Stearidonic Acid Concentration by Urea Complexation from Echium Oil

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Abstract: Concentration of polyunsaturated fatty acids ethyl esters (FAEE) by urea complexation from Echium oil was studied. Different variables involved in the process were investigated: amount and particle size of urea, solvent volume and ratio (hexane/ethanol), load of FAEE and reaction time. Hence, the main goal was to optimize SDA concentration (%) and yield (%) of stearidonic acid (SDA, 18:4 ω-3) and other bioactive FAEE. Similar behaviors were observed in fractionation between α-linolenic (ALA)-linoleic (LA), and γ-linolenic (GLA)-stearidonic (SDA) acids, attributed to similarities on their chemical structures, due to the position of the double bonds. At laboratory scale, the optimal conditions were 3 g urea (powder), 3.6 mL of hexane, 0.54 mL of ethanol and 800 mg of FAEE, during 20 h at 25℃. A scaling-up at pilot plant was carried out twice, obtaining more than 100 g of a final product, with ~29% SDA concentration and ~78% yield. Besides, after two washings with water, ethyl carbamates (urethanes) were not detected in the final product. Thus, a mixture of FAEE with about 85% of bioactive fatty acids with anti-inflammatory properties was obtained, which can be a high added-value product with great potential for the synthesis of functional lipids and nutraceuticals.

Key words: fatty acid ethyl ester (FAEE), ethyl carbamate, PUFA concentration, stearidonic acid (SDA), urea adduct

1 Introduction

Omega-3 polyunsaturated fatty acids cannot be synthesized by humans, so they are considered essential nutrients. They act on the cardiovascular system through their antithrombotic effect, by inducing vasodilation and inhibiting platelet aggregation, inhibiting atheroma plaque formation, preventing arrhythmias and decreasing serum triglycerides and VLDL. They have an anti-inflammatory effect, owing to the production of anti-inflammatory mediators, such as eicosanoids and cytokines, induction of the expression of adhesion molecules and formation of anti-inflammatory mediators (resolvines). Moreover, they have antitumor activity and several effects during pregnancy, lactation and infancy. Therefore, a great deal of studies suggests they are able to prevent or enhance diseases like autoimmune disorders (e.g. lupus or nephropathy), coronary heart diseases, wrong retinal and brain development, Crohn disease, cancer (breast, colon, prostate), hypertension or rheumatoid arthritis1-4.

Unlike ω-3 fatty acids, some ω-6 fatty acids induce the formation of pro-inflammatory mediators. Nowadays, Western diets present a high amount of calories, with high levels of ω-6 fatty acids, saturated fats and trans fatty acids. Thus, ω-6/ω-3 ratio has increased from ~1/1 to 15/1-16,7/1 in the last years, promoting the pathogenesis of cardiovascular, anti-inflammatory and autoimmune diseases, or cancer5-10.

Nevertheless, recent studies suggest the role of γ-linolenic acid (GLA, C18:3 ω-6) in different health risk factors. It is effective against rheumatoid arthritis, development of atherosclerosis and also could normalize nerve conduction velocity, among other effects. Hence, it might be interesting the application of GLA in food, nutraceutical products or pharmaceutical industry7.

Regarding the family of ω-3 fatty acids, α-linolenic acid (ALA, C18:3 ω-3) is the first step in the biosynthetic pathway to longer polyunsaturated fatty acids (PUFA). Major dietary sources of ALA are vegetable oils (e.g linseed, rapeseed or walnuts)8,9. ALA results in eicosapentaenoic acid (EPA; C20:5 ω-3) and docosahexaenoic acid (DHA;
C22:6 ω-3), both precursors of anti-inflammatory metabolites. They can be found mainly in marine sources, causing an increase of fish and PUFA supplements consumption in the last decades. This fact has promoted an overexploitation of fisheries which provides fish for human consumption, feeds to industrial fish farms and fish oil supplements, causing a critical problem to several marine species.

