparticles (Li et al. 2009), microemulsions (Rogerio et al. 2010), lecithin-based cationic nanocarriers (Leciplex) (Date et al. 2011), quercetin/ polyvinylpyrrolidone Kollidon® 25 solid dispersions (Mello-Costa et al. 2010) and quercetin/ $\beta$ -cyclodextrin solid complexes (Borghetti et al. 2009). However, there are a few studies about chemical stability of quercetin into these formulations. The chemical instability of quercetin is almost completely suppressed during 3-month storage at room temperature in the dark by its encapsulation into tristearin-based microparticles (Scalia & Mezzena 2009). However, the size of the microparticles in the range 10-45  $\mu$ m limits their application, e.g., for intravenous route. The chemical and photo-stability of quercetin is also reported during 30 days and 10 days, respectively, after the formulation of Pluronic-rich nanosuspension. The size of these nanosuspensions is small, around 200 nm, but the drug release is very fast. The API incorporated in nanosuspensions is totally released after 30 min., limiting the benefit of the encapsulation after administration (Gao et al. 2011).

Beside its own pharmacological properties, quercetin could also be used as an attractive model of poorly water stable molecules to design and develop new delivery systems. The present paper describes the preparation of drug-loaded nanodispersions by high-pressure homogenization (HPH). In HPH process, microdispersions is passed at high pressure through homogenization gaps with high velocities to obtain nanodispersions having narrow size distribution (Muller et al. 1999). HPH is usually used to formulate nanocarriers generally loading poorly soluble drugs in aqueous media as well as organic solvents. It is a simple process available at laboratory and industrial scale (Shegokar et al. 2011). In the present work, both oily phase and surfactants were optimized to prepare quercetin stable nanodispersions. Their physico-chemical characteristics were also thoroughly investigated.

## 2. Materials and methods

### 2.1. Materials

Quercetin ( $\geq$  98% purity), pepsin, sodium chloride, sodium bicarbonate, sodium citrate, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), hydrochloric acid and

trifluoro-acetic acid were supplied from Sigma-Aldrich (Saint Quentin-Fallavier, France). Phospholipon® 90G (soybean lecithin at 94 – 102% of phosphatidylcholine) was provided by Phospholipid GmbH, Lipoid Group (Köln, Germany). Gelucire® 50/13 (stearoyl macroglycerides), Labrafil® M 2125 CS (linoleoyl polyoxylglycerides), Labrasol® (caprylocaproyl polyoxyl-8 glycerides) and Labrafac® CC (capric and caprylic triglycerides) were supplied by Gattefossé S.A.S. (Saint-Priest, France). Sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ) was purchased from Fluka Chemika (Buchs, Switzerland). Methanol and acetonitrile for HPLC were obtained from Carlo Erba, Italy. Pure water was filtered through a Milli-Q water system (Millipore, France). All other solvents (analytical grade) and other chemicals (reagent grade) purchased from standard suppliers.

## 2.2. HPLC analysis

HPLC analysis were performed on a Waters HPLC system (Waters 1525 Binary HPLC pump, Waters 2487 Dual  $\lambda$  Absorbance Detector, Waters 717 plus Autosampler, Waters In-Line Degasser AF and Breeze software). Chromatographic separation was carried out with a Modulo-cart QK strategy 3 C18-2 column (150 mm  $\times$  3 mm, Interchim). The mobile phase was acetonitrile – water – trifluoroacetic acid (30:70:0.1, v/v). The detection wavelength was set at 371 nm. The flow rate was 0.5 ml/min. The sample volume was 20  $\mu\text{l}$ . The calibration curve was linear in the range of 50 – 5000 ng/ml ( $y = 166694x$ ,  $r^2 = 0.9998$ ). Before HPLC analysis, all samples were dissolved in methanol at concentrations within the linear range, and then filtered through a membrane of 0.2  $\mu\text{m}$  (Minisart® High-Flow Hydrophilic 16532K, Sartorius Stedim Biotech GmbH, Goettingen, Germany). The API peak was identified on the chromatograms at a retention time of 7.4 min.

## 2.3. Solubilisation capacity in solvents and aqueous media

The drug solubility in solvents was quantified by HPLC. An excess amount of drug was dissolved at room temperature in 10 ml of methanol or ethanol with a stirring speed of 800 rpm during 24 h. Similarly, it was dissolved in 10 ml of aqueous media including Milli-Q water, HEPES buffer, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) with a stirring speed of 100 rpm during an hour at 37 °C. The samples were filtered through a membrane of 0.2  $\mu\text{m}$  (Minisart® High-Flow Hydrophilic 16532K, Sartorius Stedim Biotech GmbH, Goettingen, Germany) and diluted appropriately in methanol before HPLC analysis ([section 2.2.](#)).

HEPES buffer contained 2.4 g of HEPES, 6.3 g of sodium chloride, 20 mM sodium citrate (the pH was adjusted with 1N NaOH to 7.4) ([Urbinati et al. 2010](#)). SGF was prepared with 2 g of sodium chloride, 3.2 g of pepsin (385 units/mg), 7 ml of hydrochloric acid (37% hydrochloric acid to adjust pH to 1.2) and completed with water to 1 l. SIF was a mixture of 10.3 g of sodium dihydrogen phosphate, 12.4 g of sodium chloride, 35 ml of sodium hydroxide solution 1 N, 7 g of sodium bicarbonate (to adjust pH to 6.5 when mixed with SGF in a ratio of 50/50; v/v) and completed with water to 1 l ([Hamoudi et al. 2011](#)).

#### 2.4. Solubilisation capacity in excipients

The drug solubility in some excipients at room temperature was determined by HPLC. To 2.5 g of excipients, drug was gradually added, from 1 mg to 25 mg, until saturation. Each vial was stirred for 800 rpm within 24 h with a magnetic stirrer (Intern. Laborat. App. GmbH, Dottingen, Germany). After visual analysis, the over saturated mixtures were centrifuged (LE-80K Ultracentrifuge Optima™, Rotor 701Ti - 93E502, Coulter™ Beckman, Villepinte, France) for 20000 rpm during an hour at 25°C. The supernatants were diluted appropriately in methanol and the drug concentration was determined by HPLC analysis as mentioned above.

#### 2.5. Stability of quercetin in different media

Stability of drug dissolved in Milli-Q water (pH = 5.6), HEPES buffer (pH = 7.4), SIF (pH = 6.8) at room temperature and SGF (pH = 1.2) was evaluated over 24 h at room temperature and 37°C. At room temperature, about 1 mg of quercetin was dissolved in 10 ml of different media with a stirring rate of 800 rpm during 30 min, and then filtered through a membrane of 0.2 µm (Minisart® High-Flow Hydrophilic 16532K, Sartorius Stedim Biotech GmbH, Goettingen, Germany). The drug contents were analyzed by HPLC analysis ([see section 2.2.](#)) according to incubation time (0 – 24 h) at the desired temperatures.

#### 2.6. Surface pressure measurements

A homemade autorecording Langmuir-type film trough (160 cm<sup>3</sup>, 217.2 cm<sup>2</sup>) was used to record monolayer compression isotherms of Phospholipon® 90G, Gelucire® 50/13 and their mixtures (G/P). This apparatus was equipped with an R&K Wilhelmy pressure transducer (Riegler and Kirstein, GmbH, Germany) in a Plexiglas box to limit surface

contamination. In these experiments, a chloroform solution containing the individual excipients or their mixtures was deposited onto pure water subphase by means of a micropipette (Microman<sup>®</sup>, Gilson, France). For all studied systems, the same concentrations (3.0 mg/ml) were spread at the air/water interface with the volume of 7.5  $\mu$ l at  $22 \pm 1^\circ\text{C}$ . After the deposition of an amphiphilic solution, the solvent was allowed to evaporate for 10 min. The compression cycle was conducted at a speed of 9.57  $\text{cm}^2/\text{min}$ . The water surface was swept with a capillary pipette connected to a vacuum pump prior to another monolayer spreading.

## 2.7. Preparation of nanodispersions by HPH process

Gelucire<sup>®</sup> 50/13 and Phospholipon<sup>®</sup> 90G were dispersed in Milli-Q water as aqueous phase thanks to an Ultra-Turrax T25 (Janke & Kunkel GMBH & Co.KG, IKA<sup>®</sup>-Labortechnik, Germany) at 9500 rpm during 5 min at  $70^\circ\text{C}$  to facilitate stabilizer dispersion. Quercetin was dissolved at  $70^\circ\text{C}$  in a mixture containing 10 ml of ethanol and 10 g of Labrafil<sup>®</sup> M 2125 CS to increase its dissolution rate in lipid. The ethanol content was then evaporated under reduced pressure (Rotavapor, Büchi, Switzerland). This lipid phase was added slowly into the aqueous phase under continuous stirring at 13500 rpm by Ultra-Turrax for 5 min at the same temperature to obtain an oil-in-water pre-emulsion. This pre-emulsion was quickly introduced into a two-stage homogenizer (APV 2000, Invensys, Albertslund, Denmark) previously heated to  $70^\circ\text{C}$  during 5 min to form a hot oil-in-water nanoemulsion. The pressure of the first floor was 600 bars and that of the 2nd floor was 200 bars. Finally, nanodispersions were kept at room temperature.

## 2.8. Particle size and zeta potential analysis

The particle size and zeta potential of nanodispersions were measured at  $25^\circ\text{C}$  using a Nano-ZS90 apparatus (Malvern Instruments, Orsay, France). The average hydrodynamic diameter and polydispersity index (Pdl) determined by *quasi*-elastic light scattering. The zeta potential determinations were performed using electrophoretic laser Doppler velocimetry. The samples were diluted to 1/300 in Milli-Q water for size analysis and in 1 mM NaCl for zeta potential measurements.

## 2.9. Drug loading and encapsulation efficiency

After the preparation, the quercetin-loaded nanodispersions were purified by

ultracentrifugation (LE-80K Ultracentrifuge Optima™, Rotor 701Ti - 93E502, Coulter™ Beckman, Villepinte, France) for 30000 rpm during one hour at 4°C. After ultracentrifugation, the colloidal suspensions could be separated into three parts: nanodispersions (top layer), aqueous phase eventually containing micelles or phospholipidic vesicles (middle layer) and precipitate (bottom layer). The top layer was used for API quantification by HPLC analysis as mentioned above in order to determine the amount of entrapped drug in nanodispersions. The drug loading and encapsulation efficiency were calculated using the formula:

$$\text{Drug loading (\%)} = \frac{\text{Amount of entrapped drug}}{\text{Initial amount of lipid in the formulation}} \times 100$$

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Amount of entrapped drug}}{\text{Initial amount of drug in the formulation}} \times 100$$

After ultra-centrifugation, an aliquot of the top layer weighed and dissolved in the mobile phase at a concentration in the linearity range of the HPLC assay. The solution was filtered through a membrane with a pore size of 0.2 µm (Minisart® High-Flow Hydrophilic 16532K, Sartorius Stedim Biotech GmbH, Goettingen, Germany) before injection into HPLC system ([section 2.2.](#)). Amount of entrapped drug in lipid nanodispersions was calculated according to the regression line and humidity of the sample.

## 2.10. Transmission electron microscopy (TEM) and cryogenic transmission electron microscopy (Cryo-TEM)

The morphology of nanodispersions was observed using a TEM system (JEM-1400, JEOL, Japan) and a cryo-TEM system (JEM 2100, JEOL, Japan). 10 µl of diluted samples (1/100; v/v in Milli-Q water) were placed on a copper grid, and the solution excess was carefully removed by a filter paper. For the TEM, fine-grain TEM specimen support film was dried using a JEE-420 vacuum evaporator (JEOL, Japan) before the observation. For the cryo-TEM, vitrification of the film was achieved by rapidly plunging the grid into liquid ethane held just above its freezing point. The vitrified sample was then transferred to the microscope. The temperature was kept about -180°C during both the transfer and the viewing procedures in order to prevent sample perturbation and the formation of ice crystals.

### 2.11. *In vitro* release studies

The *in vitro* release of the quercetin-loaded nanodispersions and the raw API suspensions was performed in 500 ml of SGF (pH = 1.2) using the dialysis bag method. The dialysis bags (MWCO 6-8000, Spectrum<sup>®</sup>Laboratories, Inc., Rancho Dominguez, CA) were soaked in Milli-Q water for one hour and then washed before use. Samples equivalent to 0.25 mg of quercetin were put into dialysis bags. The magnetic stirring speed was set at 100 rpm. The temperature was maintained at  $37 \pm 0.5^{\circ}\text{C}$ . Dissolution media samples of 0.5 ml were withdrawn at time intervals of 0, 15, 30, 45, 60, 120, 180, 240, 300, 360 min and 24 h. The drug concentrations were determined by HPLC analysis as mentioned above. In the same condition, samples were directly put into dissolution medium. The particle size was measured at time intervals of 0, 2, 4, 6, 8, 10 and 24 h. All the operations were carried out in triplicate.

### 2.12. Long-term stability

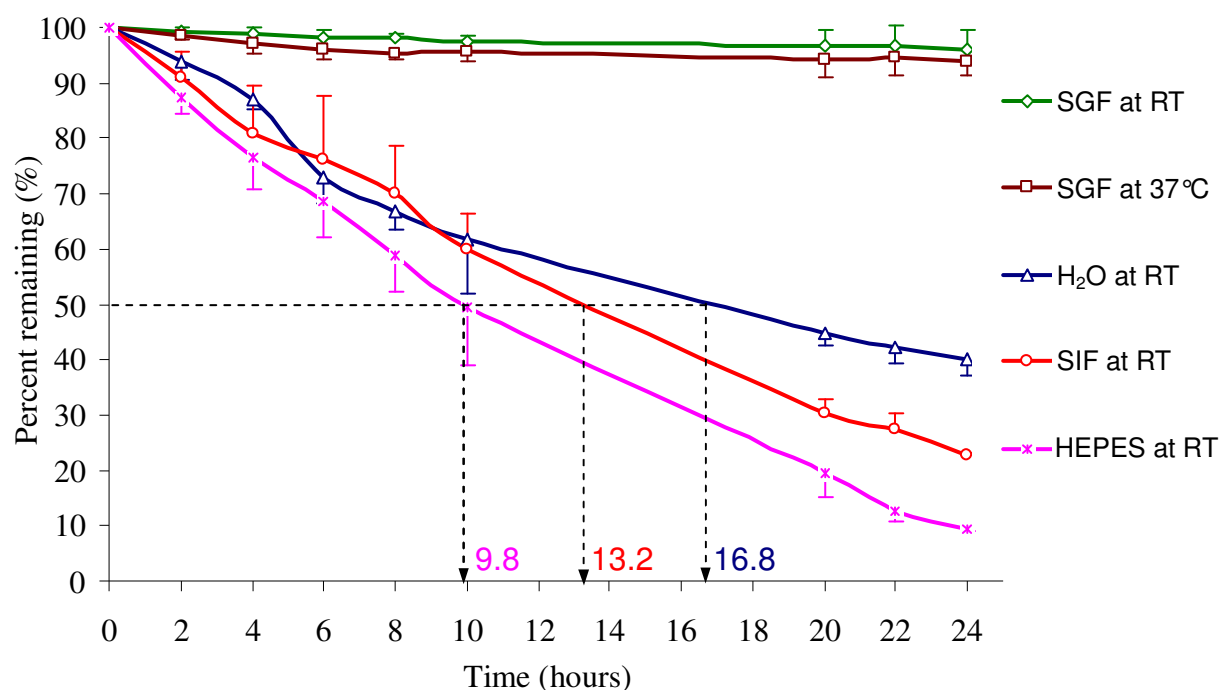
The quercetin-loaded nanodispersions were stored in the dark at both room temperature and  $4^{\circ}\text{C}$ . Concerning the physical stability, the droplet size was measured by *quasi*-elastic light scattering using a Nano-ZS90 apparatus (Malvern Instruments, Orsay, France) over 3 months. For chemical stability, the drug content was determined by HPLC analysis as mentioned above.

## 3. Results and discussion

### 3.1. Solubility and stability of quercetin dissolved in different aqueous media

To get more insights about the stability of quercetin in different aqueous media, its stability was investigated in Milli-Q water, SGF, SIF and HEPES buffet. The API concentration in SGF (pH = 1.2) remained relatively stable with  $96.1 \pm 3.6\%$  at room temperature and  $93.9 \pm 2.9\%$  at  $37^{\circ}\text{C}$  versus initial concentration within 24h. However, this molecule was unstable in Milli-Q water (pH = 5.6), HEPES buffer (pH = 7.4) and SIF (pH = 6.5) at room temperature. In fact, the half-lives of degradation ( $t_{1/2}$ ) were short, around 10 h, 13 h and 17 h in HEPES buffet, SIF and Milli-Q water, respectively. It was clear that  $t_{1/2}$  decreased with increasing pH (Figure 2.2). This is convenient with literature, notably stability studies at different pH values (pH 2.7, 7 and 10) and different temperatures (room temperature,  $4^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ ) in which quercetin is unstable under basic conditions but stable under acidic conditions (Moon et al. 2008). The instability of quercetin under basic conditions should be due to the auto-oxidation of the center ring (C-ring), resulting in fragmentation

of the molecule (Lei et al. 2008). Concerning the solubility, quercetin was practically insoluble in aqueous media as described in Table 2.1. The solubility at 37°C varied from 0.44 to 3.53 µg/ml depending on pH. The significant gap observed between the solubility into HEPES buffer versus the other media could be correlated to the values of pK<sub>a</sub>, 6.6 and 9.7, determined for quercetin. The first one corresponds to the hydroxyl group in the γ-pyrone fragment (3-OH) while the other one agrees with typical pK<sub>a</sub> values for phenol groups (Zenkevich & Guschina 2010). Probably because of the API chemical instability, a large range of quercetin water solubility is noted from 0.17 to 7.7 µg/ml in previous studies (Lauro et al. 2002; Priprem et al. 2008; Gao et al. 2009; Srinivas et al. 2010). Our data were in the same order of magnitude but should be taken with care due to the pH effect.



**Figure 2.2.** Stability of quercetin dissolved in water (pH = 5.6), HEPES buffer (pH = 7.4), SIF (pH = 6.8) at room temperature (RT) and SGF (pH = 1.2) at RT and 37°C, evaluated over 24 h.

**Table 2.1.** Solubility of quercetin in aqueous media at 37°C.

| Aqueous media    | µg/g      | %       |
|------------------|-----------|---------|
| H <sub>2</sub> O | 0.4 ± 0.3 | 0.00004 |
| SGF              | 0.8 ± 0.3 | 0.00008 |
| SIF              | 1.0 ± 0.2 | 0.00010 |
| HEPES buffer     | 3.5 ± 0.4 | 0.00035 |

µg/g: weight of quercetin / total weight (quercetin and excipients); %: weight of quercetin (g) / 100 g of solvent.

One strategy to overcome low solubility and/or high instability is the confinement of the drugs into a particulate carrier, e.g. polymer nanoparticles or lipidic systems (liposomes, nanoemulsions, solid lipid nanoparticles, and nanostructured lipid carriers). Indeed, incorporation of the drug into a nanoparticulate carrier can increase its apparent solubility and protect it against degradation, both *in vitro* and *in vivo* (Muller & Keck 2004). In order to reduce the water diffusion into the nanodispersions, and efficiently protect quercetin against hydrolysis, lipid-based nanosystems have been developed in the present work.

### 3.2. Solubility of quercetin in excipients and organic solvents

**Table 2.2.** Solubility of quercetin in excipients and solvents at room temperature

| Excipients and solvents                                          | Solubility  |        |
|------------------------------------------------------------------|-------------|--------|
|                                                                  | mg/g        | %      |
| PEG 400                                                          | 206.1 ± 0.4 | 26.0   |
| Labrasol <sup>®</sup> (HLB = 14)                                 | 112.9 ± 0.2 | 12.7   |
| Labrafil <sup>®</sup> M 2125 CS (HLB = 4)                        | 9.1 ± 0.4   | 0.9    |
| Labrafac <sup>®</sup> CC (HLB = 1)                               | 1.6 ± 0.1   | 0.2    |
| Natural oils                                                     | < 0.7       | < 0.07 |
| EtOH                                                             | 60.6 ± 0.6  | 6.5    |
| MeOH                                                             | 29.1 ± 2.0  | 3.0    |
| Traited Labrafil <sup>®</sup> M 2125 CS (HLB = 4) <sup>(*)</sup> | 17.0 ± 0.5  | 1.7    |

<sup>(\*)</sup>Traited Labrafil<sup>®</sup> M 2125 CS: an excess amount of quercetin was dissolved in a mixture containing ethanol and Labrafil<sup>®</sup> M 2125 CS (1:1) and then ethanol content was totally evaporated under reduced pressure before ultracentrifugation and HPLC analysis; mg/g: weight of quercetin / total weight (quercetin and excipients); %: weight of quercetin (g) /100 g of excipients.

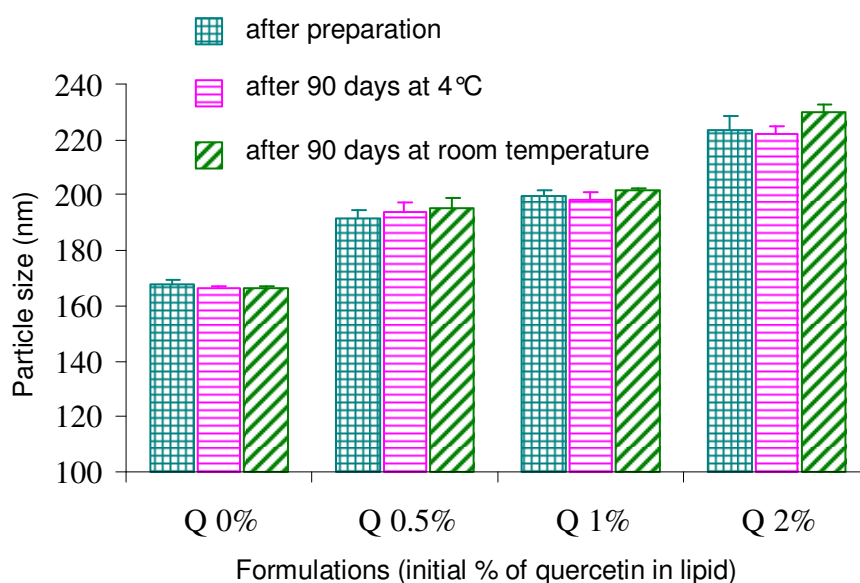
As expressed in Table 2.2, the best solvent for quercetin was PEG 400. So, depending on the PEG/lipid ratio, quercetin was freely soluble in Labrasol<sup>®</sup>, sparingly soluble in more lipophilic polyoxyglycerides (Labrafil<sup>®</sup> M2125 CS), but slightly soluble in lipophilic excipients as Labrafac<sup>®</sup> CC (HLB = 1) or natural oils (data not shown). PEG 400 and Labrasol<sup>®</sup> are too hydrophilic to give nanodispersions into aqueous phase. Thus, Labrafil<sup>®</sup> M2125 CS was chosen as oil phase of the oil-in-water nanodispersions. Preliminary work in our research group proved the interest of Gelucire<sup>®</sup> 50/13 and Phospholipon<sup>®</sup> 90G to stabilize lipid nanoparticles (Truong Cong et al., to be submitted).

Based on it, formulation containing 2g of Gelucire® 50/13, 1 g of Phospholipon® 90G, 20 g of Labrafil® M 2125 CS and 77 g of water, was taken to encapsulate the API of interest. In addition, quercetin was rapidly soluble in ethanol and methanol, especially at high temperatures. For this reason, ethanol was used as an intermediate solvent to reduce the solubilization time of the API in lipid phase for preparation process. The solubility in methanol was also studied for the preparation of the HPLC analysis samples. Fortunately, this API had not only high solubility but also good stability in methanol, ethanol as well as PEG 400 (data not shown).

### 3.3. Physico-chemical properties of nanodispersions

#### 3.3.1. Size analysis, zeta potential measurements and physical stability

Depending on the loaded quercetin amount, from 0% to 2% (w/w), the average particle sizes of nanodispersions were ranging from 165 to 230 nm with narrow size distribution (Pdl between 0.05 and 0.11). At room temperature and at 4°C, the size remained constant during three months, showing that the nanodispersions were physically stable for at least the same duration (Figure 2.3).

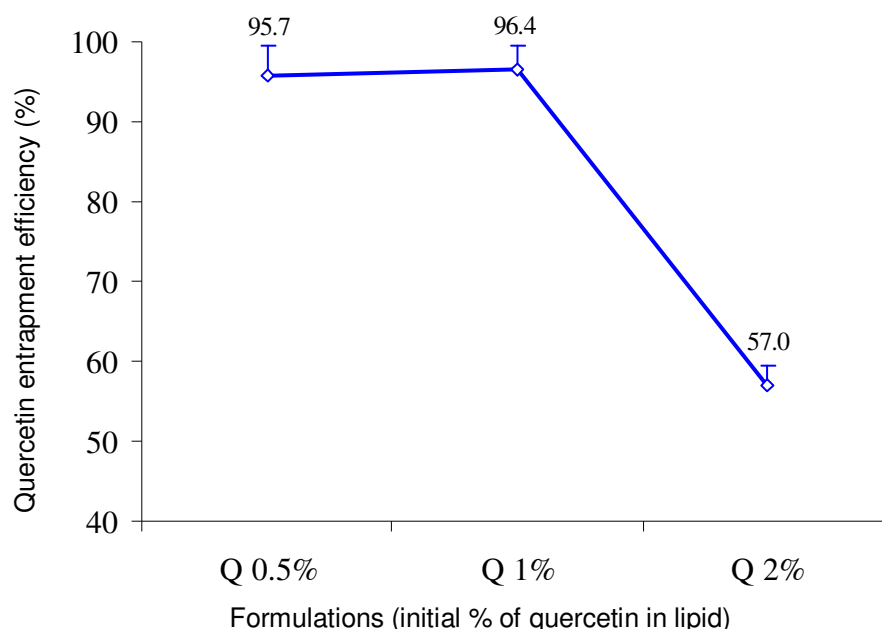


**Figure 2.3.** Dependency of particle size of nanodispersions on the loaded amount of quercetin from 0.5% to 2% (w/w) after preparation and after 90 days at room temperature and 4°C.

All the loaded nanodispersions had zeta potential similar to the unloaded one. This value was relatively low, around – 6 mV. This strongly suggested the presence of Gelucire® 50/13 at the surface of the nanoparticles which led to a charge masking effect by the PEG chains. When API amount increased, the zeta potential did not

change. It could be said that the API didn't adsorb at the interface. It is currently admitted that zeta potentials under  $|30|$  mV, optimum  $> |60|$  mV, are required for full electrostatic stabilization; potentials between  $|5|$  and  $|15|$  mV are in the region of limited flocculation; and below  $|5|$  mV of maximum flocculation (Heurtault et al. 2003). The results confirmed that Gelucire® 50/13 was important to ensure a long-time steric stability to the present nanodispersions.

### 3.3.2. Drug payload



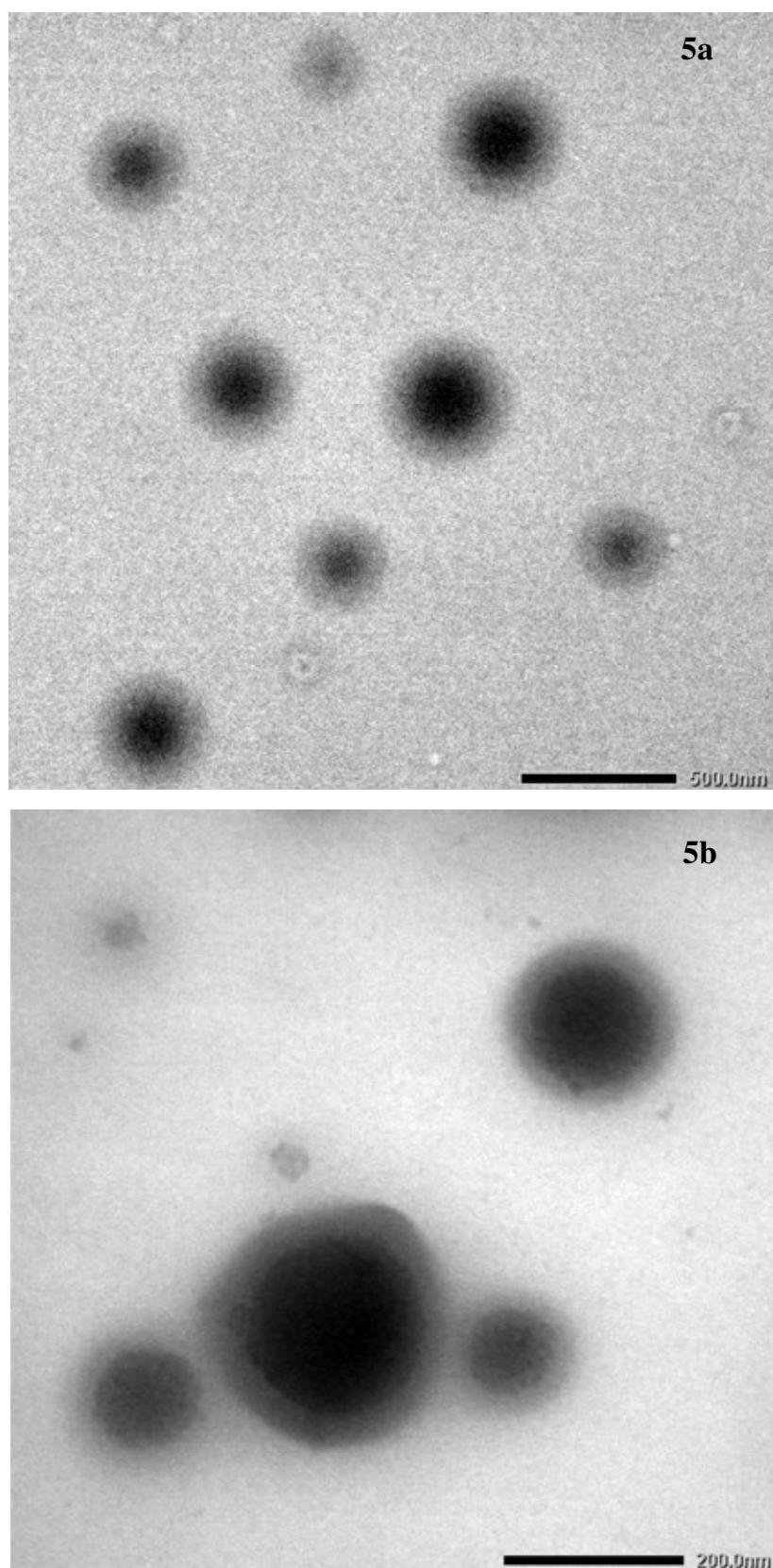
**Figure 2.4.** Quercetin entrapment efficiency (%) in nanodispersions.

The drug payload results are summarized in Figure 2.4. For formulations in which 0.5 or 1% of API have been introduced, the drug entrapment efficiency was not significantly different to 100%, at  $95.7 \pm 3.8\%$  and  $96.4 \pm 3.0\%$ , respectively. For Q2% formulation, the drug entrapment efficiency had a sudden drop to 57.0%. Percentages of quercetin loading in oil phase were equal to 0.48%, 0.96% and 1.14% for Q0.5%, Q1% and Q2% formulations, respectively. The solubility in Labrafil® M2125 CS was close to 1% (Table 2.2). These results clearly suggested that the API incorporation was limited by its solubility in the oil phase. Beyond the drug saturation threshold of the lipidic phase, it was probable that precipitate appeared in the aqueous phase. Practically, after ultracentrifugation for Q2% formulation, the colloidal suspensions were clearly separated into three parts: lipid nanoparticles at top layer, transparent aqueous phase potentially containing surfactant micelles at middle layer and precipitate at bottom layer confirming

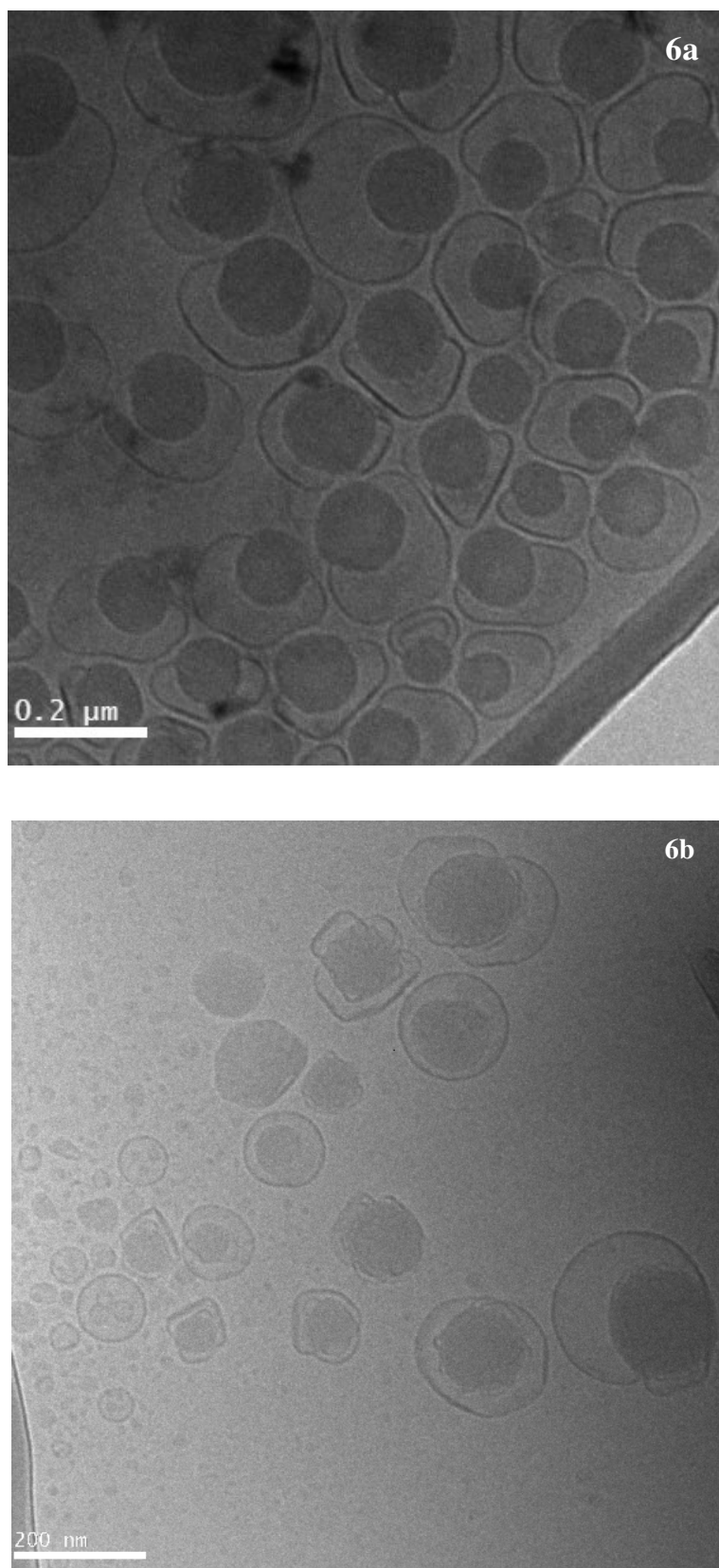
this hypothesis. Furthermore, as the encapsulation efficiency in Q1% formulation was around 100%, the API amount into the aqueous phase should be low. This was expected due to the poor-water solubility of the API but several parameters would have been able to increase this solubility. It was possible to mention the presence of free PEG coming from the excipients into the aqueous phase or the co-existence of other carriers such as liposomes (Phospholipon® 90G) or micelles (Gelucire® 50/13). As it was reasonable to assume that more or less all the API was associated with lipid droplets, Q1% formulation was taken for next investigations. The apparent solubility of quercetin in Q1% nanodispersions was 4500-fold higher compared with that of the original API ( $0.44 \pm 0.25$  µg/ml). Clearly, nanodispersions could be potential delivery systems to enhance the apparent solubility of poorly water-soluble molecules.

