Characterization and comparison of the functionality of fractionated lecithin from different sources

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Gent, June 2015

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Preface

Lecithin is defined as a complex mixture of acetone-insoluble phosphatides such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, combined with other substances such as triglycerides, fatty acids, carbohydrates and other minor compounds. There are two major sources of lecithin: animal and vegetal. Lecithin coming from the latter source was evaluated in this work.

Lecithin can be used in its native form or it can be modified. Modification is a process of which the aim is to improve the emulsifying properties of the lecithin. The idea behind modification is to separate specific compounds from the lecithin matrix to obtain tailor-made products. In this work, a physical modification technique was used.

To be able to follow the influence of this physical modification process, different characterization techniques were evaluated. The composition of the lecithin was determined using both simple, total phosphorous and nitrogen content, and very precise techniques namely $^{31}\text{P}-\text{NMR}$. Both approaches were used in order to show that simple and well known methods might be a good alternative for industries and small laboratories (especially in developing countries) in getting an idea of the phospholipid content and composition of lecithin. To assess the oil-in-water emulsion stabilizing functionality of the obtained lecithin both the emulsion droplet size distribution and the cream layer profile were followed over storage time.

Finally also the effect of mono and multi-valent cations on water-in-oil emulsions which are stabilized using lecithin was examined.

In the following lines, the reader will find four major chapters. The first chapter compiles the most important literature about the topic. The second chapter goes through the materials and methods which were used throughout the work. The third chapter goes over the results which were obtained and also gives some interpretation and comments about the results. Finally, the fourth chapter gives an overview of the research and some conclusions in addition to some further recommended research.
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LIST OF ABBREVIATIONS

GM = genetically modified
GMO = genetically modified organism
N = nitrogen
N-PL = nitrogen-containing phospholipids
O/W = oil-in-water
P = phosphorus
PL = phospholipid
PA = phosphatidic acid
PC = phosphatidylcholine
PE = phosphatidylethanolamine
PI = phosphatidylinositol
PS = phosphatidylserine
W/O = water-in-oil
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Chapter I

Literature review
1. Introduction

In general as well as in scientific literature, the term lecithin is used with a variety of different meanings.

In food technology, lecithin is defined as an additive, used as an emulsifier, instantiser, antioxidant and flavour protector (ELMA). It was originally discovered (in 1846) by the French chemist Maurice Gobley, described as a viscous matter containing fatty acids. He named it with the Greek word for egg yolk, "lekithos", based on his principal experiments on hen’s egg yolks (Sourkes, 2004).

The Food Chemical Codex (2003) defines lecithin as a complex mixture of acetone-insoluble phosphatides that consists of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, combined with other substances such as triglycerides, fatty acids, carbohydrates and other minor compounds. Lecithin is only partially soluble in water, but it readily hydrates to form emulsions. This definition is the one used along this text.

It should be noted that there are also other uses of the term “Lecithin”. According to Szuhaj (2005), the term phosphatide was assigned by Thudichum (1930) to “lipids containing phosphorus”, whereby lecithin is chemically the phosphatide fraction (phospholipid) of egg yolk and of several oilseeds. Moreover, the term “lecithin” is sometimes used as a synonym for phosphatidylcholine, which is the major component of the phosphatide fraction isolated from either egg yolk or soybean oil (Wabel, 1998). Chemically, phosphatidylcholine is a mixture of differently substituted sn-glycerol-3-phosphatidylcholine backbones (Rossi, 2007).

2. Composition and Sources

Lecithin has been in commercial use for more than a century, but since the extraction process from egg yolk proved costly, lecithin is today not only extracted from eggs but also from soybeans and other sources (ELMA).

However, there are two major differences between plant-based-lecithin and animal-based-lecithin. Firstly, the phospholipid composition is different and secondly, the plant-based-lecithin has a higher unsaturated fatty acid content and no cholesterol as compared to egg yolk lecithin (Szuhaj, 2005).

As mentioned previously, lecithin is mainly composed of phospholipids, which are a class of lipids that are formed of a phosphate-containing polar head-group attached to non-polar hydrocarbon chains. The nature of the head-group is diverse, with different
functional groups attached to the phosphate groups (phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol). The structure of these head-groups is shown in Figure 1. The type of fatty acid chains also varies and depends on the chain length and the carbon saturation.

Phospholipids are amphiphilic compounds: the phosphate-containing polar head-group composes the hydrophilic part, while the glycerol-backbones, as well as the fatty acids compose the hydrophobic part. This amphipathic property provides the basis for the compartmentalization of cells (phospholipids are the main constituents of biological membranes) and is the reason why phospholipids are considered as surfactants (Pichot et al., 2013).

The name ‘surfactant' is a contraction of the term surface-active agent. Surfactants are compounds that lower the surface tension of a liquid, because of their amphiphilic properties, allowing easier spreading. It also lowers the interfacial tension between two liquids or between a liquid and a solid (Rosen & Kunjappu, 2012).

Figure 1: Structure and major classes of phospholipids (AOCS, 2011)

The solubility of phospholipids in water depends on the head-group type and the hydrocarbon chain length. Four classes of phospholipids (PLs) can be distinguished as a function of PL solubility (Pichot et al., 2013). Class I includes Phospholipids which are...
insoluble in water (do not absorb water at all). Class II consists of Phospholipids with very low solubility in water, which swell in water. Examples are long-chain phosphatidylcholine, phosphatidylethanolamine or sphingomyelin (SM). Class III A includes Phospholipids, such as lysolecithins, which are soluble in water forming lyotropic liquid crystals at low water content. Finally, Class III B is relatively rare, and contains Phospholipids which are soluble in water forming micelles above the critical micelle concentration (CMC), but no crystalline structure. Examples of this class are saponins.

2.1 Occurrence of phospholipids

The exact composition of lecithin depends on the source as well as on the method of extraction and purification. Although lecithin can be found in all kinds of living matter, it is predominantly manufactured from plant seeds. The main plant seeds that are used are soybeans and to a lesser degree peanut, and cotton-, sunflower- and rape-seeds. As animal sources, egg yolk and brain tissues (bovine brain) are primarily used (Bueschelberger, 2004).

Essentially, however, the only raw materials suitable for commercial use are oilseeds and egg yolk. Plant raw materials have a low phospholipid content, which (as a percentage of dry matter) does not exceed 3%. By contrast, raw materials of animal origin have a much higher phospholipid content. In dried whole milk, for example, it is around 2% and in dried egg yolk around 17% (Bueschelberger, 2004).

The phospholipid composition generally gives a clear indication of the origin of the product, which is seen in table 1.

Table 1: Average composition of plant and animal oil-free phospholipid extracts (Bueschelberger, 2004; Szuhaj, 2005).

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Soybean</th>
<th>Rapeseed</th>
<th>Sunflower</th>
<th>Corn</th>
<th>Egg yolk</th>
<th>Milk</th>
<th>Cottonseed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>24</td>
<td>25</td>
<td>25</td>
<td>30</td>
<td>74</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>22</td>
<td>22</td>
<td>11</td>
<td>3</td>
<td>19</td>
<td>36</td>
<td>13</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>15</td>
<td>15</td>
<td>19</td>
<td>16</td>
<td>1</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>7</td>
<td>3</td>
<td>3</td>
<td>9</td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Lyso-phospholipids</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Other phospholipids</td>
<td>5</td>
<td>19</td>
<td></td>
<td>1</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

*Glandless cottonseed (values based on total phosphorus)
2.1.1 Animal based lecithin

Almost all body cells contain phospholipids. The common animal phospholipids are sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and other glycerol phospholipids of complex fatty acid composition.

Besides egg yolk, also milk and brains have served as animal sources of lecithin. In some instances, isolated and purified lecithins have been developed for clinical nutritional uses (Szuhaj, 2005).

The following lines give some additional information on animal sources of lecithin.

2.1.1.1 Eggs

The phospholipids in eggs are mainly found in the yolk. Phospholipids represent approximately 10% of the wet weight of the egg yolk. The main components of egg-lecithin are phosphatidylcholine and phosphatidylethanolamine (Szuhaj, 2005). Egg yolk lecithin also contains lysophosphatidylcholine, sphingomyelin, and neutral lipids in minor quantities (Palacios & Wang, 2005). Egg yolk lecithin as a commercial-industrial ingredient is too expensive, but it is used in some cases, such as medical feeding programs and infant formulas (Szuhaj, 2005).

2.1.1.2 Milk

Milk has a phospholipid content of about 1% of the total lipid content (Bitman & Wood, 1990). Phospholipids are fundamental in milk for the emulsification of fat in water.

Glycerophospholipids (70%) and sphingolipids (29%) are the two main groups belonging to the class of phospholipids in milk (Fagan & Wijesundera, 2004).

2.1.1.3 Brain

The brain is a rich source of phospholipids, and together with the spinal cord, it probably possesses the highest phospholipid content of any of the organs. There are many different types of phospholipids in the central nervous system (Kuksis, 1985) and in other animal organs (Szuhaj, 2005).

2.1.2 Plant based lecithin

2.1.2.1 Soybean

The major commercial source of lecithin is soybean. Soybean lecithin contains ca. 65-75% phospholipids (Scholfield, 1981). It is mainly used because of its availability and excellent emulsifying behaviour, colour and taste (Cherry & Kramer, 1989).
Cherry & Kramer (1989) stated that variability in the composition of phospholipids in soybean lecithin exists due to different manufacture conditions. For instance, purification steps used to produce quality oil may affect the lecithin components. Also, soybeans exposed to frost damage, or subjected to prolonged storage, have reduced lecithin yields. Phospholipases, which produce phosphatidic acid, are active during storage and may reduce the yield of lecithin. During the maturation process, the major phospholipids (PC, PE, and PI) increase, and others decrease or remain constant.

2.1.2.2 Corn

Depending on the variety of maize, the amount of phospholipids varies from 2% to 5% (Szuhaj, 2005). The amount of phospholipids found in corn lecithin is about 61% (on dry basis) according to Weber (1981).

Similar chemical compositions were noted for corn and soy phosphatidylcholine and phosphatidylinositol. Phosphatidic acid and glycolipids represent a higher proportion of phospholipids in corn than in soybean lecithin. The percentage of minor components in corn, such as steryl-glycoside esters and other glycolipids, are more than twice that found in soybean lecithin, which explains why the physical properties (emulsifying properties) of corn lecithin differ from those of soybean lecithin (Cherry & Kramer, 1989).

Corn glycolipids and phospholipids have lower percentages of linolenic acid (18:3) and are more saturated than those in the soybean. In general, crude corn and soybean lecithins are equal in linoleic acid (18:2) content, but linoleic acid in corn varies from 42% to 70% depending on the variety of maize (Cherry & Kramer, 1989).

Another compound, phytic acid, of which 88% can be found in the germ of the corn, is extracted as part of the lecithin fraction. Its elimination is desirable because it binds zinc, magnesium, and calcium. This binding decreases the nutritional availability of these minerals (Szuhaj, 2005).

2.1.2.3 Cottonseed

The phospholipids in cottonseed are similar in many respects to those of soybeans, with the exception of their lower level of linolenic acid and higher level of saturated fatty acids. Because of their low amount of linolenic acid, they are more stable to oxidation and rancidity than soybean lecithin (Szuhaj, 2005).

In ginned cottonseed, gossypol binds to lecithin during oil extraction. Gossypol is a phenolic compound, which is toxic, and is produced by cotton plants to protect them against insects (Adams, et al., 1960). This economically negates its use as a commercial
source. Cultivars of glandless or gossypol-free cottonseed may have some potential for providing commercial edible lecithins (Szuhaj, 2005).

2.1.2.4 Rapeseed

The major phospholipids present in commercial rapeseed lecithin are enumerate in table 1. Among the minor fractions, lysophosphatidyl ethanolamine accounts for about 2%. Solvent-extracted rapeseed oil contains more phosphorus than other oils obtained with other extraction methods (Szuhaj, 2005). Although a percentage of erucic acid is present, the major fatty acids are palmitic, oleic and linoleic acids (Persmark, 1968). Since canola is out on the market, the applications of rapeseed lecithin have developed positively (Szuhaj, 2005).

2.1.2.5 Sunflower

Sunflower lecithin has a mild taste and similar emulsifying properties as soybean lecithin. Its use in the manufacture of foods and cosmetics can be increased by refining and fractionation and/or modifications. This makes sunflower lecithin interesting for food manufacturers, particularly in Europe, the biggest sunflower seed producing/processing continent (Szuhaj, 2005).

Sunflower lecithin contains 43% phospholipids, which composition is mentioned in table 1, as well as 33% oil and 23% of other compounds (glycolipids, complex carbohydrates) (Cabezas et al., 2011).

2.1.2.6 Other plants

Other sources of lecithin that have been tested are rice bran, barley-, palash-, carrot-, coriander-, and papaya-seeds, as well as avocado, olive fruit and others (Szuhaj, 2005).

2.1.3 Microorganisms

Microorganisms, especially those that are acid-fast (resistant to decolourization by acids during staining procedures), also contain large amounts of lipids, including phospholipids. These entities are of interest for clinical research (Szuhaj, 2005). A survey of microbiological sources of phospholipids has been published by Ratledge (1989).

3. Manufacture, purification and modification

The industrial process to obtain lecithin depends on the raw material.

Vegetable lecithins are manufactured exclusively as by-products of the vegetable oil refining process, shown in figure 2 (van Nieuwenhuyzen, 2014). For animal lecithins,
extraction processes have been developed and are applied commercially. Egg yolk lecithin, for instance, is obtained by combined extraction with ethanol and acetone (Whitehourst, 2004).

Considering the scope of this text, only vegetable lecithin manufacture will be discussed. During water-degumming, phospha- and glyco-lipids are removed to stabilize vegetable oils against sedimentation and also to enable further refining steps. The process consists in heating the crude oil to about 70ºC, after which the crude oil is mixed with 2% water and subjected to thorough stirring for about half an hour to one hour. This addition of water to the oil hydrates the polar lipids in the oil, making them insoluble. The resulting lecithin sludge is then separated by centrifugation. The sediment contains water, phospholipids and glycolipids, some triglycerides, carbohydrates, traces of sterols, free fatty acids and carotenoids. The crude lecithin is subsequently obtained by careful drying (Whitehourst, 2004).