ALA could be an alternative to these marine sources, but some studies indicate the biosynthetic conversion pathway of ALA into EPA and DHA, which includes elongations and desaturations steps, is slow and inefficient. Moreover, this metabolic route from ALA to EPA and DHA competes with the one from LA to araquidonic acid (AA; 20:4 ω-6), as they use the same set of elongases and desaturases enzymes. Human studies shows that Δ6 desaturase, which catalyzes the formation of EPA and DHA values of 85%, can be carried out by two different methodologies: (A) at high temperatures (~75°C), using ethanol, which dissolve urea and fatty acids (free form or methyl/ethyl ester) completely, followed by rapid cooling crystallization; and (B) at ~ 21°C, using hexane/ethanol mixtures, which dissolve totally all the fatty acids (free form or methyl/ethyl ester), but partially the urea.

In recent years, several studies of ω-3 PUFA concentrations have used this technology, principally, using the procedure with high temperatures and ethanol. For example, Wanasundara & Shahidi optimized EPA and DHA from seal blubber oil, with values around 88.2%25. Liu et al. obtained EPA and DHA values of 85% with tuna oil26. Shahidi & Spurvey also optimized the different reaction conditions getting 91% of GLA from borage oil27. In addition, urea complexation also has been used combined with other techniques to obtain better purifications, such as molecular distillation28, supercritical fluid extraction29 or enzymatic processes30.

The principal drawback of this technology appeared when Canas & Yuriwecz suggested the formation of ethyl carbamates or urethanes in the process31. These potential carcinogenic compounds could appear due to the presence of ethanol and urea in the reaction media. However, a recent study suggested that formation of ethyl carbamates can be reduced by performing the methodology at room temperature with a minimum amount of ethanol32 and that subsequent washings with water were effective to remove this compound from the final products33.

Thus, the main goal of this study was to optimize the concentration of PUFA with bioactive properties (anti-inflammatory), mainly SDA, from Echium oil, at laboratory and pilot plant scale.

2 Materials and methods
2.1 Materials

Echium oil (Echium plantagineum) was supplied by Harke Nutrition (Mülheim an der Ruhr, Germany). Ethyl oleate (>98%), urethane (>99%) and sodium ethoxide (>95%) were purchased from Sigma Aldrich (St. Louis, USA). Anhydrous sodium sulfate, sodium chloride and sulfuric acid 96% were purchased from Panreac (Castellar del Valles, Spain). Hexane 99% HPLC grade was obtained from Lab-Scan (Gliwice, Poland). Finally, absolute ethanol and urea were purchased from Scharlau (Sentmenat, USA).
2.2 Methods

2.2.1 Echium oil FAEE obtained by transesterification

Echium oil, composed mainly by triacylglycerols, was transformed into their corresponding ethyl esters fatty acids. For this matter, a transesterification by ethanolysis reaction was carried out, following a methodology previously described by Vázquez and Akoh\(^{20}\). In this reaction, 400 g of Echium oil, 120 mg of tocopherol to prevent possible oxidation, 126 g of absolute ethanol and 6 g of sodium ethoxide were mixed for 40 minutes at 50°C, under nitrogen and with constant stirring. The reaction was carried out in a Buchi kihloclave reactor (Uster, Switzerland) using a double-jacketed tank with stirring volume of 1 L, coupled to a cryothermostat Julabo F32-HE (Seelbach, Germany).

After reaction, the process was stopped by adding 40% (w/w) of 0.5 M sodium chloride solution at 50°C. The mixture was transferred to a decanting funnel (1L) and decanted for 5 min. Once the phases were separated, lipid fraction (upper phase) was recovered and washed with 40% (w/w) of distilled water at 50°C. The mixture was decanted into a decanting funnel (1L) for 5 min and lipid fraction was recovered. This fraction was dried in a rotary evaporator Buchi B-480 (Uster, Switzerland) coupled to a vacuum controller Buchi B-720 (Uster, Switzerland) system at 40°C and 10 mbar, without light until a constant weight was attained. Vacuum was broken with N\(_2\) stream, to finally obtain the FAEE mixture of interest.