### 3.3.3. Morphological studies

The [Figure 2.5 and 2.6](#) described TEM and cryo-TEM observations on unloaded and loaded formulations. First of all, the pictures confirmed the droplet size measured by *quasi*-elastic light scattering (around 200 nm). More interesting, the most typical shapes were multi-compartment spherical objects as observed in TEM ([Figure 2.5](#)). Independently on the presence of API, several domains were clearly visible on the cryo-TEM pictures ([Figure 2.6](#)): a central region dense to the electrons, a medium region with a contrast similar to the vitrified water and a dense surrounding layer. The less dense domain certainly corresponds to polyoxyl chains which are numerous in the present formulation. The dense central region is independent on the presence of API and should probably correspond to acyl-chain rich domains, i.e. Labrafil droplets. This external layer looks like liposome bilayers, suggesting a complex supramolecular organization into the formulation as already described in the laboratory with Labrafil® M 2125 CS ([Truong Cong, to be submitted](#)). The phospholipid seemed not anchored into the oil droplet surface but self-organized, at least partially, onto bilayers which surrounded the oil droplet. Due to the extent physical stability of the particles and their zeta potential, it was possible to assume that the phospholipid bilayer incorporate Gelucire® 50/13, stabilizing the nanodispersions. From the cryo-TEM images, it resulted that less than 3% of non multi-compartmented particles could be counted, essentially liposomes and classical nanoemulsion droplets. This was relatively low considering that a significant excess of surfactant is generally required in the continuous phase in order to rapidly covered new surface area created during coarse emulsion size reduction (homogenization).



**Figure 2.5.** TEM photographs of blank nanodispersions (5a) and quercetin-loaded nanodispersions (5b)



**Figure 2.6.** Cryo-TEM photograph of blank nanodispersions (6a) and quercetin-loaded nanodispersions (6b).

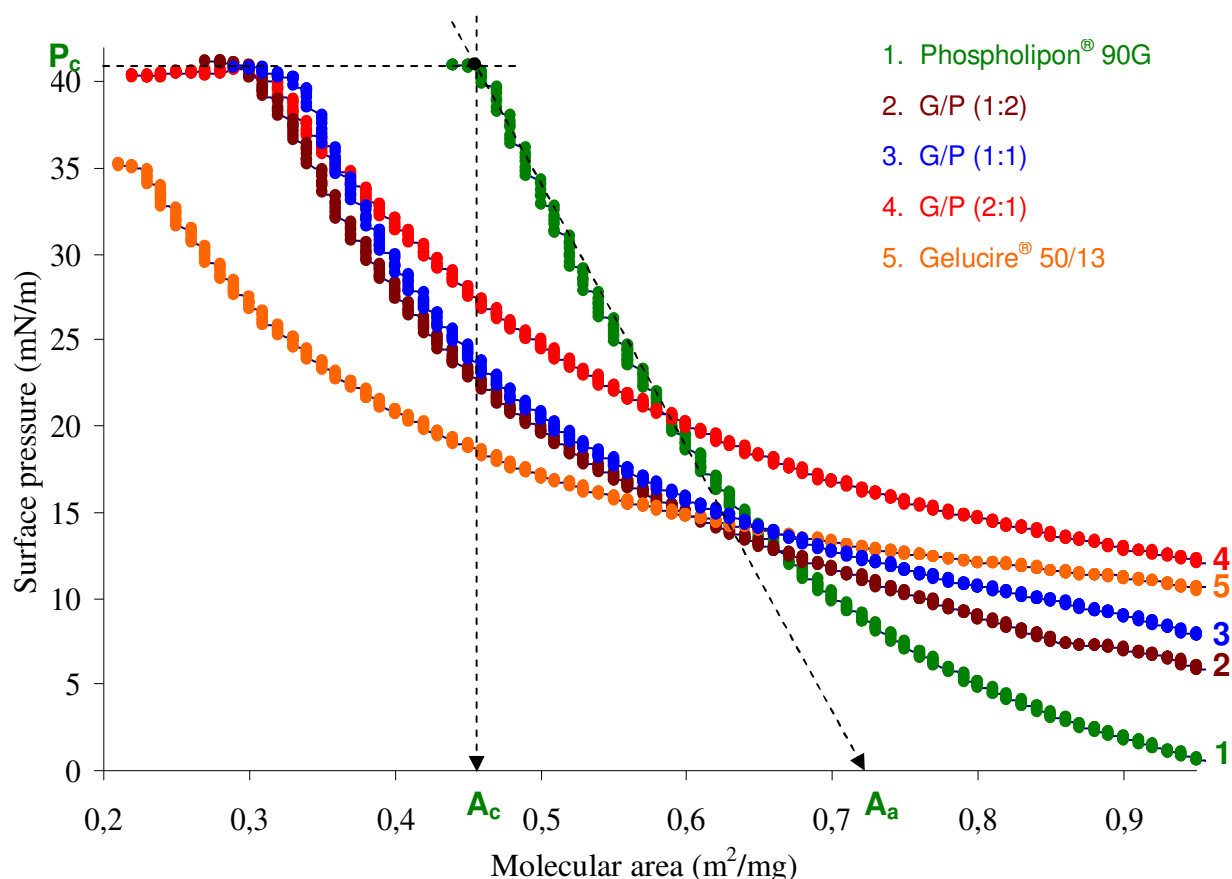
Further investigations had been done in order to correlate the amount of stabilizer (2:1) mixture and the nanodispersion surface area. First of all, the optimal amount of Gelucire® 50/13 – Phospholipon® 90G (2:1) mixture had been determined. G/P(2:1) – A,B,C,D formulations were established as reported in Table 2.3. G/P (2:1) mixtures varied from 1.5 % to 3.75 % and the nanodispersions were prepared by HPH as described in section 2.7. The results demonstrated that G/P (2:1) – A,B formulations were unstable over time. In contrast, G/P(2:1) – C,D formulations had similar sizes and remained stable at least one month. The particle size was about 170 nm with a small polydispersity index (0.1). These clearly indicated that the optimal stabilizer mixture amount is around 3g (per 20 g of Labrafil® M 2125 CS and a total dispersion mass of 100 g): lower amounts led to larger and unstable nanodispersions while higher amount had no effect on the nanodispersion diameter, and would probably results on an excess of equilibrium structures such as liposomes.

**Table 2.3.** The comparison for characteristics of G/P (2:1) formulations.

| Formulations  | G<br>(g) | P<br>(g) | L<br>(g) | Eau<br>(g) | Zeta<br>potential<br>(mV) | After preparation    |        | After 30 days        |        |
|---------------|----------|----------|----------|------------|---------------------------|----------------------|--------|----------------------|--------|
|               |          |          |          |            |                           | Droplet<br>size (nm) | PdI    | Droplet<br>size (nm) | PdI    |
| G/P (2:1) – A | 1        | 0.5      | 20       | 78.5       | -5.50                     | 410.4                | 0.13   | 802.0                | 0.77   |
|               |          |          |          |            | ± 0.25                    | ± 2.5                | ± 0.03 | ± 96.1               | ± 0.25 |
| G/P (2:1) – B | 1.5      | 0.75     | 20       | 78         | -5.96                     | 329.6                | 0.12   | 544.4                | 0.24   |
|               |          |          |          |            | ± 0.18                    | ± 2.3                | ± 0.01 | ± 47.1               | ± 0.14 |
| G/P (2:1) – C | 2        | 1        | 20       | 77         | -7.59                     | 167.8                | 0.11   | 164.9                | 0.10   |
|               |          |          |          |            | ± 0.23                    | ± 1.7                | ± 0.02 | ± 2.3                | ± 0.01 |
| G/P (2:1) – D | 2.5      | 1.25     | 20       | 76         | -5.89                     | 169.7                | 0.11   | 177.4                | 0.11   |
|               |          |          |          |            | ± 0.15                    | ± 1.2                | ± 0.03 | ± 1.2                | ± 0.03 |

*G : Gelucire® 50/13 ; P : Phospholipon® 90G ; G/P : Gelucire® 50/13 – Phospholipon® 90G ; L : Labrafil® CS M2125*

Then surface pressure experiments were done with a Langmuir-type film trough to determine the surface occupied by the stabilizers. The compression isotherms of Gelucire® 50/13, Phospholipon® 90G and their mixtures were described in Figure 2.7. Because these two pharmaceutical excipients are complex mixtures, the results are expressed as surface area per amount (m<sup>2</sup>/mg) and not per molecule. Phospholipon® 90G reached a surface pressure of 41 mN/m at collapse, while the maximum pressure obtained with Gelucire® 50/13 was around 35 mN/m.



**Figure 2.7.**  $\pi$ -A isotherms for the G/P mixtures at the air/water interface.

Furthermore, the later described extended isotherm certainly due the polymeric nature of its hydrophilic head group. Interestingly, the behavior of the two individual excipients could be observed in their mixtures. Indeed, the collapse surface pressures of the mixed monolayers were equal to Phospholipon® 90G collapse surface pressure suggesting a stabilizing effect of the phospholipids. On the contrary, the mixtures had a long expanded state similar to Gelucire® 50/13. These surface pressure results confirmed previous trends obtained by Differential Scanning Calorimetry and X-ray Diffraction which showed a clear interaction between the two stabilizers (Truong Cong et al., to be submitted). With simple geometrical consideration, the average area ( $A_a$ ) of the stabilizer mixture was then used to calculate the surface area potentially covered by the stabilizer and compared to the surface area developed by the nanodispersions. With an average area of 0.68 m<sup>2</sup>/mg (Table 2.4), 3 g G/P (2:1) mixture occupied a total surface area of 2040 m<sup>2</sup>. An estimation of the interface area developed by the nanodispersion was done taking into account the nanoparticle diameter (168 nm), the density of oil phase (0.944 g/cm<sup>3</sup>), the amount of lipophilic phase (20 g) and the number of stabilizer layer. Considering 1, 2 or 3 stabilizer

layers, the developed area were around 758 m<sup>2</sup>, 1516 m<sup>2</sup> and 2274 m<sup>2</sup> respectively. The total surface area occupied by the stabilizer mixture (2040 m<sup>2</sup>) was definitely compatible with the “three layers model” which required the covering of 2274 m<sup>2</sup>, all the more it was not taking into account for the calculation that inner layers were smaller than the nanodispersion diameter. The stabilizer amount seemed then optimized, explaining also why the drug payload experiments suggested that the API was totally associated with the lipid droplets. It could be assumed that with the composition used, the equilibrium structures were sparse.

**Table 2.4.** Collapse pressure ( $\pi$ ), collapse areas ( $A_c$ ) and average areas ( $A_a$ ) of G/P mixtures (n = 3).

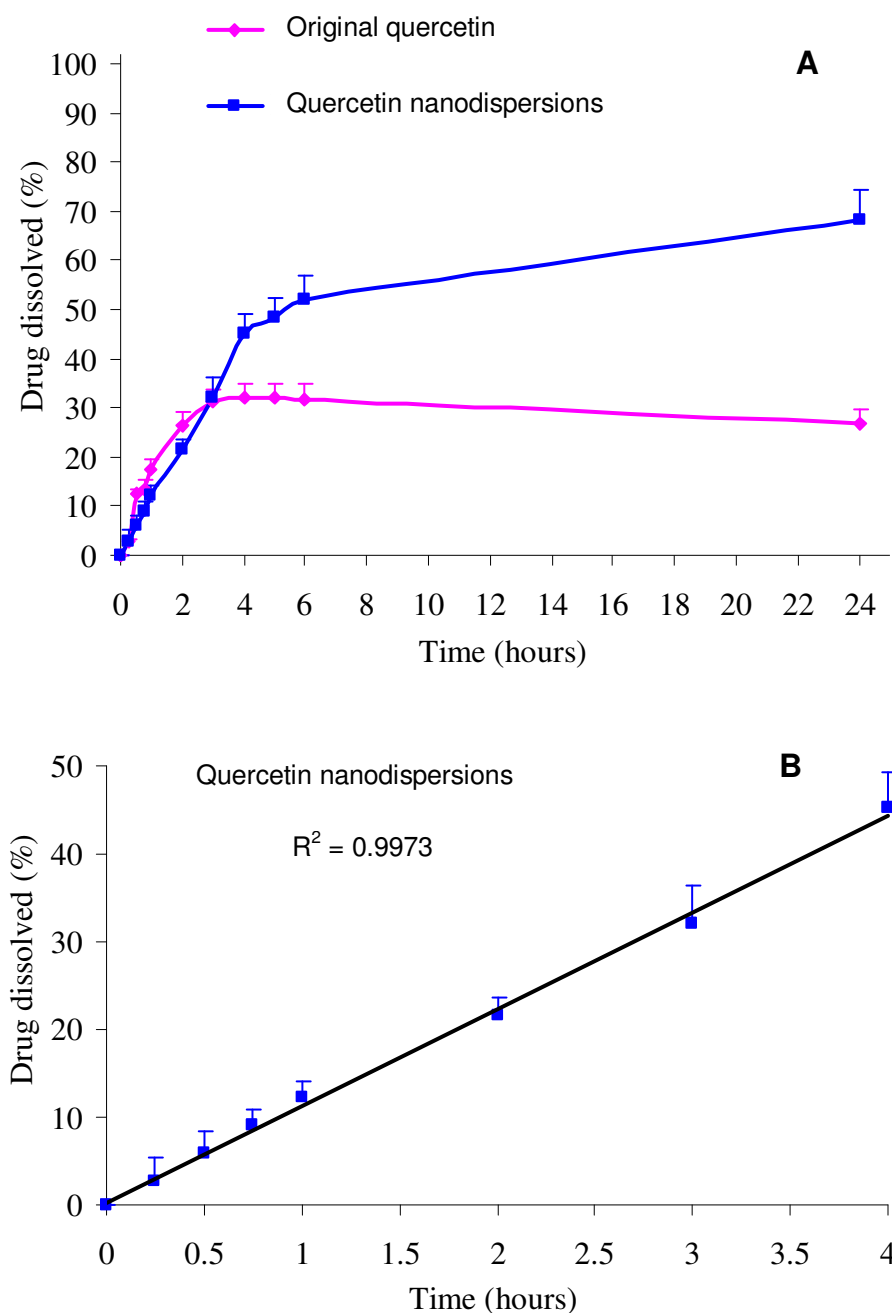
| Surfactants | $\pi$ (mN/m) | $A_c$ (m <sup>2</sup> /mg) | $A_a$ (m <sup>2</sup> /mg) |
|-------------|--------------|----------------------------|----------------------------|
| G           | 34.7 ± 0.3   | 0.22 ± 0.01                | 0.55 ± 0.01                |
| G/P (2:1)   | 40.3 ± 0.8   | 0.31 ± 0.01                | 0.68 ± 0.01                |
| G/P (1:1)   | 41.2 ± 0.3   | 0.32 ± 0.01                | 0.60 ± 0.03                |
| G/P (1:2)   | 41.0 ± 0.9   | 0.31 ± 0.01                | 0.55 ± 0.02                |
| P           | 41.2 ± 0.3   | 0.46 ± 0.01                | 0.72 ± 0.01                |

*G : Gelucire<sup>®</sup> 50/13 ; P : Phospholipon<sup>®</sup> 90G ; G/P : Gelucire<sup>®</sup> 50/13 – Phospholipon<sup>®</sup> 90G.*

### 3.4. *In vitro* release

The dissolution profiles of raw quercetin and quercetin-loaded nanodispersions in SGF (pH = 1.2) were shown [Figure 2.8 \(A,B\)](#). The dissolution rate of quercetin in nanodispersions was higher than that in the original API suspensions. Quercetin release from nanodispersions reached 51.8% ± 5.1% at 6h and about 70% at 24h. Whereas, raw quercetin suspensions released to 32.2 ± 2.6% at 4h and the dissolution slightly decreased until 26.9 ± 2.6% at 24h. The nanodispersions were easily dispersed in SGF to increase the available surface, so that the dissolution rate would be enhanced. Moreover, quercetin was dissolved in oil phase and more readily diffused in dissolution media than raw API in crystalline form. On the contrary, raw quercetin precipitated quickly in dialysis bag during the test, reducing the contact surface area with the dissolution medium. At longer times, the slight decrease of quercetin concentrations with the raw crystals could be due to its chemical instability.

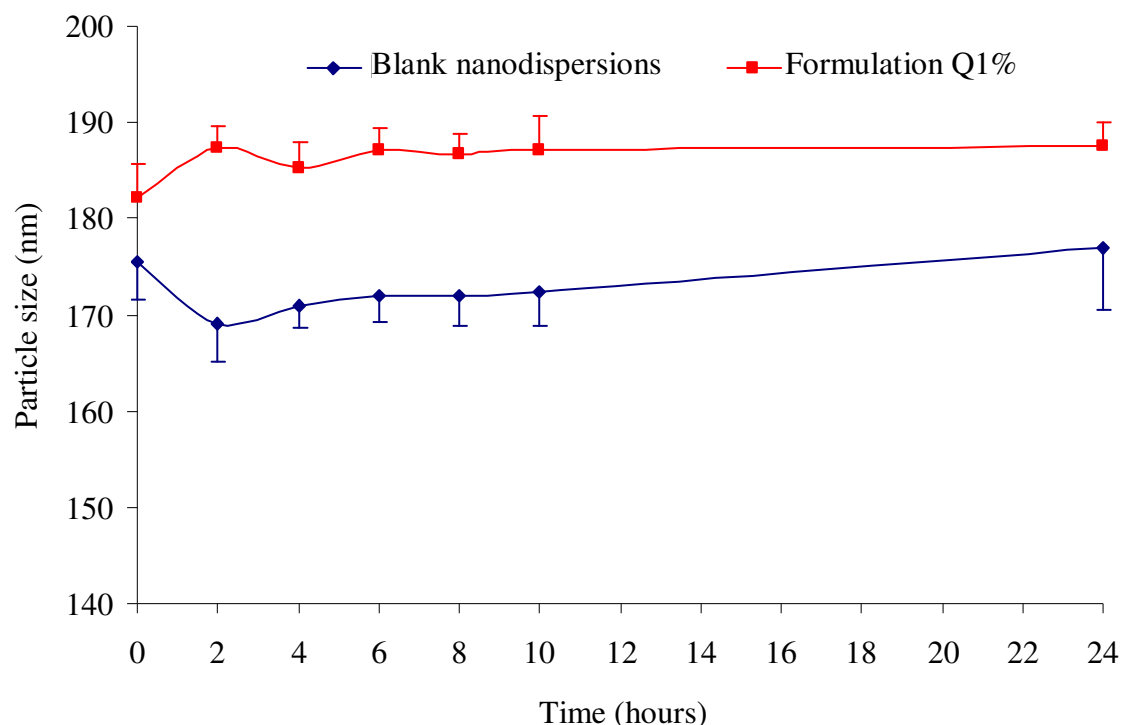
Interestingly, a fine analysis of the release profiles during the first hours suggested a zero-order kinetics in the case of the nanodispersions ([Figure 2.8B](#)).



**Figure 2.8.** *In vitro* dissolution profiles for original quercetin and quercetin nanodispersions (A) and a zero-order kinetics in the case of the nanodispersions (B).

In case of spherical device, such kind of kinetics could be observed with reservoir model in which the drug was confined by a spherical shell having a constant thickness, and as long as the drug concentration within the delivery device was significantly higher than that in the release medium. This model was convenient with the TEM and cryo-TEM observations which clearly showed a layered organisation into the nanodispersions. The absence of lag-time and burst-effect also improved the knowledge on the nanoparticles.

Indeed, a burst-effect would be the signature of an important accumulation of the API on the external layer of the particles, while a lag-time would result from a lack of API in the diffusion controlled layers. Regarding this release profile, the API seems to be distributed homogeneously into the nanoparticles. In the conditions of *in vitro* release, the size of nanodispersions remained stable during 24h in dissolution medium (Figure 2.9). This confirmed that diffusion was the mechanism involved in the drug delivery.

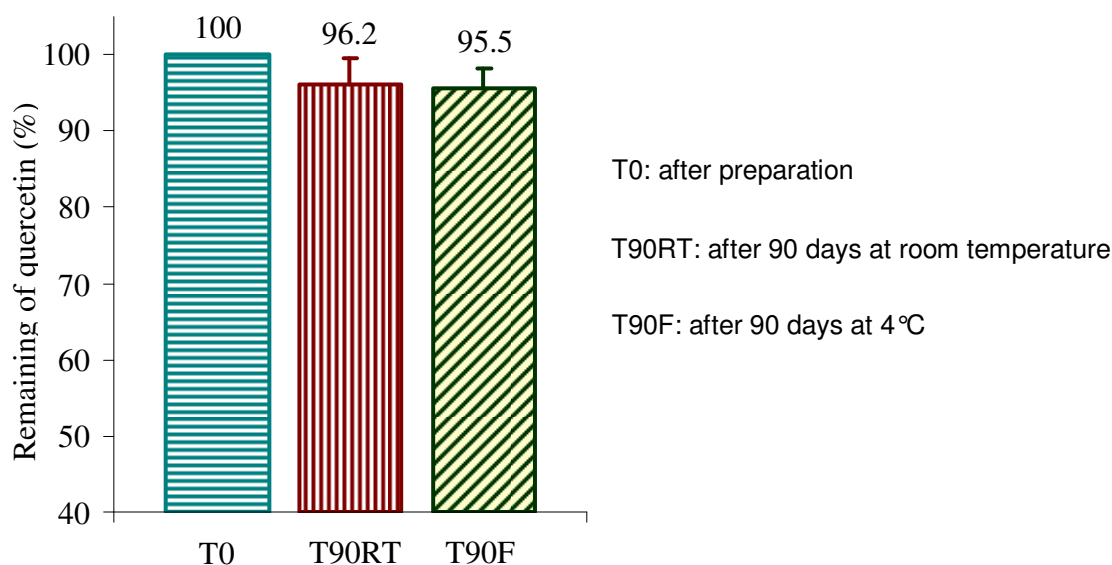


**Figure 2.9.** Size analysis of nanodispersions in dissolution medium (SGF, pH 1.2) during 24 h.

### 3.5. Long-term stability of quercetin-loaded nanodispersions

For Q1% formulation, the drug entrapment efficiency attained 96.4% confirming that quercetin was almost incorporated in the nanoparticles. Testing for chemical stability in the nanodispersions over 3 months, the quercetin content was retained 96.2% at room temperature and 95.5% at 4°C versus the initial content (Figure 2.10). Encapsulation significantly improved the chemical stability of quercetin in water in which its half-life was around 17h at room temperature. As expected, the API has been protected by its incorporation into polyoxylglyceride-based nanodispersions. The global hydrophobicity of nanoparticles was probably sufficient to limit water diffusion process into the drug-containing droplets. The slight decrease in API amount could be due to drug molecule incorporated into the outer layer on the surface of oil droplets which were accessible to

water and light. This confirmed that the most important fraction of API was included in the inner parts of the nanodispersions as shown by the release profile and then was protected against hydrolysis.



**Figure 2.10.** Stability of quercetin in nanodispersions for Q1% formulation stored at room temperature and 4°C after 90 days.

#### 4. Conclusion

Quercetin, a chemically unstable and poorly-water soluble API, was successfully encapsulated in nanodispersions using high-pressure homogenization. The use of polyoxylglyceride-based excipients allowed to overcome the two unfavourable properties and to formulate stable multi-compartmented nanoparticles which had an average size about 200 nm and were stable at least 3 months at room temperature and 4°C. Thanks to drug solubility into polyoxyl chains, the apparent solubility of the API has been significantly increased (x4500) with the entrapment efficiency close to 100%. On the other hand, the hydrophobic properties of the excipients reduced water diffusion in the particles and prevent API hydrolysis. The results clearly show that polyoxylglycerides could be efficient excipients to protect chemically labile compounds.

#### Acknowledgement

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## References

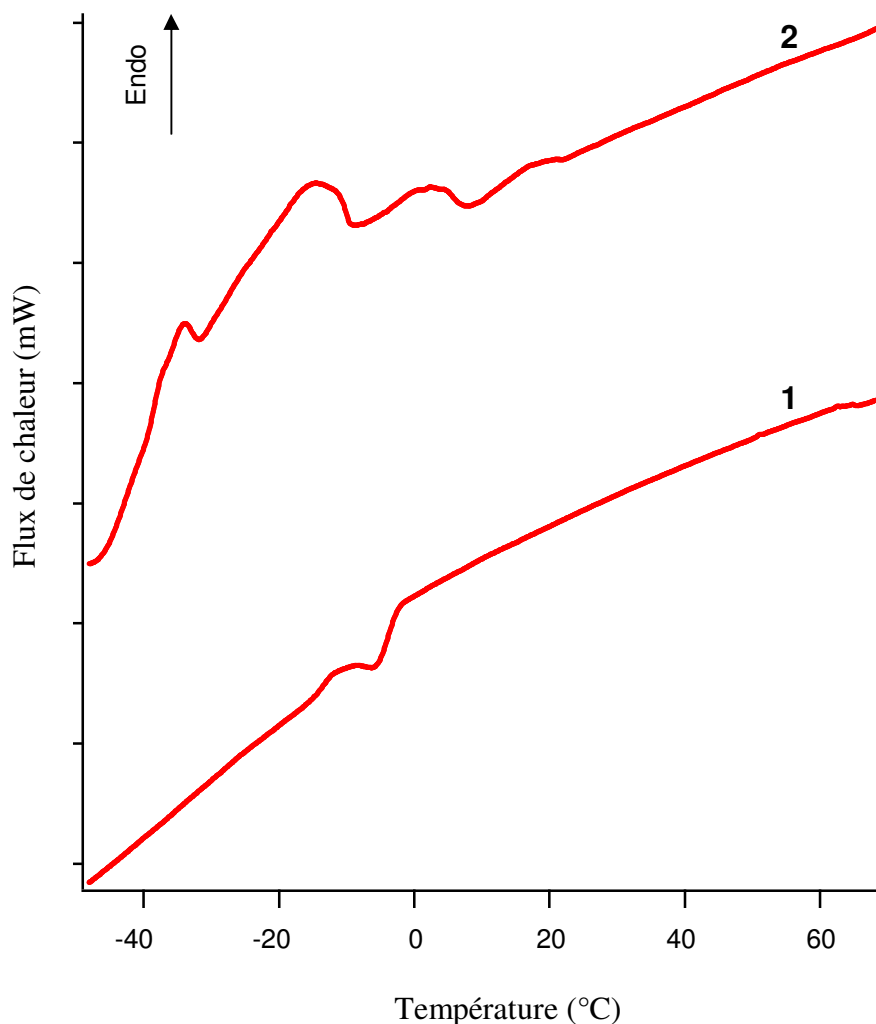
1. Azuma, K. et al., 2002. Combination of lipids and emulsifiers enhances the absorption of orally administered quercetin in rats. *Journal of Agricultural and Food Chemistry*, 50(6), pp.1706–1712.
2. Barras, A. et al., 2009. Formulation and characterization of polyphenol-loaded lipid nanocapsules. *International Journal of Pharmaceutics*, 379(2), pp.270–277.
3. Boots, A.W., Haenen, G.R.M.M. & Bast, A., 2008. Health effects of quercetin: From antioxidant to nutraceutical. *European Journal of Pharmacology*, 585(2-3), pp.325–337.
4. Borghetti, G.S. et al., 2009. Quercetin/beta-cyclodextrin solid complexes prepared in aqueous solution followed by spray-drying or by physical mixture. *AAPS PharmSciTech*, 10(1), pp.235–242.
5. Chen, C., Zhou, J. & Ji, C., 2010. Quercetin: A potential drug to reverse multidrug resistance. *Life Sciences*, 87(11-12), pp.333–338.
6. Cushnie, T.P.T. & Lamb, A.J., 2005. Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*, 26(5), pp.343–356.
7. Dajas, F., 2012. Life or death: Neuroprotective and anticancer effects of quercetin. *Journal of ethnopharmacology*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22820241>.
8. Date, A.A. et al., 2011. Lecithin-based novel cationic nanocarriers (Leciplex) II: improving therapeutic efficacy of quercetin on oral administration. *Molecular Pharmaceutics*, 8(3), pp.716–726.
9. Gao, L. et al., 2011. Preparation of a chemically stable quercetin formulation using nanosuspension technology. *International Journal of Pharmaceutics*, 404(1-2), pp.231–237.
10. Gao, Y. et al., 2009. Formulation optimization and in situ absorption in rat intestinal tract of quercetin-loaded microemulsion. *Colloids and Surfaces B: Biointerfaces*, 71(2), pp.306–314.
11. Ghosh, A. et al., 2009. Nanoencapsulation of quercetin enhances its dietary efficacy in combating arsenic-induced oxidative damage in liver and brain of rats. *Life Sciences*, 84(3-4), pp.75–80.
12. Gordon, M.H. & Roedig-Penman, A., 1998. Antioxidant activity of quercetin and myricetin in liposomes. *Chemistry and Physics of Lipids*, 97(1), pp.79–85.
13. Gugler, R., Leschik, M. & Dengler, H.J., 1975. Disposition of quercetin in man after single oral and intravenous doses. *European Journal of Clinical Pharmacology*, 9(2-3), pp.229–234.
14. Hamoudi, M. et al., 2011. Beads made of cyclodextrin and oil for the oral delivery of lipophilic drugs: In vitro studies in simulated gastro-intestinal fluids. *International Journal of Pharmaceutics*, 416(2), pp.507–514.

15. Heurtault, B. et al., 2003. Physico-chemical stability of colloidal lipid particles. *Biomaterials*, 24(23), pp.4283–4300.
16. Khaled, K.A., El-Sayed, Y.M. & Al-Hadiya, B.M., 2003. Disposition of the flavonoid quercetin in rats after single intravenous and oral doses. *Drug Development and Industrial Pharmacy*, 29(4), pp.397–403.
17. Kleemann, R. et al., 2011. Anti-inflammatory, anti-proliferative and anti-atherosclerotic effects of quercetin in human *in vitro* and *in vivo* models. *Atherosclerosis*, 218(1), pp.44–52.
18. Kumari, A. et al., 2010. Development of biodegradable nanoparticles for delivery of quercetin. *Colloids and Surfaces B: Biointerfaces*, 80(2), pp.184–192.
19. Lauro, M.R. et al., 2002. Fast- and slow-release tablets for oral administration of flavonoids: rutin and quercetin. *Drug Development and Industrial Pharmacy*, 28(4), pp.371–379.
20. Lee, D.H. et al., 2007. Preparation and characterization of quercetin-loaded polymethyl methacrylate microcapsules using a polyol-in-oil-in-polyol emulsion solvent evaporation method. *The Journal of Pharmacy and Pharmacology*, 59(12), pp.1611–1620.
21. Lei, R. et al., 2008. A method to determine quercetin by enhanced luminol electrogenerated chemiluminescence (ECL) and quercetin autoxidation. *Talanta*, 75(4), pp.1068–1074.
22. Li, H. et al., 2009. Enhancement of gastrointestinal absorption of quercetin by solid lipid nanoparticles. *Journal of Controlled Release*, 133(3), pp.238–244.
23. Mello Costa, A.R. et al., 2010. Quercetin-PVP K25 solid dispersions. *Journal of Thermal Analysis and Calorimetry*, 104(1), pp.273–278.
24. Moon, Y.J. et al., 2008. Quercetin pharmacokinetics in humans. *Biopharmaceutics & Drug Disposition*, 29(4), pp.205–217.
25. Muller, R.H. et al., 1999. Pharmaceutical nanosuspensions for medicament administration as systems with increased saturation solubility and rate of solution. Available at: <http://www.freepatentsonline.com/5858410.html>.
26. Muller, R.H. & Keck, C.M., 2004. Challenges and solutions for the delivery of biotech drugs--a review of drug nanocrystal technology and lipid nanoparticles. *Journal of Biotechnology*, 113(1-3), pp.151–170.
27. Pinelo, M. et al., 2004. Solvent effect on quercetin antioxidant capacity. *Food Chemistry*, 88(2), pp.201–207.
28. Priprem, A. et al., 2008. Anxiety and cognitive effects of quercetin liposomes in rats. *Nanomedicine: Nanotechnology, Biology and Medicine*, 4(1), pp.70–78.
29. Rogerio, A.P. et al., 2010. Anti-inflammatory effect of quercetin-loaded microemulsion in the airways allergic inflammatory model in mice. *Pharmacological Research*, 61(4), pp.288–297.

30. Rothwell, J.A., Day, A.J. & Morgan, M.R.A., 2005. Experimental determination of octanol-water partition coefficients of quercetin and related flavonoids. *Journal of Agricultural and Food Chemistry*, 53(11), pp.4355–4360.
31. Russo, M. et al., 2012. The flavonoid quercetin in disease prevention and therapy: facts and fancies. *Biochemical Pharmacology*, 83(1), pp.6–15.
32. Sahoo, N.G. et al., 2011. Preparation and characterization of quercetin nanocrystals. *Journal of Pharmaceutical Sciences*, 100(6), pp.2379–2390.
33. Sansone, F. et al., 2011. Flavonoid microparticles by spray-drying: Influence of enhancers of the dissolution rate on properties and stability. *Journal of Food Engineering*, 103(2), pp.188–196.
34. Scalia, S. & Mezzena, M., 2009. Incorporation of quercetin in lipid microparticles: Effect on photo- and chemical-stability. *Journal of Pharmaceutical and Biomedical Analysis*, 49(1), pp.90–94.
35. Scarfato, P. et al., 2008. Quercetin microspheres by solvent evaporation: Preparation, characterization and release behavior. *Journal of Applied Polymer Science*, 109(5), pp.2994–3001.
36. Shegokar, R., Singh, K.K. & Müller, R.H., 2011. Production & stability of stavudine solid lipid nanoparticles-from lab to industrial scale. *International Journal of Pharmaceutics*, 416(2), pp.461–470.
37. Srinivas, K. et al., 2010. Solubility and solution thermodynamic properties of quercetin and quercetin dihydrate in subcritical water. *Journal of Food Engineering*, 100(2), pp.208–218.
38. Thapa, M. et al., 2012. Synthesis and antiviral activity of substituted quercetins. *Bioorganic & medicinal chemistry letters*, 22(1), pp.353–356.
39. Truong Cong, T. et al., Nanodispersions stabilized by PEG diesters and phospholipids: towards compartmented lipid nanoparticles. *to be submitted*.
40. Urbinati, G. et al., 2010. Liposomes loaded with histone deacetylase inhibitors for breast cancer therapy. *International Journal of Pharmaceutics*, 397(1-2), pp.184–193.
41. Wu, T.H. et al., 2008. Preparation, physicochemical characterization, and antioxidant effects of quercetin nanoparticles. *International Journal of Pharmaceutics*, 346(1-2), pp.160–168.
42. Zenkevich, I. & Guschina, S., 2010. Determination of dissociation constants of species oxidizable in aqueous solution by air oxygen on an example of quercetin. *Journal of Analytical Chemistry*, 65(4), pp.371–375.

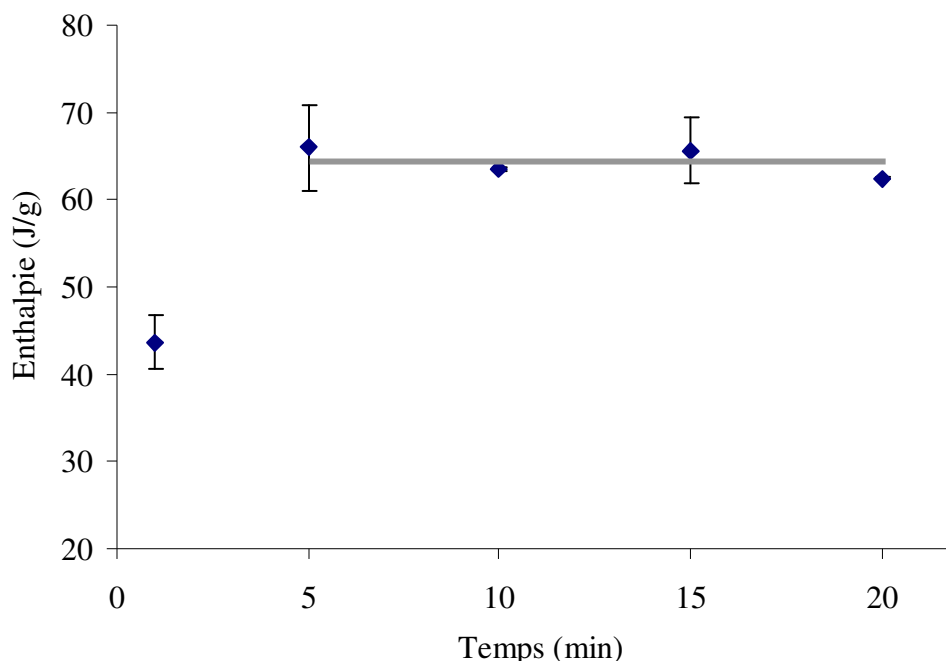
Comme les résultats expérimentaux précédents l'attestent, les nanodispersions compartimentées proposées dans ce travail présentent un intérêt potentiel pour l'encapsulation et la stabilisation de la quercétine dans les milieux aqueux. D'autres études relatives à l'interaction entre le principe actif et les excipients de la formulation ont été menées mais n'ont pu trouver leur place dans ce projet d'article.