The conditions during the degumming process, quality and origin of the oilseeds, have a considerable influence on the composition and quality of the crude lecithin (Szuhaj, 2005).

![Flow chart of oilseed lecithin manufacture](van Nieuwenhuyzen, 2014). *By-products are obtained as well

### 3.1 Modifications of lecithins

The process of modification produces products with improved emulsifying properties besides increased dispensability in aqueous systems.
The basic idea behind modification of lecithin revolves around recognition of the crude lecithin starting material as a mixed emulsifier. Therefore, lecithin can be used for both, water-in-oil and oil-in-water type emulsions, but each type of phospholipid has other emulsification properties.

Phosphatidylcholine has oil-in-water emulsification-promoting characteristics. Phosphatidylethanolamine and, to a lesser extent, phosphatidylinositol have water-in-oil emulsifying properties (Joshi et al., 2006).

There are three important steps during modification of the complex mixture of crude lecithin (Joshi et al., 2006). Standardization of the raw materials, in order to guarantee a consistent composition, and thereby its functionality is the first step. This involves selection and blending of crude lecithins of different origins according to the phospholipid composition, adjustment of the viscosity and bleaching (e.g. using hydrogen peroxide). Modification of the crude lecithin (by physical, chemical and enzymatic methods) is a second step. A third step includes the production of enriched phospholipid fractions (Whitehourst, 2004).

3.1.1 Physical modification

The principle behind physical modification consists in the separation of individual components or component groups from the matrix to obtain materials with widely different functionalities. It also involves carrying out modifications in the polar head group distribution of the native phospholipids (Joshi et al., 2006).

3.1.1.1 Separation of neutral and polar lipids

The separation of neutral and polar lipids (de-oiling) is the most important fractionation process. It is based on the fact that polar lipids (glycolipids and phospholipids) are insoluble in acetone whereas neutral lipids are soluble in acetone (Joshi et al., 2006). The aim of de-oiling is to concentrate and purify phospholipids that results in significantly lower dosage requirements and higher functionality. Moreover, de-oiled products have a more neutral taste than the corresponding liquid products.

3.1.1.2 Supercritical carbon dioxide extraction

CO₂ at a temperature of about 40°C and at a pressure of about 300 bar has solubility characteristics similar to liquid acetone. It dissolves neutral lipids, leaving behind the polar substances. Furthermore, CO₂ and oil are easy to separate, and the recovered gas is reused in the process (Schneider, 1989). The advantages of this process are the absence of oxygen and solvent residues. The low oil-dissolving capacity of CO₂, the
subsequent high solvent-to-feed ratios and the low yield need further process development before a plant-scale de-oiling operation may become economically interesting (van Nieuwenhuyzen et al., 2008).

3.1.1.3 Fractionation of de-oiled lecithin with alcohols

Hereby, ethanol or ethanol–water mixtures are used, because of the differences in solubility of the phospholipids in ethanol. Phosphatidylcholine in particular is readily soluble, whereas phosphatidylinositol and phosphatidic acid are virtually insoluble. Phosphatidylethanolamine, such as the neutral lipids, is found in both fractions. Alcohol fractionation is interesting because of the different technological properties of the fractions obtained: the alcohol-soluble fraction has improved emulsification capabilities in oil-in-water emulsification properties, whereas the insoluble fraction provides better water-in-oil emulsion. The fractionation method with alcohol, in principle, can be used on lecithins of natural composition (used in food industry), on modified lecithins and on de-oiled lecithins (Bueschelberger, 2004).

Sipos et al. (1996) evaluated the composition of a commercially prepared ethanol-soluble fractionated de-oiled lecithin, and reported a phosphatidylcholine fraction containing 40–60% PC, and the ethanol-insoluble phosphatidylinositol fraction containing 40–60% PI. A more recent study reported an ethanol-soluble phosphatidylcholine fraction containing 37% PC and 6.5% PE, the ethanol-insoluble phosphatidylinositol fraction containing 27% PI, 15% PE, and 3.3% PC (Wu et al., 2004).

Cabezas et al. (2011) also determined the phospholipid composition from modified sunflower lecithins, obtained by deoiling, fractionation with absolute ethanol (PC and PI enriched fractions), and enzymatic hydrolysis with phospholipase A. They reported a marked difference regarding the phospholipid composition between these samples and native sunflower lecithin. Phosphatidylcholine and phosphatidylinositol enriched fractions exhibited the highest concentration of phosphatidylcholine (44.3%) and phosphatidylinositol (35.2%), respectively.

Values corresponding to fractionated corn lecithin were not found in literature.

3.1.1.4 Membrane technology

Hexane-resistant (ceramic) membranes remove phospholipids from the crude oil miscella feed. The phospholipids (retentate) are bound in large micelles, which do not pass the membrane, while the triacylglycerols pass with the hexane through the membrane in the permeate (van Nieuwenhuyzen et al., 2008).
3.1.2 Chemical modification

Lecithin properties can be improved by chemical treatment, but for safety reasons several of them are not allowed in food applications. Nonetheless, hydrogenation, hydroxylation, acetylation, and others can produce modified lecithin with improved heat resistance, emulsifying properties, and increased dispensability in aqueous systems (Szuhaż, 2005).

3.1.2.1 Hydroxylation

This process involves the addition of hydroxyl groups at the points of unsaturation of the phospholipids' fatty acid chains, using high concentrations of hydrogen peroxide in combination with acids (e.g. acetic acid or lactic acid), increasing the hydrophobicity of the phospholipids, i.e. improving emulsifying properties of lecithins for O/W emulsions (Sai Prasad et al., 2011, van Nieuwenhuyzen et al., 2008, Whitehourst, 2004).

3.1.2.2 Acylation

In this process, an acyl group is introduced to reduce the amino group of the phospholipid. The most common acylation is done with acetyl group (acetylation), mainly because of its availability. A common practice is to treat lecithin with 2–5% acetic anhydride (Joshi et al., 2006). Acetylated lecithin mixtures are excellent O/W emulsifiers and also exhibit good thermal stability, due to the fact that there is no longer a primary amino-group available for Maillard reactions with the carbohydrate constituents of the lecithin (Whitehourst, 2004).

3.1.2.3 Hydrogenation

This process is done to convert unsaturated fatty acids to saturated fatty acid. In this way, oxidation is avoided (Whitehourst, 2004). Hydrogenation is performed by treating lecithin with catalysts such as nickel, palladium, rhodium or platinum at high temperature and pressure, until the iodine number (which is a measure of the degree of unsaturation) drops (Joshi et al., 2006).

3.1.3 Enzymatic modification

The use of enzymes offers possibilities of greater selectivity and can yield products that cannot be made by other methods. The added value of using enzymes is its substrate specificity (Joshi et al., 2006). Another advantage of using enzymes is that these type of reactions (hydrolysis) are often conducted under mild conditions, giving the possibility to retain the original properties of those heat- or oxygen-sensitive phospholipids (Guo, et al., 2005). Enzymatic modification is mostly meant to obtain more hydrophilic lecithin with
better oil-in-water emulsifying properties (van Nieuwenhuyzen & Tomas, 2008). Lipolytic enzymes are used, mainly phospholipases and lipases.

The source of enzymes is diverse, but mainly they are obtained from microorganisms (Joshi et al., 2006).

Phospholipases are named according to the ester bond they hydrolyse. Figure 3 shows schematically how enzymes work (the substrates and the positions where they cleave).

However, enzymatic modification suffers from drawbacks such as the requirement for the use of purified starting materials, low reaction quantities and a certain degree of undesired hydrolysis, besides its high cost (Joshi et al., 2006).

![Diagram of enzymes and substrates](image)

**Figure 3: Positional specificity of phospholipases (Guo, et al., 2005)**

### 4. Applications and uses

The main functional properties of lecithin are emulsification, anti-spatter, instantizing, wetting, dispersing, release/parting, and viscosity modification. These functional characteristics are primarily derived from the chemical structures of lecithin’s major phospholipids. These functional properties give lecithin a broad range of applications in food industry, such as chocolate and confectionery products, margarine, bakery goods, and pasta products. Lecithin is also used in other fields, for instance textiles, insecticides and paints (Szuhaj, 2005).

As described before, a variety of methods are available for modifying lecithin, which generates an unlimited potential for improved, tailor-made, functional products. The
following commercial lecithin modifications were described in a publication from Central Soya Co., Inc.:

1. The use of oil-free lecithins as emulsifiers, lubricity enhancing agents, and blending aids.
2. Producing low-viscosity, fluid lecithins as wetting, dispersing, and release agents.
3. Hydroxylated lecithins with enhanced emulsification, dispersing, and wetting properties.
4. Highly filtered lecithins for use in health food applications.
5. Special heat-resistant lecithins for release applications.
7. Enzyme-hydrolyzed lecithin for bakery, and emulsification applications.

One of the major functions of commercial lecithins is to emulsify fats. Commercial lecithins are used in both water-in-oil (W/O) and oil-in-water (O/W) emulsions. For W/O emulsions, like margarine or ready-to-use frostings, oil-loving, lipophilic lecithins are typically used. For O/W emulsions like sauces or infant formulas, water-dispersible, hydrophilic lecithins are typically used (Szuhaj, 2005).

5. Genetically modified foods

Genetically modified (GM) foods are foods derived from organisms whose genetic material (DNA) has been modified in a way that does not occur naturally, e.g. through the introduction of a gene from a different organism. Most existing genetically modified crops have been developed to improve the yield, through the introduction of resistance to plant diseases or of increased tolerance to herbicides (WHO). The most known GMO crop is soybean, and since soybean is the major source of lecithin, industries are hardly working to find alternatives to meet market and European Union (EU) regulations.

Worldwide legislation on GMO’s is limited. The European Food Safety Authority (EFSA) states that genetically modified (GM) foods can only be authorized in the EU if they have passed a rigorous safety assessment, which is an ongoing process (Szuhaj, 2005). Currently, EFSA approved around 150 GM crops to be cultivated and/or consumed in the EU territory. This status can easily be checked on the official European Commission website¹.

Consumers' perception is the most important issue. The concern of some consumers, as well as environmental and scientific organizations is focused on the risks of mass commercialization of GM products for human health, the environment and the socio-economic impacts that may result (Schaper et. al, 2001; Bawa et. al, 2013). Although there is no enough prove to say that GMO’s represent a risk for consumers, sometimes resistance is based on a perceived risk, which can be very far from a rational, science based assessment of the risk (Valletta, 2010).
CHAPTER II

Materials and methods
1. Lecithin samples

Three sources of lecithin were used:

- Soybean (Emulpur IP, Cargill; lot: 133209).
- Sunflower (Emulpur SF, Cargill; lot: 133273),
- Corn germ (Oilmills Vandamme, Deinze, Belgium)

The first two samples were de-oiled powdered lecithins while the third sample was a wet and oily lecithin sludge, directly obtained from the corn oil degumming process.

2. Fractionation

A mixture of hexane/iso-propanol/water was used to fractionate de-oiled powdered lecithin. A mixture of 100 mL hexane and 80 mL iso-propanol (ratio 5/4) was prepared as base solvent.

500 mg of lecithin was dissolved in 18 mL of base solvent. This was done for each of the de-oiled lecithin samples. In each of these solutions, a specific amount of water was added: 0 mL, 0.4 mL, 0.8 mL or 1.6 mL. After water addition, these samples were stirred for 1 hour. The obtained mixtures were then left to settle overnight.

The resulting supernatant was sampled with a syringe and filtered using a 25 mm VWR® syringe filter which contained a 0.45 µm nylon membrane. From this supernatant, samples were taken to determine the phosphorus content, the nitrogen content and to prepare O/W emulsions.

3. Lecithin characterization

3.1 Quantification of phospholipid phosphorus

3.1.1 Acid digestion with subsequent colorimetric determination (Van der Meeren et al., 1988)

3.1.1.1 Sample preparation

The native sample was prepared by adding 0.1 g of pure lecithin to 4 mL of water, after which an ultrasonication bath was used to obtain a homogeneous solution.

For the fractionated lecithin samples, 100 µL of supernatant (prepared in section 2) was poured into a Kjeldahl flask, after which the solvent was allowed to evaporate.
3.1.1.2 Colouring solution

A colouring mixture was prepared by adding one volume of 2.5% ammonium molybdate to 3 volumes of 4N H$_2$SO$_4$. This solution was mixed and one volume of 10% ascorbic acid was added. The colouring solution was prepared freshly every time it was used.

The used solutions were prepared as follows:

4N H$_2$SO$_4$: 20 mL of H$_2$SO$_4$ (96%) was diluted with 180 mL deionised water.

10% ascorbic acid: 10 g of ascorbic acid was dissolved in 100 mL of deionised water. It was stored in the refrigerator for stability reasons (prevent oxidation).

2.5% ammonium molybdate: 2.5 g of (NH$_4$)$_6$Mo$_7$O$_{24}$.H$_2$O was dissolved in 100 mL deionised water.

3.1.1.3 Procedure

3.1.1.3.1 Digestion of phospholipids

The first step in the procedure consisted in the release of phosphate from the phospholipids. Therefore, the lecithin sample was mixed in a Kjeldahl flask with 2 mL of H$_2$SO$_4$ (96%), 2 mL of HClO$_4$ (60%) and 4 mL of deionised water (unless the PL were already suspended in water, such as in the native samples). A funnel was placed at the top of the flask to enhance refluxing. The digestion was carried out under a fume hood and was completed after about 2 hours.

3.1.1.3.2 Neutralization

Prior to the P-determination, the digested samples were neutralized with NaOH (40%), in two steps: first, about 6 mL of NaOH was added, and after cooling, the neutralization was completed using phenolphthalein as indicator (the colour of the solution changed from colourless to pink).

After neutralization, the sample was transferred into a 100 mL volumetric flask to adjust the volume in order to contain about 5 µg P per 10 mL of solution.