2.2.2 Urea complexation processes at laboratory scale

The FAEE mixture obtained was used as starting material in the fractionation processes by urea complexation. Different amounts of hexane, absolute ethanol, FAEE and urea were mixed in a 120 mL vial, placed in an incubator 4000ic IKA (Staufen, Germany) with orbital shaking. Particle size of urea was reduced under 250 μm in a knife mill Grindomix GM200 Restch (Haan, Germany) for 1 min at 1000 rpm. The amount intervals of the different ingredients used in the study were: Urea (granulated) from 0.5 to 5 g; hexane from 2.667 to 8 mL; absolute ethanol from 0 to 2.4 mL; and FAEE from 200 to 1200 mg. Reactions took place at room temperature (~25°C) with stirring of 200 rpm. The reaction time was also studied from 1 min to 72 h.

When the reaction finished, the product was recovered by adding 4 mL hexane (2 times) and it was transferred to a pressurized filter glass (Aldrich) coupled to a kitasato flask with a N\(_2\) stream. In the filter, 4 mL of hexane were added again (2 times) to recover effectively the FAEE dissolved in the liquid phase. Two phases were obtained: a solid phase, constituted by urea and fatty acids which formed adducts with urea (mainly saturated and unsaturated); and a liquid phase, constituted by the solvent (hexane and ethanol) and fatty acids which did not form adducts (mainly polyunsaturated).

Afterwards, the liquid phase was washed twice with 40% (v/v) distilled water. These washes were performed to remove rest of urea and other polar compounds. The organic phase obtained was dried with anhydrous sodium sulfate. Finally, the product was filtered and the liquid phase was evaporated on a Buchi B-480 rotary evaporator (Uster, Switzerland) coupled to a vacuum controller system Buchi B-720 (Uster, Switzerland) at 40°C and 10 mbar, in absence of light and breaking the vacuum with a N\(_2\) stream, thus obtaining the FAEE of interest.

In order to close effectively the mass balance, several experiments were selected to recover and analyze the FAEE that were complexed with urea. Thus, the solid phase was washed with acidic water (1% H\(_2\)SO\(_4\), v/v in distilled water) until the solid was completely dissolved. Then, two extractions were carried out with 4 mL of hexane. Anhydrous sodium sulfate was added to the organic phase to remove water traces. Then the liquid phase was filtered and evaporated on a rotary evaporator at the previously mentioned conditions, obtaining the fraction of FAEE.

2.2.3 FAEE analysis

The FAEE obtained were dissolved in hexane at 15 mg/mL, and analyzed by gas chromatography (GC). FAEE quantification was performed on Agilent GC (Avondale, PAEEU) (6850 System Network GC) coupled to an autosampler (Agilent 6850) with 30 m capillary column HP-88 (Avondale, EEUU) (0.25 mm id and 0.20 μm film) and FID detector. The injection volume was 1 μL and split ratio 1:20 was used. Injector and detector temperatures were 220°C and 250°C, respectively. The temperature program used was the following one: at first 50°C rising to 220°C at 15°C min\(^{-1}\). The final temperature, 220°C, held for 10 min. Identification of different FAEE was based on the retention times and relative area percentages of a standard PUFA No. 3 (# 4-7085) obtained from Supelco (Bellefonte, USA). For quantification, a calibration curve with different concentrations of ethyl oleate was used.

2.2.4 Pilot plant scale processes

The optimal conditions defined at laboratory scale were used for the pilot plant scale processes. Thus, 500 g of urea (powdered), 600 mL of 99% hexane, 90 mL of absolute ethanol and 133 g of FAEE were mixed in a Buchi kihloclave reactor (Uster, Switzerland) using a double-jacketed tank with stirring volume of 5 L, coupled to a cryothermostat Julabo F32-HE (Seelbach, Germany). The process was performed at 25°C with continuous and gentle mechanical agitation. After 20 h, the product was recovered by adding 1 L of hexane, and filtered through a stainless steel filter coupled to the reactor. Subsequently, the liquid phase obtained was washed 2 times (with acidic water and distilled water) in a separatory funnel of 2 L capacity, and finally, the organic layer was evaporated at the same conditions described at laboratory scale. The scaling process at pilot