Tout d'abord, la calorimétrie différentielle à balayage a été utilisée pour approcher cette interaction, notamment entre la quercétine et le Labrafil® M2125 CS. La [Figure 2.11](#) représente un cycle de chauffage – refroidissement, entre -50°C et +70°C de l'excipient seul. Liquide à température (un léger trouble lié à la cristallisation des glycérides libres peut parfois apparaître avec le temps à température ambiante), la cristallisation du Labrafil® M2125 CS ne se produit que pendant la période d'isotherme à -50°C du cycle.



**Figure 2.11.** Thermogrammes du Labrafil® M2125 CS lors d'un cycle de refroidissement (1) - chauffage (2) entre + 70°C et -50°C à la vitesse de 5°C/min.

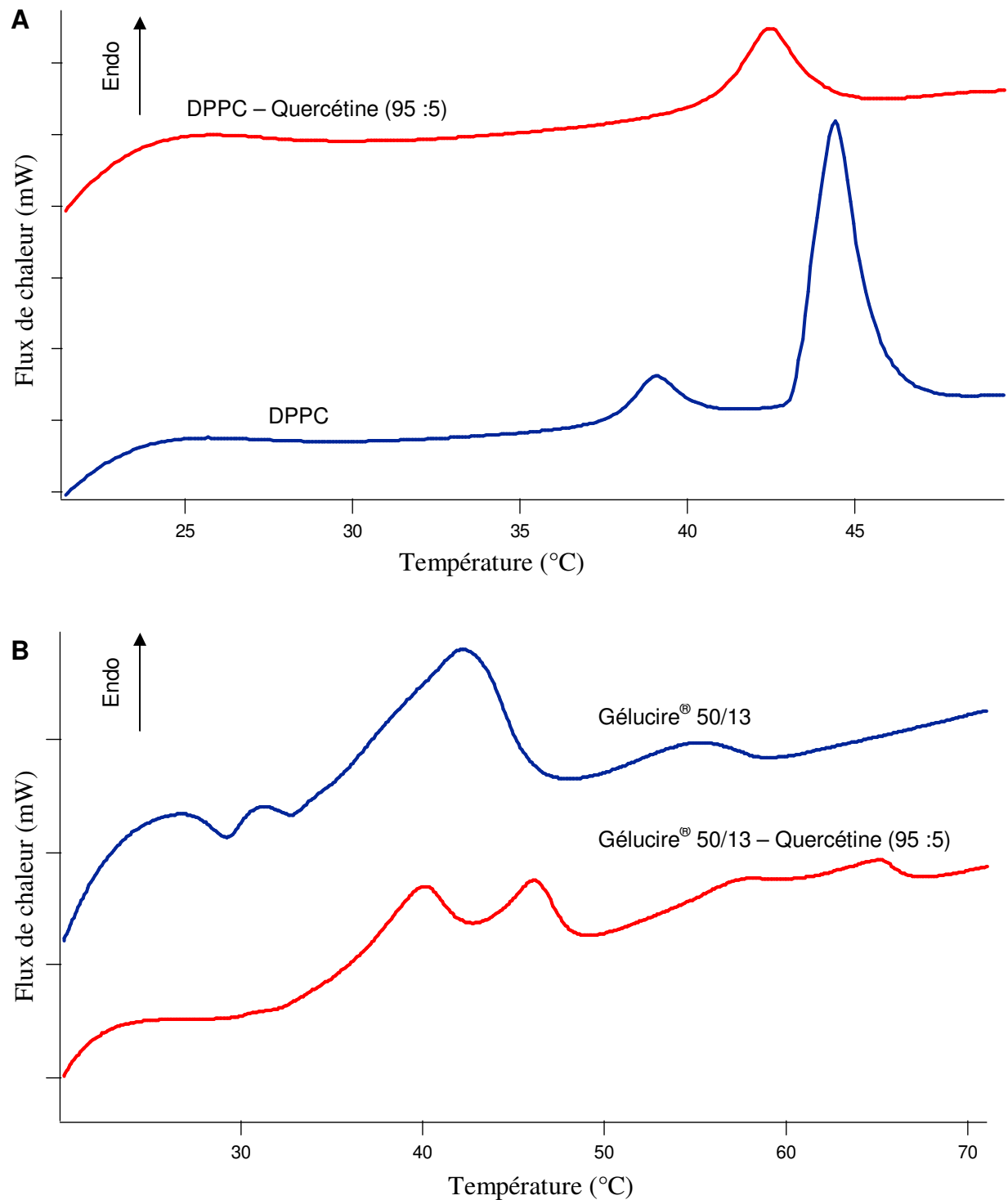
La [Figure 2.12](#), représentant l'évolution de l'enthalpie de fusion de cet excipient en fonction de la durée de cet isotherme, montre qu'un plateau de 5 minutes est nécessaire pour avoir une cristallisation totale du Labrafil® M2125 CS. En incorporant des quantités croissantes de quercétine dans le Labrafil® M2125 CS, nous n'avons pu observer de modifications significatives, ni de la durée minimum nécessaire de l'isotherme, ni de l'enthalpie de fusion de l'excipient. Ceci peut très probablement s'expliquer par les faibles quantités de principe actif incorporées, entre 1 et 5 % (m/m), et par la difficulté de déterminer précisément les paramètres thermodynamiques de la fusion ( $T_{\text{onset}}$ , enthalpie), l'évènement thermique étant très proche de la zone de mise en régime de l'appareil, très étendu (entre -30 et +20 °C) et constitué de plusieurs transitions.



**Figure 2.12.** Enthalpie de fusion du Labrafil® M2125 CS en fonction du temps d'isotherme à -50°C durant les cycles de refroidissement – chauffage. La cristallisation de l'excipient n'étant pas visible dans les cycles, l'étude de ce temps pourrait être informative sur le processus de cristallisation en présence d'une substance exogène, ici la quercétine.

L'essentiel des investigations était axé sur la solubilisation de la quercétine dans le cœur des nanoparticules, donc dans le Labrafil® M2125 CS. Toutefois, quelques expériences complémentaires ont été effectuées sur la solubilisation possible du principe actif dans les autres excipients de la formulation, le Gélucire® 50/13 et le Phospholipon® 90G. Pour le Gélucire® 50/13, une étude de solubilisation à 70°C a montré qu'il était possible d'incorporer ~53 mg de quercétine par gramme d'excipient

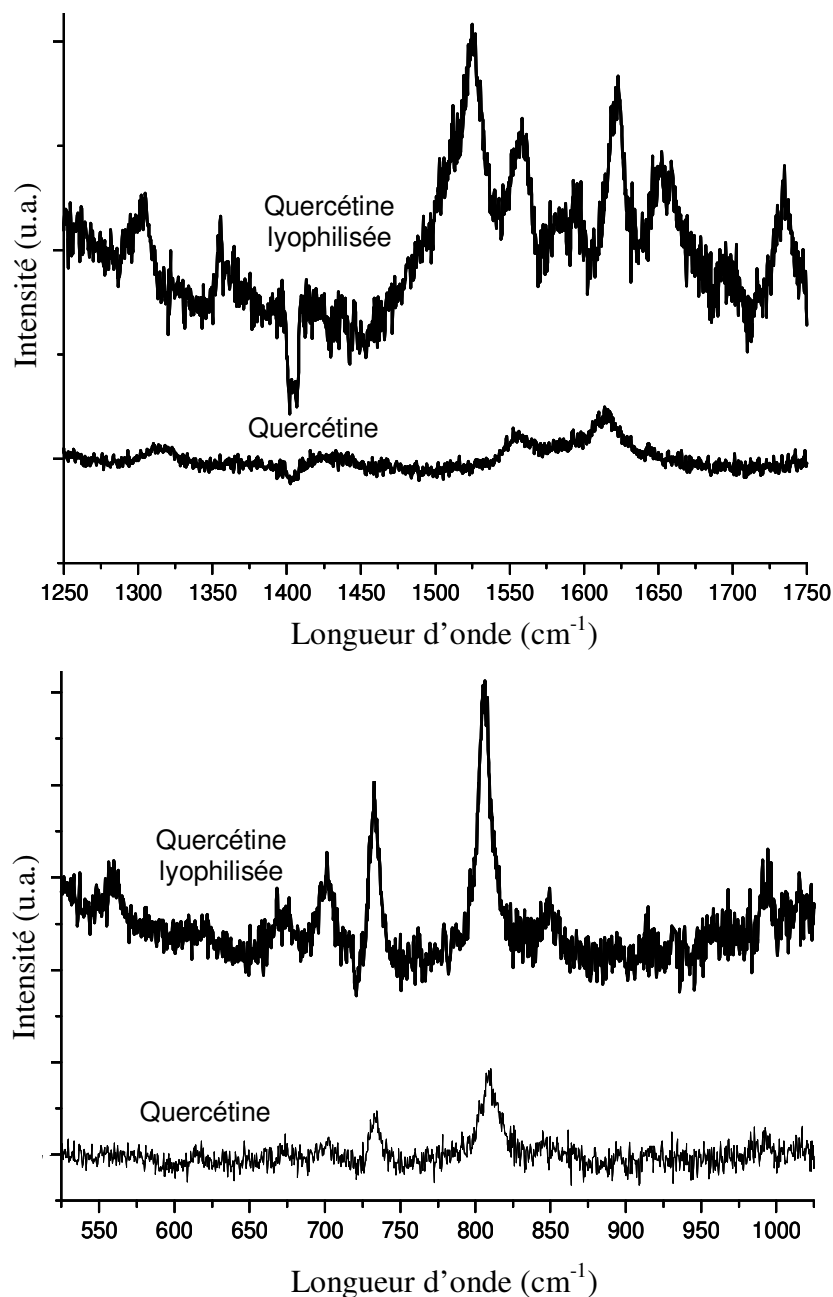
fondue, soit plus que dans le Labrafil® M2125 CS à la même température (24 mg/g). Cette solubilité s'explique par la plus grande proportion de chaîne PEG, le meilleur solvant du principe actif, dans le Gélucire® 50/13 comme l'indiquent les valeurs de HLB données par le fournisseur (selon l'échelle de Griffin, 13 pour le Gélucire® 50/13 et 4 pour le Labrafil® M2125 CS). La [Figure 2.13](#) confirme cette interaction, le thermogramme du Gélucire® 50/13 étant significativement modifié par la présence de quercétine à hauteur de 5% (m/m). Cette même figure décrit également les résultats obtenus lors du chauffage d'une bicouche phospholipidique hydratée à 80% en présence et absence de quercétine (5%). Pour accéder aux températures de transition caractéristiques des phospholipides, le mélange Phospholipon® 90G a été remplacé ici par de la DPPC (la phosphatidylcholine constituant plus de 94% du Phospholipon® 90G). Dans l'échantillon contenant le principe actif, la pré-transition des phospholipides disparaît totalement alors que la transition principale, correspondant à la fusion des chaînes grasses, est élargie et commence à une température plus basse. Ceci est le signe d'une baisse de coopérativité dans le phénomène de fusion, probablement par insertion de molécules de quercétine dans la bicouche phospholipidique. Ces résultats montrent que la quercétine est susceptible d'interagir avec les tensioactifs de la formulation et pourrait se trouver dans la couche périphérique des particules, soit solubilisée dans le PEG externe, soit incorporée dans la bicouche phospholipidique. Nous l'avons vu dans le projet d'article de revue bibliographique, une telle localisation périphérique pourrait limiter l'intérêt de l'encapsulation vis-à-vis de l'instabilité chimique de la quercétine. Les résultats de stabilité décrits dans le chapitre précédent montrent que la dégradation de la quercétine est très faible après 3 mois de stockage de la suspension de nanoparticules. Ceci suggère fortement l'absence d'accumulation de principe actif à la surface des nanoparticules compartimentées et confirme les résultats de libération (absence de *burst-effect*).



**Figure 2.13.** Thermogrammes de la DPPC (dipalmitoylphosphatidylcholine) (A) et du Gélucire® 50/13 (B) en l'absence et en présence de 5% (m/m) de quercétine. Ces enregistrements ont été obtenus à l'aide de Microcalix, un calorimètre original développé au laboratoire et capable de s'intégrer dans une ligne de diffraction de rayon X. Seul le signal de calorimétrie est présenté ici.

Toujours dans le cadre des investigations sur les interactions principe actif – excipients, des études de spectroscopie Raman ont également été initiées avec S. Bresson

(Université de Picardie). Essentiellement deux zones spectrales sont intéressantes dans le cas de la quercétine, 500-1000 et 1200-1800  $\text{cm}^{-1}$ . La [Figure 2.14](#) montre également que des différences peuvent être perçues selon le traitement subi par le principe actif. Toutefois, deux difficultés sont apparues lors des essais impliquant cette molécule : une dégradation de l'échantillon pour des temps d'exposition trop longs (plus de 30 s) et une fluorescence relativement importante masquant le signal Raman. Des études complémentaires sont toutefois en cours dans ce domaine.



**Figure 2.14.** Spectres Raman de la quercétine dihydrate, avant et après lyophilisation, dans deux régions d'intérêt, 1250 – 1750  $\text{cm}^{-1}$  et 500 – 1100  $\text{cm}^{-1}$ .

La concentration en quercétine dans les suspensions de nanoparticules compartimentées est de l'ordre de 2 g/L, soit plus de 3 Log supérieure à la solubilité aqueuse du principe actif. Pour augmenter encore cette concentration en flavonoïde, deux stratégies principales ont été envisagées: l'utilisation d'un dérivé de la quercétine aux propriétés physico-chimiques différentes, et l'incorporation de nanocristaux dans la formulation.

La rutine, ou quercétine-3-O-rutinoside, est un dérivé O-glycosidique de la quercétine ([cf Introduction bibliographique](#)). Certes moins actif que la quercétine, ce composé est intéressant car la glycosylation apporte une stabilisation chimique de la molécule. Plusieurs expériences (DSC, DRX, Raman...) ont donc été conduites pour évaluer son intérêt dans le cadre de cette thèse. Parmi ces expériences complémentaires, les essais de solubilisation ont entraîné l'abandon de cette voie. En effet, la solubilité à 70 °C de la rutine dans le PEG 400 est supérieure à celle de la quercétine (265 mg/g contre 222 mg/g) mais elle reste inférieure en « équivalent – quercétine ». De plus, la solubilité de la rutine est inférieure à celle de la quercétine dans les excipients de type polyoxylglycérides. Ceci est par exemple très net dans le Gélucire® 50/13 où la solubilité de la rutine est proche de 10 mg/g et celle de la quercétine de l'ordre de 50 mg/g. L'utilisation de cet hétéroside de la quercétine n'a donc pas été envisagée plus en avant pour enrichir la formulation en flavonoïdes.

La seconde approche, à savoir la formation de nanocristaux, sera l'objet du troisième chapitre expérimental de ce manuscrit.

## **Enrichment of quercetin-loaded nanodispersions with nanocrystals: preparation and physico-chemical characterization**

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**Abbreviations:** API, active pharmaceutical ingredient; Cryo-TEM, cryogenic transmission electron microscopy; DSC, differential scanning calorimetry; HPH, high pressure homogenization; PXRD, powder X-ray diffraction; QAD, quercetin anhydrous after drying; QAR, quercetin anhydrous recrystallized from ethanol; QHD, quercetin hydrate after drying; QHR, quercetin hydrate recrystallized from ethanol; SGF, simulated gastric fluid; TEM, transmission electron microscopy; XRD, X-ray diffraction.

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**Abstract**

Quercetin is a well known antioxidant used in pharmaceutical and nutraceutical industry. However, the poor solubility and chemical instability in water have limited the potential application of this molecule in the disease prevention and therapy. An approach for enhancing water solubility and stability is the production of drug nanocrystals. Quercetin nanocrystals were successfully prepared using high pressure homogenization (HPH) process on the basis of polyoxylglyceride nanoparticles. The physico-chemical properties of the nanodispersions have been investigated regarding size analysis, zeta potential measurement, drug payload, morphology, stability and *in vitro* dissolution. The nanodispersions incorporating 4% (w/w) of quercetin in lipid phase had the size of  $220.4 \pm 4.7$  nm with a narrow distribution (Pdl  $\sim 0.1$ ). However, the TEM and Cryo-TEM images presented at least two populations in nanodispersions: multi-compartmented nanospheres and drug nanocrystal sticks. The quercetin-loaded nanosuspensions had good *in vitro* release compared with coarse quercetin suspensions. They were stable physically and chemically over 90 days at room temperature and 4°C. In addition, quercetin crystallinity was also studied to understand the polymorphic transformation in preparation process and the physical stability of nanocrystals towards stable form.

**Keywords:** crystallinity, drug nanocrystals, high pressure homogenization, nanodispersions, nanosuspensions, polyoxylglyceride nanoparticles, polymorphism, quercetin, solubility and stability.

## Contents

1. Introduction
2. Materials and methods
  - 2.1. Materials
  - 2.2. HPLC analysis
  - 2.3. Solubility in solvent and excipients
  - 2.4. Preparation of quercetin nanocrystals by HPH process
  - 2.5. Lyophilisation
  - 2.6. Differential scanning calorimetry
  - 2.7. Powder X-ray diffraction
  - 2.8. X-ray diffraction of the nanocrystal suspensions
  - 2.9. Particle size and zeta potential measurements
  - 2.10. Drug loading and encapsulation efficiency
  - 2.11. Polarized light microscopy
  - 2.12. Dark field microscopy
  - 2.13. Transmission electron microscopy (TEM) and cryogenic transmission electron microscopy (Cryo-TEM)
  - 2.14. *In vitro* release studies
  - 2.15. Long-term stability
3. Results and discussion
  - 3.1. Particle size, zeta potential and drug payload analysis
  - 3.2. Particle morphology
  - 3.3. Crystallinity and polymorphism of quercetin
    - 3.3.1. Shape observation by polarized-light microscopy
    - 3.3.2. DSC analysis
    - 3.3.3. XRD characterization
  - 3.4. *In vitro* release of nanodispersions
  - 3.5. Long-term stability of quercetin in nanodispersions
4. Conclusion
- Acknowledgement
- References

## 1. Introduction

Drug nanocrystals are sub-micron particles made from 100% active pharmaceutical ingredient (API), which are stabilized by surfactants or polymeric steric stabilizers. Nanocrystals can be used for compounds that are insoluble in water or in both water and oil ([Rabinow 2004](#); [Müller, Gohla, et al. 2011](#)). Candidates emerging for formulation of these particles have high molecular mass and high logP, both of which are factors that contribute to insolubility (less than 0.1 mg per ml solubility in water) ([Lipinski 2000](#)). The nanocrystals are typically produced in a liquid dispersion medium called nanosuspensions ([Müller, Gohla, et al. 2011](#)). The nanocrystalline drug particles can be also dispersed into inert carriers after a drying process, such as spray drying or lyophilization. Herein, the solidified nanocrystal formulations can be defined as crystalline solid dispersion ([Kawabata et al. 2011](#)). Nanocrystals have been used for various application routes including parenteral, peroral, dermal, ocular, pulmonary and targeted drug delivery ([Shegokar & Müller 2010](#)).

Nanocrystals can solve the biopharmaceutical delivery limitations due to increased saturation, increased dissolution velocity, and increased adhesiveness to surfaces/cell membranes compared with the micro-sized drug powders ([Müller, Shegokar, et al. 2011](#)). Reviewing the literature, nanocrystal formulations have been found to show 1.7–60-fold and 2–30-fold enhancement in C<sub>max</sub> and AUC, respectively, compared with micrometer particle size ([Kawabata et al. 2011](#)). According to the modification of the Noyes-Whitney equation, the factors affecting the drug dissolution rate are defined as the effective surface area, the diffusion coefficient, the diffusion layer thickness, the saturation solubility, the amount of dissolved drug, and the volume of dissolution media ([Hörter & Dressman 2001](#)). The effective surface area is clearly increased with nanocrystals compared to more classical powders or suspensions. It is also frequently mentioned in the field of nanodispersed crystals that in the nano-range domain, the saturation solubility increases with decreasing particle size according to the Ostwald-Freundlich equation, also known as the Gibbs-Thomson or Kelvin equation. An enhanced dissolution rate may not be the only factor for the nanoparticles to achieve better oral absorption. It is well known that Peyer's patches may uptake nanoparticles, and the nanoparticle ability to diffuse in the mucous layer may affect the absorption ([Kawakami 2012](#)). Therefore, nanocrystal serves as ideal delivery system for oral drugs having the dissolution velocity as rate limiting step for absorption, i.e. drugs of the biopharmaceutical classification system class

II and IV (Shegokar & Müller 2010). Among all the nanocrystal formulations, compounds such as danazol (Liversidge & Cundy 1995), cilostazol (Jinno et al. 2006), tranilast (Kawabata et al. 2010), and curcumin (Onoue et al. 2010) showed better improvements in the pharmacokinetic parameters than basic compounds by using nanocrystal technologies. Currently, five nanocrystal oral formulations using NanoCrystal® (Elan Drug Technologies) and IDD-P® (SkyePharma) technologies are available on the market (Kawabata et al. 2011; Shegokar & Müller 2010).

For the manufacturing of nanosuspensions, there are two basic approaches to produce drug nanocrystals: the bottom-up (precipitation) and the top-down (high pressure homogenization (HPH) and bead/pearl milling) technologies. HPH, available at both laboratory and industrial scale, is widely used to formulate nanocrystals. HPH is a high energy process generally using piston-gap homogenizers. The microsuspensions pass at high pressure through a narrow homogenization gap with a very high velocity. In the gap, the objects of interest are diminished in size by cavitation, collision of crystals with each other and the steel wall, and shear forces of the liquid. All these homogenization processes are performed with water suspensions (Rabinow 2004).

Quercetin is an excellent antioxidant (Boots et al. 2008; Russo et al. 2012; Dajas 2012) but practically insoluble and unstable in aqueous media (water, intestinal fluids, and blood) at room temperature and 37°C (Moon et al. 2008; Truong Cong, Faivre, et al. to be submitted). A new approach for enhancing water solubility and stability is the production of drug nanocrystals. These particles possess a 100% drug payload in contrast to other nanodispersions consisting micelles, polymeric nanoparticles, liposomes, microemulsions, nanoemulsions, solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), and lipid nanocapsules. The high loading makes them easily reaching a sufficiently high therapeutic concentration for the pharmacological effect. Moreover, the crystalline nature and the surface coverage by stabilizers as barriers to diffusion of oxygen and/or water into particles improve the chemical stability of API.

In a submitted paper (Truong Cong, Faivre, et al. to be submitted), the formulation of nanodispersed lipid particles containing quercetin has been described. The aim of the present one was to increase the drug concentration into the nanosuspension with quercetin nanocrystals successfully prepared by HPH. The properties of the quercetin

nanocrystals have been investigated regarding size analysis, zeta potential measurement, drug payload, morphology, physico-chemical stability and *in vitro* dissolution. Furthermore, crystallinity and polymorphism were also studied to understand the structure and physical stability of nanocrystals based on morphology of crystals, X-ray scattering and thermal analysis.

## 2. Materials and methods

### 2.1. Materials

Anhydrous quercetin ( $\geq 98\%$  purity), pepsin, sodium chloride, hydrochloric acid and trifluoroacetic acid were supplied from Sigma Aldrich (Saint-Quentin Fallavier, France). Quercetin hydrate (96.3% purity) was obtained from Faculty of Pharmacy, Ho Chi Minh City Medicine and Pharmacy University, Vietnam. Phospholipon<sup>®</sup> 90G (soybean lecithin at 94–102% of phosphatidylcholine) was provided by Phospholipid GmbH, Lipoid Group (Köln, Germany). Gelucire<sup>®</sup> 50/13 (stearoyl macrogol-glycerides), Labrafil<sup>®</sup> M 2125 CS (linoleoyl polyoxylglycerides) were supplied by Gattefossé S.A.S. (Saint-Priest, France). Methanol and acetonitrile for HPLC was obtained from Carlo Erba, Italy. Deionised and filtered water was obtained through a Milli-Q water system (Millipore, France). Other solvents used without further purification were of analytical grade and other chemicals were reagent grade from standard suppliers.

### 2.2. HPLC analysis

HPLC analysis was performed on a Waters HPLC system (Waters 1525 Binary HPLC pump, Waters 2487 Dual  $\lambda$  Absorbance Detector, Waters 717 plus Autosampler, Waters In-Line Degasser AF and Breeze software). Chromatographic separation was carried out using a Modulo-cart QK strategy 3 C18-2 column (150 mm  $\times$  3 mm, Interchim). The mobile phase was acetonitrile – water – trifluoroacetic acid (30:70:0.1, v/v). The detection wavelength was set at 371 nm. The flow rate was 0.5 ml/min. The sample volume was 20  $\mu$ l. The calibration curve was linear within the range of 50 – 5000 ng/ml ( $y = 166694x$ ,  $r^2 = 0.9998$ ) in methanol containing blank nanoparticles. Before HPLC analysis, all samples were dissolved in methanol at concentrations of linear range, and then filtered through a membrane of 0.2  $\mu$ m (Minisart<sup>®</sup> High-Flow Hydrophilic 16532K, Sartorius Stedim Biotech GmbH, Goettingen, Germany). The peak of quercetin was identified on the chromatograms at a retention time of 7.4 min.

### 2.3. Solubility in solvent and excipients

The drug solubility in ethanol and Labrafil® M2125 CS at room temperature by HPLC and at 70 °C by visual analysis was determined. To amount of excipients or solvent known, drug was gradually added, from 1 mg to 25 mg, until saturation. Each vial was stirred for 800 rpm within 24 h with a magnetic stirrer (Intern. Laborat. App. GmbH, Dottingen, Germany). For samples at room temperature, the over saturated mixtures after visual analysis were centrifuged (LE-80K Ultracentrifuge Optima™, Rotor 701Ti - 93E502, Coulter™ Beckman, Villepinte, France) for 20000 rpm during an hour at 25 °C. The supernatants were diluted appropriately in methanol and the drug concentration was determined by HPLC analysis as mentioned above.

### 2.4. Preparation of quercetin nanocrystals by HPH process

Gelucire® 50/13 and Phospholipon® 90G were dispersed in Milli-Q water as aqueous phase thanks to an Ultra-Turrax T25 (Janke & Kunkel GMBH & Co.KG, IKA®-Labotechnik, Germany) for 9500 rpm during 5 min at 70 °C. Quercetin was dissolved in a mixture (10 ml of ethanol and 10 g of Labrafil® M 2125 CS) at 70 °C. The ethanol content was then decreased under reduced pressure (Rotavapor, Büchi, Switzerland) to a very low residual level corresponding to the quercetin solubility limit. At the end of the process, the maximal ethanol content varies from  $0.6 \pm 0.3$  % to  $1.2 \pm 0.3$  % with increasing amount of quercetin (from 3 to 5% of quercetin compared with lipid phase). The lipid phase was added slowly into the aqueous phase under continuous stirring at 15000 rpm by Ultra-Turrax for 5 min at the same temperature. The dispersions was quickly introduced into the two stage homogenizer (APV 2000, Invensys, Albertslund, Denmark) previously heated to 70 °C, under a dual effect of pressure (the pressure of the first floor is 600 bar and that of the 2nd floor is 200 bar) during 5 min to form hot (o/w) nanodispersions. Finally, it was kept at room temperature to obtain quercetin nanocrystals.

### 2.5. Lyophilisation

Nanodispersions in dispersion systems were separated by ultracentrifugation (LE-80K Ultracentrifuge Optima™, 701Ti rotor - 93E502, Coulter™ Beckman, Villepinte, France), for a speed of 35,000 rpm during 1 h at 4 °C. The lipid nanoparticles were floating on the surface of the centrifuge tube, while the free quercetin and/or nanocrystals aggregated or precipitated in the bottom. After separation, nanocrystals were isolated in a 1.5 ml microtube

(Trefflab, Degersheim, Schweiz, Switzerland). They were lyophilized for 24 h by a freeze dryer ALPHA 1-2 LD plus (Fisher Scientific Bioblock, Innovation Park - BP 50111 - Illkirch F67403 cedex, France). The system temperature was between -55 and -65 °C at a pressure below 0.5 mbar vacuum. The dried powder was stored in a desiccator.

## 2.6. Differential scanning calorimetry (DSC)

Thermal analysis has been carried out using a Diamond DSC system (Perkin-Elmer, USA) associated to Pyris software version 9. This system was equipped with a cooling device (Intracooler 2P) in dry air atmosphere. The instrument was standardized using the enthalpy and melting point of lauric acid (99.5% purity,  $T_m = 43.6^\circ\text{C}$ ,  $\Delta H_m = 167.75$  J/g). For the sample preparation of quercetin recrystallized from ethanol, native quercetin samples were dissolved in ethanol, then evaporated under reduced pressure during 24 h to completely remove traces of solvent. The samples (about 2 to 10 mg) were loaded into hermetically sealed aluminium pans with holes (50  $\mu\text{l}$ , Perkin-Elmer, USA). Concerning the temperature program, samples were held for 5 min at 20 °C and then heated from 20 °C to 350 °C with a scan rate of 10 °C/min.

## 2.7. Powder X-ray diffraction (PXRD)

The data were collected at room temperature on a Rigaku D/max-B goniometer with a radius of 50 cm, associated with a Rigaku RA HF 18 rotating anode. The power of the X-ray generator was set to 50 kV and 200 mA. The  $K\alpha$  ( $K\alpha_1 + K\alpha_2$ ) copper radiation wavelength was selected through a graphite monochromator. The powder, after grinding, was glued with vaseline on a home-made sample holder consisting of a silicon wafer. The scans were recorded with the following setting: 2-theta angle ( $2\theta$ ) from 0.5 to 50°, a step size of 0.02°, and a counting time of 2 seconds per step.

## 2.8. X-ray diffraction (XRD) of the nanocrystal suspensions

X-ray scattering experiments were performed on the nanocrystal suspension with the help of the high-flux 5.2L beamline at ELETTRA (Trieste, Italy). The energy and wavelength of the incident X-ray beam were 8 KeV and 1.54 Å, respectively. The samples were thermostated at 20 °C in the laboratory made sample holder, Microcalix (Ollivon et al. 2006), allowing both sample temperature control and simultaneous DSC analysis. Small angle (SAXS) and wide angle (WAXS) X-ray scattering patterns were

recorded simultaneously using two position-sensitive linear gas detectors filled with an argon-ethane mixture. As no Bragg reflections were detected in the SAXS region, only the WAXS scattered intensities are reported as a function of the scattering vector  $q = 4\pi\sin\theta/\lambda = 2\pi/d$ , where  $2\theta$  is the scattering angle,  $\lambda$  is the wavelength and  $d$  is the repeat distance between two reticular plans. The calibration of the  $q$ -range was achieved using pure tristearine (2L $\beta$  form) characterized by short spacings of 4.59, 3.85 and 3.70 Å ( $\pm 0.01$  Å). The samples were loaded into quartz capillaries (Quarzkapillaren, Germany) with the external diameter of  $1.4 \pm 0.1$  mm and the wall thickness of 0.01 mm. The data were analyzed by the IGOR Pro software (WaveMetrics, Inc. USA).

## 2.9. Particle size and zeta potential measurements

The particle size and zeta potential of nanodispersions were measured at 25°C using a Nano-ZS90 apparatus (Malvern Instruments, Orsay, France). The average hydrodynamic diameter and polydispersity index (Pdl) were determined by *quasi*-elastic light scattering while the zeta potentials were quantified from electrophoretic mobility using laser Doppler velocimetry. The samples were diluted to 1/300 in Milli-Q water for size analysis and in 1 mM NaCl for zeta potential measurements.

## 2.10. Drug loading and encapsulation efficiency

After the preparation, the quercetin-loaded nanodispersions were purified by an ultracentrifugation (LE-80K Ultracentrifuge Optima™, Rotor 701Ti - 93E502, Coulter™ Beckman, Villepinte, France) for 30000 rpm during an hour at 4°C. After ultracentrifugation, the colloidal suspensions could be separated into three parts: lipid nanodispersion (top layer), aqueous phase (middle layer) and precipitate (bottom layer). The top layer was used for quercetin quantification by HPLC analysis as mentioned above in order to determine the amount of entrapped drug in lipid nanoparticles. The drug loading and encapsulation efficiency were calculated using the formula:

$$\text{Drug loading (\%)} = \frac{\text{Amount of entrapped drug}}{\text{Amount of lipid in the formulation}} \times 100$$

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Amount of entrapped drug}}{\text{Total amount of drug in the formulation}} \times 100$$

### 2.11. Polarized light microscopy

The crystallinity of the lipid mixtures was observed using a polarized light microscope equipped with a heating stage (Eclipse E600, Nikon, Japan) under x40 objective. Micrographs were recorded using a digital camera (Nikon Coolpix, Japan). For treated samples, quercetin was dissolved in ethanol or ethanol – Labrafil® M2125 CS (1:1) at 70°C, then evaporated under reduced pressure during 24 h to completely remove traces of solvent. Quercetin recrystallized in Labrafil® M2125 CS was equivalent to 4% (w/w).

### 2.12. Dark field microscopy

The lipid colloidal systems were observed using a dark field microscope (Eclipse E600, Nikon, Japan) under x40 objective. Before the observation, the samples were diluted to 1/20 in Milli-Q water. Micrographs were recorded using a digital camera (Nikon Coolpix, Japan).

### 2.13. Transmission electron microscopy (TEM) and Cryogenic transmission electron microscopy (Cryo-TEM)

The nanodispersion morphologies were observed using a TEM system (JEM-1400, JEOL, Japan) and a cryo-TEM system (JEM 2100, JEOL, Japan). 10 µl of samples diluted in Milli-Q water (1/100; v/v) was placed on a copper grid and excess solution was removed with a filter paper. For the TEM, specimen support film was rapidly dried by carbon evaporation thanks to a JEE-420 vacuum evaporator (JEOL, Japan) before the observation. For the cryo-TEM, vitrification of the film was achieved by rapidly plunging the grid into liquid ethane held just above the freezing point. The vitrified sample was then transferred at low temperature to the microscope. The temperature was kept about –180°C during both the transfer and the viewing procedures in order to prevent sample perturbation and the formation of ice crystals.

### 2.14. *In vitro* release studies

The *in vitro* release of quercetin-loaded nanodispersions containing nanocrystals and lipid nanoparticles in comparison with coarse quercetin suspensions was performed in 500 ml of SGF using the dialysis bag method. The dialysis bags (MWCO 6-8000, Spectrum® Laboratories, Inc., Rancho Dominguez, CA) were soaked in Milli-Q water for one hour and then washed before use. Samples equivalent to 0.25 mg of quercetin were put into dialysis bags. The rotation was set at 100 rpm. The temperature was maintained at 37 ± 0.5°C. The

dissolved solution samples of 0.5 ml were withdrawn at time intervals of 15, 30, 45, 60, 120, 180, 240, 300, 360 min and 24 h. The concentrations of drug were determined by HPLC analysis (section 2.2). All the operations were carried out in triplicate.

### 2.15. Long-term stability

The quercetin nanodispersions were stored at room temperature and at 4°C in the dark. The quercetin content was determined by HPLC analysis as mentioned above. The size analysis was measured by *quasi*-elastic light scattering using a Nano-ZS90 (Malvern Instruments, Orsay, France) over 3 months.