3.1.1.3.3 Colorimetric determination

Ten mL of the solution was sampled for the P-determination and 4 mL of the colouring mixture was added. After incubation during 20 minutes at 45°C, the absorbance was measured at a wavelength of 820 nm and compared to a standard series containing 0 to 8 µg P per 10 ml. Finally, the amount of phospholipids was calculated based on an average phospholipid molar mass of 750 g/mol (containing 31 g of P per mol). Hence,
the P-content must be multiplied by about 25 \((\approx 750/31)\) to obtain the PL-content, using equation 1:

\[
\frac{\mu g P}{10 mL} \times \frac{100 mL}{0.1 mL} \times \frac{18 mL}{0.5 g} \times \frac{750 g}{30.97 g} \times 100
\]

(1)

Herein:

a) The first term corresponds to the value obtained during colorimetry and interpolated using a calibration curve.

b) The second term corresponds to the final volume (100 mL) after neutralization, and to the volume of sample (0.1 mL) taken for the digestion step.

c) The third term corresponds to the sample preparation, as specified in section 2 (18 mL/0.5 g).

d) The fourth term corresponds to the molecular weight of lecithin and phosphorus, respectively.

Overall, equation (1) indicates that the experimentally determined P-content (in \(\mu g P/10 mL\)) must be multiplied by a factor of about 9 to obtain the phospholipid content of the lecithin (expressed in %) provided that 0.1 mL is sampled of a solution of 500 mg per 18 mL of solvent.

3.1.1.4 Inorganic phosphorus determination

In order to assess some hypotheses about the former method, the influence of acids, triglycerides and lecithin on the detection method described in section 3.1.1.3 was examined.

Tests on samples containing lecithin were run to evaluate the influence of digestion time on the recovery of phosphorus. Moreover, samples containing inorganic phosphorus (mono-potassium phosphate) in combination with acids (either including or excluding the digestion step (heating)), and samples containing inorganic phosphorus in combination with acids and triglycerides were also evaluated.

3.1.1.4.1 Procedure

An aqueous phosphate solution was prepared: 10.98 mg of \(KH_2PO_4\) was dissolved in 200 mL of deionised water (sample without acids).

To 4 mL of the previous aqueous phosphate solution, 2 mL of \(H_2SO_4\) (96%) and 2 mL of \(HClO_4\) (60%) was added. This solution was diluted to 200 mL using deionised water (sample with acids).
Once the samples were prepared, the colorimetry step was carried out for both samples, as described in section 3.1.1.3.3.

To prepare the third sample, 4 mL of the aqueous phosphate solution prepared previously were mixed with 2 mL of H₂SO₄ (96%) and 2 mL of HClO₄ (60%) in a Kjeldahl flask (sample without triglycerides). Then, to prepare the fourth sample, 0.5 g of sunflower oil was dissolved in 18 mL of solvent (hexane). An amount of 100 µL was mixed with 4 mL of aqueous phosphate solution, 2 mL of H₂SO₄ (96%) and 2 mL of HClO₄ (60%) in a Kjeldahl flask (sample with triglycerides).

The influence of digestion time was examined. Samples were taken after 15, 30, 60 and 120 minutes of heating.

The digested samples were neutralized as explained in section 3.1.1.3.2 and the colorimetric determination was carried out as in section 3.1.1.3.3

3.1.2 Phosphorus determination using TNT Reagent Set, Low Range (HACH®)

3.1.2.1 Sample preparation

3.1.2.1.1 Native sample

The de-oiled powdered samples were dissolved in two solvents (water and hexane). For the water dilution, 0.5 g of powdered lecithin was dispersed in 18 mL of ultrapure water (Milli-Q), using ultra-sonication. Ultra-sonication is defined as the irradiation of a liquid sample with ultrasonic (>20 kHz) waves resulting in agitation. Sound waves propagate into the liquid media and result in alternating high-pressure (compression) and low-pressure (rarefaction) cycles, creating high local temperatures (Royal Society of Chemistry).

For the hexane dilution, 0.5 g of powdered lecithin was dissolved in 18 mL of hexane by magnetic stirring.

Afterwards, the sample was transferred into a volumetric flask and the volume was adjusted in order to contain a concentration of 0.06-3.5 mg/L of PO₄³⁻, as required by the specifications of the Reagent Set. To that end, 100 µL of the mixture was first evaporated, and subsequently dispersed in 200 mL of ultrapure water (Milli-Q). If the lecithin would only contain phospholipids (with a molar mass of 750 g/mol), this procedure would give rise to a dispersion that contained 1.76 mg/L of phosphate, i.e. half of the specified maximum.
For the crude lecithin samples, 1.5 g was dispersed in 18 mL of deionised water. This amount was calculated taking into account the water content (about 50%) and the oil content (about 35% of the dry matter) of lecithin sludges (Scholfield, 1981).

### 3.1.2.1.2 Fractionated sample

100 µL of filtered supernatant (prepared in section 2) was transferred into a 200 mL volumetric flask. After evaporation of the solvent, deionised water was added up to the mark in order to stay within the specified range (0.06-3.5 mg/L of phosphate) of the Reagent Set.

### 3.1.2.2 Procedure

The procedure followed corresponds to the Total Phosphorus determination by PhosVer3 Ascorbic Acid Method with acid persulfate digestion (HACH® method 8190). Hereby, 5 mL of the previously prepared aqueous sample was added to the Total Phosphorus Test vial, together with the packet of potassium persulfate powder. The vial was shaken to dissolve the sample and the powder and then was inserted into the reactor (HACH® slot COD Chemical Oxygen Demand reactor), pre-heated at 150ºC for sample destruction. After 30 minutes, the vial was taken out and was cooled to room temperature. Subsequently, 2 mL of 1.54 N NaOH was added to the vial and it was shaken. Once the HACH® DR/2010 Spectrophotometer was set up with a wavelength of 890 nm, the vial was cleaned (to avoid fingerprints and any other impurities that could interfere with the UV measurement) and inserted into the 16 mm cell holder and zeroed. Afterwards the content of one PhosVer3 powder packet (containing ascorbic acid) was added to the vial, it was shaken and after 2 min reaction time, the vial was cleaned and inserted into the 16 mm cell holder to read the result.

### 3.2 Phosphorus nuclear magnetic resonance measurement (\textsuperscript{31}P-NMR Spectroscopy)

Phosphorus nuclear magnetic resonance (\textsuperscript{31}P-NMR) was used to analyse native and fractionated lecithin samples.

#### 3.2.1 Materials

- NMR tubes (Routine NMR tubes 600 MHz; 5x178 mm (7"), Bruker)
- Deuterated water (99.8 atom%; Armar Chemicals)
- Sodium deoxycholate (98%, dry matter; Sigma-Aldrich Bioxtra)
- Glyphosate Pestanal ® (Sigma-Aldrich)
3.2.2 Sample preparation

3.2.2.1 Method I

Samples were prepared adding 200 mg (20 mg.mL\(^{-1}\)) lecithin to a mixture of 5 mL deionised water and 5 mL D\(_2\)O (ratio 1:1), which also contained 1.11 g of sodium deoxycholate (10% (w/w)), 7.44 mg of Na-EDTA (2 mM; Acros Organics) and 1.69 mg glyphosate (1 mM) as internal standard for quantification, as described previously by Kasinos et al. (2014). The samples were then stirred until a homogeneous solution was obtained, after which the pH was adjusted to 8.0 with NaOH (1M).

3.2.2.2 Method II

Method II is based on a method developed by SynBioc at Ghent University (Stevens et al., 2014). Samples were prepared weighing an amount of lecithin between 700 mg and 900 mg, to which 5.3 mg triphenyl phosphate (internal standard) was added. To this mixture, 1 mL of chloroform and 1 mL of Cs-EDTA (0.2 M, pH 8) were added. This mixture was vortexed until obtaining a homogeneous solution. Then, after addition of 1 mL of methanol and two droplets of D\(_2\)O, it was stirred (magnetic stirrer) for 1 min and centrifuged for 4 min at 4000 rpm. The lower phase (organic phase) is sampled and poured into an NMR tube for analysis.

3.2.3 Procedure

The NMR tubes were filled (over a height of about 4 cm) with the samples. \(^{31}\)P NMR spectra were obtained using a Bruker Ascend 400 (Rheinstetten, Germany) operating at a \(^{31}\)P frequency of 161.98 MHz, equipped with a BBOFO 5 mm probe. Inverse gated proton decoupling was used for suppression of the Nuclear Overhauser Effect (NOE). Measurements were performed at 25ºC and the temperature was controlled to within ± 0.01ºC. The following instrument settings were used: 131072 data points, 30 excitation pulse, and number of scans = 256 with a 10.00 s. relaxation delay time and a decay acquisition time of 5.38 s. The solvent for method I was D\(_2\)O and for method II chloroform.

3.3 Determination of the nitrogen content using the Kjeldahl method

The Kjeldahl-method is based on the wet combustion of a sample by heating with concentrated sulphuric acid in the presence of metallic catalysts to effect the reduction of organic nitrogen in the sample to ammonia, which is retained in a solution as ammonium sulphate. It is commonly used for protein quantification, but in this case, it is used to quantify the nitrogen-containing phospholipids.
3.3.1 Materials

3.3.1.1 Reagents

- Concentrated sulphuric acid (density: 1.84 g/ml)
- K₂SO₄
- CuSO₄
- NaOH (50%) + thiosulphate solution (8 w/w %)
- Deionised water
- Boric acid indicator solution (2 % boric acid (W/V) + 0.75 % Mish indicator (V/V)
- Hydrochloric acid (0.05 N)

3.3.1.2 Apparatus

- Kjeldahl tube
- Destruction equipment
- Steam distillation equipment (Kjeltec)

3.3.2 Sample preparation

For soybean and sunflower lecithin, 15 mL of supernatant (prepared as described in section 2) was taken for the analysis. Hereby, the solvent was evaporated once the supernatant was in the Kjeldahl tube.

For corn lecithin, 0.5 g of lecithin was taken for the analysis. The water that was present in the sludge was evaporated before weighing and adding the reagents.

3.3.3 Procedure

3.3.3.1 Destruction

Samples were transferred in a Kjeldahl tube. A glass pearl was added, as well as 10 mL H₂SO₄, 0.5 g CuSO₄ and 5 g K₂SO₄. The destruction was carried out in a destruction block until a clear solution was obtained, to get complete breakdown of all organic matter (about 1.5 h). The tubes rested until room temperature was reached.

3.3.3.2 Distillation

The tubes were placed in the distillation equipment. Here, 30 mL of a NaOH/thiosulphate solution was added, and the ammonia was distilled into a 20 mL boric acid indicator solution.
The resulting solution was further titrated with 0.05 N HCl, until a colour change from brilliant green to purple was observed.

### 3.3.3.3 Calculation

The amount of nitrogen-containing phospholipids (i.e. PC, PE and PS) was estimated based on an average molar mass of 750 g/mol phospholipids:

\[
\frac{g \text{ NPL}}{15 \text{ ml supernatant}} = N \times V \times 750 \quad (2)
\]

N = normality of the HCl (N)
V = volume used of HCl (L)
NPL = Nitrogen-containing phospholipids

### 3.3.4 Determination of Nitrates and Ammonia

Because of the crude nature of the corn lecithin sample, also inorganic nitrogen was measured separately.

#### 3.3.4.1 Sample preparation

Corn lecithin was diluted in order to obtain a concentration within the detection range (maximum of 20 mg N/L), in this case 1.5 g of corn lecithin (moisture content of about 50%) was dissolved in 500 mL deionised water, using ultra-sonication.

#### 3.3.4.2 Procedure

Once the sample was prepared, it was delivered to the ISOFYS department at Ghent University for its analysis. The analyses run were based on Auto-Analyser Multi-test Methods. The samples were filtered before injection into the Auto-Analyser.

For nitrate determination (G-287-02), the nitrate in the sample is reduced to nitrite by a copper-cadmium reduction coil at pH of 8.0. The nitrite ion then reacts with sulfanilamide under acidic conditions to form a diazo compound. This compound then couples with N-1-naphthylene diamine dihydrochloride to form a reddish-purple azo dye, which is colorimetrically measured at 520 nm.

For ammonia determination (G-102-93), the sample is reacted with salicylate and dichloroisocyanuric acid to produce a blue compound, which is measured at 660 nm (nitroprusside is used as a catalyst).


3.4 Moisture content of corn lecithin samples

The moisture content was determined using the oven-drying method. The method is based on measuring the mass of the sample before and after the water is removed by evaporation at 105°C:

\[
\% \text{moisture} = \frac{\text{initial mass} - \text{final mass}}{\text{initial mass}} \times 100 \tag{3}
\]

The basic principle of this technique is that water has a lower boiling point than the other major components (not taking into account volatiles, which are lost during the process and are not quantified).

3.4.1 Materials

- Aluminium petri dish
- Forced Convection Oven
- Dessicator
- Balance

3.4.2 Procedure

About 5 g of lecithin sludge was weighed in an aluminium petri dish (previously weighed). It was taken into the oven for the drying process to start. The oven was pre-heated at 105°C. The weight was determined on a daily basis until a constant mass was reached (3 days). Calculations were done using equation (3).

4. Functionality

4.1 Oil-in-Water emulsion preparation

Five wt-% oil-in-water emulsions were prepared. First, each of the supernatants (prepared in section 2) were poured in a round-bottom flask, to evaporate the solvent using a rotary evaporator (rotavapor). 0.12 g of the remaining fractionated lecithin is added to 18.88 g of a 0.02 wt-% NaN₃ solution (0.02 g NaN₃ was weighed and deionised water was added to a total of 100 g).

These samples were put in an ultra-sonication bath until the mixture was homogeneous, after which 1 g of sunflower oil (100%, Carrefour) was added and a pre-emulsion was formed by mixing at 24000 rpm for 5 minutes, using an Ultraturrax S25-10G (IKA®-Werke, Germany). This pre-emulsion was further processed by using a Microfluidizer (M-110S) at 840 bar for 1 minute of recirculation through a water bath at 25°C.

The resultant emulsions were stored at 5°C and the droplet size determined directly after preparation (day 0) and after 1, 4 and 23 days of storage.
Before each analysis, samples were shaken to homogenize the emulsion.

4.2 Droplet size distribution measurement

The droplet size of each O/W emulsion was measured using two techniques.

For the first technique, a laser diffraction particle size analyser (Mastersizer 3000, Malvern Instruments Ltd) was used. This technique is applied to measure particles with a diameter between 0.05 and 900 μm. Data-analysis was performed using the Mie theory, whereby the refractive index of the material (sunflower oil) was set to 1.465 + 0.01i, whereas the refractive index of the continuous phase was 1.33. Samples were diluted in deionised water until an obscuration between 2 and 6%, with a shear speed of 1500 rpm.