## 3. Results and discussion

### 3.1. Particle size, zeta potential and drug payload analysis

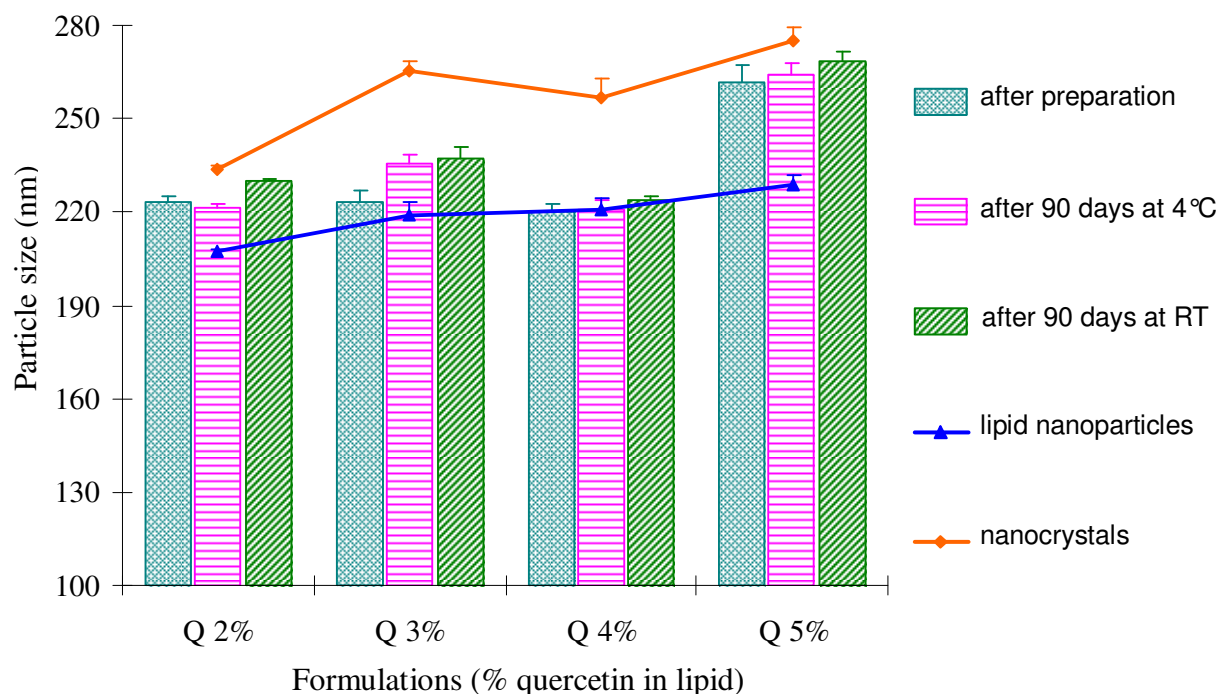
The previous work of our research group in paper “Quercetin solubility and chemical stability: enhancement with polyoxylglyceride-based nanoparticles”, a potentiel delivery system for administration of quercetin was developed. The formulation contained 20 g of Labrafil® M2125 CS as oil phase, 1 g of Phospholipon® 90G, 2 g of Gelucire® 50/13, 200 mg of quercetin, and sufficient quality of Milli-Q water for 100 g of nanodispersions (Truong Cong, Faivre, et al. to be submitted). Based on this formulation, we tried to increase the amount of quercetin in the formulations (2, 3, 4, 5% of quercetin compared with lipid phase) thanks to the increase of quercetin solubility at 70°C and/or in presence of ethanol (Table 3.1).

**Table 3.1.** Solubility of quercetin in Labrafil® M2125 CS and ethanol at room temperature determined by HPLC and 70°C by visual analysis

| Excipients and solvent | at room temperature |      | at 70°C     |      |
|------------------------|---------------------|------|-------------|------|
|                        | mg/g                | %    | mg/g        | %    |
| Labrafil® M 2125 CS    | 9.1 ± 0.4           | 0.92 | 24.3 ± 1.2  | 2.5  |
| EtOH                   | 60.6 ± 0.6          | 6.45 | 109.8 ± 3.6 | 12.3 |

*mg/g: weight of quercetin / total weight (quercetin and excipients); %: weight of quercetin (g)/100 g of excipients*

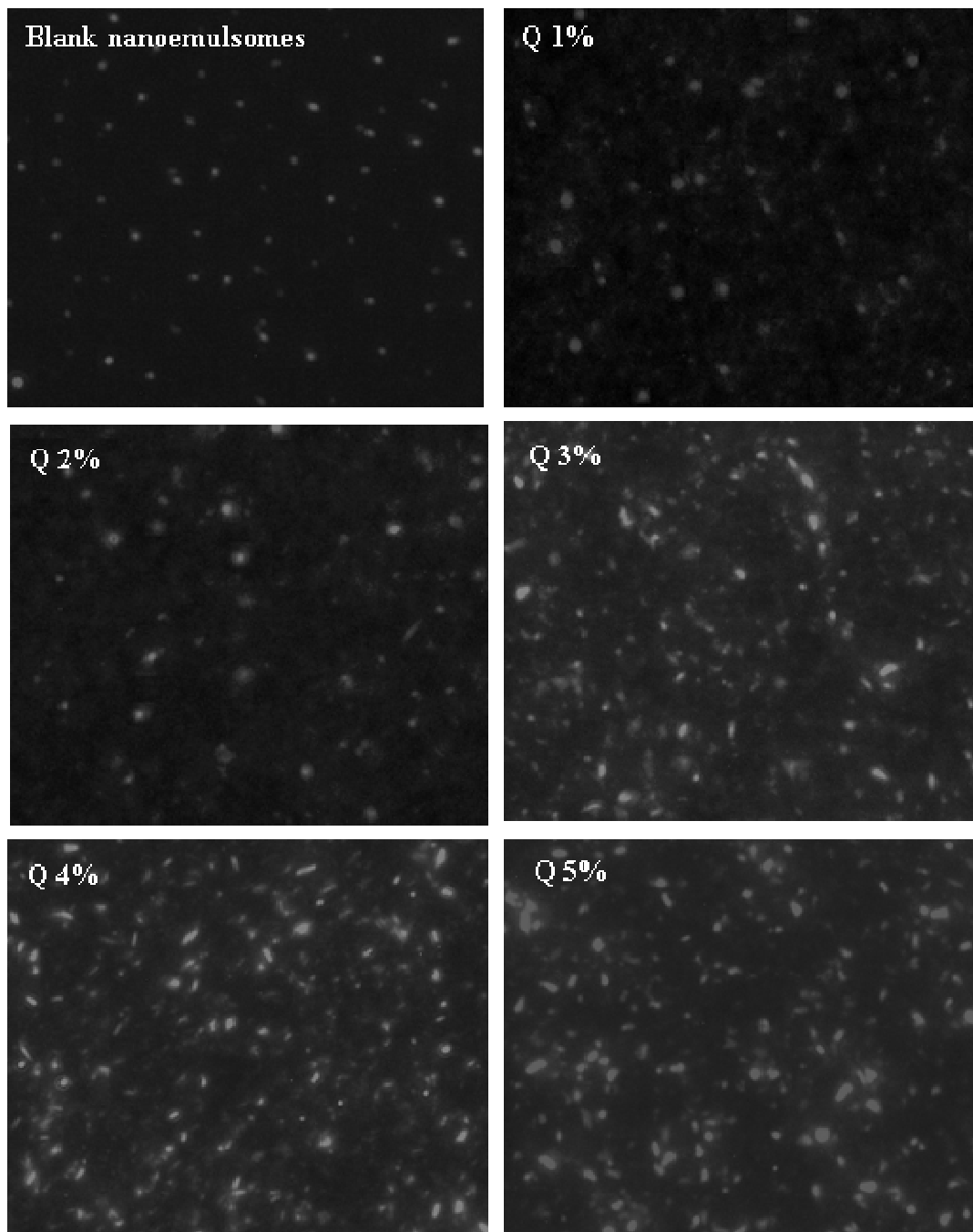
After preparation, particle average diameters were reported in the range of 223.1 – 261.8 nm with Pdl ~ 0.1, depending on the quercetin payload. The quercetin-loaded nanodispersions were stable in size at room temperature or 4°C for at least 3 months. Both hydrodynamic diameters and Pdl remained unchanged during the observation period (Figure 3.1).



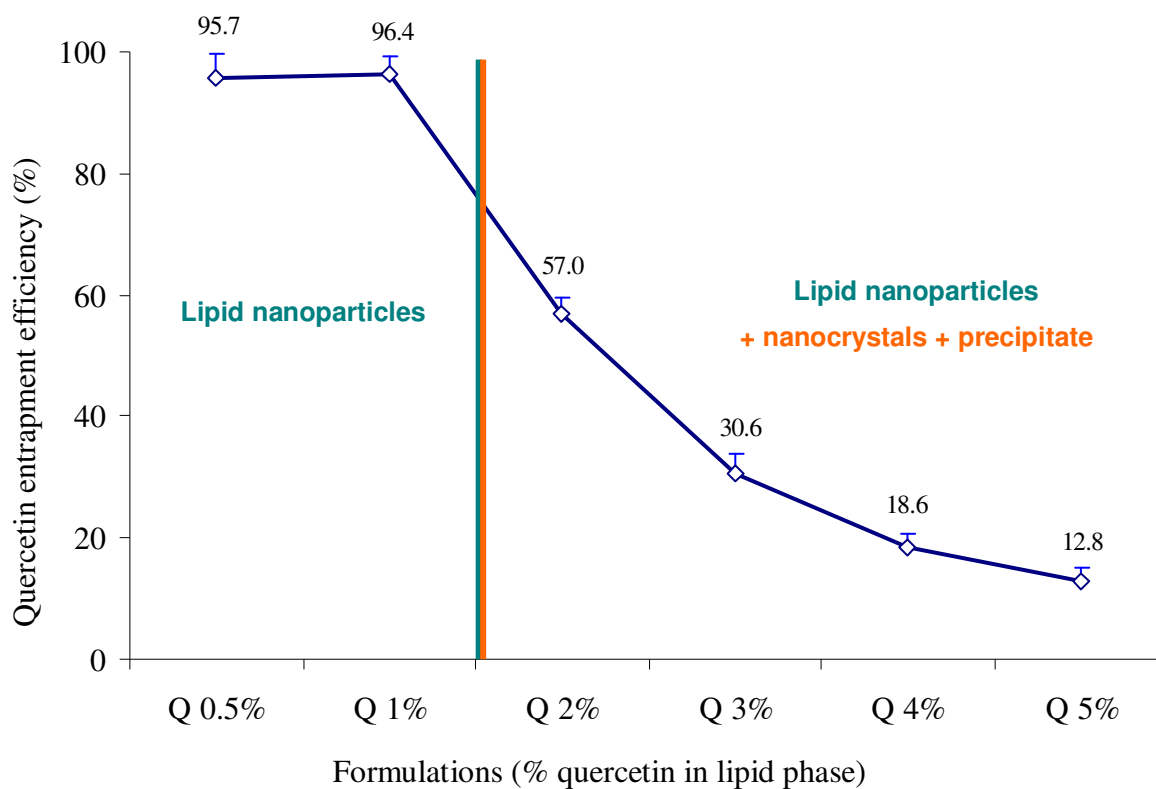
**Figure 3.1.** Particle size of nanodispersions, lipid nanoparticles and nanocrystals in formulations Q2%, Q3%, Q4% and Q5% (w/w; quercetin in lipids) after preparation and 90 days at room temperature (RT) and 4°C.

From preliminary observations by dark-field microscopy, micrographs highlighted the presence of two particle populations which were not detected with *quasi*-elastic light scattering. Stick-like shapes of particles were clearly seen in formulations Q3%, Q4% and Q5% where they coexisted with spherical nanoaggregates attributed to the presence of lipid nanoparticles (Figure 3.2). After ultracentrifugation, the colloidal suspensions were clearly separated into three parts: lipid nanoparticles at top layer, aqueous phase eventually containing surfactant micelles at middle layer and precipitate at bottom layer. For top layer, the size of lipid nanoparticles was in the range of 207.8 – 228.7 nm. It was suitable for the size of polyoxylglyceride nanoparticles containing 1% (w/w) quercetin in lipid (~ 200 nm) (Truong Cong, Faivre, et al., to be submitted). Regarding precipitate, the average size varied from 233.4 to 275.1 nm after redispersion in Milli-Q water suggesting the presence of nanocrystals (Figure 3.1). When increasing the drug payload, beyond the drug saturation threshold of the lipidic phase, nanocrystals or precipitate appeared more and more in the aqueous phase. As a result, the drug entrapment efficiency in part concerning lipid nanoparticles had a sudden drop to 57.0%, 30.6%, 18.6% and 12.8% for formulations Q2%, Q3%, Q4% and Q5%, respectively (Figure 3.3). The quercetin loading in lipid nanoparticles had fluctuation from 1.03 % to

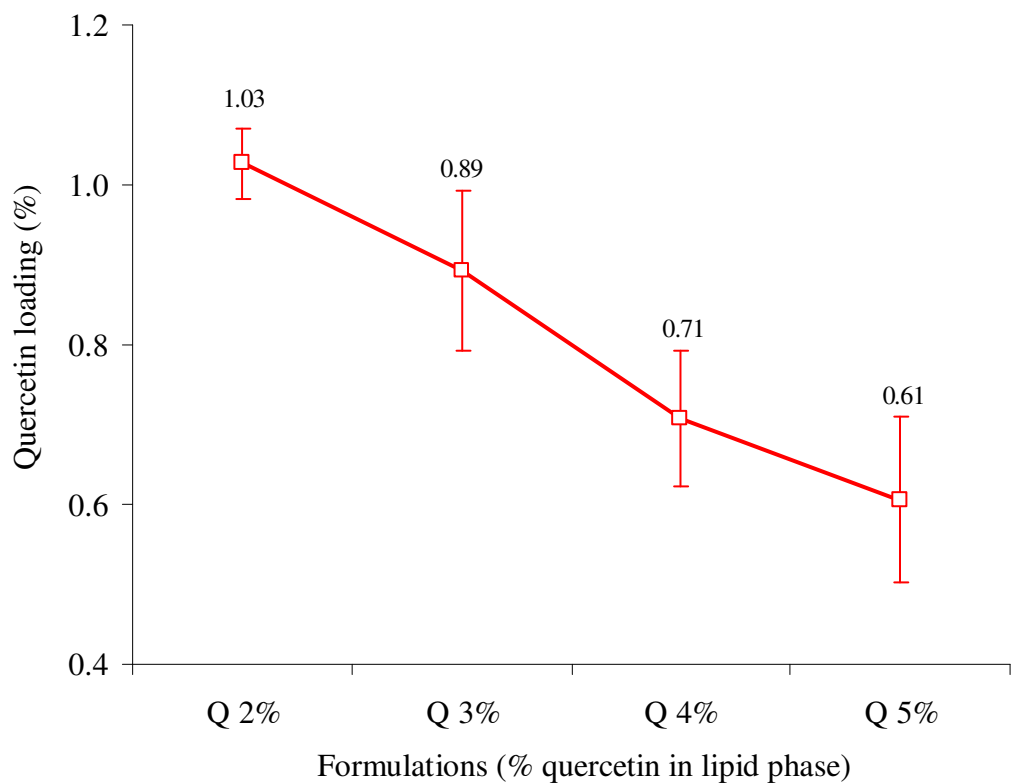
0.61 % with increasing amount of quercetin in the formulation (Figure 3.4). It corresponds to the quercetin solubility in Labrafil® M2125 CS about 1% at room temperature (Truong Cong, Faivre, et al., to be submitted). Thus, when the drug payload increased, the entrapment efficiency greatly reduced and the number of drug nanocrystals augmented.



**Figure 3.2.** Dark field microscopy of the quercetin nanodispersions in formulations Q1%, Q2%, Q3%, Q4% and Q5% versus blank nanoemulsomes.



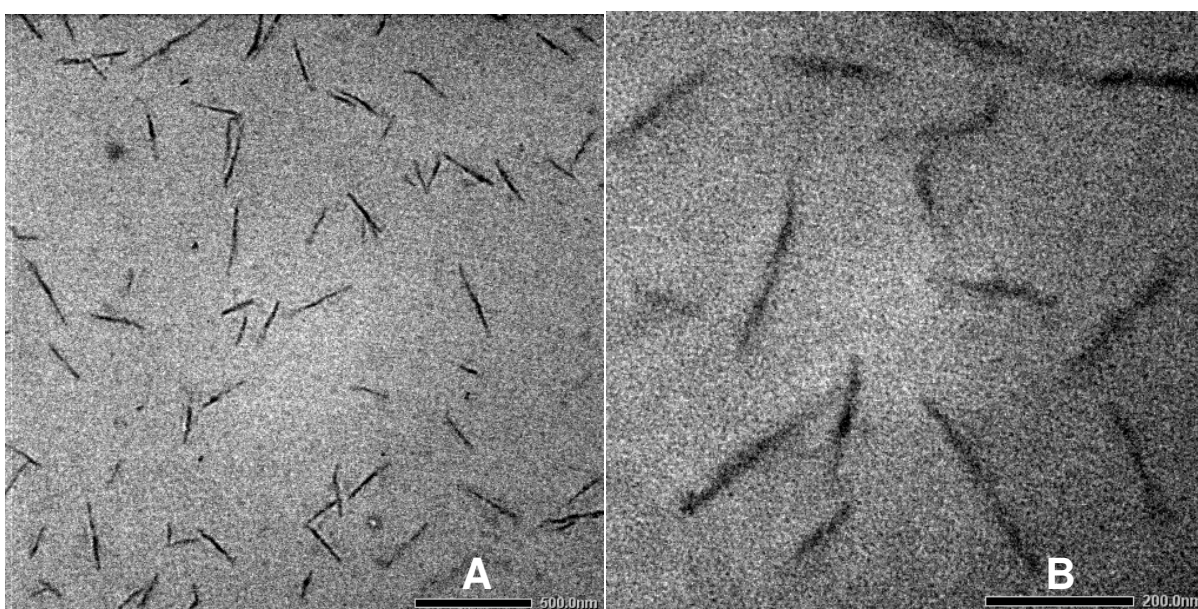
**Figure 3.3.** Quercetin entrapment efficiency (%) in fraction containing lipid nanoparticles



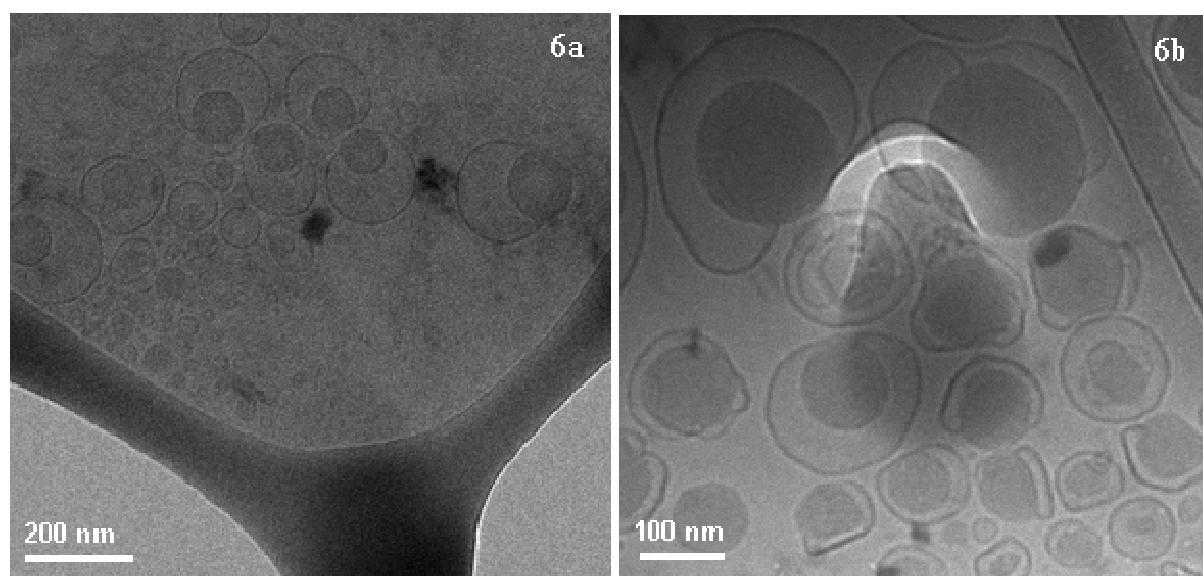
**Figure 3.4.** Quercetin payload (%) in fraction containing lipid nanoparticles for formulations Q2%, Q3%, Q4% and Q5%.

### 3.2. Particle morphology (TEM and Cryo-TEM)

In order to evaluate the ultrastructure of quercetin nanodispersions, formulation Q4% was characterized by TEM and Cryo-TEM. The TEM images presented needle-shaped nanocrystals with a length of approximately 100 – 350 nm and a width of some tens of nanometers (Figure 3.5), which was in agreement with the size analysis by *quasi*-elastic light scattering even if the nanocrystals were not spherical. As regards the lipid nanoparticles, the Figure 3.6 described cryo-TEM observations in formulations Q4% versus blank nanoparticles. The multi-compartment spherical objects were representative on the whole of the samples. As fully described in another paper (Truong Cong, Ouattara, et al, to be submitted), the typical structures of particles presented three essential parts: a central region dense to the electrons, a medium region with a contrast similar to the vitrified water and a dense surrounding layer. The dense central region is independent on the presence of API and should probably correspond to acyl-chain rich domains, i.e. Labrafil® M2125 CS droplets. The less dense domain certainly corresponds to the low contrasted polyoxyl chains, which are numerous in the present formulation, and water. The external ring-shaped surrounding layer with a comparatively high contrast looks like liposome bilayers. If the lipid bilayer is high flexible, vesicle deformation and bilayer invaginations may occur during freezing process (Kuntsche et al. 2011). Accordingly, some lipid vesicles had irregular borders. Except for multi-compartment particles, a few emulsion droplets and liposomes were sometimes detected in the samples. In the cryo-TEM, the emulsion droplets appear as circular structures with an even distribution of contrast. They can easily be distinguished from liposomal vesicles with ring-like structures (Kuntsche et al. 2011; Klang et al. 2012).



**Figure 3.5.** TEM photograph of quercetin nanocrystals. Scale bar: (A) 500 nm and (B) 200 nm.



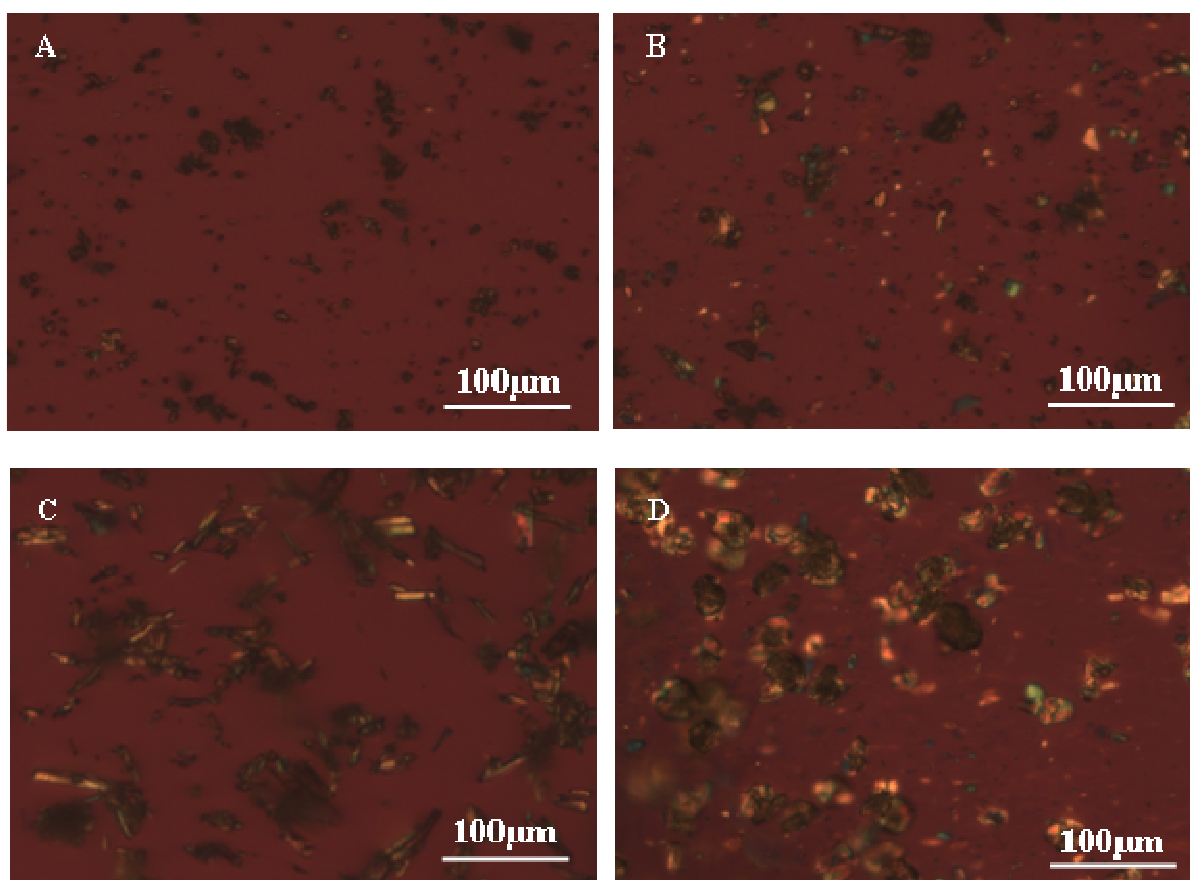
**Figure 3.6.** Cryo-TEM photograph of blank nanoparticles (6a) and quercetin loaded-nanoparticles (6b).

### 3.3. Crystallinity and polymorphism of quercetin

In order to better characterize the nanocrystals and their formation process, bulk quercetin polymorphism was analyzed by polarized light microscopy, DSC and XRD. Apart from quercetin incorporated in the preparations, quercetin hydrate and treated quercetin samples were also characterized. Treated samples were anhydrous quercetin and quercetin hydrate samples recrystallized from ethanol solution.

#### 3.3.1. Shape observation by polarized-light microscopy

The micrographs of raw anhydrous quercetin, quercetin hydrate, quercetin crystallized from pure ethanol and in ethanol / Labrafil® M2125 CS (50:50) mixture are shown in Figure 3.7. It could be observed that the quercetin samples exhibited heterogeneity in shape and size. For each sample, the raw crystals were rather large and irregular. Frequently described in the literature, the differences may be caused by the API (pseudo)polymorphism, the nature of crystallization media and the crystallization process. The SEM images show a different crystal structure for anhydrous quercetin and quercetin dihydrate maintained at 25°C (Srinivas et al. 2010). In another study, quercetin raw materials from different suppliers present various crystalline structures in size and shape, ascribed to the different degree of hydration of their crystal lattice (Borghetti et al. 2012). The SEM micrographs of raw quercetin samples, supplied from Sigma-Aldrich, present diverse textures in shape and size (Borghetti et al. 2009; Srinivas et al. 2010; Sahoo et al. 2011; Kakran et al. 2011) .

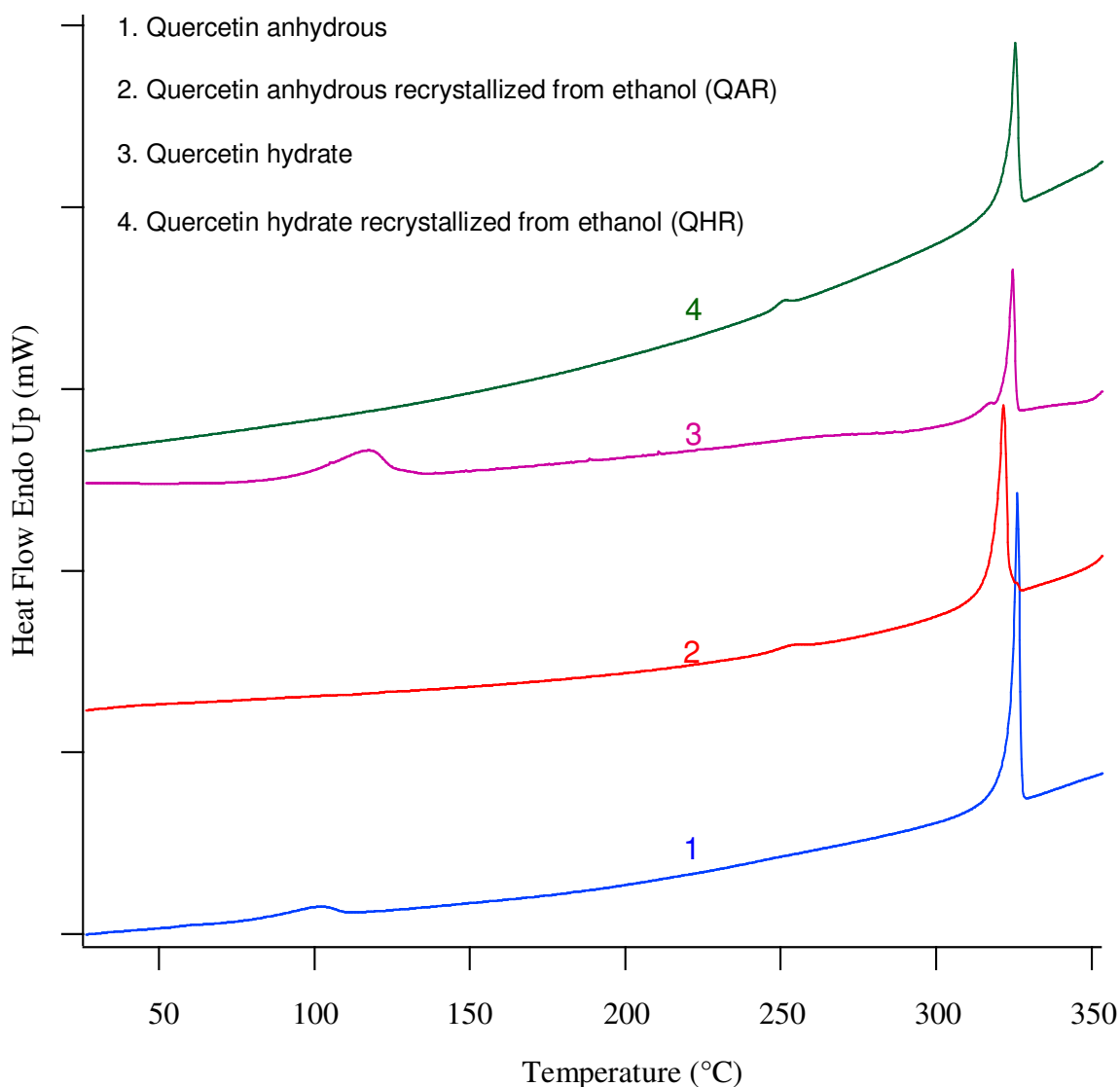


**Figure 3.7.** Micrographs of quercetin anhydrous (A), quercetin hydrate (B), quercetin crystallized from ethanol (C) and quercetin crystals in Labrafil M2125 CS (D)

### 3.3.2. DSC analysis

The DSC thermograms of anhydrous quercetin, quercetin hydrate and their materials recrystallized from ethanol are shown in Figure 3.8. In agreement with the previous investigations (Wu et al. 2008; Zhang et al. 2011; Borghetti et al. 2012), the DSC curves of native quercetin samples presented two main endothermic peaks. For both samples, the first one should correspond to the release of water. With a  $T_{\text{onset}}$  at 83°C, this peak could be referred to moisture incorporated in quercetin anhydrous powder, while it might be relative to crystallization water, entrapped in the crystal lattice, for quercetin hydrate ( $T_{\text{onset}}$  at 102°C). Hence, the stage of water loss for this quercetin hydrate sample was in the large range of temperature from 80°C to 135°C with a great enthalpy of 163.0 (J/g). The second one related to the melting points at 324°C for anhydrous quercetin and 322°C for quercetin hydrate after its dehydration. For quercetin samples recrystallized from ethanol (QAR and QHR), the DSC curves were very similar and exhibited two endothermic events (Figure 3.8). A minor endothermic event was observed at

approximately 247°C for QAR and 253°C for QHR. This thermal event, probably a solid-solid transition, was then followed by sharp melting endothermic peaks at 319°C and 323°C, respectively. In these QAR and QHR samples the peak attributing to the water loss did not appear (Figure 3.8; Table 3.2). For a defined form, the melting enthalpy ( $\Delta H$ ) is proportional to the degree of crystallinity in the samples (Kundu et al. 2003; Sahoo et al. 2011; Kakran et al. 2012). It was not significantly different here among quercetin samples.



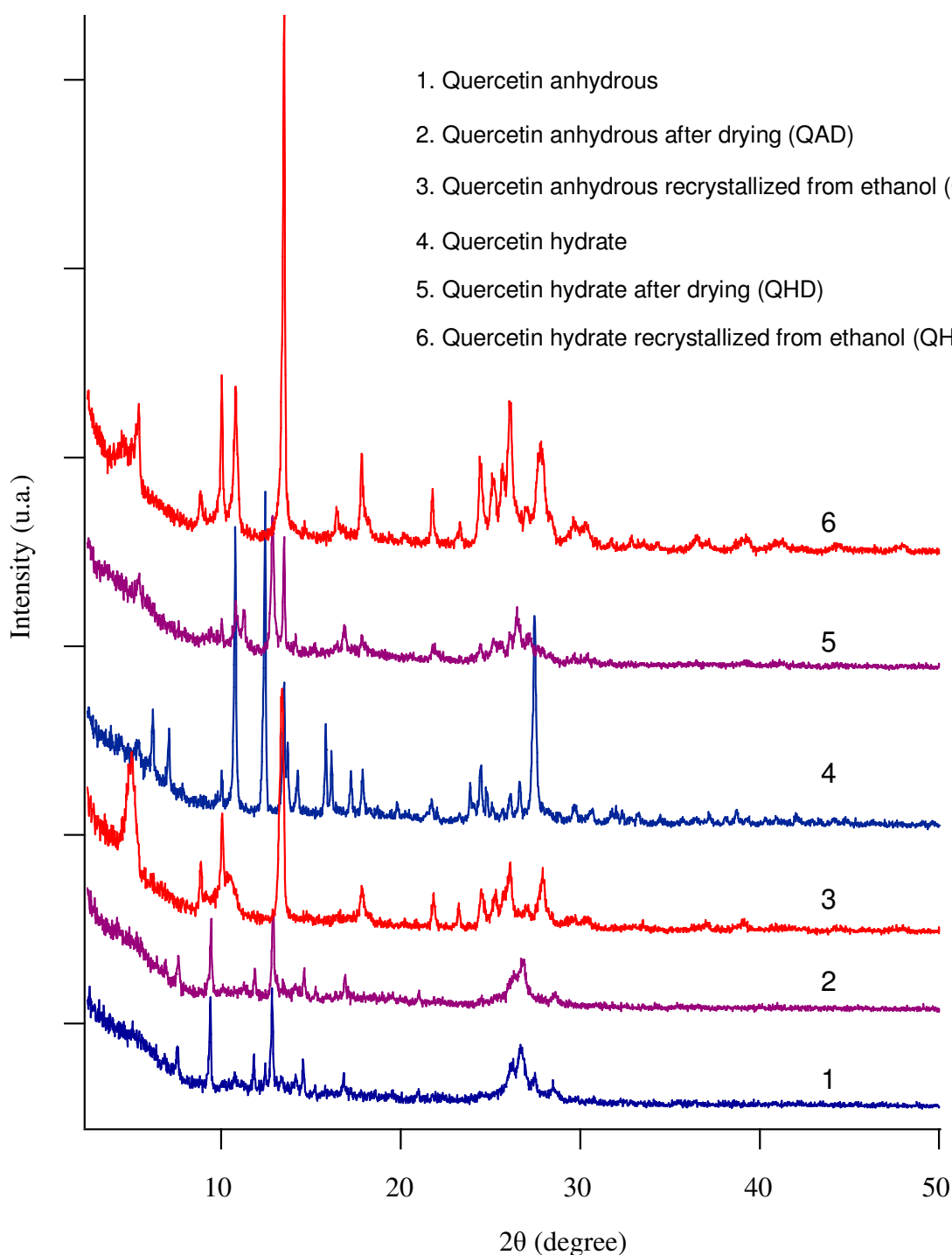
**Figure 3.8.** DSC thermograms of quercetin recrystallized from ethanol compared with native quercetin samples during the heating at 10°C/min.

**Table 3.2.** DSC parameters of quercetin samples: transition temperatures and theirs enthalpies ( $\Delta H$ )

| Quercetin samples                                           | Phase of water loss |                  | Solid-solid transition |                  | Melting point |                  |
|-------------------------------------------------------------|---------------------|------------------|------------------------|------------------|---------------|------------------|
|                                                             | °C                  | $\Delta H$ (J/g) | °C                     | $\Delta H$ (J/g) | °C            | $\Delta H$ (J/g) |
| Quercetin anhydrous                                         | 83                  | 25.0             |                        |                  | 324           | 157.3            |
| Quercetin anhydrous<br>recrystallized from ethanol<br>(QAR) |                     |                  | 245                    | 6.5              | 319           | 143.3            |
| Quercetin hydrate                                           | 102                 | 163.0            | 236                    | 23.8             | 322           | 140.6            |
| Quercetin hydrate<br>recrystallized from ethanol<br>(QHR)   |                     |                  | 247                    | 5.7              | 323           | 134.1            |

### 3.3.3. XRD characterization

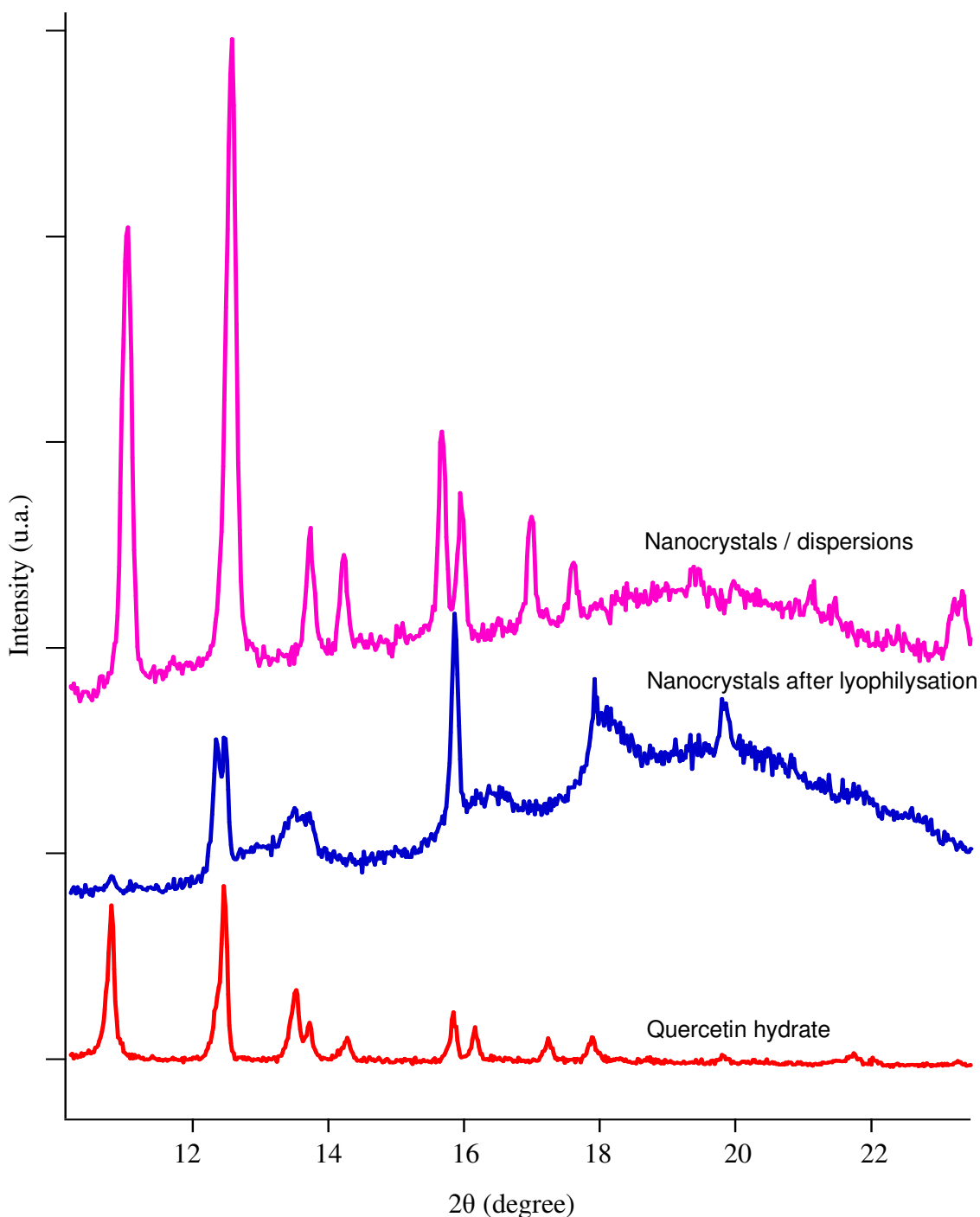
The diffractograms for quercetin samples at 25°C are illustrated in [Figure 3.9](#) and the main diffraction peaks are summarized in [Table 3.3](#). All of them presented high crystalline nature which was confirmed by a series of sharp peaks. However, the differences in the peak positions, which correspond to the periodic packing of molecules in the solid state, indicated different crystalline structures ([Borghetti et al. 2012](#)). Quercetin anhydrous and quercetin hydrate displayed different crystalline states due to the different degree of hydration of their crystal lattices. More precisely, the hydrate pattern described in [Figure 3.9](#) perfectly corresponded to the dihydrate quercetin diffractogram recorded in the Cambridge Structural Data base, and did not correspond to the monohydrate pattern also referenced. After a drying process during 2h at 150°C, the anhydrous quercetin diffractogram (QAD) remained similar to the native one while that of hydrated quercetin (QHD) significantly changed towards the anhydrous profile. It correlated well with the assumptions made from the scanning calorimetry results. A third pattern has been described by the two quercetin samples recrystallized from ethanol (QAR and QHR samples). Independently on the initial structure (anhydrous or dihydrate), quercetin was crystallized in the same form from ethanol. However, as it was suggested by the specific differential scanning calorimetry trace of these samples, the x-ray diffraction pattern did not correspond to the anhydrous one.



**Figure 3.9.** X-ray diffractograms of treated quercetin samples (after drying at 150°C/2h and recrystallizing from ethanol) compared with native quercetin samples (anhydrous and hydrate forms).

Quercetin is known to give different solvates depending on the crystallization media ([Kavuru 2008](#)), however, to our knowledge, “ethanolate” has not been described in the literature concerning this molecule. In the present work, ethanol was used initially as an intermediate solvent to reduce the solubilization time of quercetin during the preparation process of nanodispersions. Thus, it could eventually lead to a solvent-mediated

polymorphic transformation of quercetin in the nanodispersions. Thanks to Synchrotron radiations (Elettra, Trieste), the crystalline structure of the nanosuspension have been determined *in-situ*, even with the dilution of the sample. In fact, quercetin nanocrystals in suspension state existed in a crystalline form which totally corresponded to the dihydrate form as proved by the similarities between the x-ray diffraction patterns (Figure 3.10).



**Figure 3.10.** X-ray diffractograms of quercetin hydrate, quercetin nanocrystals before and after lyophilization.

Quercetin dihydrate, considered as a stable form, is reported as the most appropriate crystalline form for the development of pharmaceutical products (Borghetti et al. 2012). However, in order to obtain a dry powder, which should improve the chemical stability of the API, lyophilization assays have been done on the nanocrystals. Only the structural point of view, and not the reconstitution properties, had been checked in these preliminary investigations (Figure 3.10). The broad peaks observed in the lyophilized sample strongly suggested an amorphisation of the powder during this operation. Amorphous solid should be interesting to improve the aqueous solubility, the dissolution rate and then the bioavailability of poorly-water soluble API. However, in the case of chemically instable molecules such as quercetin, the high chemical reactivity of the amorphous solids, explaining why they are more soluble, compared to crystalline solids is a major drawback of this solid state. From chemical and physical stability considerations, dihydrate quercetin nanocrystals should be more interesting than amorphous nanosuspensions and lyophilization step must be improved.

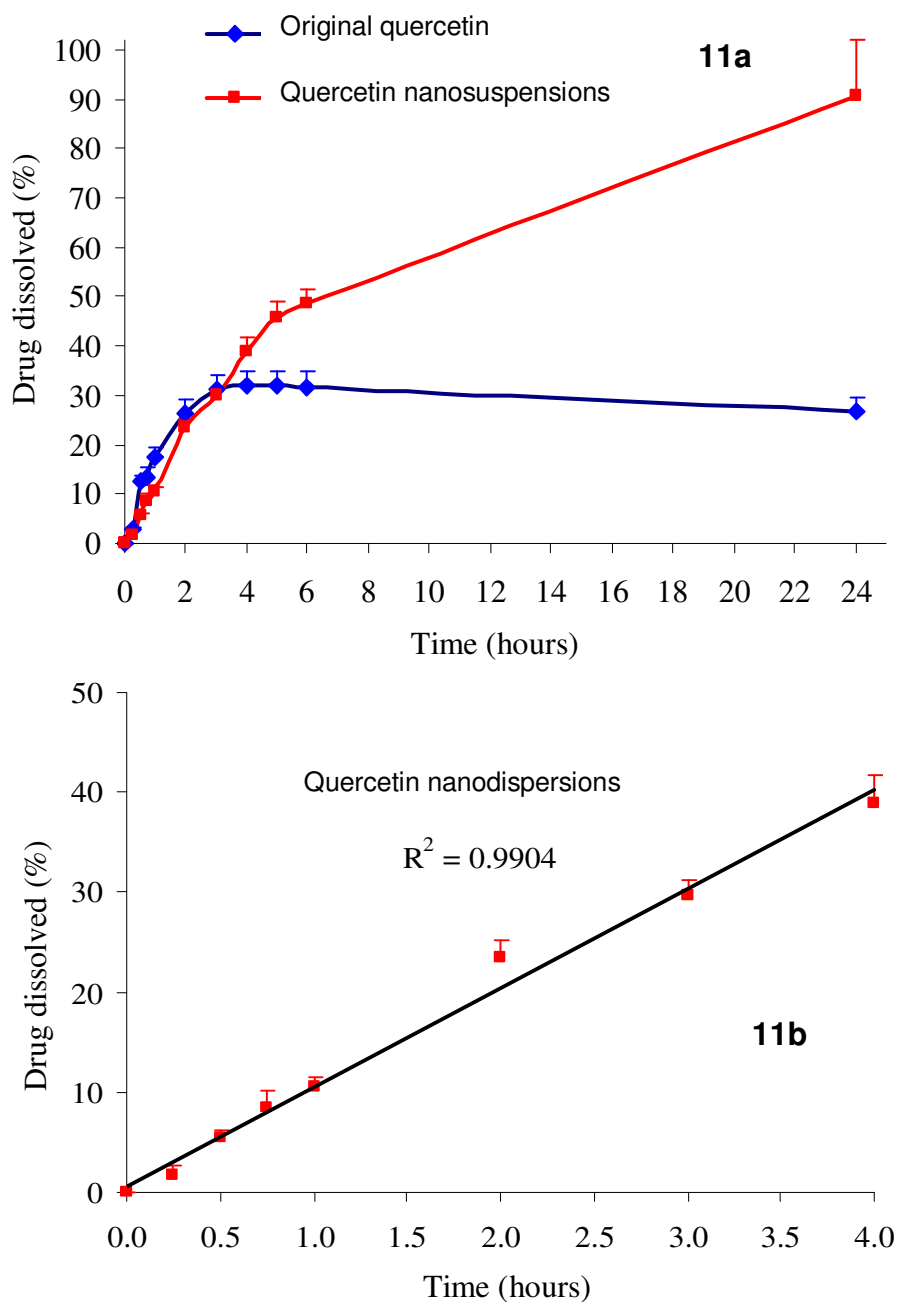
The process used here to enrich the quercetin nanodispersion with nanocrystals was a mix of the bottom-up and top-down processes classically developed in the field of nanocrystals. In the bottom-up way, poorly-water soluble compounds should be dissolved in an organic solvent, as we partially did in the Labrafil® M2125 CS / ethanol mixture, and then precipitated after addition of a non-solvent (water in our case). Contrary, high pressure homogenization is a milling technique which allows to reduce and homogenize the size of particles in a top-down approach. Several papers deal with quercetin nanocrystals. Following a bottom up process implying ethanol and water, Kakran et al. obtained acicular nanocrystals having mean sizes around 11  $\mu\text{m}$  (Kakran et al. 2011). With HHP, Sahoo et al obtained crystals with same shape but with sizes in the range 0.5 to 1  $\mu\text{m}$  (Sahoo et al. 2011). At last, smaller sizes, around 200 nm, were reached by Gao et al. with both bottom-up and top-down methods (Gao et al. 2011). In the later case, the nanocrystals were more spherical and important amount of surface active agents, Pluronic® F68 and lecithin, were incorporated in the formulation.

The nanocrystals described in our results had a very thin width, some tens of nanometers, suggesting their appearance after the homogenization step. Indeed, high-pressure systems induce high fluid velocities, cavitation, high-shear and turbulences which seem unfavourable for the production of such kind of so thin needle-shape

crystals and the process trends to give more or less spherical material (Kluge et al. 2012). From the Figure 3.4, it seemed that the amount of API within the lipid nanodispersion phase decreased with the total amount of API in the preparation suggesting an “equilibrium” between the polyoxylglyceride-based dispersion and the whole suspension. The importance of this excipient was confirmed by the preparation of nanocrystal with similar process, with the stabilizers used in the formulation, Gelucire® 50/13 and Phospholipon® 90G, but without Labrafil® M2125 CS (data not shown). The mean size and the Pdl of the nanocrystals reached 1µm and 0.9 respectively in those cases. The role of potential ethanol traces and temperature, which decreased from 70°C to room temperature during the preparation process, should be clarified but one hypothesis is that nanocrystals are generated from the Labrafil® M2125 CS droplet through the aqueous phase. The crystallization into quercetin dihydrate also seems to suggest a crystallization from the surface of this droplet (in contact with the external aqueous phase).

### 3.4. *In vitro* release of nanodispersions

The dissolution profiles of raw quercetin suspensions and quercetin-loaded nanodispersions containing lipid nanoparticles and nanocrystals in SGF (pH = 1.2) were shown in the Figure 3.11. For the quercetin nanodispersions, the dissolution rate of drug significantly improved versus raw quercetin suspensions. Quercetin-loaded nanoparticles reached  $48.7\% \pm 2.6\%$  dissolution at 6 h and about 90% dissolution at 24%. In contrast, raw quercetin released to  $32.2 \pm 2.6\%$  at 4 h and the dissolution slightly decreased until  $26.9 \pm 2.6\%$  at 24 h. According to the Noyes-Whitney equation, particle size reduction could lead to an increase of the surface area and a decrease of the diffusion layer thickness, which could provide an enhanced dissolution rate for drugs (Kawabata et al. 2011). For the polyoxylglyceride-based nanovectors, particle surface covered by hydrophilic PEG chains resulted in greater wetting and reduced interfacial tension. Particles were easily dispersed in the dissolution medium to increase the available surface for dissolution medium. For original quercetin, raw crystals precipitated quickly in dialysis bag reducing thus the contact surface area with the dissolution medium. At longer times, the slight decrease of quercetin concentrations with the raw crystals could be due to its chemical instability. In short, the reduction in the particle size, increased dispersibility and wettability of nanovectors contributed to enhancement of the dissolution rate of drug.

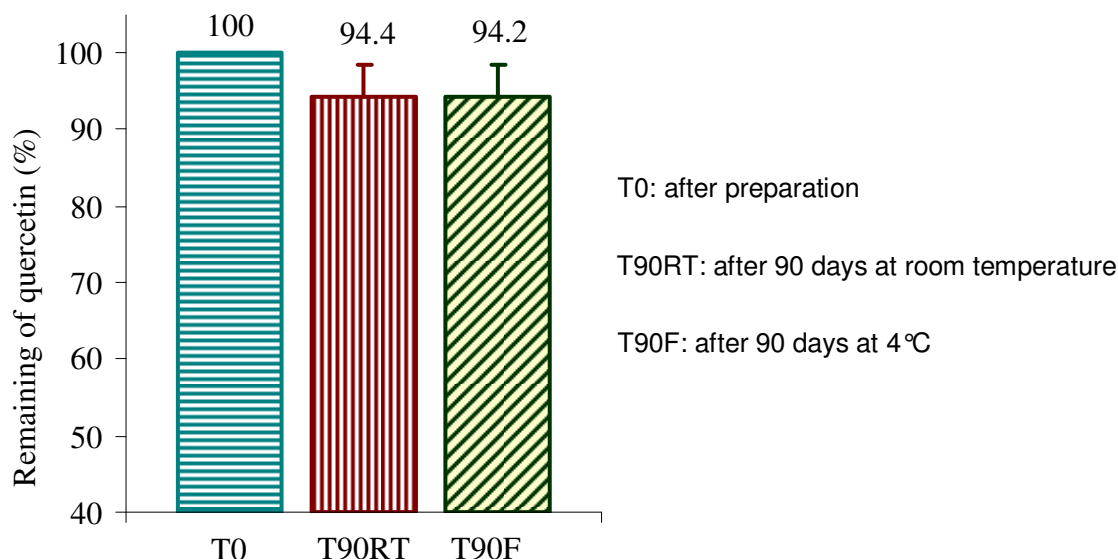


**Figure 3.11.** *In vitro* dissolution profiles for original quercetin, quercetin nanodispersions (11a) and a zero-order kinetics in the case of the nanodispersions (11b).

### 3.5. Long-term stability of quercetin in nanodispersions

To validate the effect of nanocarriers on the chemical stability of quercetin, a long-term study was performed by HPLC on formulation Q4%. The drug content in nanodispersions reduced about 6% after 3 months at room temperature and 4°C in the dark (Figure 3.12). Because of the diffusion into aqueous phase and/or the adsorption at surface of particles, the drug loss might take place. In comparison to chemical stability of quercetin in water ( $t_{1/2} = 16.8$  h, the half-life of degradation), quercetin-loaded nanodispersions showed no

significant alteration in drug content. For polyoxylglyceride-based nanoparticles, quercetin was dissolved in lipid cores of particles and protected against hydrolysis in aqueous phase (Truong Cong, Faivre, et al. to be submitted). For nanocrystals, the chemical stability is explained by two effects. Firstly, the molecules of the surface stabilizer could shield the chemical compound. Secondly, the crystalline structure in nanocrystals results in higher drug stability (Liversidge & Wei 2003; Möschwitzer et al. 2004).



**Figure 3.12.** Stability of quercetin in nanodispersions for formulation Q4% stored at room temperature and 4°C after 90 days.

#### 4. Conclusion

Nanocrystals was successfully developed on the basis of polyoxylglyceride-based nanoparticles by HPH process. Nanodispersions containing needle-like nanocrystal and lipid nanoparticles improved the physico-chemical stability, the dissolution rate and the drug. The XRD, DSC and polarized-light microscopy results presented the solvent-mediated polymorphic transformation of quercetin towards stable form during preparation process. This study suggested that nanocrystals and polyoxylglyceride-based nanodispersions could be efficient to protect chemically sensitive compounds and then could probably enhance bioavailability.

#### Acknowledgement

The authors are grateful to Ghislaine FREBOURG (Paris VI University, France) for cryo-TEM images, Quoc Bao NGUYEN (Ho Chi Minh City University of Technology, Vietnam) for TEM images and Heinz AMENISTSCH (ELETTRA synchrotron, Italy) for XRD technique.

## References

1. Boots, A.W., Haenen, G.R.M.M. & Bast, A., 2008. Health effects of quercetin: From antioxidant to nutraceutical. *European Journal of Pharmacology*, 585(2-3), pp.325–337.
2. Borghetti, G.S. et al., 2012. Physicochemical properties and thermal stability of quercetin hydrates in the solid state. *Thermochimica Acta*, 539(0), pp.109–114.
3. Borghetti, Greice S et al., 2009. Quercetin/beta-cyclodextrin solid complexes prepared in aqueous solution followed by spray-drying or by physical mixture. *AAPS PharmSciTech*, 10(1), pp.235–242.
4. Dajas, F., 2012. Life or death: Neuroprotective and anticancer effects of quercetin. *Journal of ethnopharmacology*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22820241>.
5. Gao, L. et al., 2011. Preparation of a chemically stable quercetin formulation using nanosuspension technology. *International Journal of Pharmaceutics*, 404(1-2), pp.231–237.
6. Hörter, D. & Dressman, J.B., 2001. Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. *Advanced drug delivery reviews*, 46(1-3), pp.75–87.
7. Jinno, J. et al., 2006. Effect of particle size reduction on dissolution and oral absorption of a poorly water-soluble drug, cilostazol, in beagle dogs. *Journal of controlled release: official journal of the Controlled Release Society*, 111(1-2), pp.56–64.
8. Kakran, M., Sahoo, N.G. & Li, L., 2011. Dissolution enhancement of quercetin through nanofabrication, complexation, and solid dispersion. *Colloids and Surfaces B: Biointerfaces*, 88(1), pp.121–130.
9. Kakran, M. et al., 2012. Fabrication of quercetin nanocrystals: comparison of different methods. *European journal of pharmaceutics and biopharmaceutics: official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V*, 80(1), pp.113–121.
10. Kavuru, P., 2008. Crystal engineering of flavonoids. *Graduate School Theses and Dissertations*. Available at: <http://scholarcommons.usf.edu/etd/325>.
11. Kawabata, Y. et al., 2011. Formulation design for poorly water-soluble drugs based on biopharmaceutics classification system: basic approaches and practical applications. *International journal of pharmaceutics*, 420(1), pp.1–10.
12. Kawabata, Y. et al., 2010. Novel crystalline solid dispersion of tranilast with high photostability and improved oral bioavailability. *European journal of pharmaceutical sciences: official journal of the European Federation for Pharmaceutical Sciences*, 39(4), pp.256–262.

13. Kawakami, K., 2012. Modification of physicochemical characteristics of active pharmaceutical ingredients and application of supersaturatable dosage forms for improving bioavailability of poorly absorbed drugs. *Advanced drug delivery reviews*, 64(6), pp.480–495.
14. Klang, V. et al., 2012. Electron microscopy of nanoemulsions: an essential tool for characterisation and stability assessment. *Micron (Oxford, England: 1993)*, 43(2-3), pp.85–103.
15. Kluge, J., Muhrer, G. & Mazzotti, M., 2012. High pressure homogenization of pharmaceutical solids. *The Journal of Supercritical Fluids*, 66(0), pp.380–388.
16. Kundu, P.P. et al., 2003. Influence of film preparation procedures on the crystallinity, morphology and mechanical properties of LLDPE films. *European Polymer Journal*, 39(8), pp.1585–1593.
17. Kuntsche, J., Horst, J.C. & Bunjes, H., 2011. Cryogenic transmission electron microscopy (cryo-TEM) for studying the morphology of colloidal drug delivery systems. *International journal of pharmaceutics*, 417(1-2), pp.120–137.
18. Lipinski, C.A., 2000. Drug-like properties and the causes of poor solubility and poor permeability. *Journal of pharmacological and toxicological methods*, 44(1), pp.235–249.
19. Liversidge, E. & Wei, L., 2003. Stabilization of chemical compounds using nanoparticulate formulations. Available at: <http://www.freepatentsonline.com/y2003/0054042.html>.
20. Liversidge, G. G. & Cundy, K.C., 1995. Particle size reduction for improvement of oral bioavailability of hydrophobic drugs. I: Absolute oral bioavailability of nanocrystalline danazol in beagle dogs. *International journal of pharmaceutics*, 125(1), pp.91–97.
21. Moon, Y.J. et al., 2008. Quercetin pharmacokinetics in humans. *Biopharmaceutics & Drug Disposition*, 29(4), pp.205–217.
22. Möschwitzer, J. et al., 2004. Development of an intravenously injectable chemically stable aqueous omeprazole formulation using nanosuspension technology. *European Journal of Pharmaceutics and Biopharmaceutics: Official Journal of Arbeitsgemeinschaft Für Pharmazeutische Verfahrenstechnik e.V.*, 58(3), pp.615–619.
23. Müller, R.H., Shegokar, R., et al., 2011. Nanocrystals: Production, Cellular Drug Delivery, Current and Future Products. In A. Prokop, ed. *Intracellular Delivery*. Dordrecht: Springer Netherlands, pp. 411–432. Available at: <http://www.springerlink.com/content/h7t0l64651080398/>.
24. Müller, R.H., Gohla, S. & Keck, C.M., 2011. State of the art of nanocrystals - Special features, production, nanotoxicology aspects and intracellular delivery. *European Journal of Pharmaceutics and Biopharmaceutics*, 78(1), pp.1–9.

25. Ollivon, M. et al., 2006. DSC and high resolution X-ray diffraction coupling. *Journal of Thermal Analysis and Calorimetry*, 85(1), pp.219–224.
26. Onoue, S. et al., 2010. Formulation design and photochemical studies on nanocrystal solid dispersion of curcumin with improved oral bioavailability. *Journal of pharmaceutical sciences*, 99(4), pp.1871–1881.
27. Rabinow, B.E., 2004. Nanosuspensions in drug delivery. *Nature Reviews Drug Discovery*, 3(9), pp.785–796.
28. Russo, M. et al., 2012. The flavonoid quercetin in disease prevention and therapy: facts and fancies. *Biochemical Pharmacology*, 83(1), pp.6–15.
29. Sahoo, N. G et al., 2011. Preparation and characterization of quercetin nanocrystals. *Journal of Pharmaceutical Sciences*, 100(6), pp.2379–2390.
30. Shegokar, R. & Müller, R.H., 2010. Nanocrystals: Industrially feasible multifunctional formulation technology for poorly soluble actives. *International Journal of Pharmaceutics*, 399(1-2), pp.129–139.
31. Srinivas, K. et al., 2010. Solubility and solution thermodynamic properties of quercetin and quercetin dihydrate in subcritical water. *Journal of Food Engineering*, 100(2), pp.208–218.
32. Truong Cong, T., Ouattara, M., et al., Nanodispersions stabilized by PEG diesters and phospholipids: towards compartmented lipid nanoparticles. *to be submitted*.
33. Truong Cong, T., Faivre, V., et al., Quercetin solubility and chemical stability: enhancement with polyoxylglyceride-based multi-compartmented nanodispersions. *to be submitted*.
34. Wu, T.H. et al., 2008. Preparation, physicochemical characterization, and antioxidant effects of quercetin nanoparticles. *International Journal of Pharmaceutics*, 346(1-2), pp.160–168.
35. Zhang, L. et al., 2011. Preparation, physicochemical characterization and in vitro digestibility on solid complex of maize starches with quercetin. *LWT - Food Science and Technology*, 44(3), pp.787–792.

Dans le chapitre précédent, il a été montré qu'il était possible d'augmenter la concentration en quercétine dans la suspension de nanoparticules compartimentées par l'incorporation de nanocristaux. Dans nos travaux, la concentration maximale en principe actif dans la suspension est de l'ordre de 10 mg/ml, soit 5 fois plus que dans les nanoparticules multi-compartimentées seules, et ceci sans augmentation importante de la taille apparente des objets obtenus. Toutefois, si les mesures de taille, en volume de sphère équivalente, ne permettent pas de les différencier, la microscopie électronique fait clairement apparaître l'existence de deux types d'objets bien distincts : des nanoparticules sphériques (particules multi-compartimentées) et des particules fortement anisotropes (nanocristaux). Deux aspects seront principalement discutés ici, le polymorphisme et le faciès des nanocristaux.

D'une manière générale, les nanocristaux sont classiquement envisagés et générés dans le cadre de travaux visant à augmenter la solubilité et/ou la vitesse de dissolution de principes actifs peu solubles ([Rabinow 2004](#); [Keck & Müller 2006](#); [Kawabata et al. 2011](#)). La solubilité à saturation augmente lorsque la taille des particules diminue selon l'équation d'Ostwald-Freundlich, également connue sous les noms d'équation de Gibbs-Thomson ou Kelvin.

$$\frac{S(d)}{S_0} = \exp \frac{\gamma V_m}{RTd}$$

avec  $S(d)$  la solubilité (mol/kg) des nanocristaux de diamètre  $d$  à la température  $T$  (K),  $V_m$  le volume molaire ( $\text{m}^3/\text{mol}$ ),  $\gamma$  l'énergie libre de surface ( $\text{mJ}/\text{m}^2$ ),  $R$  la constante des gaz parfaits et  $S_0$  la solubilité du produit en masse ( $d$  tend vers l'infini). Quand la taille des particules est dans le domaine des nanomètres,  $S(d)$  devient significativement différent de  $S_0$ . Par ailleurs, cette solubilité est d'autant plus grande que le « nanosolide » est à l'état amorphe ou cristallisé dans des formes instables.

Les choses sont un peu plus complexes dans le cas des nanocristaux de quercétine développés ici. La quercétine est effectivement un principe actif peu soluble en milieu aqueux dont la transformation en nanocristaux pourrait favoriser la solubilisation. Toutefois, l'obtention de cristaux dans une forme dihydrate plus stable et moins soluble qu'une forme anhydre ou amorphe (cf. partie bibliographique) s'oppose à ce bénéfice de la réduction de taille. De notre point de vue, cette forme physiquement stable est malgré tout un avantage dans le cas de la quercétine. En effet, instable chimiquement en milieu aqueux, le but de cette thèse n'était pas d'augmenter la vitesse de dissolution et la

solubilité du principe actif mais bien d'assurer sa protection. Une solubilisation lente à partir des nanocristaux comme l'indique la cinétique de libération présentée dans le projet d'article suggère qu'il serait possible de maintenir une concentration contrôlée du principe actif dans le milieu biologique d'intérêt malgré sa dégradation parallèle. En ce sens, la lyophilisation de la suspension qui tend à « amorphiser » le produit ne semble pas être un avantage dans la problématique considérée.

Second point très intéressant, la morphologie des cristaux. Si les mesures de diffusion *quasi*-élastique de la lumière ont donné un diamètre de sphère équivalente de l'ordre de 250 nm ([Figure 3.1](#)), la microscopie a montré des images de cristaux fortement anisotropes, dont la longueur est d'environ 200-500 nm alors que la largeur est de quelques dizaines de nanomètres. Les études sur les particules non-sphériques sont en plein essor actuellement, la morphologie pouvant avoir un effet significatif sur le comportement des particules dans les fluides biologiques et au contact des tissus. Par exemple, les particules non-sphériques ont tendance à dériver latéralement dans un flux de liquide laminaire alors que des particules sphériques suivront les lignes de courant ([Lee et al. 2009](#)). Par augmentation des surfaces de contact et diminution de l'effet des forces hydrodynamiques, des particules cylindriques ou oblates adhéreront plus fortement à des tissus cibles qu'une sphère de volume équivalent ([Decuzzi & Ferrari 2006](#)). On peut noter que cette adhérence supérieure pourrait expliquer pourquoi nous n'avons pas visualisé les nanocristaux lors des expériences de cryo-TEM. L'incidence de cette morphologie des cristaux serait certainement à approfondir dans des travaux ultérieurs.

La présence de traces d'éthanol est le dernier point à commenter par rapport à cette publication n°3. L'une des limites de cette partie est l'absence de quantification de l'éthanol en fin de procédé. Après préparation de la phase lipidique et évaporation partielle de l'éthanol, les concentrations finales qui resteraient dans la préparation varient de 0,6 à 1,2 % selon la quantité de quercétine incorporée. Toutefois, le passage à l'ultra-turrax et dans l'homogénéiseur haute-pression se faisant à 70°C pendant plusieurs minutes, il est très probable que ces valeurs soient surestimées. Une quantification précise, par exemple par chromatographie en phase gazeuse, aurait permis de lever un doute sur ce point et peut-être d'apporter un éclairage supplémentaire sur le mécanisme d'apparition de ces nanocristaux. Probablement à l'état de trace, la présence d'alcool dans la suspension ne devrait toutefois pas s'opposer à son administration chez l'homme, quelque soit la voie choisie.

## Références

1. Decuzzi, P & Ferrari, M, 2006. The adhesive strength of non-spherical particles mediated by specific interactions. *Biomaterials*, 27(30), pp.5307–5314.
2. Kawabata, Y. et al., 2011. Formulation design for poorly water-soluble drugs based on biopharmaceutics classification system: basic approaches and practical applications. *International journal of pharmaceutics*, 420(1), pp.1–10.
3. Keck, C.M. & Müller, R.H., 2006. Drug nanocrystals of poorly soluble drugs produced by high pressure homogenisation. *European Journal of Pharmaceutics and Biopharmaceutics: Official Journal of Arbeitsgemeinschaft Für Pharmazeutische Verfahrenstechnik e.V*, 62(1), pp.3–16.
4. Lee, S.Y., Ferrari, Mauro & Decuzzi, Paolo, 2009. Shaping nano-/micro-particles for enhanced vascular interaction in laminar flows. *Nanotechnology*, 20(49), p.495101.
5. Rabinow, B.E., 2004. Nanosuspensions in drug delivery. *Nature Reviews Drug Discovery*, 3(9), pp.785–796.

# DISCUSSION GÉNÉRALE

La majorité des résultats de ce travail de thèse ont déjà été discutés dans les projets de publications et dans les transitions entre chacun des chapitres. La discussion générale de ce manuscrit se « détachera » donc parfois des résultats et portera plus sur les limites et perspectives de cette thèse, beaucoup sous l'angle du vecteur de médicament, un peu sous celui du principe actif.

### Sur le choix des stabilisants

Le Gelucire® 50/13 appartient à la famille des polyoxyglycérides qui sont des mélanges de glycérides, minoritaires dans la composition, et de mono- et diesters de PEG. Selon la nature des acides gras estérifiés et la longueur de la chaîne de PEG, ces produits seront considérés comme plutôt lipophiles ou plutôt hydrophiles. Le Gélucire® 50/13 appartient aux composés parmi les plus hydrophiles dans cette famille en raison de la longueur de la chaîne PEG (PEG 1500). Dans le domaine des dispersions colloïdales, essentiellement deux approches sont utilisées pour stabiliser les nanoobjets et éviter leur coalescence : une stabilisation par l'ajout de charges de surface ou une stabilisation stérique. Cette dernière possibilité a été privilégiée dans ce travail et a été à l'origine du choix du Gelucire® 50/13.

Stables en taille depuis pratiquement 5 ans pour les plus anciennes (à température ambiante), cette option s'est avérée efficace dans le cas des nanoparticules développées dans cette thèse malgré un potentiel zeta de l'ordre de quelques mV incompatible avec une stabilisation électrostatique. Ceci est une confirmation indirecte de la présence de chaînes de PEG en surface des nanoparticules. La déformation des nanoparticules accolées dans les images de cryo-TEM va également dans le sens de la présence de PEG en surface des particules.

### Des triglycérides aux polyoxyglycérides

Comme fréquemment rencontré dans la littérature, des triglycérides ont été initialement utilisés pour formuler des nanodispersions lipidiques à l'aide des tensioactifs décrits précédemment. En utilisant le Labrafac® CC, le beurre de cacao et leur mélange, nous avons pu formuler des nanodispersions de type nanoémulsions, nanoparticules lipidiques solides (*Solid Lipid Nanoparticles*, SLN) et particules lipidiques nanostructurées (*Nanostructured Lipid Carriers*, NLC) respectivement ([Souto et al.](#)

2004). Les deux difficultés décrites fréquemment dans le domaine des particules lipidiques solides (SLN) sont une aggrégation des particules (gélification) et un relargage prématuré du principe actif pendant le temps de stockage des formulations (Freitas & Müller 1999). L'origine de ces deux phénomènes est commune : la transition polymorphique de la matrice lipidique depuis une structure instable ( $\alpha$  en général) vers des structures plus stables ( $\beta'$  ou  $\beta$ ). Cette transition s'accompagne d'un changement morphologique des particules, d'une sphère vers une « plaquette », qui auront ensuite tendance à s'agréger par augmentation des surfaces de contact (Bunjes et al. 2007). Au niveau moléculaire, cette transition polymorphique entraîne une « compaction » de la matrice lipidique avec expulsion du principe actif. En incorporant du liquide dans le solide, c'est le passage des SLN au NLC proposé par le laboratoire de R.H. Müller (Berlin), des défauts sont introduits dans la matrice lipidique, ralentissant ainsi sa transition polymorphique. Redouté dans le monde pharmaceutique en raison de son comportement polymorphique complexe (cf l'annexe sur les matières premières), le beurre de cacao peut s'avérer très intéressant dans ce cadre. En effet, la richesse de sa composition (même si 3 triglycérides monoinsaturés sont majoritaires) couplée à sa très grande dispersion à l'état de nanoparticules vont fortement ralentir sa transition polymorphique. Par exemple, des expériences menées au laboratoire de tempérages d'échantillons de beurre de cacao en « bulk » ou sous forme de nanoparticules ont clairement montré que cet excipient était en masse susceptible de recristalliser vers ses formes les plus stables (Formes V et VI) selon les conditions de stockage alors qu'à l'état de nanoparticules, la forme la plus stable obtenue dans les mêmes conditions était la Forme IV (celle présentée dans le projet de Publication n°1). Ceci explique en partie la grande stabilité physique (taille) des nanoparticules de beurre de cacao décrites dans ce manuscrit. Cette stabilité n'est donc pas seulement due au couple de tensio-actifs choisi comme l'ont montré des expériences complémentaires impliquant d'autres mélanges de glycérides (Compritol®, Précirol®) à la place du beurre de cacao. Pour ces deux excipients, une gélification est apparue relativement rapidement.