The values taken into account for the discussions correspond to the volume-weighted mean diameter ($D_{4,3}$) of all the particle volumes forming the entire emulsion, which is calculated according to equation (4):

$$D_{4,3} = \frac{\sum n_i \times d_i^4}{\sum n_i \times d_i^3}$$  \hspace{1cm} (4)

Where $n = \text{number of particles}$ and $d = \text{diameter}$.

Second, an analysis with Photon Correlation Spectroscopy (PCS) was performed, which is also known as Dynamic Light Scattering. The latter technique can only be used to evaluate the size of nanoparticles. The equipment consisted of a 15 mW He-Ne laser, a spectrometer PCS-100 SM (Malvern) and digital correlator K7032CN (Malvern). Analyses were performed at 25°C and at a scattering angle of 150°.

4.3 1D pfg NMR profilometry of Oil-in-Water emulsions

One dimensional pulsed field gradient NMR profilometry (1D pfg NMR) is used to characterize the cream layer of the O/W emulsion. The advantages of this method are that it is fast and non-destructive (Vermeir, 2011).

The application of low-field 1D pfg NMR profilometry is based on the manipulation of the strength of the applied magnetic field on the precession of the spin, derived from the Larmor frequency. Hence, the water content at different positions is correlated to the NMR signal intensity at different frequencies. Due to the exponential decay of transverse relaxation, the acquired signal intensity ($I$) is a function of the signal intensity at time zero ($I_0$), of the time at which the signal is acquired ($t$), and of the transverse relaxation time ($T_2$) of the sample (Wuxin, et al., 2011):
Profilometric analysis was performed with a Maran Ultra 23 spectrometer, using a script in the RINMR software, as described by Vermeir (2011). Measurements were performed at 5°C, with a frequency offset of 46508.22 Hz and an inversion time (τ-value) of 6 ms, unless stated differently.

As water and oil have different $T_2$-relaxation times, it should be possible to filter out the oil signal in 1D pfg-NMR profilometry. Theoretically, increasing the inversion time will lead to a complete annihilation of the oil signal, as shown in figure 4, where $t = 2 * \tau$. At a τ-value of at least 100 ms, the oil signal ($I/I_o$) should be nearly completely eliminated, whereas the water signal is still relatively unaffected. The triangles in figure 4 represent the ratio of the relative water to oil signal. Whereas this ratio increases with τ, still a too large τ-value is not desirable as the water signal itself becomes reduced and hence more susceptible to experimental noise.

![Figure 4: Simulated relative response of oil and water as a function of twice the inversion time (tau-value)](image)

To confirm this simulated response, the optimal τ-value was determined.

As the measurement window is located between 22 and 47 mm (Wuxin et al., 2011), the tubes were filled up to 40 mm with a sample containing 30 mm of deionised water and 10 mm of sunflower oil. Afterwards the optimal settings were applied to characterize the cream layer of O/W emulsions.

### 4.3.1 Sample preparation

Emulsions were prepared as indicated in section 4.1, after which they were poured into NMR tubes until a height of about 40 mm. Tubes were kept at 5°C, to allow them to cream.

1D pfg NMR profiles were determined at day 4, day 22, day 30 and day 50.
4.4 Effect of mono- and multi-valent ions on lecithin-stabilized Water-in-Oil emulsions

Lecithins are widely used as O/W emulsifiers. Previous research indicated that their emulsifying activity in W/O applications was poor. As multivalent ions are known to bind strongly to the phosphate headgroup of phospholipids, this might reduce the negative charge of the headgroup and as such the degree of hydration. According to the oriented wedge theory, the subsequent increase in surfactant number should be beneficial for the W/O emulsifying properties of the lecithins.

W/O-emulsions containing different concentrations of mono- and multi-valent ions in the water phase were prepared in order to qualify the effect of these elements on the emulsion’s stability.

In addition, different concentrations of lecithin in the oil phase were used to evaluate the influence of lecithin concentration on the stability of emulsions containing mono- and multi-valent ions.

4.4.1 Emulsion preparation

The emulsions were prepared by mixing an oil phase (70% w/w) with a water phase (30% w/w) previously prepared as described below.

4.4.1.1 Oil phase

12 g of soybean lecithin was weighed after which sunflower oil (100%, Carrefour) was added until a total mass of 150 g (8 wt-%). This mixture is heated until 60°C and stirred for 10 minutes using a magnetic stirrer. The same procedure was used to obtain 0.01, 0.05, 0.1, 0.5, 2 and 4 wt-% lecithin in oil.

4.4.1.2 Water phase

Different concentrations of salts were prepared:

Sodium chloride (NaCl) was used as mono-valent salt. Hereby, 374.08 mg NaCl was dissolved in 50 mL of deionised water (128 mM). This solution was further diluted to obtain concentrations of 8 mM and 32 mM.

Calcium chloride (CaCl₂·2H₂O) was used to have di-valent counterions: 470.4 mg CaCl₂·2H₂O was dissolved in 50 mL of deionised water (64 mM). This water phase was then diluted to obtain 1 mM, 2 mM, 4 mM, 8 mM and 16 mM.
As tri-valent salt, cerium chloride (CeCl$_3$.7H$_2$O) was used: 525 mg of CeCl$_3$.7H$_2$O was dissolved in 50 mL of deionised water (28.18 mM). This water phase was then diluted to obtain concentrations of 7.05 mM, and 1.76 mM.

4.4.1.3 Preparation

14 gram of oil phase at 60 °C was mixed at 6500 rpm with an Ultra-Turrax S25-10G (IKA®-Werke, Germany) for 1 minute while adding gradually 6 gram of the water phase. The emulsion was further homogenized by mixing for five additional minutes at 17500 rpm.

4.4.2 Centrifugation

Centrifugation is one of the most important techniques used in analytical chemistry, which uses the centrifugal force to separate 2 phases based on their density (Orozco, 1998). In this case, it is used to accelerate the phase separation of W/O emulsions prepared with soybean lecithin in combination with mono and multivalent ions, with the purpose to have a descriptive evaluation of the separated phases.

In this sense, W/O emulsions were centrifuged at 4200 rpm for 1 hour (Sigma 3-16P, rotor 11180, VWR® 50 mL tubes).

4.4.3 Light Microscopy

W/O emulsions were evaluated under the microscope to assess the droplet size in the different concentrations. Image acquisition was conducted using an optical microscope (Olympus Cx40) with a 100x magnification coupled with a digital camera (AxioCam ERc 5S).

4.5 Electrophoretic mobility

Electrophoresis is defined as the migration of charged colloidal particles or molecules through a stationary medium under the influence of an applied electric field. The electrophoretic mobility ($\mu$) of a charged particle is defined as the ratio between its stationary velocity ($v_c$) and the applied electric field (E) (Landers, 2007),

$$\mu = \frac{v_c}{E} \left[ \frac{\mu m + c m}{s + V} \right]$$  \hspace{1cm} (6)

Different Ce$^{3+}$ to lecithin ratios were prepared in order to see the influence of tri-valent ions on the charge of lecithin in dispersion.
4.5.1 Sample preparation

A suspension containing 0.2 wt-% lecithin in 30 mL of water containing different CeCl₃ concentrations was prepared.

To prepare the CeCl₃ stock solutions, 0.993 g CeCl₃·7H₂O was weighed and dissolved in 1 L deionised water. Four additional CeCl₃ solutions were prepared from this stock solution by diluting 2, 3, 4 and 5 times.

The electrophoretic mobility of each sample was measured five times at 25°C.

5. Statistical analysis

To evaluate the phospholipids content in soybean lecithin and sunflower lecithin, a paired test was performed, to assess the difference between both, based on TNT Reagent Set (HACH®) test results.

To evaluate the influence of fractionation on the stability of the O/W emulsion over time, a two way Analysis of Variance (ANOVA) was performed, assessing the effect of the amount of water added and of time on the droplet size. Also interaction between amount of water added and time was checked. After which a post hoc analysis with 2-sided Tukey’s HSD with a family-wise error type at the 95% confidence level was performed, comparing in first place, the droplet size of samples with water added to the droplet size of native samples and in second place, comparing droplet size over time to droplet size at day 0.

All tests were run with a 5% level of significance. The software used for the analysis was Spotfire S+ 8.2.
CHAPTER III

Results and discussion
1. Fractionation

Edible oils are degummed after extraction as one of the first steps of the refining process, with the aim of removing phosphatides and other impurities.

The degumming process consists of adding water to the oil at high temperature. In this sense, phosphatides will become hydrated and thereby, oil-insoluble. A further centrifugation step will separate the gum phase from the oil phase.

Münch (2007) mentioned that the hydration of phosphatides depends on the phosphorus containing-head group, the larger the head group the more hydratable is the phosphatide, i.e. PC is the largest, hence the most hydratable and PA is the smallest, hence the least hydratable.

In this work, lecithin was dissolved in a hexane/isopropanol mixture after which different amounts of water were added. Once the water was added, the samples were stirred for one hour obtaining a transparent yellow brownish solution, which was kept overnight to settle, allowing compounds to hydrate, which forced their separation from the solvent phase (which contained the phospholipids). In this case, the intention was to hydrate residual impurities and separate them from the solvent phase.

It is clear that the objective was to enhance the lecithin emulsification properties. Nevertheless, some phospholipids may have migrated to the water phase because of their amphiphilic behaviour and some of them may be lost since equilibrium needs to be reached to have a complete phase separation (Dijkstra & Van Opstal, 1989).

After settling, a visual observation revealed that some sediment was formed at the bottom of the lecithin solutions, as expected. The amount of sediment seemed to increase with increasing water content. The supernatant was collected and filtered.

In order to give more comprehensible and easy reading results, some abbreviations are used regarding samples that have been fractionated. In this sense, 0 mL corresponds to the fractionated sample with 0 mL water added, 0.4 mL corresponds to the fractionated sample with 0.4 mL water added, and the same principle is applied to 0.8 mL and 1.6 mL. The native sample corresponds to the lecithin sample that has not been fractionated neither filtrated and that was dispersed in deionised water.

The corn lecithin sample could not be fractionated as this crude lecithin sample already contained about 50% water. Therefore, only native samples were analysed.
2. Lecithin characterization

2.1 Moisture content of corn lecithin sludge

As the corn lecithin was sampled directly after the wet degumming of corn oil, it still contained a significant amount of water. In order to correct for the water content, the loss of mass on drying at 105°C was determined. After 3 days, a stable mass was obtained, from which the moisture content was finally determined (oven-drying method). The analysis was done in quadruplicate, and resulted in water content percentages of 52.18, 55.57, 52.14 and 53.44. Based on these four values, the average water content and the standard deviation was calculated to be 53.33 ± 1.39 %.

2.2 Quantification of phospholipid phosphorus

2.2.1 Acid digestion with subsequent colorimetric determination (Van der Meeren et al., 1988)

First, a calibration curve was determined, using potassium di-hydrogen phosphate (KH₂PO₄). The curve is shown in figure 5. It is relevant to mention that for each determination, a new calibration curve was determined.

Subsequently, the content of phospholipids was determined in the different fractionated lecithin samples. Therefore, 100 µL of supernatant of each sample was used in the analysis. For this analysis, only soybean lecithin was evaluated. Following 2 hours of digestion, the phosphate content of the diluted samples was determined by comparing the experimentally determined absorbance values with the data of the calibration curve.

In a next step, the phospholipid content was calculated, taking into account the dilution factors, the initial sample amount, and the molecular weight of phospholipids and phosphorus, as expressed by equation (1).
Assuming that the total amount of phosphorus is equal (on a molar basis) to the amount of phospholipids in the lecithin sample, figure 6 shows the results for the phospholipids content in the different fractions for different days of analysis, which were carried out always with the same sample and same conditions.

![Graph showing phospholipid content in different fractions for different days of analysis.](image)

**Figure 6: Amount (%) of PL in soybean lecithin samples. Analyses of same samples were carried out on different days (shows the variability of the method)**

Whereas the calibration curves were highly reproducible (with slopes of 0.059, 0.064, 0.058 and 0.060 on 09/09, 06/10, 25/10 and 13/11, resp.), the phospholipid content results were not reproducible, as reflected by the large variance between the different repetitions, considering that the conditions and the soybean lecithin source were the same. This variability is shown in table 2, which specifies the average, as well as the standard deviation, based on four individual values.

**Table 2: Amount (%) of PL in the soybean lecithin samples plus standard deviation (SD). Analyses of the same samples were carried out on different days**

<table>
<thead>
<tr>
<th>SAMPLE (ml of water)</th>
<th>% PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>46.2 ± 10.0</td>
</tr>
<tr>
<td>Fractionated (0.0)</td>
<td>52.7 ± 9.2</td>
</tr>
<tr>
<td>Fractionated (0.4)</td>
<td>49.0 ± 5.1</td>
</tr>
<tr>
<td>Fractionated (0.8)</td>
<td>48.2 ± 8.9</td>
</tr>
<tr>
<td>Fractionated (1.6)</td>
<td>55.8 ± 15.6</td>
</tr>
</tbody>
</table>

Van der Meeren et al. (1988) reported a content of more than 86% of phospholipids in powdered soybean lecithin. In contrast, in this measurement, it was only possible to recover about 50% in the native lecithin.
In the case of the fractionated samples, taking into account the average percentages, no reliable trend has indicated that the phospholipids content decreased or increased with the amount of water added, and the variance between same samples is high.

The causes of the large variance observed may be due to an incomplete conversion of the phospholipids to phosphate, affected by digestion conditions like time of digestion or temperature variance during digestion, to the neutralization step, to the incubation period before absorbance measurement or to the preparation of a homogeneous solution of the samples during dilutions steps (before or after digestion).

To test these hypotheses, some additional tests were performed.

Digestion of native lecithin samples (dissolved in hexane, 2.7% lecithin) was evaluated over time, in duplicate (Series 1 and Series 2). Results are shown in figure 7.