Le mélange Gelucire® 50/13 – Phospholipon® 90G – Beurre de cacao permet de fabriquer des particules lipidiques stables mais sa très grande lipophilie ne permet pas d'envisager la formulation d'une large gamme de principes actifs, même en présence de Labrafac® CC susceptible d'aider légèrement à la solubilisation de ces derniers. Pour

élargir ce pouvoir solubilisant, le Labrafil® M2125 CS a donc été introduit dans la formulation. Cet excipient appartient, comme le Gelucire® 50/13, à la famille des polyoxyglycérides mais son HLB, selon les informations du fournisseur, est beaucoup plus bas (~4). Ceci est notamment lié à la longueur, relativement courte, des chaînes de PEG (PEG 300) et permet d'envisager sa dispersion, et pas sa solubilisation, dans l'eau. Ce choix s'est avéré positif dans le cas de la quercétine mais il conviendrait de tester la solubilisation d'autres principes actifs. Par ailleurs, des études sont en cours dans le laboratoire pour tester d'autres lipides polyoxyéthylénés, différents par leur HLB (entre 2 et 9) et/ou leur état physique à température ambiante (solide ou liquide).

### Sur l'intérêt de la compartimentation des nanoparticules

La principale innovation émanant de cette thèse est la production de nanoparticules compartimentées possédant un noyau dense aux électrons, donc *a priori* essentiellement constitué de lipides, et une couronne peu dense, donc *a priori* constituée d'eau et/ou de PEG. Le tout étant entouré par une membrane à base de phospholipides. Cette morphologie originale de particules a donc mené à une procédure de prise de brevet (en cours) et un nom pourrait être proposé pour ces objets dont l'organisation rappelle celle des cellules, les « cellisomes ».

La multifonctionnalité est une approche en pleine expansion dans le domaine de la vectorisation. L'idée est de donner plusieurs fonctions à un même vecteur de médicament (Torchilin 2006; Kim et al. 2010), par exemple une capacité diagnostique et thérapeutique (la « théragnostique ») ou d'associer plusieurs principes actifs aux activités complémentaires. Dans ce cadre, un vecteur capable d'incorporer simultanément des principes actifs hydrosolubles et liposolubles pourrait s'avérer très intéressant. Dans les poursuites de ce travail, des expériences de co-encapsulation de molécules de solubilités différentes devraient être effectuées pour confirmer cette double fonctionnalité.

La présence d'une membrane phospholipidique en périphérie de la particule est également un atout de cette formulation. En effet, il est aisément possible d'envisager une fonctionnalisation en surface par l'insertion de ligands en profitant d'un ancrage lipidique au sein de cette bicouche. Ceci est déjà largement décrit pour les liposomes dits de « troisième génération » et dotés de propriétés de ciblage actif par le greffage par exemple de glycolipides ou d'autres amphiphiles.

### **Du choix de l'origine des matières premières**

Il a été décidé dans ce travail de privilégier des matières premières disponibles en quantités relativement importantes pour des coûts modérés. Ce choix se justifie essentiellement par l'utilisation d'un homogénéiseur haute-pression relativement consommateur de matière première, le volume minimum utilisable étant de l'ordre de 50 ml. Des excipients procurés par des fournisseurs classiques de l'industrie pharmaceutique ont donc été utilisés. Ceci présente l'avantage de rapprocher ce travail des conditions du monde industriel mais apporte une limite certaine dans la compréhension de la physico-chimie de ces objets, notamment à cause de la pureté « moyenne » et de la complexité, en termes d'espèces moléculaires présentes, des matières premières utilisées.

L'acquisition prochaine d'un homogénéiseur permettant de manipuler des plus petits volumes (jusqu'à 2 ml) devrait permettre d'approfondir certains de ces aspects physico-chimiques. Parmi ces études complémentaires, il est important de citer le remplacement du Labrafil® M2125 CS et du Gelucire® 50/13 par des monoesters et diesters de PEG parfaitement définis pour préciser l'incidence de chacun de ces produits sur la morphologie et la stabilité des nanoparticules finales. Ceci est un point fondamental pour comprendre l'organisation originale des nanoparticules compartimentées.

### **De la voie d'administration**

Il a été peu question dans ce manuscrit de la voie d'administration choisie, l'idée étant de développer un vecteur ubiquitaire susceptible d'être administré par différentes routes, notamment orale, parentérale et locale. Du point de vue de leur taille, les nanoparticules compartimentées répondent à cette attente. Toutefois, dans le cadre d'une utilisation par chacune de ces voies, certaines spécificités seront à considérer avec une grande attention. Les polyoxylglycérides ont été largement utilisés par voie orale notamment pour leur pouvoir solubilisant de principes actifs peu solubles. Compte tenu du riche milieu enzymatique rencontré tout au long du tractus digestif, une conversion des phospholipides en lyso-phospholipides et une digestion progressive des polyoxylglycérides avec libération notamment d'acides gras sont à attendre. Cette digestion devrait aboutir à la dégradation progressive des particules et à la modification transitoire et locale de la composition du liquide gastro-intestinal. Cependant, plusieurs études ont montré que malgré cette sensibilité enzymatique, les polyoxylglycérides permettaient

d'améliorer significativement la biodisponibilité de principes actifs peu solubles comme le danazol par exemple (Larsen et al. 2008). Au-delà de la seule solubilisation des principes actifs, certains polyoxylglycérides, les plus hydrophiles comme le Labrasol® non utilisé ici, ont été proposés comme promoteur d'absorption par perturbation transitoire de la paroi intestinale (Li & Zhao 2007; Buggins et al. 2007). Parmi les constituants de nos formulations, le Gelucire® 50/13 pourrait avoir un tel effet.

Par voie injectable, la problématique à clarifier en priorité sera la sensibilité des particules compartimentées à la présence des constituants sanguins, et notamment des protéines sériques. Il est largement connu dans la littérature que l'administration IV de nanoparticules (lipidiques ou polymériques) est suivie par leur élimination rapide de la circulation sanguine par le système réticulo-endothélial si elles ne sont pas rendues furtives par une couche périphérique de polymère hydrophile capable de limiter leur opsonisation. Dans notre cas, cette couche semble présente et suffisamment dense en PEG (en fonction du ratio Phospholipon® 90G – Gelucire® 50/13) pour assurer cette fonction. Bien évidemment, ceci devra être confirmé expérimentalement.

### De la toxicité

Comme décrit dans le projet d'article sur les nanoparticules blanches, des essais préliminaires ont été conduits sur la toxicité des particules à base de mélange du beurre de cacao – Labrafac® CC (75 :25) suggérant leur faible toxicité. Une première étude *in vivo* chez la souris (non montrée) a également été effectuée pour évaluer la tolérance chez l'animal. Jusqu'à la dose maximale utilisée (1g de nanoparticules/kg), aucun signe d'intolérance (perte de poids, trouble du comportement) n'a été observé pour cette même formulation blanche. L'ensemble de ces résultats suggère que l'éventuelle perturbation des membranes biologiques induites par le Gélucire® 50/13 évoquée dans le paragraphe précédent ne s'avère pas délétère pour une administration IV.

Ces problèmes de toxicité seront également à approfondir. En effet, parmi les familles de produits constituant nos formulations, l'innocuité des glycérides et des phospholipides a été largement validé par les autorités internationales autour du médicament (FDA, EMA, ANSM,...) et par la pratique clinique, quelque soit la voie d'administration. Dans le cas des polyoxylglycérides, et notamment le Labrafil M2125 CS présent en grande quantité, la situation est plus complexe. D'après le *Handbook of pharmaceutical excipients* (6<sup>ème</sup>

édition), ces produits sont considérés comme des matériaux relativement non toxiques et non irritants mais ils n'ont été validés par les différentes pharmacopées (USP 32, Ph.Eur 6.0) que pour des applications essentiellement orales et locales actuellement (Rowe et al. 2009). Cependant, divers travaux de recherche utilisent ces excipients pour la voie intraveineuse (Li & Zhao 2007). Eventuellement, de nombreuses autres familles d'excipients dérivés de PEG étant approuvées pour une utilisation IV, des études pourraient être envisagées pour leur remplacement le cas échéant.

### Sur la quercétine

Au-delà de ses effets biologiques, de sa faible solubilité aqueuse et de son instabilité chimique, ce principe actif avait le double avantage d'être étudié au Vietnam dans mon laboratoire d'origine et de remplir la condition de coût préalable à son utilisation dans l'homogénéiseur haute pression.

Dans ces travaux, l'accent a été mis sur la solubilité, la stabilité et la cinétique de libération de la quercétine. Par contre, aucune expérience n'a été faite pour évaluer l'influence de l'encapsulation sur son activité. Initialement, dans le cadre d'un projet déposé en 2011 auprès d'Egide-Volubilis, cette activité devait être évaluée en collaboration avec un partenaire marocain (J. Abrini, Laboratoire de Biologie et Santé, Université de Tétouan). Faute de financement, ce volet a été mis de côté en attendant les résultats d'un nouveau dépôt en 2012. Au regard de l'importante littérature publiée autour de la quercétine pendant les années dernières, il semble que son encapsulation soit favorable à son activité (Wu et al. 2008; Priprem et al. 2008; Kumari et al. 2010; Rogerio et al. 2010; Date et al. 2011).

Pour compléter ce travail à court terme, les méthodes suivantes de quantification de l'activité antioxydante pourraient être testées :

- Etude de la décoloration du  $\beta$ -carotène par une méthode spectrophotométrique.
- Mesure du pouvoir réducteur par détermination de l'aptitude à réduire une solution de ferricyanure de potassium.
- Mesure du pouvoir de piégeage du radical DPPH basé sur la réduction d'une solution méthanolique de 1,1-diphenyl-2-picryl-hydrazyl (DPPH).

Dans le cas de la quercétine, les particules multicompartimentées pourraient s'avérer particulièrement intéressantes. En effet, la littérature fait état à plusieurs reprises

(Ramos 2007) d'effets synergiques entre cet actif et d'autres flavonoïdes ou le cisplatine. Cette dernière molécule, utilisée en thérapie anti-cancéreuse (sarcomes, carcinomes, lymphomes), voit son effet anti-prolifératif amélioré par la présence concomitante de quercétine. Possédant une solubilité aqueuse de l'ordre de 2,5 g/L, ce principe actif alkylant pourrait être un très bon candidat pour une co-encapsulation avec notre principe actif d'intérêt.

## Références

1. Buggins, T.R., Dickinson, P.A. & Taylor, G., 2007. The effects of pharmaceutical excipients on drug disposition. *Advanced drug delivery reviews*, 59(15), pp.1482–1503.
2. Bunjes, H., Steiniger, F. & Richter, W., 2007. Visualizing the structure of triglyceride nanoparticles in different crystal modifications. *Langmuir: the ACS journal of surfaces and colloids*, 23(7), pp.4005–4011.
3. Date, A.A. et al., 2011. Lecithin-based novel cationic nanocarriers (Leciplex) II: improving therapeutic efficacy of quercetin on oral administration. *Molecular Pharmaceutics*, 8(3), pp.716–726.
4. Freitas, C. & Müller, R.H., 1999. Correlation between long-term stability of solid lipid nanoparticles (SLN) and crystallinity of the lipid phase. *European journal of pharmaceutics and biopharmaceutics: official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V*, 47(2), pp.125–132.
5. Kim, K. et al., 2010. Tumor-homing multifunctional nanoparticles for cancer theragnosis: Simultaneous diagnosis, drug delivery, and therapeutic monitoring. *Journal of controlled release: official journal of the Controlled Release Society*, 146(2), pp.219–227.
6. Kumari, A. et al., 2010. Development of biodegradable nanoparticles for delivery of quercetin. *Colloids and Surfaces B: Biointerfaces*, 80(2), pp.184–192.
7. Larsen, A. et al., 2008. Lipid-based formulations for danazol containing a digestible surfactant, Labrafil M2125CS: in vivo bioavailability and dynamic in vitro lipolysis. *Pharmaceutical research*, 25(12), pp.2769–2777.
8. Li, P. & Zhao, L., 2007. Developing early formulations: practice and perspective. *International journal of pharmaceutics*, 341(1-2), pp.1–19.
9. Priprem, A. et al., 2008. Anxiety and cognitive effects of quercetin liposomes in rats. *Nanomedicine: Nanotechnology, Biology and Medicine*, 4(1), pp.70–78.
10. Ramos, S., 2007. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. *The Journal of nutritional biochemistry*, 18(7), pp.427–442.

11. Rogerio, A.P. et al., 2010. Anti-inflammatory effect of quercetin-loaded microemulsion in the airways allergic inflammatory model in mice. *Pharmacological Research*, 61(4), pp.288–297.
12. Rowe, R.C. et al., 2009. *Handbook of pharmaceutical excipients.*, London; Chicago; Washington, DC: Pharmaceutical Press; American Pharmacists Association.
13. Souto, E.B. et al., 2004. Development of a controlled release formulation based on SLN and NLC for topical clotrimazole delivery. *International journal of pharmaceutics*, 278(1), pp.71–77.
14. Torchilin, V.P., 2006. Multifunctional nanocarriers. *Advanced Drug Delivery Reviews*, 58(14), pp.1532–1555.
15. Wu, T.H. et al., 2008. Preparation, physicochemical characterization, and antioxidant effects of quercetin nanoparticles. *International Journal of Pharmaceutics*, 346(1-2), pp.160–168.

# CONCLUSION GÉNÉRALE

Au cours de cette thèse, nous avons exploré la possibilité de développer des nanoformulations encapsulant des flavonoïdes peu solubles dans l'eau et instables chimiquement et physiquement, la quercétine dans le cas présent, afin d'augmenter sa solubilité apparente et de la protéger contre sa dégradation.

Dans la première partie de ce travail, nous nous sommes intéressés à la formulation et la caractérisation physico-chimique des nanodispersions lipidiques blanches. Différents systèmes dispersés (nanoémulsions, SLN, NLC et nanoparticules multicompartimentées) ont été préparés par homogénéisation haute pression à chaud avec une taille de particules entre 100 - 200 nm. Ces nanodispersions, très stables sur plusieurs années à température ambiante, pourraient encapsuler effectivement les molécules lipophiles. Après avoir analysé leur structure microscopique et leur organisation supramoléculaire, nous avons constaté que les nanoparticules multicompartimentées à base de polyoxylglycérides semblaient particulièrement intéressantes pour encapsuler non seulement les molécules lipophiles mais également des molécules plus hydrophiles. En outre, leur procédé de fabrication est simple et rapide avec une haute reproductibilité, sans utilisation de solvant organique et sa transposition à l'échelle industrielle est possible. Les excipients utilisés (triglycérides, polyoxylglycérides et phospholipides) sont actuellement appliqués largement dans les industries pharmaceutiques, cosmétiques et agroalimentaires.

Dans un deuxième temps, nous avons étudié l'encapsulation de la quercétine dans ces nanoparticules lipidiques multicompartimentées. Ces particules avaient une taille moyenne d'environ 200 nm avec un Pdl  $\sim 0,1$  et une efficacité d'encapsulation de 96,4%. La solubilité apparente de la quercétine dans les nanodispersions était environ 4500-fois plus élevée que dans l'eau ( $0,4 \pm 0,3$  mg/ml). Les études de stabilité ont montré que la dégradation de la quercétine était très faible après 3 mois de stockage de la suspension de nanoparticules versus son temps de dégradation de 16,8h dans l'eau. Ces résultats ont confirmé que ces nanodispersions améliorent fortement la solubilité apparente et la stabilité chimique de la quercétine.

Enfin, la dernière partie a concerné la préparation et la caractérisation de nanocristaux permettant d'augmenter la teneur en quercétine au sein des suspensions colloïdales. Les nanodispersions contenant 4% (m/m) de quercétine par rapport à la phase lipidique

avaient une taille moyenne d'environ 220 nm avec une distribution étroite ( $PdI \sim 0,1$ ). La concentration en quercétine était 4 fois plus importante que dans les dispersions de nanoparticules lipidiques multicompartimentées. Leurs images de MET et cryo-MET ont montré au moins deux populations nanoparticulaires différentes dans ce système : les nanoparticules lipidiques multicompartimentées et des nanocristaux en aiguilles. En outre, la cristallinité de la quercétine a été également étudiée pour comprendre sa transformation polymorphique dans le processus de préparation. Ces particules étaient stables physiquement et chimiquement plus de 90 jours à température ambiante et à 4 °C.

En résumé, les caractéristiques des nanoparticules préparées, notamment, leurs taille et distribution granulométrique, potentiel zêta, morphologie, cristallinité, charge en cholestérol ou en quercétine et leur stabilité physico-chimique ont confirmé les potentialités très prometteuses de ces systèmes comme vecteurs de principes actifs peu solubles dans l'eau et/ou fragiles.

# **ANNEXE**

## **MÉTHODES EXPÉRIMENTALES & MATIÈRES PREMIÈRES**

|                                                                      |     |
|----------------------------------------------------------------------|-----|
| 1. Homogénéisation haute pression (HHP)                              | 204 |
| 2. Calorimétrie différentielle à balayage (DSC)                      | 206 |
| 3. Diffractométrie des rayons X (DRX)                                | 209 |
| 4. Microscopie électronique en transmission (MET) et Cryo-MET        | 215 |
| 5. Balance de Langmuir                                               | 221 |
| 6. Dosage de la quercétine par HPLC                                  | 224 |
| 7. Excipients : triglycérides, polyoxyl glycérides et phospholipides | 226 |

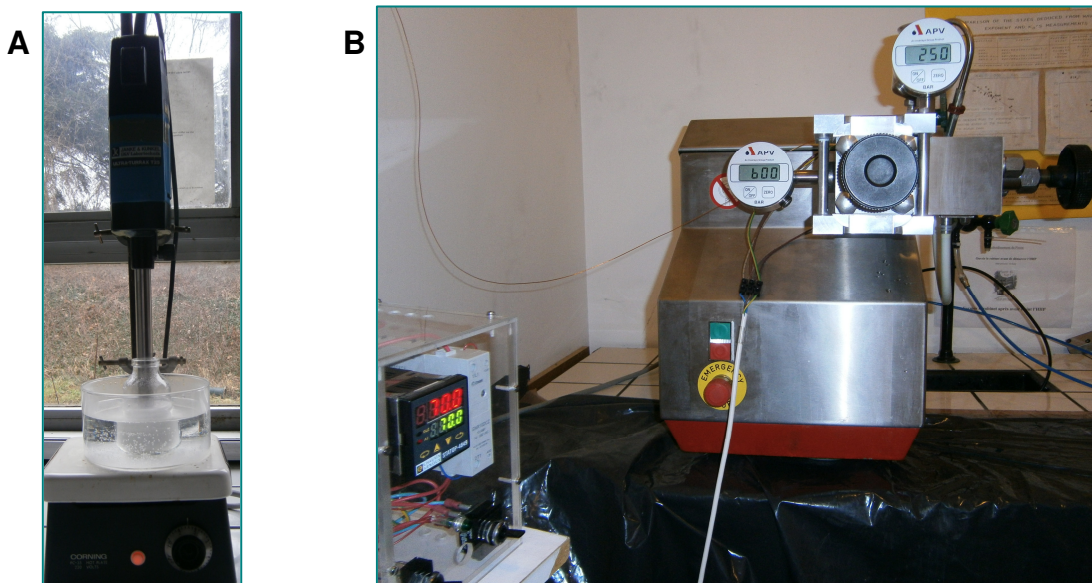
## 1. Homogénéisation haute pression (HHP)

### 1.1. Principe

L'homogénéisation haute pression permet une dispersion liquide à travers un orifice très étroit et éventuellement réglable (quelques microns) sous haute pression (100-2000 bar). Le fluide s'accélère sur une très courte distance à une grande vitesse (plus de 1000 km/h). La contrainte de cisaillement et les forces de cavitation diminuent la taille des particules jusqu'à une gamme de taille submicronique (Mehnert & Mäder 2001).

### 1.2. Dispositif expérimental

Les nanodispersions ont été préparées à l'aide d'un homogénéisateur à 2 étages (APV 2000, Invensys, Albertslund, Danemark) (Figure A1B) équipé d'un système de chauffage et associé à un ultra-turrax T25 (Janke & Kunkel GMBH & Co.KG, IKA®-Labortechnik, Allemagne) (Figure A1A) pour la préparation de l'émulsion primaire.



**Figure A1.** Ultra-turrax T25 (A) et homogénéisateur à 2 étages (B)

### 1.3. Procédé de préparation par HHP à chaud

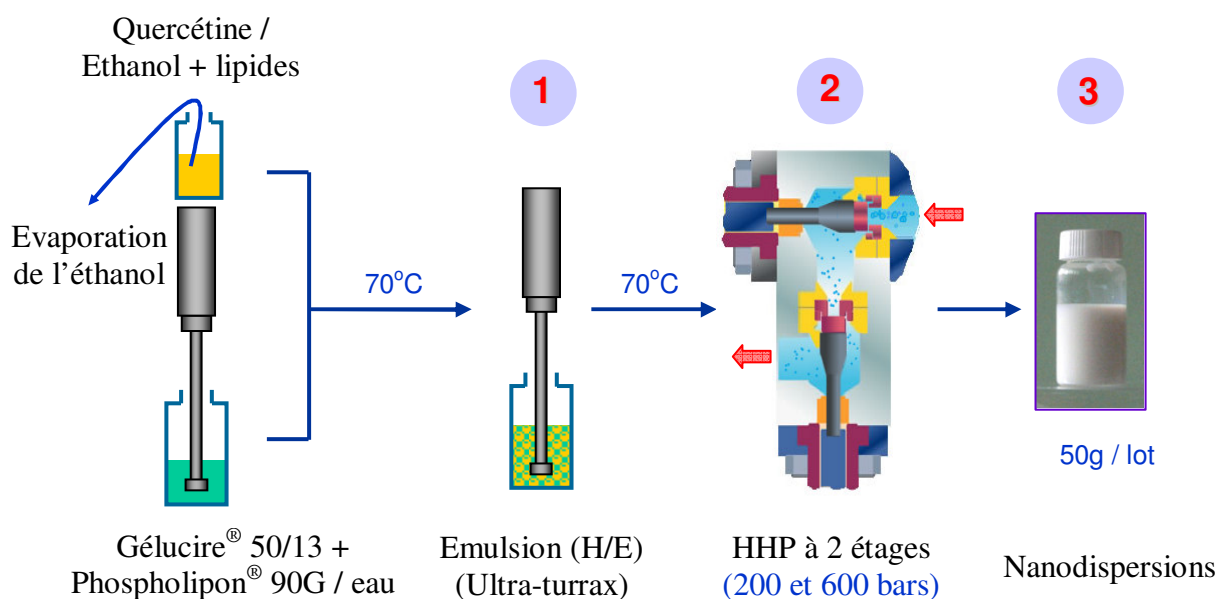
Pour chaque lot, 50 g de nanodispersions ont été préparés selon les trois étapes suivantes (Figure A2):

**Emulsification :** Tout d'abord, les lipides (Labrafac® CC, Labrafil® M2125 CS, le beurre de cacao ou le mélange du beurre de cacao - Labrafac® CC 75 :25) et les tensioactifs

(Gélucire 50/13 et Phospholipon® 90G) ont été pesés et introduits dans des flacons de 20 ml et 100 ml, respectivement. Pour les préparations contenant le principe actif, la quercétine a été dissoute dans un mélange (10 ml d'éthanol et 10 g de Labrafil M CS 2125) à 70°C puis l'éthanol a été évaporé sous pression réduite (Rotavapor, Büchi, Suisse). Ensuite, à la même température, la suspension de tensioactifs a été préparée sous agitation mécanique (Ultra-turrax T25, Janke & Kunkel GMBH & Co.KG, IKA®-Labortechnik, Allemagne), à la vitesse de 9500 rpm pendant 5 min. Enfin, le mélange lipidique a été ajouté dans la phase aqueuse et agité par ultra-turrax à la vitesse de 13500 rpm pendant 5 min, pour obtenir une pré-émulsion (H/E).

**Diminution et homogénéisation de la taille des particules par HHP :** La pré-émulsion obtenue est rapidement introduite dans l'homogénéisateur à deux étages (APV 2000, Invensys, Albertslund, Danemark) chauffé au préalable à 70°C. Elle y subit plusieurs cycles d'homogénéisation continue pendant 5 minutes, sous un double effet de pression (la pression du 1<sup>er</sup> étage est de 600 bar et celle du 2<sup>nd</sup> étage est de 200 bar), pour former une nanoémulsion (H/E) à chaud.

**Refroidissement des particules :** Les nanodispersions ont été ensuite maintenues à température ambiante et à 4°C.



**Figure A2.** Schéma synthétique du procédé de préparation des nanodispersions

## 2. Calorimétrie différentielle à balayage (DSC : Differential Scanning Calorimetry)

L'analyse par calorimétrie différentielle à balayage a été utilisée pour étudier le comportement thermique au cours de cycles de chauffage-refroidissement de composés purs, de mélanges ou encore du produit fini (les nanoparticules lipidiques).

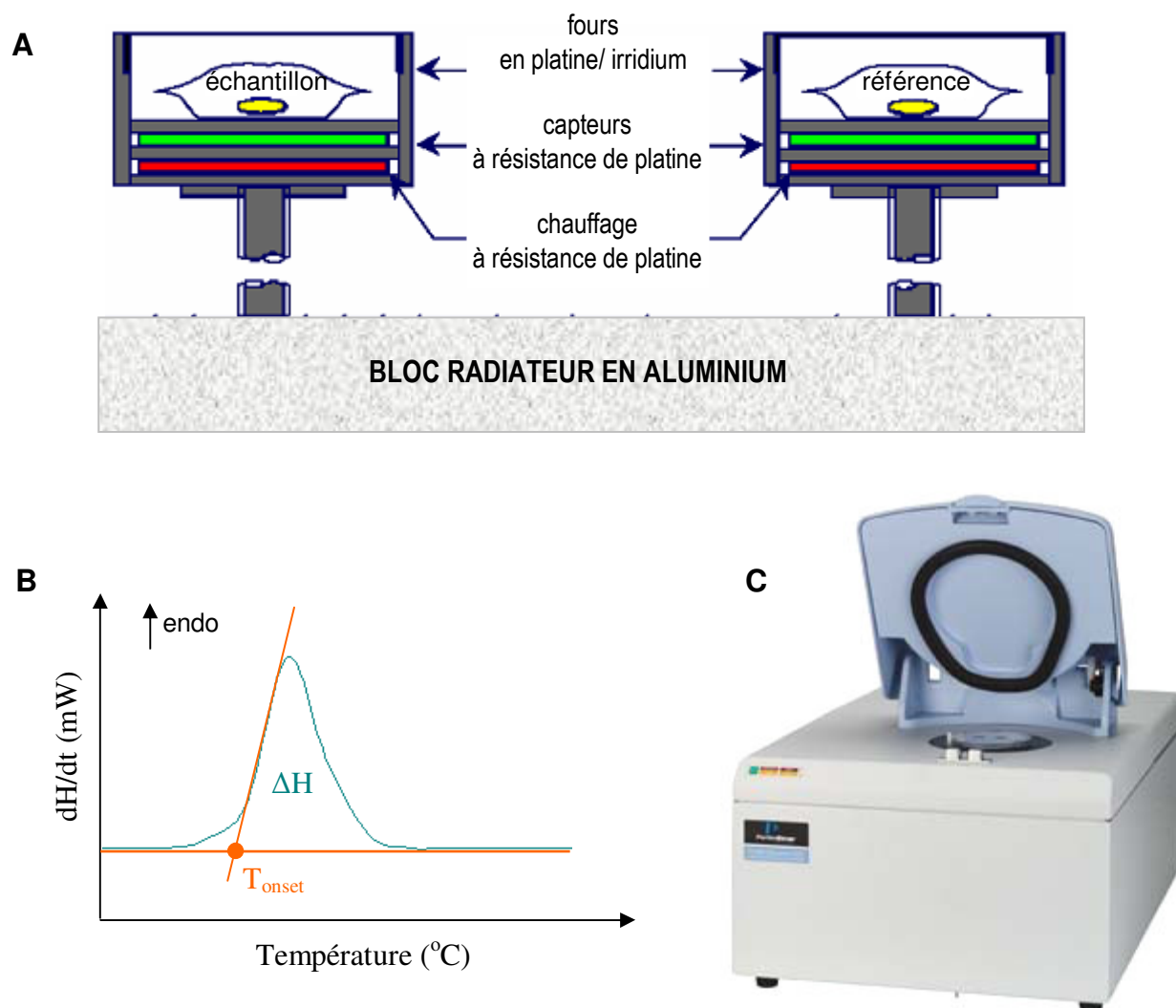
### 2.1. Principe

La technique de DSC permet de mesurer les échanges d'énergie sous forme de chaleur lors de transformations d'un système telles que des transitions de phase (fusion, cristallisation) ou des réactions chimiques et de déterminer les températures critiques auxquelles ont lieu ces événements.

Le principe consiste à enregistrer la différence d'énergie (différence de flux de chaleur) fournie à un échantillon et à une référence pour les maintenir à la même température au cours d'un chauffage ou d'un refroidissement à une vitesse parfaitement contrôlée. La cellule échantillon et la cellule référence possèdent chacune leur propre élément chauffant et système de contrôle de température. Le contrôle des températures est assuré par des sondes thermiques (Figure A3A). En l'absence d'événement endothermique ou exothermique, l'énergie supplémentaire à fournir à l'échantillon est proportionnelle à sa capacité calorifique,  $C_p$ , et les températures de la référence et de l'échantillon évoluent toutes deux linéairement en fonction du temps. Lorsqu'un événement thermique survient, l'appareil doit fournir plus ou moins de chaleur à l'échantillon pour le maintenir à la même température que la référence. Cette différence entre les flux de chaleur est enregistrée en fonction du temps converti en température à partir de la rampe de balayage thermique imposée. C'est le principe du calorimètre à compensation de puissance. Les résultats, sous forme de thermogrammes, représentent le flux de chaleur (mW) en fonction de la température (°C) (Figure A3B). Dans le cas de transformations dites de premier ordre, l'enregistrement de l'excès de quantité de chaleur en fonction de la température se traduit par un pic dont l'aire est proportionnelle à l'enthalpie de transition ( $\Delta H$ ). Une aire positive correspond à un phénomène endothermique (fusion d'une variété cristalline, par exemple), une aire négative indique un événement exothermique (cristallisation ou transition polymorphique).

La DSC permet donc de déterminer les températures de fusion et de cristallisation, la chaleur spécifique, l'entropie et l'enthalpie d'une transition dans un échantillon. De plus,

la variation de ces paramètres permet de détecter des changements de structure lors de l'ajout de différents composants qui vont perturber les interactions entre les molécules et modifier ainsi l'énergie engagée dans les événements thermiques (Pivette 2011).



**Figure A3.** Principe de fonctionnement de la tête de mesure d'un calorimètre à compensation de puissance (A), exemple de thermogramme représentant le flux de chaleur en fonction de la température (B) et image du Diamond DSC (C) (d'après Perkin Elmer 2006).

## 2.2. Dispositif expérimental

La différence d'énergie fournie entre un échantillon analysé et une cellule de référence a été mesurée par un équipement Diamond DSC (Perkin Elmer, USA) (Figure A3C), équipé d'un système de refroidissement (Intracooler 2P) sous une purge d'azote et associé à un logiciel d'acquisition et d'analyse Pyris (version 9, Perkin Elmer, USA). La calibration du Diamond DSC est réalisée à l'aide d'un échantillon d'acide laurique pur (pureté 99.9% déterminée par chromatographie en phase gazeuse) dont la température de fusion de

43,7°C et l'enthalpie correspondante,  $\Delta H_f = 151,15 \text{ J/g}$ , ont été déterminées selon le protocole décrit par Grabielle-Madelmont ([Grabielle-Madelmont & Perron 1983](#)).

### 2.3. Mesures expérimentales

Pour chaque mesure, une masse connue d'échantillon (1 à 10 mg pesés précisément à  $\pm 0,01 \text{ mg}$ ) est introduite dans des capsules en aluminium (Perkin Elmer, Courtaboeuf, France) d'une contenance de 50  $\mu\text{L}$ . Ces capsules sont ensuite scellées hermétiquement et introduites dans la tête de mesure. Une capsule vide est utilisée comme référence.

Le comportement thermique des échantillons a été étudié à différentes vitesses de refroidissement et de chauffage (de 2°C/min à 10°C/min), selon différents cycles de chauffage/refroidissement, sous un balayage constant d'azote sec.

La température de début de transition ( $T_{\text{onset}}$  exprimée en °C) correspond à l'intersection entre la tangente de la plus forte pente du pic et la ligne de base. Les aires mesurées en fonction du temps, sous les pics des thermogrammes, correspondent à des énergies et sont reliées aux enthalpies de transition  $\Delta H$  (exprimée en J/g). Les enthalpies sont normalisées par rapport à la masse de l'échantillon analysé.

### 3. Diffractométrie des rayons X (DRX)

La diffraction des rayons X est une technique de choix pour l'identification des différentes structures et formes polymorphiques présentes dans les composés purs, des mélanges ou dans le produit fini (les nanoparticules lipidiques).

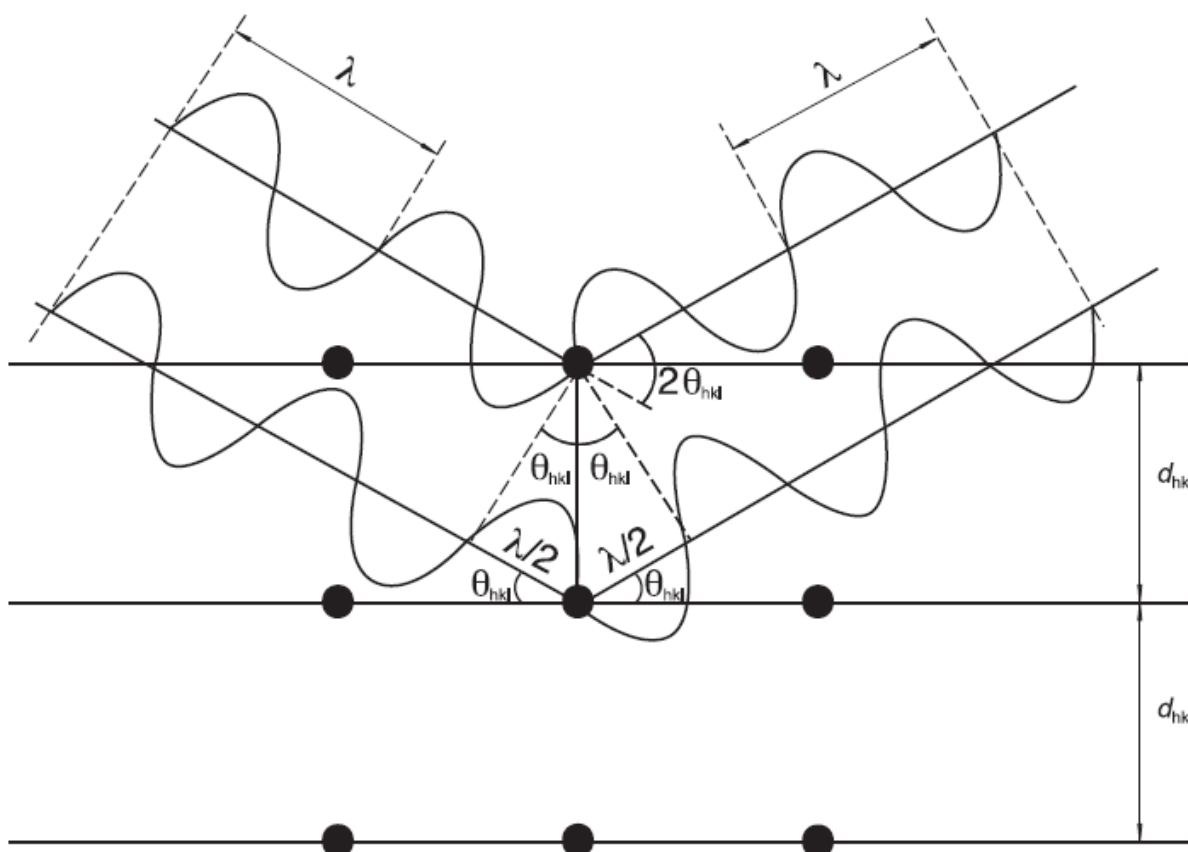
#### 3.1. Principe

Les rayons X sont des ondes électromagnétiques dont la longueur d'onde  $\lambda$  est de l'ordre de l'Angström. Ils permettent l'étude de motifs de répétition dont les dimensions sont du même ordre de grandeur que la longueur d'onde ( $\lambda$ ). Lorsque les photons X rencontrent les électrons des atomes constituant le matériau qu'ils traversent, ils sont déviés de leur trajectoire incidente dans toutes les directions de l'espace produisant un rayonnement diffusé de faible intensité. Toutefois, lorsque les atomes ou molécules possèdent une organisation dans l'espace, si la condition de Bragg est satisfaite, la diffusion à un angle égal à l'angle d'incidence s'effectue sous forme d'interférences constructives, donc avec une intensité beaucoup plus importante. Pour que la condition de Bragg soit remplie, il faut que la différence entre les chemins optiques parcourus par deux ondes réfléchies par des atomes ou molécules situés sur des plans parallèles et équidistants (distance entre plan réticulaire  $d$ ), soit égale à un nombre entier ( $n$ ) de fois la longueur d'onde du rayonnement (Figure A4):

$$2d \sin \theta = n \lambda \text{ (Loi de Bragg)}$$

La relation de Bragg permet de relier, la distance réticulaire  $d$  (en Å) d'une famille de plans et la longueur d'onde  $\lambda$  des rayons X, à l'angle de réflexion  $\theta$  (en degré) du faisceau sur ces plans ;  $n$  est un nombre entier représentant l'ordre de la réflexion. L'ordre 1 ( $n = 1$ ) détermine la distance réticulaire entre deux plans consécutifs et renseigne sur l'épaisseur de la strate considérée. En général, plusieurs réflexions correspondant à des ordres différents sont observées et permettent de préciser l'épaisseur de la strate.

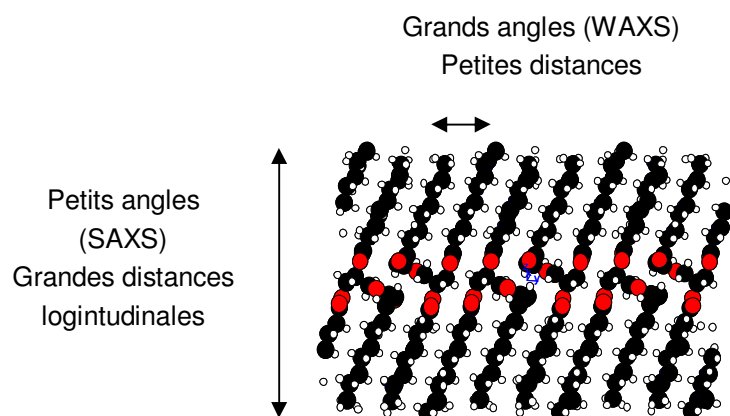
La détermination des angles de Bragg permet ainsi de mesurer des distances interatomiques ou intermoléculaires du même ordre de grandeur que la longueur d'onde du rayonnement X, classiquement entre 1 et 100 Å.



**Figure A4.** Principe de la diffraction de rayons X selon la loi de Bragg représentant le chemin optique des rayons X se diffractant sur un échantillon cristallin avec l'angle  $\theta$  correspondant à l'angle d'incidence du rayonnement sur les plans atomiques de l'échantillon. L'angle de diffraction entre la direction du faisceau incident et celle du faisceau réfléchi est égal à  $2\theta$  (d'après la Pharmacopée Européenne 7.5 2012).

Pour l'analyse, la direction de propagation du faisceau de rayons X est fixe par rapport à l'échantillon et l'on mesure l'angle entre cette direction et celle du faisceau diffracté, égale à  $2\theta$ . Les angles de diffraction peuvent être exprimés en degré ou par le vecteur de diffusion  $q = 4\pi \sin \theta / \lambda$  avec  $d = 2\pi / q$ .

A une longueur d'onde donnée,  $\lambda$ , les angles de diffraction du faisceau, par rapport au plan d'atomes ou de molécules, sont donc inversement proportionnels aux distances réticulaires  $d$ . Pour cette raison, les grandes distances longitudinales, par exemple les phases lamellaires des formes cristallines des lipides, sont observées aux petits angles tandis que les petites distances, par exemple l'arrangement transversal caractéristique des sous cellules cristallines des lipides, seront détectées aux grands angles (Figure A5).



**Figure A5.** Schéma représentant les distances inter-lamellaires observées aux petits angles (small-angle X-ray scattering, SAXS) et les distances inter-chaines détectées aux grands angles (wide-angle X-ray scattering, WAXS) pour la trilaurine (d'après Pivette 2011).

### 3.2. Dispositif expérimental

Les analyses par DRX ont été réalisées sur deux équipements différents, soit sur l'appareillage du laboratoire UMR CNRS 8612, soit sur la ligne SAXS du synchrotron Elettra (Trieste, Italie).

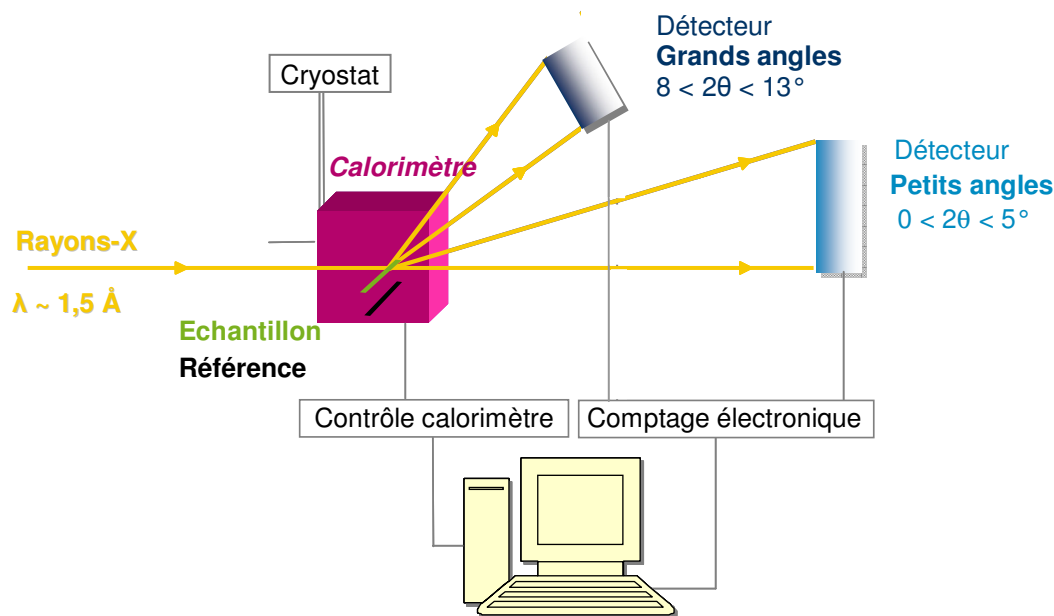
Pour ces deux installations, les échantillons sont placés dans un microcalorimètre, Microcalix, développé au sein du laboratoire (Keller et al. 1998; M. Ollivon et al. 2006). Microcalix permet de coupler les mesures de diffusion des rayons X aux petits et grands angles à la microcalorimétrie différentielle. L'appareil actuel permet en utilisant le rayonnement du générateur de RX du laboratoire ou le rayonnement synchrotron, d'étudier l'évolution des structures et des propriétés thermodynamiques d'un seul et même échantillon de petit volume, entre -30 et +230 °C, à des vitesses de balayage choisies entre 0,01 et 20 °C/min, avec une très grande sensibilité dans les deux techniques. Le calorimètre peut aussi être utilisé simplement comme un porte échantillon thermostaté.

#### ***Appareillage DRX du laboratoire UMR CNRS 8612***

Les analyses de diffraction des rayons X aux petits et grands angles ont été réalisées sur un montage composé d'un générateur de RX, d'un système de monochromatisation et de focalisation, de fentes, d'un microcalorimètre et d'un système de détection et d'acquisition des données (Figure A6). Le générateur de rayons X (Enraf Nonius) est un

appareil utilisant une anode de cuivre émettant une radiation  $\text{Cu } K\alpha$  ( $\lambda=1,542 \text{ \AA}$ ). Il a été utilisé à une tension de 40 kV et une intensité de 5 mA. Le faisceau incident est focalisé par un miroir multicouche (courbure elliptique en W/ Si, Osmic) et la collimation est réalisée par des systèmes de fentes placées avant l'échantillon.

La diffusion de l'échantillon aux petits (SAXS) et grands angles (WAXS) est mesurée simultanément par deux détecteurs linéaires placés horizontalement et de façon perpendiculaire à la direction du faisceau. Ce sont des compteurs proportionnels à gaz à localisation (1024 canaux de 0.0542 mm). La détection repose sur l'ionisation d'atomes de gaz (Argon) par effet photoélectrique et sur la localisation de l'avalanche électronique. Ces détecteurs ont une efficacité de 50%, un taux de comptage total maximum de 50 000 coups par seconde et une résolution spatiale voisine de 80  $\mu\text{m}$ .



**Figure A6.** Représentation schématique du montage de DRX disponible au laboratoire UMR CNRS 8612 (d'après Pivette 2011)

### ***Ligne synchrotron SAXS (Elettra, Trieste)***

Certaines analyses ont été effectuées sur la ligne SAXS utilisant le rayonnement synchrotron Elettra (Trieste, Italie). Le faisceau de rayons X est produit par le rayonnement de particules chargées (positrons) contraintes à se mouvoir sur une trajectoire circulaire à une vitesse proche de celle de la lumière. Au moment du passage par un aimant de courbure, un rayonnement de photons très énergétique est émis. Il est

rendu monochromatique par un cristal de germanium choisi pour son fort pouvoir réflecteur. Ce cristal permet de focaliser le faisceau latéralement. L'extension latérale du faisceau incident frappant le monochromateur est limité par une fente de 20 mm d'ouverture. La taille du faisceau est également définie par deux jeux de doubles fentes verticales et latérales, limitantes et anti-diffusantes. Le faisceau utilisé avait une énergie de 8 keV, soit une longueur d'onde de radiation  $\lambda$  de 1.549 Å.

L'intensité de diffraction est enregistrée simultanément par un détecteur 2D à comptage de photons uniques (Platus 100K) basé sur la technologie à pixels hybrides (DECTRIS, Suisse) pour les SAXS et un détecteur linéaire rempli par un mélange éthane-argon pour les WAXS.

Ils sont situés dans l'axe du faisceau à une distance fixe de l'échantillon et placés derrière un tube sous vide (équipé de fenêtres en kapton) permettant de s'affranchir de la diffusion par l'air. Le détecteur aux grands angles était placé à 55° par rapport au faisceau incident (distance échantillon-détecteur 33 cm) et le détecteur aux petits angles était perpendiculaire au faisceau (distance échantillon-détecteur 100 cm). Dans cette configuration les intervalles du vecteur de diffusion  $q$  étaient 0.03-0.55 Å<sup>-1</sup> et 0.70-1.75 Å<sup>-1</sup>.

L'intensité détectée sur chaque canal est transmise au système d'acquisition. Un piège de tungstène de quelques millimètres de hauteur est placé sur le chemin optique du faisceau direct (non diffracté) afin d'éviter qu'il vienne frapper directement le détecteur. Les diagrammes enregistrés contiennent donc tous une remontée en intensité aux très petits angles correspondant aux bords du piège. Pour plus de clarté, les diagrammes de diffraction sont reportés au-delà de cette zone angulaire ([Pivette 2011](#)).

### 3.3. Mesures expérimentales

Les échantillons (10 à 20 mg) sont introduits dans des capillaires de Lindemann en quartz ou en verre possédant un diamètre externe de 1,5 mm et une épaisseur de 0,01 mm (GLAS Muller, Berlin, Allemagne).

L'acquisition donne l'intensité diffusée (en nombre total de coups) enregistrée sur chaque canal du détecteur. La conversion des numéros de canaux en vecteur de diffusion  $q$  (Å<sup>-1</sup>) est réalisée à l'aide de deux corps cristallins étalons : la tristéarine (SSS) cristallisée sous

forme  $\beta$  et le b  h  nate d'argent (AgBe). La forme  $\beta$  de la trist  arine pr  sente des pics correspondant aux distances caract  ristiques suivantes : 3,7   , 3,85    et  $4,59 \text{   } \pm 0,01 \text{   }$  pour les grands angles et 44,95    pour les petits angles (Ollivon & Perron 1992). Le b  h  nate d'argent pr  sente des pics aux petits angles (3    5 ordres de diffraction) correspondant    la distance caract  ristique 58,38    (Huang et al. 1993).

La mesure de l'intensit   diffract  e est report  e en fonction du vecteur de diffusion  $q$  ( $q = 4\pi \sin \theta / \lambda$ ). Les distances de r  p  tition,  $d$ , caract  ristiques des arrangements structuraux, sont donn  es par la relation  $q \text{ (  }^{-1}\text{)} = 2\pi / d \text{ (  )}$ . Le traitement des donn  es a   t   r  alis      l'aide du logiciel Igor Pro (version 6.03).

## 4. Cryo - microscopie électronique en transmission (cryo-MET)

Le cryo-MET est une technique de choix pour l'investigation directe des ultrastructures et de l'organisation des composants dans les nanoparticules lipidiques.

### 4.1. Principe

La microscopie électronique en transmission (MET) (Figure A7) est proche dans son principe de la microscopie optique. Il existe une certaine analogie entre la MET et le microscope optique à lumière directe.

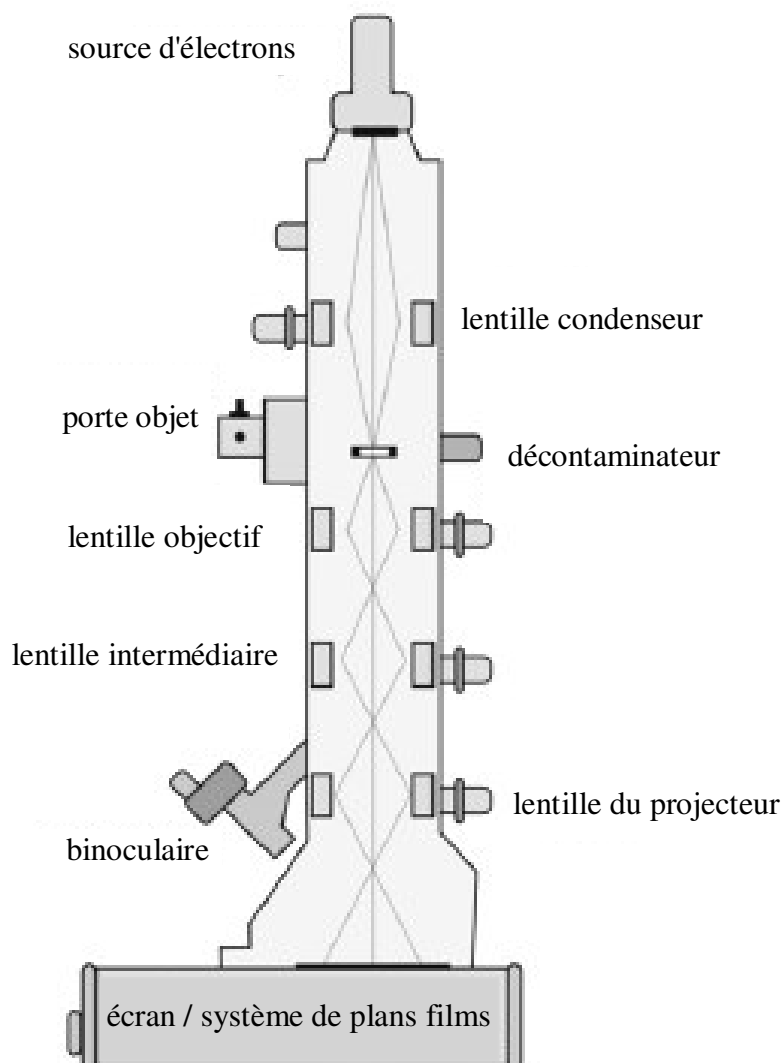
C'est le rayonnement utilisé qui diffère principalement dans les deux cas :

- Le microscope optique utilise comme rayonnement des photons (lumière extérieure). Un système de lentilles optiques permet de dévier ou focaliser le rayon lumineux qui traverse un échantillon "relativement fin". L'image obtenue se forme directement sur la rétine de l'observateur.
- Le MET utilise, lui, comme rayonnement des électrons. Un système de lentilles magnétiques permet de dévier ou focaliser le faisceau d'électrons sur un échantillon "extrêmement fin". L'image (ou cliché de diffraction) obtenue peut être vue sur un écran fluorescent, enregistrée sur un film photographique ou bien détectée par un capteur CCD.

Le MET a deux principaux modes de fonctionnement suivant que l'on obtient une image ou un cliché de diffraction :

- Mode image : Le faisceau d'électrons traverse l'échantillon. Suivant l'épaisseur, la densité et la nature chimique de celui-ci, les électrons sont plus ou moins absorbés. En plaçant le détecteur dans le plan image, on peut, par transparence, observer une image de la zone irradiée. C'est ce principe qui est utilisé, en outre, en biologie, pour observer des cellules ou des coupes minces d'organes.
- Mode diffraction : Ce mode utilise le comportement ondulatoire des électrons modélisé par la physique quantique (onde de Broglie). Lorsqu'ils rencontrent de la matière organisée (des cristaux), ils vont donc être diffractés, c'est-à-dire déviés dans certaines directions dépendant de l'organisation des atomes. Le faisceau est diffracté en plusieurs petits faisceaux, et ceux-ci se recombinent pour former l'image, grâce à des lentilles magnétiques (électro-aimants qui dévient les électrons) (<http://www.techno-science.net>).

La différence majeure entre la cryo-MET et la MET tient dans la préparation des échantillons. En effet, ils peuvent être détériorés par la déshydratation et la fixation des sels de métaux dans la MET. Ces étapes sont évitées dans la cryo-MET grâce au phénomène de vitrification de l'eau. En cryo-MET, l'échantillon est visualisée directement à l'état hydrate congelé (Kuntsche et al. 2011; Klang et al. 2012).



**Figure A7.** Schéma représentant les principaux éléments et le faisceau d'électrons dans un MET (d'après Kuntche et al. 2011)

#### 4.2. Dispositif expérimental

La morphologie et la structure des nanoparticules ont été observées en utilisant un système de cryo-MET (JEM 2100, JEOL, Japan).

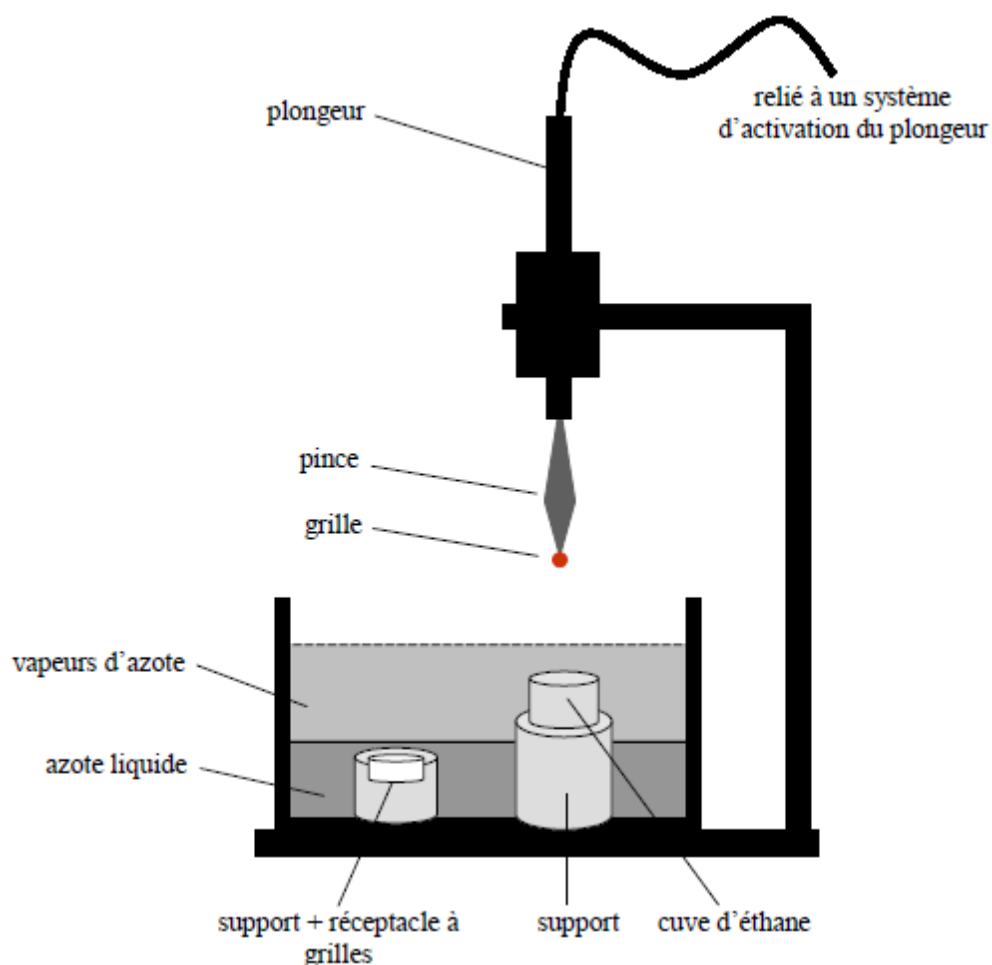
### 4.3. Mesures expérimentales

#### *Préparation des échantillons*

Les nanodispersions lipidiques sont diluées dans l'eau Milli-Q ( $1/50 - 1/100$ , v/v) à une concentration en lipides équivalente à environ  $2 - 3$  mg/ml.

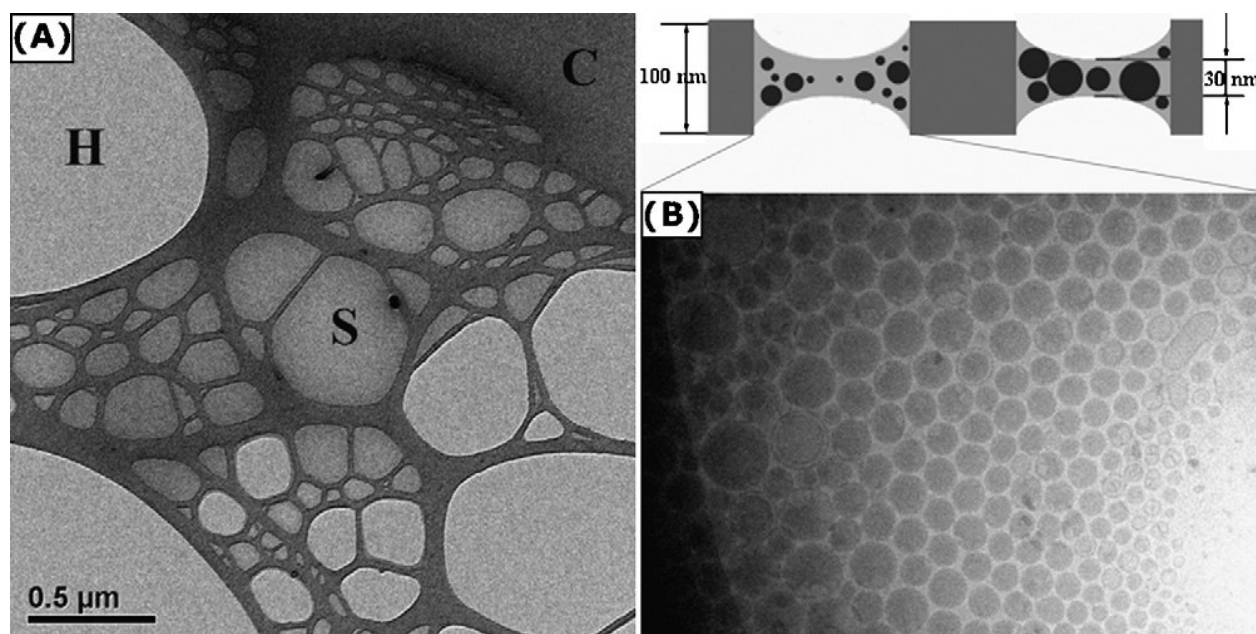
#### *Congélation rapide des échantillons*

La deuxième étape consiste à congeler très rapidement l'échantillon sur la grille de microscopie électronique afin de former une couche de glace vitreuse. Cet état vitreux correspond à un état amorphe métastable de l'eau et évite la formation de glace cristalline, opaque aux électrons. Le support technique utilisé est appelé une guillotine ou un plongeur. Ce dispositif se compose d'une cuve comprenant un récipient rempli d'éthane liquide et un support de réceptacle à grilles, la cuve étant remplie d'azote de façon à recouvrir le support et affleurer le récipient d'éthane (Figure A8).



**Figure A8.** Schéma illustrant le principe d'une guillotine à déclenchement manuel (d'après Mouche 2001)

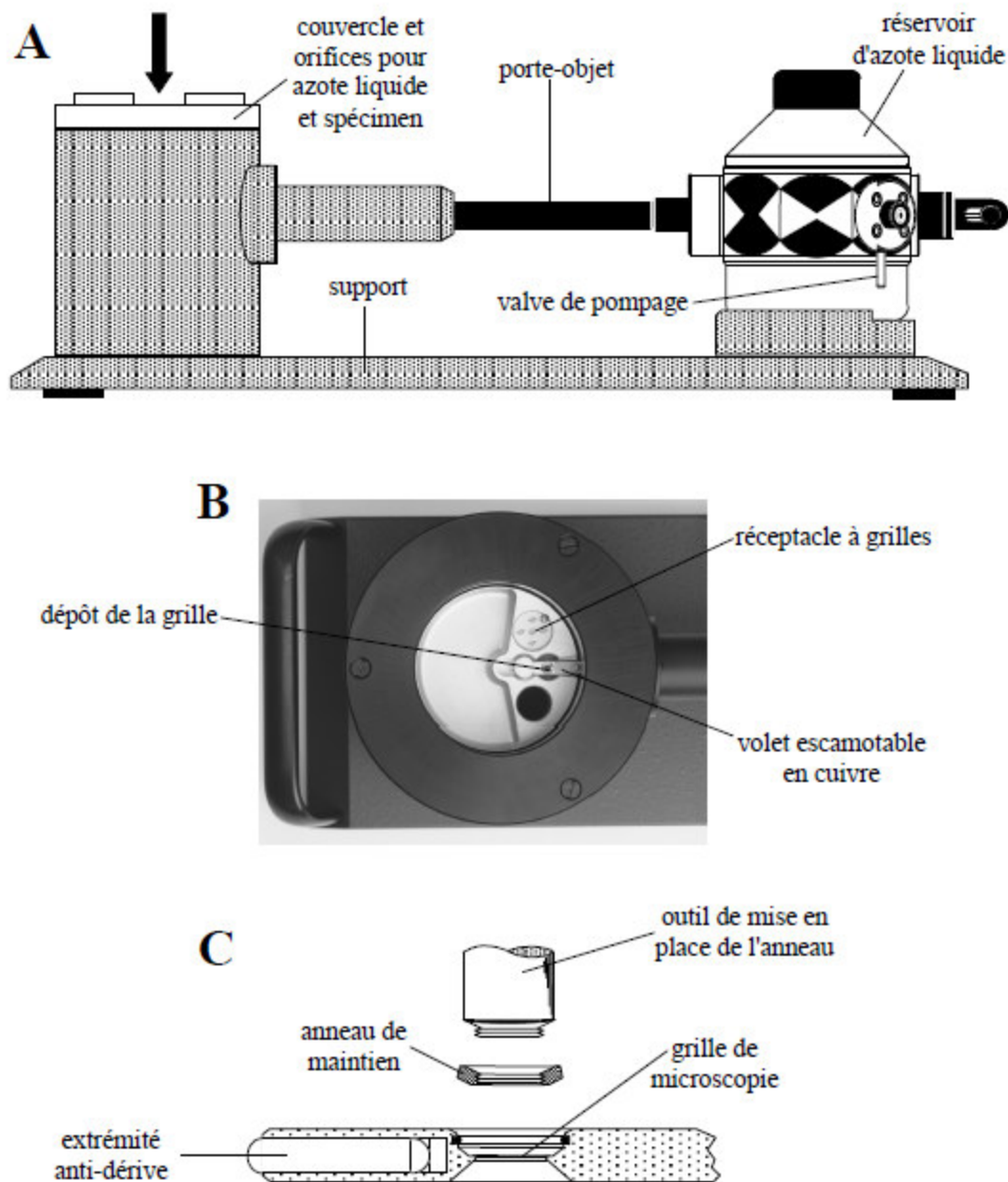
Comme les points d'ébullition et de fusion de l'éthane sous une pression de 1 atmosphère sont de  $-89^{\circ}\text{C}$  et de  $-183^{\circ}\text{C}$ , l'éthane reste liquide car il est entouré de vapeurs d'azote liquide (points d'ébullition  $-190^{\circ}\text{C}$ , et de fusion  $-196^{\circ}\text{C}$ ), ce qui maintient sa température aux alentours de  $-175^{\circ}\text{C}$ . La pince supportant la grille de microscopie électronique est fixée à un plongeur muni d'un dispositif d'activation. Une goutte de  $5\text{ }\mu\text{l}$  de l'échantillon est disposée sur la grille recouverte d'un film de carbone à trous. Le surplus d'échantillon est absorbé à l'aide d'un papier-filtre pour ne laisser qu'un mince film aqueux au niveau des trous dans le carbone, et l'activation du plongeur est déclenchée. La grille pénètre rapidement dans l'éthane liquide, entraînant une vitrification immédiate de l'échantillon. L'épaisseur de la fine couche de glace, formée dans les trous du film de carbone, est de l'ordre de 30 à 100 nm (Figure A9). La grille est alors transférée dans le réceptacle à grilles: l'ensemble des manipulations a donc lieu sous azote liquide ou sous vapeurs d'azote, afin d'éviter un réchauffement de la grille qui conduirait par exemple à la formation de glace cubique à partir de  $-155^{\circ}\text{C}$  (Mouche 2001).



**Figure A9.** Images d'une nanoémulsion typique analysée par cryo-MET après congélation. (A) Une grille TEM classique revêtue d'un film de carbone à trous est représentée. Les lettres indiquent la couche de carbone (C), les trous dans le film de carbone (H) et l'échantillon congelé hydraté (S). Le schéma à droite représente la formation de la couche mince d'échantillon liquide qui reste après essuyage avec un filtre en papier juste avant le processus de congélation. (B) La ségrégation des gouttelettes de nanoémulsions: les petites gouttelettes restent dans les parties les plus fines de film de l'échantillon tandis que les plus grands ne se trouvent que dans les parties les plus épaisses de la couche d'échantillon (d'après Klang et al. 2012).

***Mise en place des grilles dans le microscope***

A l'étape suivante, l'utilisation d'une station de cryotransfert permet de maintenir la chaîne du froid et d'éviter la contamination lors du transfert de la grille dans le microscope. Cette station se compose d'un porte-objet froid et de son support (Figure A10A). Le support comporte une chambre permettant de faire le transfert de la grille du réceptacle à grilles vers le porte-objet froid, sous azote liquide et vapeurs d'azote (Figure A10B). Le porte-objet froid est schématiquement composé d'un réservoir d'azote liquide, d'un logement à grilles situé à l'extrémité d'une tige métallique, qui par convection thermique maintient le logement de la grille à une température inférieure à -155 °C. La grille est donc transférée de ce réceptacle vers le logement du porte-objet froid, et est fixée à l'aide d'un anneau de maintien ou "clip ring" (Figure A10C). Un volet escamotable en cuivre vient couvrir la grille afin de la protéger de toute contamination extérieure (vapeur d'eau ou poussière) au cours du transfert dans le microscope. Le porte-objet froid est ensuite introduit rapidement dans le microscope au niveau du goniomètre, le volet escamotable est alors retiré afin de permettre l'observation (Mouche 2001).



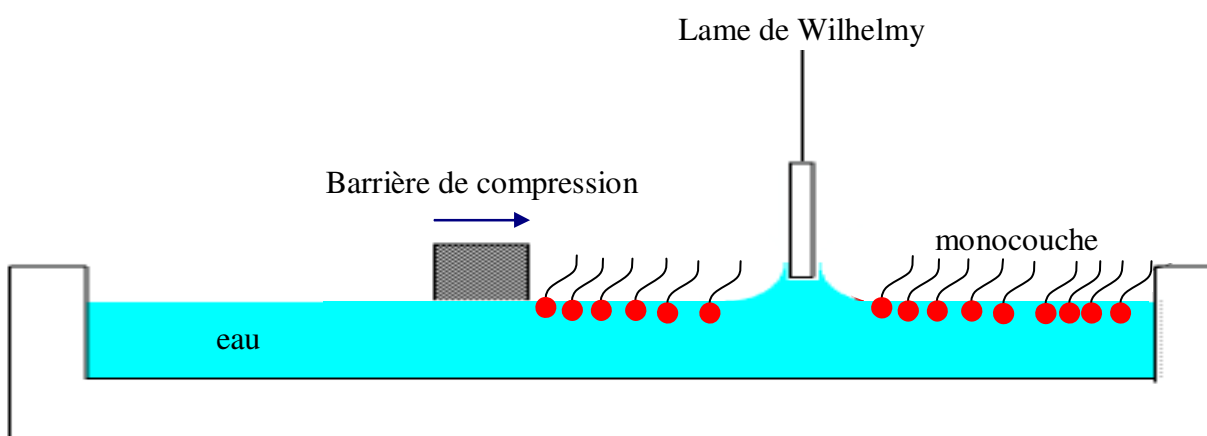
**Figure A10.** Illustration du transfert d'échantillon en cryo-MET. **(A)** Schéma d'une station de cryo-transfert. Elle est composée d'un porte-objet et d'un support. **(B)** Illustration du transfert de la grille de microscopie du réceptacle à grilles vers le porte-objet. Vue correspondant à la flèche du schéma A, sans le couvercle. **(C)** Schéma du système de fixation de l'échantillon sur la pointe du porte-objet (d'après Mouche 2001).

## 5. Balance de Langmuir

### 5.1. Principe

Cette technique permet d'étudier les caractéristiques des films formés à l'interface air/eau par des amphiphiles insolubles ou peu solubles dans l'eau. La monocouche se forme en déposant à la surface de l'eau (dénommée sous-phase) une faible quantité d'une solution de l'amphiphile à étudier dans un solvant volatil (chloroforme, méthanol ou éther).

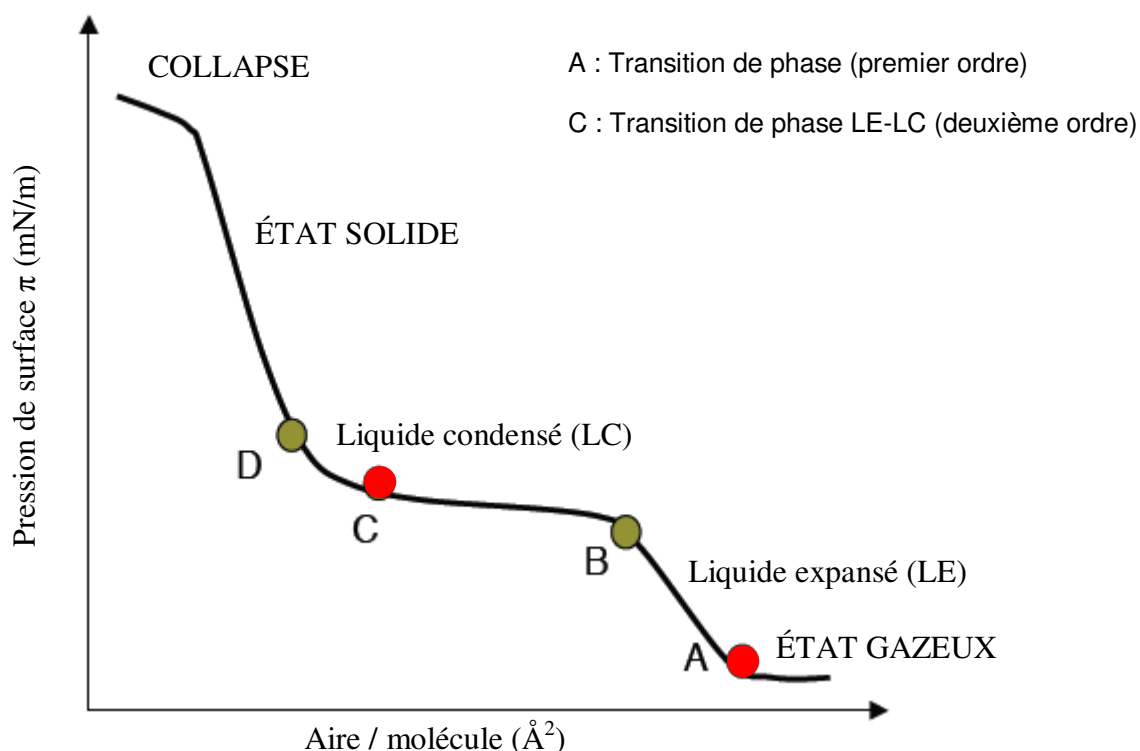
L'évaporation du solvant disperse les molécules amphiphiles à l'interface, sur toute l'aire disponible, formant ainsi une monocouche qui pourra par la suite être comprimée par le déplacement, à la surface de la sous-phase (eau ou tampon), d'une barrière mobile en téflon. Tout en diminuant l'aire de l'interface grâce à la barrière, on mesure la variation de tension de surface (en mN/m) provoquée par la force exercée par le film de molécules sur le capteur de pression, une lame de Wilhelmy (en platine ou en papier), placée à l'interface air/eau (Figure A11).