![Figure 7: Amount (%) of phospholipids (native soybean lecithin) as a function of the digestion time](image_url)

The effect of an increase of the digestion time is clearly a decrease in the percentage of phospholipids. On the contrary, Van der Meeren et al. (1988) obtained a complete digestion after 15 min, and the values were constant when further increasing the digestion time. Yet, in this case, the high percentage obtained after 15 min could be explained due to the high organic matter still present, which may interfere during colorimetry. After 3 hours of digestion, the phospholipid content was still decreasing, which is another indication of some interference between other components and the colorimetric method. As these components probably degrade over time, the absorbance also decrease.

Furthermore, the effect of acids on the detection method was assessed. To do so, a test on samples without lecithin was run.

The colorimetric step of the samples was performed without the digestion step, i.e., without heating the samples. The results are shown in Table 3. The variability is about...
1% (based on absorbance), with a loss of phosphorus (100% corresponds to 5µg per 10 mL), which indicates that acids have no interference on the detection method.

Table 3: Amount of inorganic phosphorus measured using colorimetry without heating step, where 100% equals 5 µg P per 10ml

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance (-)</th>
<th>Concentration of P (µg P/10ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With acid added</td>
<td>0.270</td>
<td>4.46</td>
</tr>
<tr>
<td></td>
<td>0.285</td>
<td>4.71</td>
</tr>
<tr>
<td>Without acid added</td>
<td>0.280</td>
<td>4.63</td>
</tr>
<tr>
<td></td>
<td>0.300</td>
<td>4.96</td>
</tr>
</tbody>
</table>

Additionally, the influence of digestion time was evaluated on samples without lecithin, but containing inorganic phosphorus with and without triglycerides.

The phosphorus content of the samples without triglycerides over time is shown in figure 8, from where it is possible to see that the concentration of phosphorus is on average, 12% less than expected (not considering the samples at 15 min), i.e., less than 5 µg per 10 mL. However, after 30 min of digestion, the recovery was almost constant, with an average and standard deviation of 4.41±0.01 µg P/10mL.

The sample taken after 15 min digestion cannot be considered reliable. At simple visual inspection, the sample still was yellowish coloured.

![Figure 8: P-concentration as a function of digestion time when no triglycerides were added](image)

For the samples with triglycerides, the phosphorus content over time is shown in Figure 9. The graph clearly shows a decrease of phosphorus content over time, which was the same trend observed with lecithin samples. The expected concentration was 5 µg per 10 mL.
CHAPTER III. RESULTS & DISCUSSION

After digestion for 15 min, the obtained P-concentration is 8% lower than expected. After 30 min, 17% less, after 60 min, 29% less and after 120 min, 30% less phosphorus was obtained.

A possible explanation of this experience is that phosphorus bind to other compounds, which are no longer digested, the detection method (colouring solution) is no longer able to detect it, and hence, low concentrations are obtained.

In conclusion, it can be said that some components that are inherent to the lecithin or the triglycerides play an important role on the recovery and/or detection of phospholipids over time.

2.2.2 Phosphorus determination by TNT Reagent Set, Low Range (HACH®)

The phospholipid content was determined in the different samples, following the protocol of the Total Phosphorus determination method using the TNT Reagent Set (HACH®). The instrument’s results are directly expressed in mg of phosphate per litre. As 1 µg/10 mL corresponds to 0.1 mg/L, and considering the molar mass of phosphorus (31 g/mol) and phosphate (95 g/mol), the slope (mg PO₄³⁻/L read versus µg P/10 mL added) is expected to be equal to (95/31)/10=0.306. The experimental curve is shown in figure 10, which slope (0.288) is indeed close to the expected value.

In a second step, the different lecithin samples were analysed. The phospholipid content was calculated by comparing the experimental readings to the standard series (calibration curve), taking into account the dilution factors, the initial sample amount and the molar weight of phospholipids and phosphorus.
Assuming that the total amount of phosphorous is equal (on a molar basis) to the amount of phospholipids in the different lecithin samples, figure 11 shows the results for the phospholipid content in the native lecithins (dissolved in water and in hexane), and the fractionated lecithins with different amounts of water (in mL) added to 18 mL of hexane/iso-propanol solutions.

The PL content of the soybean lecithin sample decreased with the amount of water added to a hexane/iso-propanol solution. The no-water-added sample had a phosphorus content higher than the water-added samples. It can be said that the decrease is of about 30% in the 1.6 mL fractionated sample.

The native samples dispersed in water and dissolved in hexane had a PL content of 86% and 82%, respectively. This difference may be due to the difference in solubility in both solvents during the dilutions steps. The 100 µL sample dissolved in water, had no problem to be diluted in water, whereas for the 100 µL sample dissolved in hexane, there may be difficulties to dilute in water although the solvent was evaporated.
The PL content of the sunflower lecithin followed the same trend, i.e. it decreased with the amount of water added during fractionation. In this sense, the decrease is about 25% in the case of 1.6 mL sample. The native samples dispersed in water and dissolved in hexane had a PL content of 83% and 74%, respectively.

Furthermore, the PL content of the corn lecithin was lower compared with the other lecithin. Dispersing the corn lecithin in water, the phospholipid content resulted in about 50% based on dry basis.

Comparing the soybean phospholipid content to the sunflower phospholipid content, the mean sunflower phospholipids content was significantly different from the mean soybean phospholipids content (p-value=0.0162). In all samples, soybean lecithin had a higher phospholipid content. In the case of fractionated samples, both lecithins presented a decreasing trend with the addition of water during fractionation.

These results indicate that to obtain the same phospholipid content as soybean lecithin, a higher amount of sunflower or corn lecithin is necessary because the phospholipid content is lower in the latter lecithin samples.

### 2.3 Phosphorus nuclear magnetic resonance measurement ($^{31}$P-NMR Spectroscopy)

$^{31}$P-NMR Spectroscopy was used for the analysis of the phospholipid composition of native and fractionated samples of soybean lecithin, and native sunflower and corn lecithin, to see if there is a difference in phospholipid composition, next to phospholipid content. Hence, the question is whether the water-induced precipitation (fractionation) is selective for some phospholipids.

First, a comparison between the two sample preparation methods was performed. Therefore, soybean lecithin was sampled and samples for $^{31}$P-NMR were prepared following method I and method II. The peaks obtained were similar, the main phospholipids were identified in both spectra, although some chemical shifts were identified as well. It is important to mention that the chemical shifts of phospholipids depend on a series of variables, among which are temperature, pH, solvent, sample matrix, and the fatty acid chains attached to the phospholipids, which cannot be fully controlled because it is inherent to the phospholipids (Kaffarnik et al., 2013).

For the fractionated soybean lecithin samples and sunflower and corn native lecithin samples, method I was used because of its easy preparation procedure. Besides, during phase separation in method II, there could be some migration of phospholipid to the methanol phase, which is unwanted.
The spectra obtained from native lecithin samples and fractionated lecithin samples are shown in appendix I.

Concerning fractionated soybean lecithin samples, the phospholipids peaks were identified. With a simple visual inspection, there was not a big difference between the fractionated lecithin samples. Therefore, peak integration was needed. After integration, a trend could be discerned. The peak areas related to native and fractionated soybean lecithin showed the same trend that was obtained in section 3.2, a decrease of total phospholipid content as the amount of water added during fractionation increased.

The relative peak areas of individual phospholipids are shown in table 4. The PC, PI and PE peak areas of soybean lecithin were decreased as the amount of water added during fractionation increased compared to native soybean lecithin.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Native lecithin</th>
<th>Fractionated soybean lecithin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corn</td>
<td>Sunflower</td>
</tr>
<tr>
<td>PC</td>
<td>17.69</td>
<td>43.56</td>
</tr>
<tr>
<td>PE</td>
<td>26.85</td>
<td>17.88</td>
</tr>
<tr>
<td>PI</td>
<td>17.99</td>
<td>23.50</td>
</tr>
<tr>
<td>PS</td>
<td>15.19</td>
<td>3.13</td>
</tr>
<tr>
<td>PA</td>
<td>6.68</td>
<td>4.73</td>
</tr>
<tr>
<td>GPC</td>
<td>3.12</td>
<td>1.24</td>
</tr>
<tr>
<td>LPI</td>
<td>4.79</td>
<td>1.16</td>
</tr>
<tr>
<td>LPC</td>
<td>6.20</td>
<td>4.80</td>
</tr>
<tr>
<td>LPA</td>
<td>1.48</td>
<td>-</td>
</tr>
</tbody>
</table>

* from phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), lysophosphatidylycholine (LPC), lysophosphatidylinositol (LPI), lysophosphatidic acid (LPA), glycerophosphorylcholine (GPC), phosphatidyl serine (PS).

The high content of phosphatidyserine (PS) found in fractionated soybean lecithin, compared to the native sample, could be explained due to the fact that peak integration could include PS and LPC peaks, since no LPC area was possible to obtain (the peak was too small).

Furthermore, comparing soybean native lecithin with sunflower native and corn lecithin, there is also a clear difference. The PC content is higher in sunflower lecithin than in soybean lecithin and in corn lecithin. Therefore, \( \text{PC}_{\text{sunflower}} > \text{PC}_{\text{soybean}} > \text{PC}_{\text{corn}} \), whereas with PE the trend is different, \( \text{PE}_{\text{corn}} > \text{PE}_{\text{soybean}} > \text{PE}_{\text{sunflower}} \), and PI the trend is \( \text{PI}_{\text{sunflower}} > \text{PI}_{\text{soybean}} > \text{PI}_{\text{corn}} \).

The references found in literature are diverse. While van Nieuwenhuyzen & Tomás (2008), reported a PL content (only for soybean and sunflower) that indicates
PC_{sunflower} > PC_{soybean}, PE_{soybean} > PE_{sunflower} and PI_{sunflower} > PI_{soybean}. Schneider (1989) reported PC_{soybean} > PC_{corn} > PC_{sunflower}, PE_{sunflower} > PE_{soybean} > PE_{corn}, and PI_{soybean} > PI_{corn} > PI_{sunflower}.

2.4 Quantification of nitrogen-containing phospholipid

The nitrogen content was determined with the Kjeldahl method. Soybean and sunflower lecithin were used in native and fractionated form, while corn lecithin was only evaluated in native form.

The amount of nitrogen-containing phospholipids (i.e. PC, PE and PS) was estimated based on an average molar mass of 750 g/mol for phospholipids, using equation 2. The results are shown in figure 12.

![Figure 12: Amount (%) of nitrogen-containing PL in lecithin samples (soybean, sunflower and dry corn). Corn lecithin is dissected in nitrogen-containing phospholipids and ammonia content. Bars represent standard deviation](image)

The nitrogen-containing PL content in soybean samples increased with the amount of water added, but this decrease is not significant, being that there is no significant difference between the samples (p-value = 0.083). This increase is not that pronounced as it was with the decrease of total phospholipids content. $^{31}$P-NMR results did not show a reliable trend to confirm this.

In the case of sunflower samples, the case is opposite, since the nitrogen-containing PL-content decreased with the amount of water added. Here, the decrease is more marked
than in the previous case, but the decrease is not significant (p-value = 0.178), and it is the same trend observed for total phospholipids content.

Sunflower lecithin samples clearly have a higher nitrogen-containing PLs content. According to literature (Bueschelberger, 2004; Szuhaj, 2005), this difference is not that pronounced. However, this difference has been confirmed by $^{31}$P NMR results (shown in section 3.3 of results).

The nitrogen-containing PL content of the corn lecithin sample is higher than the previous lecithins, the native sample contained 74.4% of nitrogen-containing PL, which is also higher than the phospholipid content (50%). This is not a congruent result.

This result might indicate some contamination with other nitrogen-containing compounds (such as ammonia) during the wet degumming process or during analysis. It could also show that the degumming process was not completed.

To test the former hypothesis, samples have been analysed with a continuous flow analyser, following the methods G-287-02 for nitrate and G-102-93 for ammonia. Analyses were run in the ISOFYYS laboratory of Ghent University.

Results showed no nitrate content in the sample, but a 0.27 mg/L of ammonia was found, which was converted to 0.91% of ammonia of the total amount of nitrogen found in the sample, i.e., $0.27 \times \frac{0.5}{1.5 \times 0.53} = 0.17$ mg N/g lecithin, where the second term corresponds to the dilution factor (0.5 L) per the amount of lecithin used on a dry basis (1.5 g corn lecithin (53% water content) was dissolved in 0.5 L of deionised water, from which a sample was taken to be analysed).

Considering that 500 mg lecithin was used in Kjeldahl analysis, to be able to compare the same amount has to be taken into account here. Hence, $0.0849$ mg N/500 mg lecithin coming from ammonia, which corresponds to $\frac{0.0849 \times 10^{-3}}{14} \times \frac{750}{0.5} = 0.91\%$, where the denominator of the first term corresponds to the molecular weight of nitrogen and the second term corresponds to the molecular weight of lecithin per the amount of lecithin used (g).

This result may indicate that indeed some ammonia remained in the hydration water which originates from pH adjustment by ammonia addition. On the other hand, there must be another source of nitrogen in the sample, because 73.1% of nitrogen-containing phospholipids is still unrealistically high, considering that the total phospholipids content was only about 50%.
3. Functionality

The emulsifying properties of the lecithins were considered for both O/W and W/O emulsions applications.

3.1 Droplet size measurement

The droplet size distribution of O/W emulsions was followed over time. The results of the laser diffraction (Mastersizer) measurements are shown in figure 13 for soybean lecithin, figure 14 for sunflower lecithin and figure 15 for corn lecithin (including soybean and sunflower native lecithin).

Three samples were followed over time for the soybean lecithin samples and individual measurements were performed in triplicate, one independent measurement per triplicate in the corn lecithin case, and two independent measurements per triplicate in the sunflower lecithin case.

It is obvious that the emulsions behave differently depending on the amount of water that was added during lecithin fractionation. More stable emulsions were obtained when fractionated lecithin was used. Also for the fractionated samples, the size of the droplets increased over time, but the increase is less pronounced than the native samples.

![Bar chart](image_url)

Figure 13: Volume-weighted average diameter of 5% O/W emulsion prepared with soybean lecithin, as a function of time, measured by laser diffraction. Bars represent standard deviation.

Furthermore, running a statistical analysis, some conclusions about the stability of the prepared emulsions could be made. The output of the two-way ANOVA test is shown in appendix II.

Starting with soybean lecithin emulsions, fractionation had a significant effect (p-value<0.00001) on the droplet size of the emulsion, meaning that there is an effect on
O/W emulsion stability. To evaluate in which extent, a Tukey’s test was run, where a comparison between the native sample and the fractionated samples was made; the four samples (0 mL, 0.4 mL, 0.8 mL and 1.6 mL) were significantly different from the native sample. Furthermore, a comparison between all the fractionated samples was made, resulting in no significant differences between them.

Regarding the influence of time, all comparisons show a significant difference, as expected, since droplet size increased over time.

Sunflower lecithin samples had a similar behaviour as soybean lecithin samples, since fractionation has a significant effect on the droplet size of the emulsions prepared (p-value<0.0001). To evaluate in which extent this effect is a multi-comparison test was run (Tukey’s test). Here, a comparison between the native sample and the fractionated samples was made, where the four fractionated samples (0 mL, 0.4 mL, 0.8 mL and 1.6 mL) were significantly different from the native sample. When checking the comparison between all the fractionated samples, no significant differences between them could be observed, the same case as in soybean lecithin.

![Figure 14: Volume-weighted average diameter of 5% O/W emulsion prepared with sunflower lecithin, as a function of time, measured by laser diffraction. Bars represent standard deviation](image)

With respecting to the day of the measurement, the output of 2-way ANOVA showed the same effect as in soybean lecithin samples, i.e. there is a significant effect (p-value<0.00001) of time on droplet size. The multiple comparison test showed that all days are significantly different, except for day 0 and day 1, where no significant difference was found (for fractionated samples). This output is possible to corroborate looking at figure 14 and table 5.
Corn lecithin samples were only analyzed under native form, for which no comparison could be done with fractionated samples. After run a multi-comparison test (Tukey’s test) with a 95% level of confidence, a significantly difference was found with corn and soybean lecithin, and with corn and sunflower lecithin, but no significantly difference between soybean and sunflower lecithin. These results are also possible to see in figure 15, where corn lecithin emulsion’s average droplet size is slightly bigger than soybean lecithin samples at day 0, but afterwards the increase is less pronounced than in soybean samples. Compared to sunflower lecithin, corn lecithin emulsion’s droplet size is smaller, even at day 0. From here, it is also possible to conclude that soybean lecithin presented the lowest particle size at day 0, but also, the main increase after 23 days, whereas corn lecithin presented a more stable droplet size distribution over time.

Table 5 summarizes the droplet size measurements using laser diffraction (Mastersizer) of the different lecithins that were used (soybean, sunflower and corn), in their native or fractionated form.

These results lead to the conclusion that fractionation has indeed an effect on the stability of O/W emulsions, but to determine the ideal amount of water used during fractionation, more tests would be needed.

![Figure 15: Volume-weighted average diameter of 5% O/W emulsion prepared with native soybean, native sunflower and corn lecithin, as a function of time, measured by laser diffraction](image)

The droplet size as measured using PCS is shown in figure 16 for soybean lecithin, figure 17 for sunflower lecithin and figure 18 for corn lecithin (including native lecithin of soybean and sunflower). As the range of this device is a lot smaller, in some samples the results may be erratic (especially at day 23), because big particles cannot be measured using PCS.
Because of this limitation, no statistical analyses were done. The possible results could give a non-accurate conclusion.

Table 6 summarizes the droplet size measurements using PCS of the different lecithins that were used (soybean, sunflower and corn), in their native or fractionated form.

However, comparing to laser diffraction, the same trends can be distinguished, although to use this technique without other additional, it might be insufficient.

---

**Figure 16:** Z-average diameter of 5% O/W emulsion stabilized by soybean lecithin, determined by PCS. Bars represent standard deviation.

**Figure 17:** Z-average diameter of 5% O/W emulsion stabilized by sunflower lecithin, determined by PCS. Bars represent standard deviation.
Table 5: Mean droplet size and standard deviation based on volume-weighted average diameter (µm) of 5% O/W emulsion prepared with lecithin, as a function of time, measured by laser diffraction

<table>
<thead>
<tr>
<th>Sample</th>
<th>day 0</th>
<th>day 1</th>
<th>day 4</th>
<th>day 23</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOYBEAN</td>
<td>SUNFLOWER</td>
<td>CORN</td>
<td>SOYBEAN</td>
</tr>
<tr>
<td>Native</td>
<td>0.58±0.03</td>
<td>1.92±0.03</td>
<td>1.46±0.06</td>
<td>2.23±0.02</td>
</tr>
<tr>
<td>Fractionated 0mL</td>
<td>0.48±0.01</td>
<td>0.85±0.01</td>
<td>-</td>
<td>0.48±0.01</td>
</tr>
<tr>
<td>Fractionated 0.4mL</td>
<td>0.46±0.02</td>
<td>0.48±0.01</td>
<td>-</td>
<td>0.45±0.03</td>
</tr>
<tr>
<td>Fractionated 0.8mL</td>
<td>0.47±0.01</td>
<td>0.54±0.01</td>
<td>-</td>
<td>0.50±0.01</td>
</tr>
<tr>
<td>Fractionated 1.6mL</td>
<td>0.48±0.02</td>
<td>0.58±0.09</td>
<td>-</td>
<td>0.50±0.02</td>
</tr>
</tbody>
</table>

Table 6: Mean and standard deviation based on Z-average diameter (nm) of 5% O/W emulsion stabilized by lecithin, determined by PCS

<table>
<thead>
<tr>
<th>Sample</th>
<th>day 0</th>
<th>day 1</th>
<th>day 4</th>
<th>day 23</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOYBEAN</td>
<td>SUNFLOWER</td>
<td>CORN</td>
<td>SOYBEAN</td>
</tr>
<tr>
<td>Native</td>
<td>255±13</td>
<td>250±2</td>
<td>188±10</td>
<td>271±23</td>
</tr>
<tr>
<td>Fractionated 0mL</td>
<td>275±41</td>
<td>257±3</td>
<td>-</td>
<td>300±33</td>
</tr>
<tr>
<td>Fractionated 0.4mL</td>
<td>246±15</td>
<td>333±40</td>
<td>-</td>
<td>247±41</td>
</tr>
<tr>
<td>Fractionated 0.8mL</td>
<td>221±7</td>
<td>239±18</td>
<td>-</td>
<td>216±13</td>
</tr>
<tr>
<td>Fractionated 1.6mL</td>
<td>227±7</td>
<td>228±15</td>
<td>-</td>
<td>234±19</td>
</tr>
</tbody>
</table>
Comparing the native samples (figure 18), corn lecithin showed the smallest particle size, which is similar than laser diffraction results (not considering that soybean lecithin at day 0 had a slightly smaller particle size). The small variation among days in corn lecithin samples was also observed with laser diffraction. However, what is not observed in laser diffraction results is the small variation among the soybean samples, this result may be due to the big size of the particles as well.

![Figure 18: Z-average diameter of 5% O/W emulsion stabilized by native soybean, native sunflower and corn lecithin, determined by PCS](image)

**3.2 1D pfg NMR profilometry of O/W emulsions**

One dimensional pulsed field gradient NMR profilometry (1D pfg NMR) allows to analyse the extent of creaming of emulsions.

A low profilometry signal intensity is related to a small T2-relaxation time. The T2-relaxation time is the time it takes for the magnetic resonance signal to decay irreversibly to 37% of its initial value after its generation by tipping the longitudinal magnetization towards the magnetic transverse plane. Because molecules have different electron configuration, different compounds within an emulsion have different T2-relaxation times. For example, fluids have a much longer T2 than solids and water-based samples have a higher T2 than oil-based samples (Reich, 2010).

This principle is applied to describe the graphs of figure 19, but first, it is important to identify the window in which the NMR signal is visible. According to Wuxin et al., (2011), the range goes from 22 mm to 47 mm. This means that the signal detected by the device in this case is coming from the top of the filled tube, which contains about 40 mm of sample. To measure the lower part, it is necessary to elevate the tube. In figure 19, it is possible to see the different profilometry curves of the different prepared emulsions containing soybean (A, B, C), sunflower (D, E, F) and corn lecithin (G), respectively. In
these graphs, the left part (indicating negative frequencies) corresponds to the lower part of the detection window (i.e. about 22 mm sample height), whereas the right part (with positive frequencies) corresponds to the upper part of the detection window. The steep decrease in signal intensity at the right side of the graphs corresponds to the interphase between the emulsion and air. Hence, creaming should become obvious in this region. As the water content in the cream layer is smaller than in the bulk emulsion, the cream layer is expected to give rise to a lower signal intensity.

At simple visual inspection, it is possible to distinguish a cream layer on top of all the emulsions, but it is more pronounced in native samples and in 0 mL samples. However it is very hard to see this effect from pictures. Therefore, profilometry was expected to be a good alternative, and indeed, the visual inspection is corroborated with the graphs obtained. The cream layer is more pronounced in fractionated samples without added water, whereas in fractionated samples with added water a decrease in intensity of the signal is more difficult to identify, which indicates that the phase separation is less pronounced.

In the case of corn lecithin, at visual inspection, the cream layer is almost invisible, although the profilometric analysis shows an increase in the cream layer over time, as shown in figure 19.G. As the droplet size didn’t show a significant increase over time, but the cream layer was increased, there is a clear indication that coagulation is prone to occur.
Figure 19: Profilometry curves of 5% O/W emulsions measured over time. A) Fractionated soybean lecithin 0 mL water added. B) Fractionated soybean lecithin 0.8 mL water added. C) Fractionated soybean lecithin 1.6 mL water added. D) Fractionated sunflower lecithin 0 mL water added. E) Fractionated sunflower lecithin 0.8 mL water added. F) Fractionated sunflower lecithin 1.6 mL water added. G) Corn lecithin
Further efforts were done to try to minimize the oil signal, i.e. the inversion time has been increased in order to evaluate the effect on the oil signal. The results are shown in figure 20. Here, the sample containing 30 mm of water and 10 mm of oil was measured at different τ-values. A clear distinction between water and oil layer can be observed. Even at a τ of 6 ms, the trend is visible (as in figure 19).

![Figure 20: Signal intensity of the sample containing 30 mm water and 10 mm oil as a function of applied inversion time](image)

The distribution obtained for the measurement with τ=50 ms has a very low maximum, therefore this sample was measured three times, obtaining a high signal variability. To avoid this variability, one can divide the signal by the maximal value, and obtain a relative signal intensity.

At τ = 100 ms the oil signal is completely eliminated, for which reason this inversion time was selected and used to measure the lecithin-stabilized O/W-emulsions. It is important to mention that the emulsions used in this case are the same emulsions used for previous case (τ = 6 ms), but measured at day 50.

Figure 21 clearly shows the differences between the 0 mL sample, 0.4 mL and 1.6 mL of soybean lecithin, although no big difference can be seen between 0.4 mL and 1.6 mL. This effect was also visible with τ = 6 ms.
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Figure 21: Rescaled signal intensity of O/W-emulsions stabilized using (fractionated) soybean lecithin after 50 days of storage

The same trend is visible for sunflower lecithin, as shown in figure 22, although the difference is smaller than soybean lecithin and the frequency shift is bigger which hinders the comparison.

Figure 22: Rescaled signal intensity of O/W-emulsions stabilized using (fractionated) sunflower lecithin after 50 days of storage

Figure 23 show the differences between corn lecithin and 0 mL sample of soybean and sunflower lecithin. The thickest cream layer is obtained when corn or soybean lecithin are used, whereas sunflower lecithin showed the thinnest layer.
In conclusion, the signal intensity is better at $\tau = 100$ ms, since the oil signal is eliminated, but the same trends as for $\tau = 6$ ms were observed.

Further optimization is needed to minimize the frequency shifts and the variability in signal intensity.

After corn lecithin evaluation, it is possible to conclude that it is a viable alternative to soybean lecithin, although more studies and analyses have to be performed in order to get more information about the exact composition of the crude corn lecithin and/or some refining steps could be introduced in order to obtain a purer lecithin.

Corn lecithin could be a good and “clean label” alternative to soybean lecithin because of the GMO status of the latter, although about 70 maize GM varieties are approved against 25 soybean varieties (European Commission). The consumers’ perception about GM crops is of interest, and is the main issue. Valletta (2010) mentioned that there are several explanations for the dominance of negative perceptions and attitudes to GM foods. For instance, sometimes GM foods are perceived by part of the consumers as unnatural and therefore risky, without necessarily a rationally defined idea of ‘unnatural’ and even less a defined link between unnatural and risky.

### 3.3 Effect of mono- and multi-valent ions on lecithin-stabilized W/O emulsions

It is known that salts dissolve in water but not in oil. However, what is not completely understood is to what extent salts (whereby both the concentration and the type of salts may be important) affect the stability of emulsions, and the cause of the effect.
In water, lecithin can self-assemble into characteristic structures such as micelles and vesicles, driven by hydrophobic and electrostatic interactions. It is known that salts reduce the CMC (critical micelle concentration) when added to suspensions containing surfactants, reducing the electrostatic repulsions between the head-groups of the surfactants, which facilitates micelle formation and assembly into cylindrical structures (Israelachvili, 1991). But, in oil, the effect of salts is not that well studied. It is known that reverse self-assembly of amphiphilies can also occur in oil, but compared with water, lecithin in oil may not have a well-defined CMC (Lee et al., 2010).

In this case, mono- and multi-valent ions were added to the water phase of the W/O emulsions stabilized with lecithin, and a macroscopic and microscopic examination was performed. Some theories and hypotheses about the self-assembly of lecithin in the emulsion are also discussed.

All emulsions obtained corresponded to W/O emulsions, as verified with a simple drop dilution test. The latter is based on the fact that W/O emulsion droplets will not disperse in water, based on the solubility of the external phase of the emulsion. All emulsions, formed a creamy sediment, containing flocculates. These flocculates vary in size and consistency depending on the type of salt and the salt concentration.