**Figure A11.** Représentation schématique de la balance à film de Langmuir

Ainsi, en comprimant à vitesse et température constantes la monocouche, on mesure la variation de tension de surface en fonction de l'aire par molécule (en  $\text{\AA}^2$ ) afin de tracer une isotherme de compression; celle-ci a une allure caractéristique pour chaque système amphiphile / sous – phase et est dépendante de la température.

Le profil classique d'une isotherme de compression se caractérise par l'existence de différentes zones (Figure A12) :

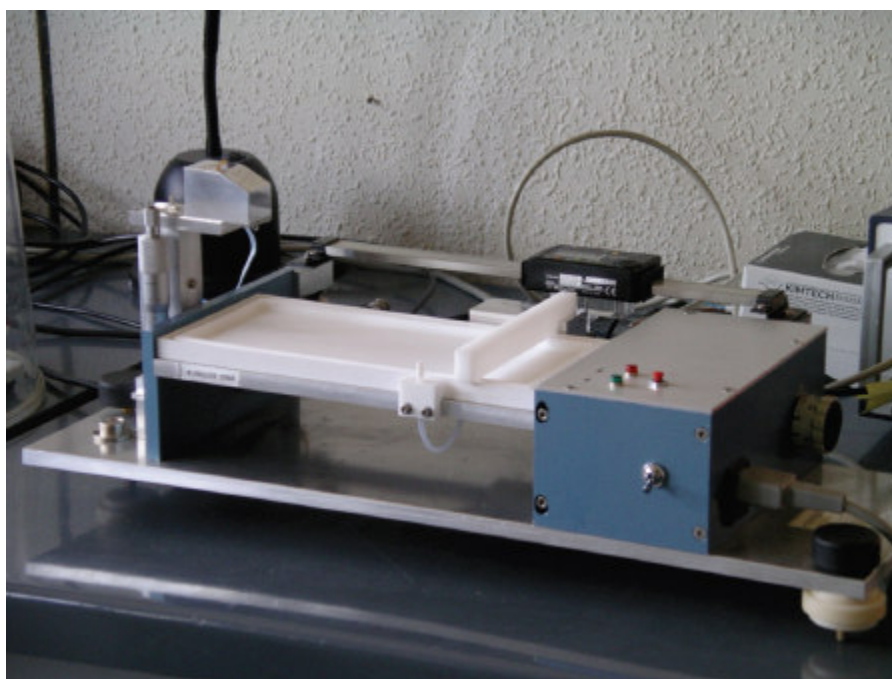


- pour de très grandes aires (hors compression), les molécules se trouvent en phase gaz, très éloignées les unes des autres et n'interagissant pas entre elles. Dans les conditions expérimentales habituelles, dès que la solution est déposée à l'interface air/eau, le film est dans un état plus dense qu'en phase gazeuse pure, car il y a déjà une coexistence entre ce type de phase et une phase dite liquide.
- la phase liquide se caractérise par un rapprochement des molécules. Le film devient plus organisé et les chaînes aliphatiques interagissent par le biais des forces de van der Waals. L'existence d'un plateau dans l'isotherme traduit la coexistence de deux phases liquides : expansée et condensée.
- la phase solide se manifeste par un changement de pente. Dans cette phase, les molécules sont encore plus compactées et organisées. Les contraintes appliquées au film étant de plus en plus importantes, la monocouche reste stable jusqu'à une certaine tension de surface critique.
- en augmentant encore la compaction et la pression de surface, la monocouche se

brise pour former de nouvelles phases (multicouches, agglomérats tridimensionnels ou dissolution des molécules dans la sous-phase), c'est le "collapse". La pression au "collapse", qui est la plus grande pression de compression du film de Langmuir, est une valeur importante caractérisant la stabilité de la monocouche (Bololoï 2008).

## 5.2. Dispositif expérimental

Une balance de Langmuir développée au laboratoire ( $160\text{ cm}^3$ ,  $217,2\text{ cm}^2$ ), équipée d'un capteur de type R&K Wilhelmy (Riegler and Kirstein, GmbH, Allemagne) à lame de Wilhelmy en papier et placée dans une enceinte en plexiglas pour limiter la contamination de surface, a été utilisée (Figure A13).



**Figure A13.** Balance de Langmuir développée par Keller et al. 2003 au laboratoire de Physico-chimie des Surfaces de l'UMR CNRS 8612.

## 5.3. Mesures expérimentales

Pour chaque étude, une solution chloroformique des tensioactifs ou de leurs mélanges a été déposée à la surface de l'eau à l'aide d'une micropipette (Microman<sup>®</sup>, Gilson, France). 7,5  $\mu\text{l}$  de solutions à 3,0 mg/ml ont été répartis sur l'interface air/eau à  $22 \pm 1\text{ }^{\circ}\text{C}$ . Après dépôt, le solvant a été laissé à évaporer pendant 10 minutes avant le début de la compression à la vitesse de  $9,57\text{ cm}^2/\text{min}$ . La surface de la sous-phase a été balayée par une pipette capillaire reliée à une pompe à vide avant de répandre la monocouche.

## 6. Dosage de la quercétine par HPLC

### 6.1. Appareillages

La quercétine a été dosée à l'aide d'un appareillage HPLC de chez Waters<sup>TM</sup>, équipé d'un détecteur UV (Waters<sup>TM</sup> 2487 Dual  $\lambda$  Absorbance Detector), d'une pompe (Waters<sup>TM</sup> 1525 Binary HPLC Pump), d'un injecteur associé à un passeur d'échantillon automatisé (Waters<sup>TM</sup> 717 plus Autosampler) et d'un dégazeur en ligne (Waters<sup>TM</sup> In-Line Degasser AF).

### 6.2. Conditions de chromatographie liquide

La quercétine a été analysée sur une colonne (Modulo-cart QK 3 C18-2 colonnes, 150 mm x 3 mm, Interchim) à 25°C avec une phase mobile acétonitrile - eau - acide trifluoroacétique (30:70:0.1, v/v). La longueur d'onde du détecteur a été fixée à 371 nm. Le débit était de 0,5 ml/min et le volume injecté de 20  $\mu$ l.

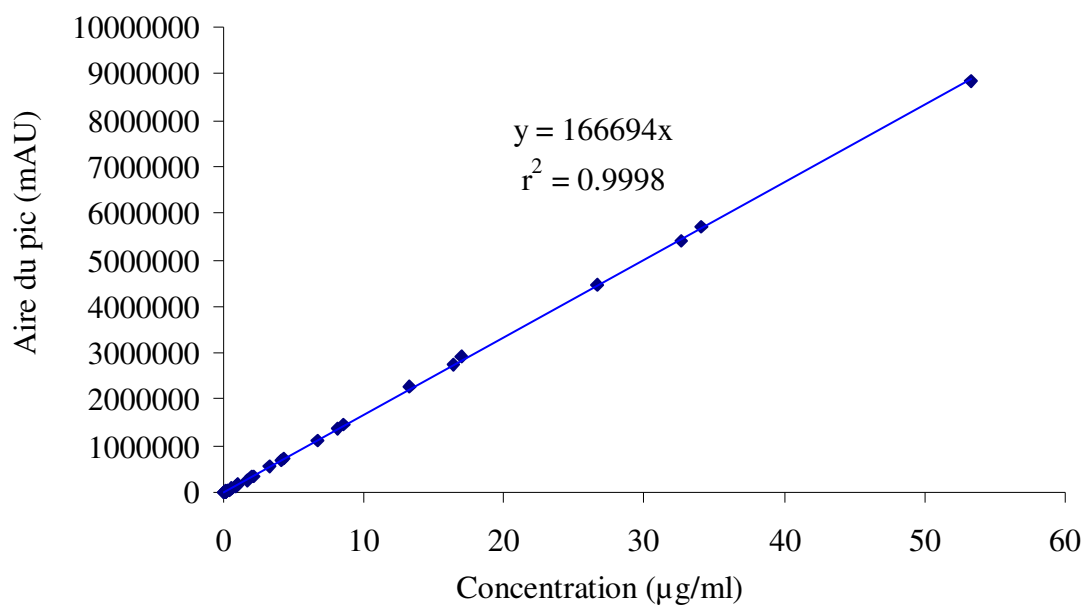
### 6.3. Gamme d'étalonnage

La gamme d'étalonnage (Figure A14) établie à partir de trois solutions mères indépendantes et contenant des particules lipidiques blanches a un coefficient de corrélation ( $r^2$ ) de 0.9998 et une zone de linéarité large de 50 à 5000 ng/ml. Ces chiffres ont confirmé que la spécificité, la sensibilité et la précision de la méthode étaient élevées dans les conditions de chromatographie déterminées.

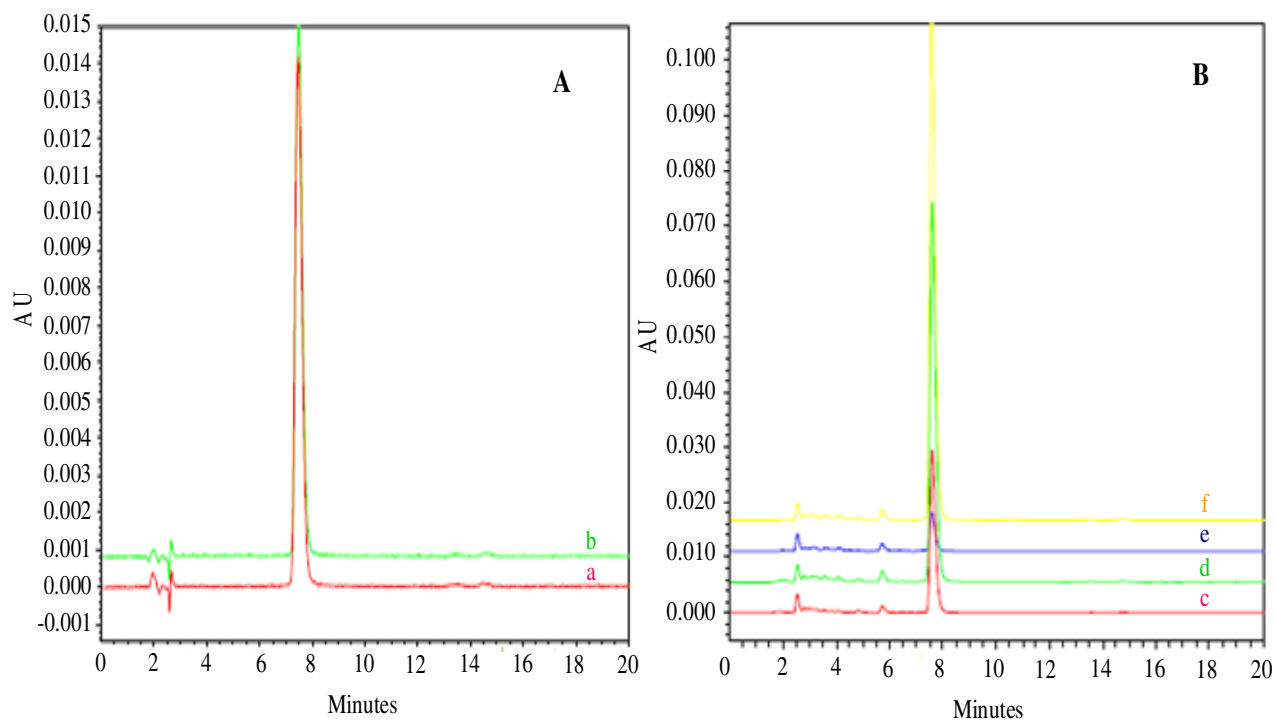
### 6.4. Préparation des échantillons

Avant l'analyse HPLC, tous les échantillons ont été dissous dans le méthanol à des concentrations comprises dans la gamme linéaire, puis filtrés à travers une membrane de porosité moyenne 0,2  $\mu$ m (Minisart<sup>®</sup> High-Flow Hydrophilic 16532K, Sartorius Stedim Biotech GmbH, Goettingen, Allemagne).

La méthode HPLC mise au point permet de séparer le PA et les excipients, et donc de déterminer directement la teneur en PA encapsulé dans les particules lipidiques, ainsi que les cinétiques de libération dans les différents milieux de dissolution (Figure A15).



**Figure A14.** Droite de régression du dosage de la quercétine par HPLC



**Figure A15.** Chromatogrammes de la quercétine (A) dans le méthanol: (a) quercétine native, (b) quercétine dans les nanodispersions et (B) dans les différents milieux aqueux: (c) SGF pH 1,2, (d) SIF pH 6,8, (e) eau Milli-Q pH 5,6 et (f) tampon HEPES pH 7,4.

## 7. Excipients : les lipides et les tensioactifs

Dans le cadre de ce travail, les nanoparticules lipidiques seront préparées à partir de trois grandes familles d'excipients lipidiques : les triglycérides (ou triacylglycérols), les polyoxyl glycérides et les phospholipides. Cette partie du rapport se concentre sur ces matières premières.

### 7.1. Triglycérides

Les triglycérides sont des esters d'acides gras qui composent entre 95 et 98 % en masse des corps gras, résultant de l'association de trois molécules d'acides gras et d'une molécule de glycérol (Figure A16). Ce sont souvent des lipides peu polaires thermotropes présentant un polymorphisme de type monotropique (Figure A17). Les trois formes polymorphes principales appelées  $\alpha$ ,  $\beta'$  et  $\beta$  sont basées sur les différentes formes d'empilement soit respectivement hexagonale (H), triclinique (T) et orthorhombique (O) (Figure A18). La température de fusion des triglycérides croît lorsque l'organisation des chaînes évolue de la forme  $\alpha$  (moins stable) vers  $\beta'$  (forme métastable), puis  $\beta$  (forme plus stable) (Figure A17) (Calvignac 2007).

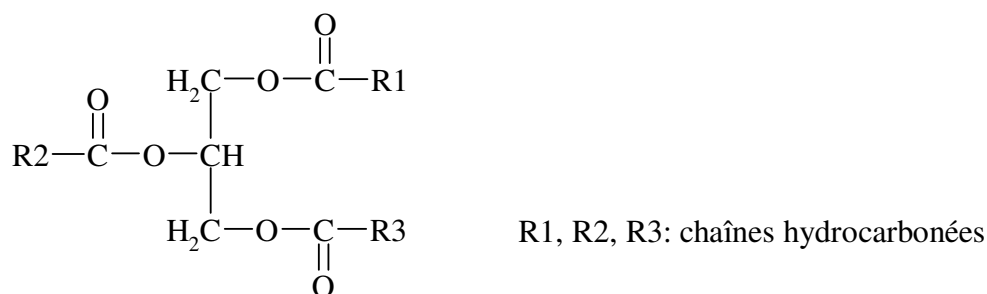


Figure A16. Structure chimique des triglycérides

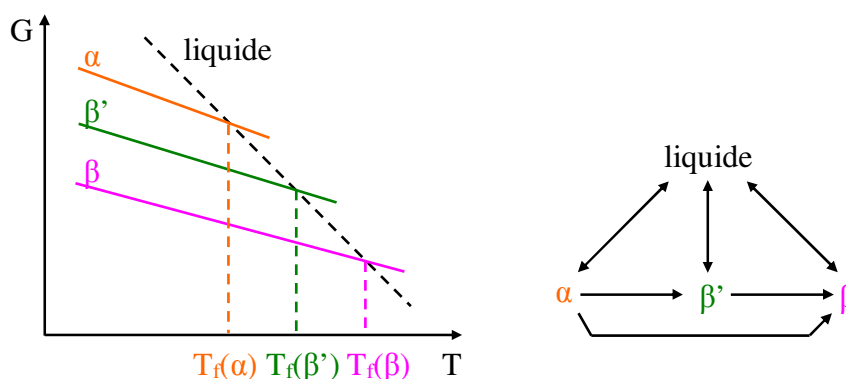
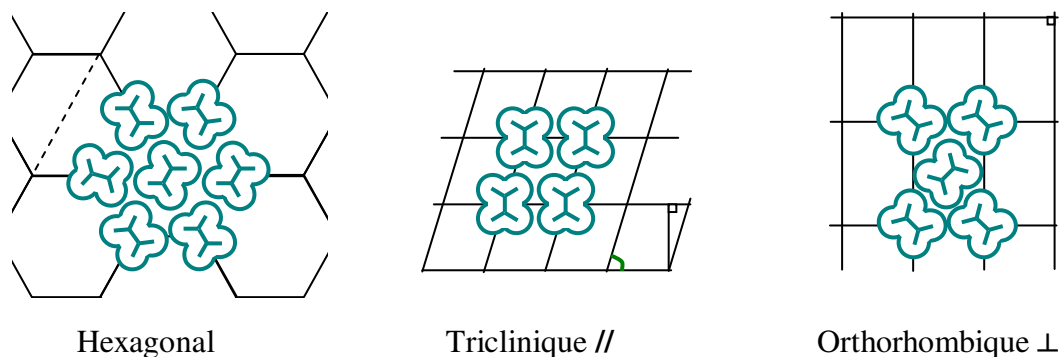
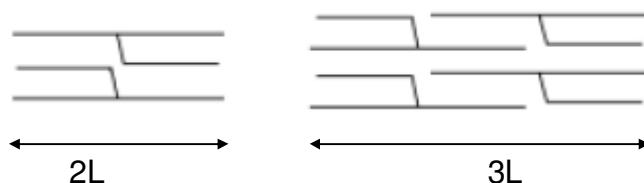


Figure A17. Transitions polymorphiques typiques des triglycérides  
 $T_f$  : transition de fusion; T : température; G : enthalpie libre (J)



**Figure A18.** Arrangement des chaînes aliphatiques

Enfin, l'arrangement des chaînes hydrocarbonées entre les triglycérides est aussi connu (Figure A19). Si R1, R2, R3 sont identiques, les chaînes grasses s'arrangent de manière à former une double couche (2L). Par contre, il se forme une structure à trois longueurs bien distinctes (triple couche, 3L) voire plus dans certains cas pour des longueurs de chaînes différentes (Calvignac, 2007).



**Figure A19.** Organisation longitudinale des triglycérides

#### 7.1.1. Beurre de cacao

Le beurre de cacao est une matière grasse végétale obtenue à partir des fèves de cacao, *Theobroma cacao* L., Sterculiaceae. Sa composition en triglycérides est supérieure à 96% (en masse). Parmi ces triglycérides, les trois composés largement majoritaires (> 80%) sont les dérivés monoinsaturés suivants: le 1,3-palmitoyl-2-oléoylglycérol (POP), le 1-palmitoyl-2-oléoyl-3-stéaroylglycérol (POS) et le 1,3-stéaroyl-2-oléoylglycérol (SOS) dont la proportion peut varier selon l'origine du beurre de cacao (Tableau A1).

Six formes polymorphiques différentes, notées de I à VI par ordre croissant de stabilité thermique, ont été décrites pour ce mélange complexe (Loisel et al. 1998). La fraction polymorphique de chaque forme influe directement sur la qualité et sur les caractéristiques du produit final. Le type de polymorphe souhaité est la forme V grâce à

sa grande résistance au rancissement contrairement à la forme VI. Ainsi pour obtenir préférentiellement la forme V, il convient d'effectuer une étape de tempérage, c'est-à-dire une pré-cristallisation du beurre de cacao par une succession de refroidissement du liquide chaud jusqu'à 27 °C qui conduit ainsi à la formation de cristaux de formes stables et moins stables. Puis, enfin, le mélange est réchauffé à 30-32 °C afin de transformer les cristaux instables en forme V (Tableau A2) (Calvignac 2007).

**Tableau A1.** Composition en triglycérides (TAG) du beurre de cacao (d'après Calvignac, 2007)

| TAG                 | %           | TAG | %         | TAG    | %         |
|---------------------|-------------|-----|-----------|--------|-----------|
| POS                 | 34,0 – 45,5 | POO | 0,8 – 5,6 | SOAr   | 0,4 – 1,0 |
| SOS                 | 21,0 – 29,5 | SLS | 1,5 – 3,7 | OOO    | 0,1 – 0,8 |
| POP                 | 12,2 – 21,5 | PLP | 0,7 – 1,7 | PSS    | 0,4 – 0,6 |
| SOO                 | 1,6 – 6,5   | PPS | 0,6 – 0,8 | SSS    | 0,2 – 0,6 |
| PLS                 | 2,5 – 4,1   | PLO | 0,4 – 1,0 |        |           |
| TAG saturés *       |             |     |           | 1,5 %  |           |
| TAG monoinsaturés * |             |     |           | 88,7 % |           |
| TAG polyinsaturés * |             |     |           | 7,2 %  |           |
| Total*              |             |     |           | 97,4 % |           |

\* : valeurs moyennes. Abréviations: Ar: acide arachidique, (C 20:0); L: acide linoléique, (C 18:2); O: acide oléique, (C 18:1); P: acide palmitique (C 16:0); S: acide stéarique (C 18:0); TAG: triacylglycérols (triglycérides).

**Tableau A2.** Six formes cristallines du beurre de cacao (d'après Calvignac, 2007; Loisel et al. 1998)

| Forme cristalline | Domaine de fusion (°C) | Organisation longitudinale | Organisation latérale      | Sous-cellule principale                                                                                 |
|-------------------|------------------------|----------------------------|----------------------------|---------------------------------------------------------------------------------------------------------|
| I                 | 13,1 – 17,6            | 2L                         | $\beta'$ - sub( $\alpha$ ) | Orthorhombique perpendiculaire (comme $\beta'$ )<br>mais perpendiculaire au plan de base comme $\alpha$ |
| II                | 17,8 – 19,9            | 2L                         | $\alpha$                   | Hexagonale                                                                                              |
| III               | 22,4 – 24,5            | 2L                         | $\beta'_2$                 | Orthorhombique                                                                                          |
| IV                | 26,4 – 27,9            | 2L                         | $\beta'_1$                 | Orthorhombique                                                                                          |
| V                 | 30,7 – 34,4            | 3L                         | $\beta_2$                  | Triclinique                                                                                             |
| VI                | 33,8 – 34,1            | 3L                         | $\beta_1$                  | Triclinique                                                                                             |

### 7.1.2. Labrafac<sup>®</sup> CC

Le Labrafac<sup>®</sup> CC est un mélange de triglycérides des acides caprique (C10, 20 - 50%) et caprylique (C8, 50 - 80%) liquide à température ambiante. Il a une valeur de HLB égale à 1 sur l'échelle de Griffin et sa viscosité se situe entre 25 – 35 mPa.s à 20°C. Le Labrafac<sup>®</sup> CC est insoluble dans l'eau à 20°C ([Tang et al. 2007](#)). Dans la formulation, le Labrafac<sup>®</sup> CC a été utilisé seul pour former des nanoémulsions ou un mélange avec le beurre de cacao pour former des particules lipidiques nanostructurées (NLC).

## 7.2. Polyoxylglycérides

Polyoxylglycérides sont un mélange de mono-, di- et triesters de glycérol, et mono- et diesters de polyéthylène glycol (PEG ou macrogol). Ils sont utilisés largement dans les domaines pharmaceutique, cosmétique et agroalimentaire comme tensioactifs et agents de solubilisation ([Rowe et al. 2009](#)).

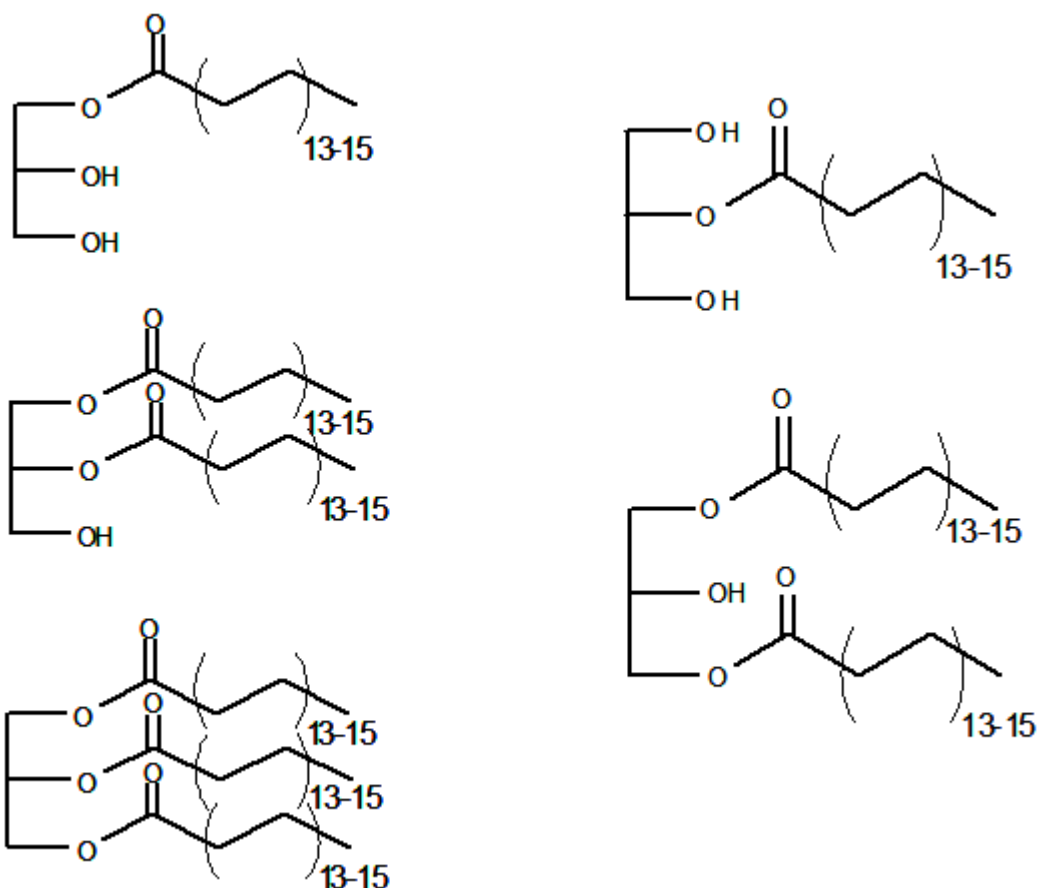
### 7.2.1. Labrafil<sup>®</sup> M2125 CS

Le Labrafil<sup>®</sup> M2125 CS (linoléoyl macrogol-6-glycérides) est un mélange de triglycérides des acides oléique (C18 :1) et linoléique (C18 :2) et de mono- diesters de PEG 300. Il a une valeur HLB égale à 4 sur l'échelle de Griffin et sa valeur de viscosité varie entre 70 – 90 mPa.s à 20°C. Le Labrafil<sup>®</sup> M2125 CS est dispersible dans l'eau à 20°C ([Tang et al. 2007](#)). Plus hydrophile que le Labrafac<sup>®</sup> CC, le Labrafil<sup>®</sup> M2125 CS a été utilisé dans la formulation pour rendre le cœur des particules moins lipophile et envisager la solubilisation d'une large gamme de principes actifs

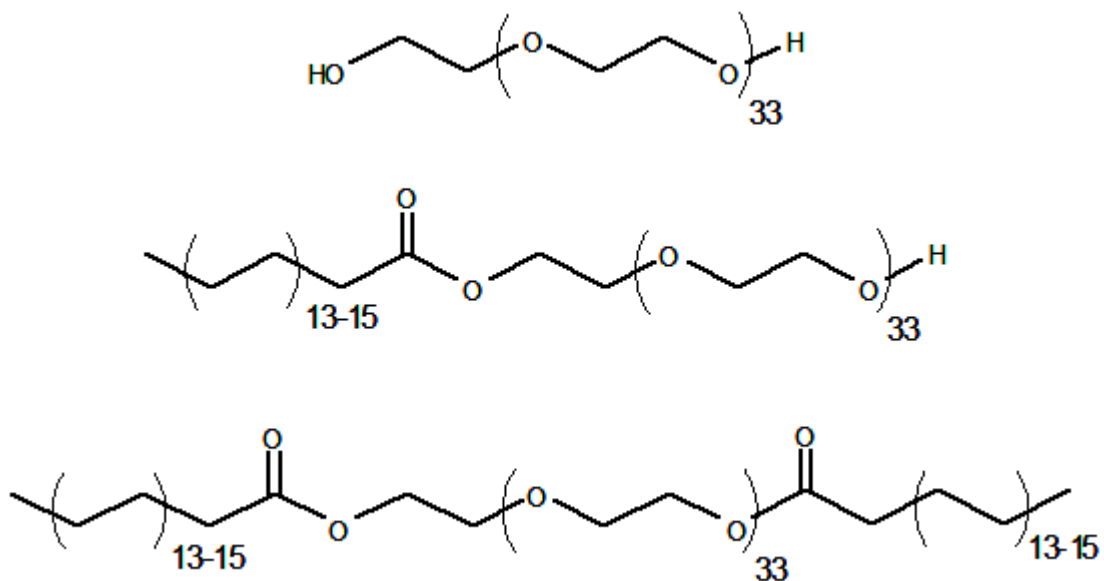
### 7.2.2. Gélucire<sup>®</sup> 50/13

Le Gélucire<sup>®</sup> 50/13 est un mélange de mono-, di- et triacylglycérides d'acides palmitique et stéarique (20%), de mono- et diesters de PEG 1500 (72% avec une majorité de diesters) et de PEG 1500 libre (8%) ([Figure A20](#)). Les deux numéros de 50/13 correspondent à la température de liquéfaction (point de goutte) de 50°C et à la valeur de balance hydrophile-lipophile (HLB) de 13 sur l'échelle de Griffin, respectivement ([Brubach et al. 2004](#)). Dans la formulation, Gélucire<sup>®</sup> 50/13 a été utilisé comme tensioactif.

A



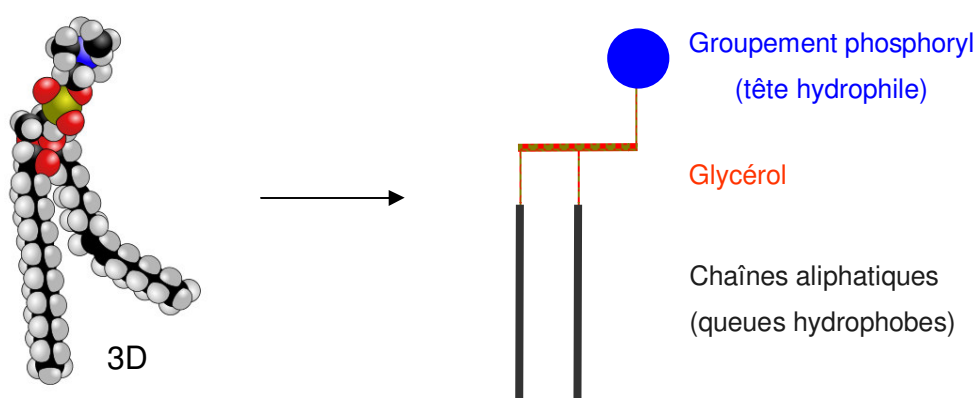
B



**Figure A20.** Structure des composés présents dans le Gélucire 50/13. **(A)** : mono-, di- et triacylglycérides d'acides palmitique et stéarique (20%), **(B)** : mono- et diesters de PEG 1500 (72% avec une majorité de diesters) et PEG 1500 libre (8%).

### 7.3. Phospholipides

La majorité des phospholipides sont des molécules amphiphiles constitués d'une molécule de glycérol sur la quelle sont estérifiés par deux acides gras et un groupement phosphoryl d'une molécule polaire (Figure A21). Les phospholipides sont des constituants très importants au niveau des membranes cellulaires. Ils sont présents chez les animaux que chez les végétaux (Le Grandois 2009). Dans la formulation galénique, ils sont fréquemment utilisés pour former les bicouches membranaires des vésicules médicamenteuses.



**Figure A21.** Structure générale des phospholipides

Le produit commercial utilisé était le Phospholipon<sup>®</sup> 90G. Ce produit est un mélange purifié à partir de la lécithine de soja. Il se compose principalement de phosphatidylcholine (94,0% - 102,0%), lysophosphatidylcholine ( $\leq 4,0\%$ ) et de tocophérol ( $\leq 0,3\%$ ). Le rôle de stabilisant a été recherché par l'emploi de cet excipient dans la formulation.

## Références

1. Bololoi, A.M.C., 2008. Nouveaux amphiphiles catanioniques analogues du galactosylceramide : corrélation structure, propriétés physicochimiques et activité anti-VIH. Université de Toulouse III.
2. Brubach, J.B. et al., 2004. Structural and Thermal Characterization of Mono- and Diacyl Polyoxyethylene Glycol by Infrared Spectroscopy and X-ray Diffraction Coupled to Differential Calorimetry. *The Journal of Physical Chemistry B*, 108(46), pp.17721–17729.
3. Calvignac, B., 2007. Mise au point et compréhension phénoménologique d'un procédé propre de recristallisation / fractionnement de corps gras et de lipides, Paris: École nationale supérieure des Mines de Paris.
4. Grabielle-Madelmont, C. & Perron, R., 1983. Calorimetric studies on phospholipid—water systems: I. DL-Dipalmitoylphosphatidylcholine (DPPC)—water system. *Journal of Colloid and Interface Science*, 95(2), pp.471–482.
5. Le Grandois, J., 2009. Caractérisation et stabilité de sources de phospholipides polyinsaturés à intérêts nutritionnels. Université de Strasbourg.
6. Huang, T.C. et al., 1993. X-ray powder diffraction analysis of silver behenate, a possible low-angle diffraction standard. *Journal of Applied Crystallography*, 26(2), pp.180–184.
7. Keller, G. et al., 1998. DSC and X-ray diffraction coupling: Specifications and applications. In *Journal of thermal analysis and calorimetry. JCAT'97 Conference on Calorimetry and Thermal Analysis*. Springer, pp. 783–791.
8. Klang, V. et al., 2012. Electron microscopy of nanoemulsions: an essential tool for characterisation and stability assessment. *Micron* (Oxford, England: 1993), 43(2-3), pp.85–103.
9. Kuntsche, J., Horst, J.C. & Bunjes, H., 2011. Cryogenic transmission electron microscopy (cryo-TEM) for studying the morphology of colloidal drug delivery systems. *International journal of pharmaceutics*, 417(1-2), pp.120–137.
10. Loisel, C. et al., 1998. Phase transitions and polymorphism of cocoa butter. *Journal of the American Oil Chemists' Society*, 75(4), pp.425–439.
11. Mehnert, W. & Mäder, K., 2001. Solid lipid nanoparticles: production, characterization and applications. *Advanced drug delivery reviews*, 47(2-3), pp.165–196.
12. Mouche, F., 2001. Étude structurale de macromolécules biologiques par cryomicroscopie électronique, reconstruction tridimensionnelle et recalage de données de cristallographie aux rayons X. Paris: Université Paris 6 - Pierre et Marie Curie.

13. Ollivon, M. et al., 2006. DSC and high resolution X-ray diffraction coupling. *Journal of Thermal Analysis and Calorimetry*, 85(1), pp.219–224.
14. Ollivon, Michel & Perron, R., 1992. Propriétés physiques des corps gras. In *Manuel des corps gras*. Paris: Lavoisier, p. 433.
15. Pharmacopée Européenne 7.5, 2012. Conseil de l'Europe.
16. Pivette, P., 2011. Microsphères lipidiques obtenues par prilling : du polymorphisme des constituants à la compréhension du mécanisme de libération d'un principe actif hydrosoluble. Châtenay-Malabry: Université Paris Sud 11.
17. Rowe, R.C. et al., 2009. *Handbook of pharmaceutical excipients*, London; Chicago; Washington, DC: Pharmaceutical Press; American Pharmacists Association.
18. Tang, J., Sun, J. & He, Z.-G., 2007. Self-emulsifying drug delivery systems: strategy for improving oral delivery of poorly soluble drugs. *Current Drug Therapy*, 2(1), pp.85–93.
19. <http://www.techno-science.net>.