Regarding the effect of bi-valent ions, shown in figure 24, the floc size decreased as the concentration of CaCl$_2$ increased, but at 32 mM CaCl$_2$ the floc size increased again, which gives an idea of a possible ideal salt concentration (16 mM). At this concentration the molar ratio of lecithin to Ca$^{2+}$ was about 15:1, which is based on the concentration and volume of lecithin solution (in oil) and the concentration and volume of Ca$^{2+}$-solution (in water): from a 30/70 (m/m) W/O emulsion, $0.3 \times C_{\text{ion}}$ (mol/kg) to $\frac{0.7 \times \text{wt}\%_{\text{lecithin}}}{100 \times 0.750}$ (mol/kg), where $C_{\text{ion}}$ corresponds to the ion concentration used, wt% lecithin corresponds to the lecithin concentration used, and 0.750 is the molecular weight of lecithin in kg/mol. This same approach is used in the following cases.
Figure 24: W/O emulsions (30/70, m/m) containing Ca$^{2+}$ right after preparation

The emulsion with 16 mM CaCl$_2$ showed a smooth creamy phase, with smaller flocs. Lower concentrations showed bigger aggregates, and higher concentrations showed more viscous and bigger aggregates.

After 2 hours, it was possible to see precipitation of the flocs obtained at higher concentrations (4, 8, 16, 32, 64, 128 mM CaCl$_2$). Lower concentrations still had flocs homogenously distributed all over the emulsion. The emulsion with 16 mM CaCl$_2$ still showed the smaller flocs.

The emulsions were kept under observation for 5 days at room temperature, after which it was possible to see that the precipitation was almost complete. At high CaCl$_2$ concentrations (32, 64 mM and 128 mM) a water layer was formed (pointed out with an arrow), indicating the destruction of the emulsion (figure 25).

Figure 25: W/O emulsions (30/70, m/m) containing Ca$^{2+}$ 5 days after preparation. Water layers are indicated with an arrow.

Petelska et al., (2010) presented evidence for the formation of phosphatidylcholine-Ca$^{2+}$ ion complexes, as LCa$^{2+}$ and L$_2$Ca$^{2+}$, where L stands for phosphatidylcholine. The ratio between Ca$^{2+}$ and phospholipids is assumed to be 1:1 and 1:2. On the other hand, Lee et al. (2010) reported a molar ratio 3:1 (lecithin:Ca$^{2+}$) as most efficient. They also
measured the area occupied by one $L_2Ca$ complex and they deduced that it was smaller than the area occupied by each component of the complex.

$Ca^{2+}$ is known to make lecithin more suitable for W/O emulsions (enhanced stabilization) due to a change in conformation. Cations like $Ca^{2+}$ bind to the phosphate portion of the headgroup. The phospholipid molecule has a conical shape and forms discrete, spherical reverse micelles in oil. $Ca^{2+}$ ions, upon binding to lecithin, cause the headgroup area to expand. The lipid tails also become more straightened, causing the tail to shrink and the micelle radius to increase. The molecular geometry becomes more like a truncated cone and in turn, the assemblies transform into cylindrical (wormlike) fibrils (Lee et al., 2010).

Emulsions containing mono-valent ions (figure 26) showed the same tendency as the bi-valent counterions: the floc size decreased as the salt concentration increased. The emulsions became more smooth and homogeneous upon NaCl addition, but not as pronounced as in the presence of bi-valent cations.

After centrifugation, the samples presented phase separation (i.e. water layer was formed), which is a clear indication about the destabilization effect of Na$^+$ ions on emulsions, whereas in the case of bi-valent and tri-valent ions, no phase separation was observed after centrifugation.

Scherze et al. (2007) reported coalescence of the droplets after 7 days in a W/O emulsion (30/70, w/w), which in this case was also visible after 5 days, and was confirmed with the phase separation after centrifugation (for all emulsions).

Petelska et al. (2013) concluded that monovalent ions have a little effect on the formation of complexes with phospholipids. Moreover, they determined that the stability constant values (which are a measure of the strength of the interaction between the reagents that come together to form the complex) of complexes being formed between the lecithin and the monovalent ions increase along with the increase of the radius of the ion ($Li^+<Na^+<K^+<Cs^+$). In this case, it can be concluded in a similar way, that Na$^+$ is not good...
as $\text{Ca}^{2+}$, because one $\text{Ca}^{2+}$ can bind two phosphate groups, thus two phospholipids, whereas $\text{Na}^+$ only can bind to one phosphate group.

The effect of tri-valent counterions was similar to the bi-valent ion effect. The emulsions obtained are shown in figure 27.

![Image: W/O emulsions (30/70, m/m) containing $\text{Ce}^{3+}$ right after preparation](image)

**Figure 27: W/O emulsions (30/70, m/m) containing $\text{Ce}^{3+}$ right after preparation**

Here, it is also possible to see how the floc size decreased as the salt concentration increased. However, as in the case of $\text{Ca}^{2+}$, at higher concentrations the flocs became bigger, which also is an indication of a possible ideal salt concentration.

In general, the emulsions containing $\text{Ce}^{3+}$ presented a better (i.e. more homogenous) macroscopic appearance. They are smoother, whiter, and appear less viscous than the ones containing $\text{Ca}^{2+}$ and $\text{Na}^+$, which could indicate that the droplet size is smaller. To verify this hypothesis, a further microscopic evaluation was performed.

A visible droplet size decrease was observed with increased $\text{Ce}^{3+}$ concentration (figure 28). However, a small increase in droplet size at 28.2 mM may be noticed, which is congruent with what was observed macroscopically. When the droplet size was followed up over time, no visible changes have been observed in a second microscopic evaluation after 3 hours of preparation.
As reported by Huang et al. (2010), the phosphate group attracts tri-valent cations. The effect on the head group is similar to Ca$^{2+}$ and consequently promotes easy formation of micelles and growth (Loshchilova, 1978). The stability constant of tri-valent ions, follows the order (observed by several methods): La$^{3+}$ < Ce$^{3+}$. Ce$^{3+}$ can bind to the polar group more closely than La$^{3+}$. Hence, its effect is stronger than the latter (Huang et al., 2001).

Consequently, the influence of lecithin concentration on W/O emulsion stability containing mono- and multi-valent cations was evaluated, in order to assess to what extent lecithin has an effect on the emulsion stability, and what can be attributed to the effect of salts.

For this, an oil phase was prepared with different lecithin concentrations (1, 2, 4% and 8%). The concentration of mono- and multi-valent cations used was 32 and 16 mM for mono- and bi-valent and 7.05 mM for tri-valent ions, considering that these concentrations showed the best results for emulsions with 8% of lecithin.

Again, all emulsions prepared were water-in-oil emulsions. The macroscopic properties were diverse. They are summarised in table 4, and shown in figure 29.
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Figure 29: W/O emulsions (30/70, m/m) containing different lecithin concentrations and A) 32 mM NaCl, B) 16 mM CaCl₂, C) 7.05 mM CeCl₃, right after preparation

In general, emulsions containing 1% of lecithin were smoother than the emulsions at higher concentrations, although the 1% emulsion containing mono-valent cations was completely destroyed after one hour, which also was observed at 2% and 4% of lecithin. This indicates that the emulsion containing NaCl at 8% of lecithin was stable because of the high lecithin concentration. Hence, mono-valent cations were not able to destabilize the emulsion.

Table 7: Macroscopic properties of emulsions prepared with an oil phase with different lecithin concentrations (1, 2, 4, and 8%), and with 32, 16 or 7.05 mM of mono-, di-, and tri-valent cations, resp.

<table>
<thead>
<tr>
<th>Salt</th>
<th>Salt Concentration (mM)</th>
<th>Lecithin (%)</th>
<th>Phase separation</th>
<th>Sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>32</td>
<td>1</td>
<td>yes</td>
<td>big flocs a</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>16</td>
<td>1</td>
<td>no</td>
<td>smooth a, d</td>
</tr>
<tr>
<td>CeCl₃</td>
<td>7.05</td>
<td>1</td>
<td>no</td>
<td>smooth c</td>
</tr>
<tr>
<td>NaCl</td>
<td>32</td>
<td>2</td>
<td>yes</td>
<td>big flocs b</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>16</td>
<td>2</td>
<td>no</td>
<td>small flocs d</td>
</tr>
<tr>
<td>CeCl₃</td>
<td>7.05</td>
<td>2</td>
<td>no</td>
<td>smooth</td>
</tr>
<tr>
<td>NaCl</td>
<td>32</td>
<td>4</td>
<td>yes</td>
<td>big flocs b</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>16</td>
<td>4</td>
<td>no</td>
<td>big flocs d</td>
</tr>
<tr>
<td>CeCl₃</td>
<td>7.05</td>
<td>4</td>
<td>no</td>
<td>partially smooth c</td>
</tr>
<tr>
<td>NaCl</td>
<td>32</td>
<td>8</td>
<td>no a</td>
<td>small flocs b</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>16</td>
<td>8</td>
<td>no</td>
<td>smooth</td>
</tr>
<tr>
<td>CeCl₃</td>
<td>7.05</td>
<td>8</td>
<td>no</td>
<td>smooth</td>
</tr>
</tbody>
</table>

a: smooth but some small flocks were formed. The emulsion was smooth and homogeneous, but not stable as the emulsion containing CeCl₃ (after 4 hours still was not showing phase separation).
b: emulsions containing Na\(^+\) showed already big flocks after preparation, and a total phase separation was visible after few hours of preparation.

c: emulsions containing Ce\(^{3+}\) were stable at all lecithin concentrations. At low lecithin concentrations, emulsions were more liquid-like and sedimentation was visible after 2 days.

d: at low lecithin concentrations, emulsions containing Ca\(^{2+}\) were totally destroyed after 2 days.

e: phase separation was possible to see after centrifugation step.

Moreover, emulsions containing 1% and 2% of lecithin were more liquid-like emulsions, while emulsions containing 4% and 8% of lecithin appeared more viscous. In addition, the flocs were bigger and compact.

Considering the good results observed with Ce\(^{3+}\), the lowest lecithin concentration where stable emulsions were obtained, was sought. In this sense, oil phases containing 0.01, 0.05, 0.1, 0.25, and 0.5% soybean lecithin were used to prepare emulsions containing 7.05 mM CeCl\(_3\). In addition, a blank was prepared, i.e. an emulsion only containing oil and a water phase with 7.05 mM CeCl\(_3\). As expected, the latter emulsion was destabilised immediately after preparation (figure 32.a): a clear water layer was immediately obvious.

Emulsions containing 0.5% and 0.05% were still stable, liquid-like emulsions, whereby sedimentation became visible after 3 hours. On the other hand, emulsions with only 0.01% lecithin showed the same effect as the blank, indicating that this small amount of lecithin was not able to stabilize the emulsion anymore, neither with the presence of tri-valent cations. In this case, the emulsion was destroyed, i.e. a phase separation was already visible some minutes after preparation.

![Image: W/O emulsions containing 7.05 mM CeCl\(_3\) and different lecithin concentrations, right after preparation.](image)

This means that the critical soybean lecithin concentration at which emulsions are stable in the presence of tri-valent cations (7.05 mM) is in the range between 0.01 and 0.05%, where the molar ratio of Ce\(^{3+}\) to lecithin is about 4.5 to 1.
W/O emulsions were clearly improved with the addition of CeCl$_3$. This effect is already seen with a lecithin content of about 0.05% and 7.05 mM of CeCl$_3$. Not only the stability was improved but also the macroscopic properties: smoother, whiter, and more homogeneous emulsions.

Huang et al. (2001) evaluated the effect of divalent and trivalent salts on the micelle formation in C$_8$-lecithin solutions using the techniques of static and dynamic light scattering. They concluded that the micelles in calcium solutions and the tri-valent solutions formed easily and were stable. They explained this effect somehow by the counterion effect between the salts and the polar head of the lecithin. The phosphatidylcholine polar head is zwitterionic at pH between 3 and 11. This means that in this pH range, the phosphate group of the polar head has a net negative charge and the choline group has an equal positive charge with a spatial separation. In aqueous solution, water molecules are bound to the phosphate group while none is bound to the choline group. When salts are added to the solution, cations bind to the phosphate group. For divalent salts, the following order of binding has been observed Ba$^{2+} <$ Mg$^{2+} <$ Ca$^{2+}$. For trivalent salts, the order is La$^{3+} <$ Ce$^{3+}$ (Loshchilova, 1978). It has also been found that Ca$^{2+}$ can even be bound to the phosphate group as close as 1–2 Å; therefore, among the divalent cations, calcium ions have the strongest effect on the surface potential of the amphiphilic molecules (Huang et al., 2001).

However, according to Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on Food Additives, specifically in Annex II (Positive List of additives), CeCl$_3$ is not listed, which means that it is not allowed to be used in foods. Nevertheless, it could be used in other technical applications.

### 3.4 Electrophoretic mobility

To see the effect of CeCl$_3$ on lecithin, the electrophoretic mobility of suspension containing soybean lecithin and different ratios of CeCl$_3$ were measured.
Figure 31: Electrophoretic mobility as function of Ce$^{3+}$:soybean lecithin ratio. Bars represent the standard deviation

The results are shown in figure 31. As the ratio of soybean lecithin to Ce$^{3+}$ increases, the net charge of the suspension decreases, leading to a change in charges which gives an idea about how Ce$^{3+}$ is binding to the phosphate group of the phospholipids.

The main (negatively) charged phospholipids present in soybean lecithin (about 2.5%) are phosphatidylinositol, phosphatidic acid and phosphatidylserine, all other main compounds are either uncharged or zwitterionic. By adding a salt, in this case Ce$^{3+}$, it starts interacting with the negative charges of the head groups and then with the phosphate groups leading to a net positive charge (as the zwitterionic phospholipids contain a positive headgroup). To see the structure of phospholipids, one can refer to figure 1.

Pure lecithin in water has a negative charge. As the ratio Ce$^{3+}$:lecithin is becoming even, Ce$^{3+}$ is able to bind all negative charged groups, leading to the uncharged result, which occurs at a ratio about 1:4.2 according to the figure 31. At higher concentrations, Ce$^{3+}$ starts to bind phosphate groups, increasing the positive charge. The ratio 1:3 may correspond to the 0.5% lecithin discussed in previous section (2.12 mmol of Ce$^{3+}$ to 6.36 mmol soybean lecithin).
CHAPTER IV

Conclusions
In this research, the characterization and functionality of native and fractionated lecithin from different sources was evaluated.

As a first step of this research, soybean and sunflower lecithin were fractionated, having as objective to improve their O/W emulsifying properties by adding amounts of water to lecithin dissolved in a hexane/isopropanol mixture that may hydrate impurities and make them insoluble in the solvent, leading to precipitation.

After fractionation, the lecithins were characterized. First, the total phospholipid content was determined. Two methods were used. The first method was acid digestion with subsequent colorimetric determination as described in Chapter II, section 3.1.1. This method has not given the results that were expected, they were not reproducible (showed with a large variance) and the expected amount of phospholipids was not recovered.

The second method used to determine phospholipids content was a commercial kit test, purchased from HACH®. This method has a fast and simple procedure, and is based on the same principles as the former method. In this case, the results were better, and the phospholipid recovery was as expected. Native soybean lecithin yielded the highest phospholipid content (87%), while native sunflower lecithin yielded 83% and corn lecithin 51%, which was expected because of its crude form. Regarding the fractionated lecithin, a decrease of phospholipid content was observed as more water was added to the mixture. The same trend has been observed for soybean and sunflower. This decrease may be due to the amphiphilic behaviour of the phospholipids. Some may have migrated to the sediments and/or separated during the filtration step.

Secondly, nitrogen-containing phospholipids (N-PL) were determined using the standard Kjeldahl method. These phospholipids are basically PC, PE and PS, which are the major components of lecithins, besides PI that has no nitrogen in its structure. The results showed that the N-PL content in sunflower lecithin is higher than in soybean lecithin but no significant differences were found between fractionated samples (for both lecithins).

The N-PL content in corn lecithin was unrealistically high. Some extra analyses (i.e. inorganic nitrogen determination) were performed. The amount of ammonia found in the sample was too low and neither nitrate nor nitrite was present. This low amount cannot explain the high N-content determined with Kjeldahl, therefore the nitrogen must come from other, non-characterized N-containing compounds in the lecithin (which was in crude form).
As a confirmation of these simple methods, a $^{31}$P-NMR measurement was performed. After obtaining the spectra and the corresponding peak integration, the peak areas showed the same trend as observed with the above mentioned methods. This leads to a conclusion that the former methods applied are a good alternative for lecithin analysis. They are easily accessible, relatively cheap and they don’t need a specially trained staff to perform them.

On the other hand, the functionality of native and fractionated lecithin samples was evaluated. The droplet size distribution of oil-in-water emulsions was followed over time. The emulsions containing fractionated lecithin were more stable ($p$-value<0.00001) than the native lecithin samples, for both sources (soybean and sunflower), although no significant differences were found within the different fractionated samples. This was confirmed with the 1D pfg NMR results, where no distinct cream layer could be observed on top of the emulsions prepared with the fractionated samples with added water.

Corn lecithin had a different behaviour. The droplet size directly after preparation was in the same range than soybean lecithin samples and smaller than sunflower lecithin samples, but the increase was not pronounced over time. However, a cream layer could be identified with profilometric analysis, which indicates that coagulation is prone to occur.

The influence of mono-valent ions on water-in-oil emulsions was evaluated, concluding that $\text{Na}^+$ has no beneficial effect on the emulsion’s stability. On the contrary, it tends to destabilize it.

On the other side, bi-valent ions showed a stabilizing effect on lecithin-stabilised W/O emulsions. Tri-valent ions showed a better stabilizing effect than bi-valent ions, even at 0.05 wt-% lecithin, where the molar ratio of $\text{Ce}^{3+}$ to lecithin is about 4.5 to 1.

Several studies describe the bi- and tri-valent ions stabilizing effect, as well as the binding between the ions and the phospholipids. At first, the cations bind to the negatively charged phospholipids, after which cations start to bind to the negatively charged phosphate group of the zwitterionic phospholipids. One can assume that one $\text{Ca}^{2+}$ is able to bind two phospholipids, and one $\text{Ce}^{3+}$ is able to bind three of them, leading to a change in the structure of the micelles formed (from spherical micelles to cylindrical fibrils). The binding of $\text{Ce}^{3+}$ to soybean lecithin was confirmed with electrophoretic mobility measurements, where a change in charge was observed from negative to positive as the $\text{Ce}^{3+}$ concentration was increased.

Further research could be done on purification and modification of corn lecithin, although the native form (containing water) performs quite well. Also, the influence of tri-valent
ions on O/W emulsions can be sought, as well as a food grade alternative for cerium. Furthermore, further research is needed to optimise 1D pfg NMR profilometry to follow the creaming behaviour of O/W emulsions, especially regarding to the frequency and signal intensity shifts. This is necessary to be able to quantify the amount of water and oil present in the cream layer.
CHAPTER V

Appendices
Appendix I

I.1 $^{31}$P-NMR spectra for native and fractionated lecithin

Phosphorus nuclear magnetic resonance spectra of native lecithin samples, **A**) Corn lecithin; **B**) Soybean lecithin, Method I; **C**) Sunflower lecithin; **D**) 0.8 mL soybean; **E**) 1.6 mL soybean; **F**) 0 mL soybean; **G**) Soybean lecithin, Method II; showing resolution of phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), lysophosphatidylinositol (LPI), lysophosphatidic acid (LPA), glycerophosphorylcholine (GPC), phosphatidyl serine (PS), phosphatidylglycerol (PG) and the internal standard (STD).

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Continued on next page…
Appendix II

II.1 Paired t-test comparing soybean and sunflower PL content

Paired t-Test

data: x: soybean in SDF27, and y: sunflower in SDF27
t = 3.56, df = 5, p-value = 0.0162
alternative hypothesis: mean of differences is not equal to 0
95 percent confidence interval:
0.05256113 0.32568946
sample estimates:
mean of x - y
0.1891253

II.2 ANOVA of droplet size results obtained with laser diffraction measures

II.2.1 Soybean lecithin

*** Analysis of Variance Model ***

Short Output:

Call:
aov(formula = droplet.size ~ sample * day, data = soybean, na.action = na.exclude)

Residual standard error: 0.2773241
Estimated effects may be unbalanced

<table>
<thead>
<tr>
<th>Df</th>
<th>Sum of Sq</th>
<th>Mean Sq</th>
<th>F value</th>
<th>Pr(F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sample</td>
<td>4</td>
<td>46.17630</td>
<td>11.54408</td>
<td>150.1012</td>
</tr>
<tr>
<td>day</td>
<td>3</td>
<td>17.45714</td>
<td>5.81905</td>
<td>75.6618</td>
</tr>
<tr>
<td>sample:day</td>
<td>12</td>
<td>4.49894</td>
<td>0.37491</td>
<td>4.8748</td>
</tr>
</tbody>
</table>

Residuals 85 6.53723 0.07691

Type III Sum of Squares

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<td>4.8748</td>
</tr>
</tbody>
</table>

Residuals 85 6.53723 0.07691

95 % simultaneous confidence intervals for specified linear combinations, by the Tukey method

critical point: 2.8035000000000001
response variable: droplet.size
intervals excluding 0 are flagged by '****'
**II.2.2 Sunflower lecithin**

*** Analysis of Variance Model ***

Short Output:

Call:
```
aov(formula = droplet.size ~ sample * day, data = sunflower, na.action = na.exclude)
```

Type III Sum of Squares
```
 Df  Sum Sq Mean Sq   F Value Pr(>F)
    4 24.7752  6.1938 1197.872  <2e-16  
    3  6.0873  2.0291  392.428  <2e-16  
   12  0.9511  0.0792   15.329 3.99e-01  
 45  0.2327  0.0051
```

95 % simultaneous confidence intervals for specified linear combinations, by the Tukey method

critical point: 2.787199999999999

---

**Estimate Std.Error Lower Bound Upper Bound**

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<th>native</th>
<th>0 mL</th>
<th>0.75100</th>
<th>0.0332</th>
<th>0.65800</th>
<th>0.84400</th>
</tr>
</thead>
<tbody>
<tr>
<td>native</td>
<td>0.4 mL</td>
<td>0.83800</td>
<td>0.0313</td>
<td>0.75000</td>
<td>0.92600</td>
</tr>
<tr>
<td>native</td>
<td>0.8 mL</td>
<td>0.82500</td>
<td>0.0308</td>
<td>0.71700</td>
<td>0.93300</td>
</tr>
<tr>
<td>native</td>
<td>1.6 mL</td>
<td>0.74900</td>
<td>0.0313</td>
<td>0.66100</td>
<td>0.83600</td>
</tr>
<tr>
<td>0 mL-0.4 mL</td>
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<td>0.0332</td>
<td>-0.00633</td>
<td>0.18000</td>
<td></td>
</tr>
<tr>
<td>0 mL-1.6 mL</td>
<td>-0.00267</td>
<td>0.0332</td>
<td>-0.09590</td>
<td>0.09050</td>
<td></td>
</tr>
<tr>
<td>0.4 mL-0.8 mL</td>
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<td>0.0384</td>
<td>-0.12100</td>
<td>0.09440</td>
<td></td>
</tr>
<tr>
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<td>0.0313</td>
<td>-0.17700</td>
<td>-0.00167</td>
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</tr>
<tr>
<td>0.8 mL-1.6 mL</td>
<td>-0.07630</td>
<td>0.0384</td>
<td>-0.18400</td>
<td>0.03130</td>
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</tr>
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</table>

95 % simultaneous confidence intervals for specified linear combinations, by the Tukey method

critical point: 2.6347

response variable: droplet.size

intervals excluding 0 are flagged by '****'

Estimate Std.Error Lower Bound Upper Bound

| 0-1 | -0.0964 | 0.0307 | -0.177 | -0.0155 |
| 0-4 | -0.2250 | 0.0320 | -0.309 | -0.1410 |
| 0-23 | -0.5130 | 0.0307 | -0.594 | -0.4330 |
| 1-4 | -0.1290 | 0.0320 | -0.213 | -0.0443 |
| 1-23 | -0.4170 | 0.0307 | -0.498 | -0.3360 |
| 4-23 | -0.2880 | 0.0320 | -0.373 | -0.2040 |
response variable: droplet.size
intervals excluding 0 are flagged by ****

<table>
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<th>Lower Bound</th>
<th>Upper Bound</th>
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<td></td>
<td></td>
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<tr>
<td>native-0.4 mL 1.68000 0.0801 1.4500 1.9000 ****</td>
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<tr>
<td>native-0.8 mL 1.65000 0.0980 1.3800 1.9200 ****</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>native-1.6 mL 1.50000 0.0801 1.2700 1.7200 ****</td>
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<td></td>
</tr>
<tr>
<td>0 mL-0.4 mL 0.17400 0.0849 -0.0629 0.4100</td>
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<tr>
<td>0 mL-0.8 mL 0.14700 0.1020 -0.1370 0.4320</td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8 mL-1.6 mL -0.15300 0.0980 -0.4260 0.121</td>
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95 % simultaneous confidence intervals for specified linear combinations, by the Tukey method
critical point: 2.6206

response variable: droplet.size
intervals excluding 0 are flagged by ****

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<th>Upper Bound</th>
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<tbody>
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<td>0-1 -0.193 0.0784 -0.398 0.0127</td>
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<td></td>
</tr>
<tr>
<td>0-4 -0.450 0.0816 -0.664 -0.2360 ****</td>
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<td></td>
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<tr>
<td>0-23 -1.030 0.0784 -1.230 -0.8210 ****</td>
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<td></td>
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<tr>
<td>1-4 -0.257 0.0816 -0.471 -0.0431 ****</td>
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</tr>
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<td>1-23 -0.834 0.0784 -1.040 -0.6280 ****</td>
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<tr>
<td>4-23 -0.577 0.0816 -0.791 -0.3630 ****</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

II.2.3 Differences between different native lecithins droplet size distribution

*** Analysis of Variance Model ***
Short Output:
Call:
aov(formula = Droplet.size ~ Day * Sample, data = SDF31, na.action = na.exclude)
Residual standard error: 0.3871124
Estimated effects may be unbalanced
  DF Sum of Sq Mean Sq F Value  Pr(F)
  Day 3 8.159959 2.719986 18.15067 0.0000000157
  Sample 2 7.057661 3.528831 23.54815 0.000000197
  Day:Sample 6 3.355726 0.559288 3.73217 0.004986824
  Residuals 39 5.844384 0.149856

95 % simultaneous confidence intervals for specified linear combinations, by the Tukey method
critical point: 2.4363
response variable: Droplet.size
intervals excluding 0 are flagged by '****'

<table>
<thead>
<tr>
<th></th>
<th>Estimate</th>
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<th>Lower Bound</th>
<th>Upper Bound</th>
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</thead>
<tbody>
<tr>
<td>Sunflower-Corn</td>
<td>0.884</td>
<td>0.151</td>
<td>0.515</td>
<td>1.250</td>
</tr>
<tr>
<td>Sunflower-Soybean</td>
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<td>0.137</td>
<td>-0.193</td>
<td>0.474</td>
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<tr>
<td>Corn-Soybean</td>
<td>-0.744</td>
<td>0.129</td>
<td>-1.060</td>
<td>-0.429</td>
</tr>
</tbody>
</table>

II.3 Nitrogen-containing phospholipids

II.3.1 Soybean

*** Analysis of Variance Model ***
Short Output:
Call:
  aov(formula = PL.content ~ Sample, data = SDF29, na.action = na.exclude)
Terms:
  Sample Residuals
  Sum of Squares 145.5338 110.0800
  Deg. of Freedom 1 4
  Residual standard error: 5.245951
Estimated effects may be unbalanced
  Df Sum of Sq Mean Sq F Value Pr(F)
  Sample 1 145.5338 145.5338 5.28829 0.08297296
Residuals 4 110.0800 27.5200

II.3.2 Sunflower

*** Analysis of Variance Model ***
Short Output:
Call:
  aov(formula = PL.content ~ Sample * PL.content, data = SDF30, na.action = na.exclude)
Type III Sum of Squares
  Df Sum of Sq Mean Sq F Value Pr(F)
  Sample 4 94.00114 23.50028 2.428885 0.17837
Sample:PL.content
  Residuals 5 48.37670 9.67534
REFERENCES

32. Reich, H. J. (2010). Relaxation in NMR Spectroscopy. Retrieved on 2015, May 4th from Chemistry Department, University of Wisconsin: 
http://www.chem.wisc.edu/areas/reich/nmr/notes-8-tech-1-relax.pdf