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*J. Oleo Sci.* 67, (9) 1091-1099 (2018)
3 Results and discussion

As previously mentioned, the main objective of this work was to optimize the concentration of SDA and other fatty acids with bioactive properties, mainly anti-inflammatory, from Echium oil. Different variables involved in the process were investigated to enhance two responses: Concentration or purity: % in composition of one compound

\[
\frac{\text{Fatty acid weight in one fraction (mg)}}{\text{Weight of the whole fraction (mg)}} \times 100
\]
Yield: % recovery of one compound

\[
\frac{\text{Fatty acid weight in one fraction (mg)}}{\text{Weight of the fatty acid in the starting material (mg)}} \times 100
\]

The procedure at room temperature (\(\sim 25^\circ\text{C}\)) was selected for the urea complexation processes, because at low temperature degradation (mainly oxidation) of polyunsaturated \(\alpha\)-3 FAEE can be avoided, lower energetic requirements are needed, and the formation of ethyl carbamates may also be reduced\(^{25}\). Hence, the initial conditions of the study were based on the procedure by Linstead et al. with slight modifications\(^{20}\): 200 mg of FAEE, 0.8 mL of absolute ethanol, 8 mL of hexane and 3 g of urea (in this order of addition), at room temperature (\(\sim 25^\circ\text{C}\)).

3.1 Reduction of the particle size of urea

It was demonstrated that fractionation was much more effective by using urea with a reduced particle size (powdered) than with granulated urea. When granulated urea was used only slight formation of adducts with saturated FAEE occurred, whereas mono and polyunsaturated composition hardly changed. On the contrary, adducts formation with saturated FAEE was complete when powdered urea was used, so these fatty acids were practically not detected in the liquid phase. Hence, oleic acid concentration decreased (from 15% to 2.5%), LA and ALA remain constant (\(~15\%\) and 34%, respectively), and GLA and SDA increased (from 11% to 21%, and from 14% to 27%, respectively) in the liquid phase. These differences on the fractionation efficiency can be explained because a reduced particle size can lead to a higher amount of urea dissolved in the medium, and also provide greater surface area, resulting in an enhanced contact between urea and FAEE.

3.2 Amount of urea

Secondly, the amount of urea used in the process was investigated (see Fig. 1).

As shown in Fig. 1, an increased amount of urea led to higher SDA concentrations and reduced SDA yields. Thus, suitable urea/FAEE ratios are necessary to attain good enrichments according to the two responses studied. If the urea/FAEE ratio is low, the amount of urea in the medium is insufficient for the complexation of saturated and mono-unsaturated FAEE. On the other hand, with a high urea/FAEE ratio, the formation of adducts is possible even with polyunsaturated FAEE, causing yield losses. We concluded that 3 g of urea can provide a good relationship between SDA concentration (\(~27\%)\) and yield (\(~89\%)\).

3.3 Volume of ethanol (first study)

At this point, the effect of the amount ethanol was studied. Figure 2A shows the results of concentration of the different FAEE. It was observed that 0.8 mL (10% v/v related to hexane) was the lowest amount of ethanol that made the fractionation possible at these conditions. With this volume of ethanol, the composition drastically decreased in saturated and monounsaturated FAEE, LA and ALA kept constant, and GLA and SDA remarkably increased in the liquid phase. Regarding the yield (Fig. 2B),

![Fig. 1 SDA concentration and yield for different amounts of urea. 8 mL of hexane, 0.8 mL of ethanol and 200 mg of FAEE were used.](image)
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Fig. 2 FAEE concentration (2A) and yield (2B) for different volumes of ethanol. 3 g of urea, 8 mL of hexane and 200 mg of FAEE were used.

~100% of saturated FAEE, 92.5% of monounsaturated, and 55% of LA and ALA, were removed, whereas more than 80% of GLA and SDA remained in the liquid phase utilizing 0.8 mL of ethanol.

Figure 2B also shows that amounts of ethanol over 0.8 mL decreased the yield of LA, ALA, GLA and SDA (~10%). Considering behavior and assuring the enrichment of SDA and GLA, the minimum amount of ethanol (0.8 mL) was selected in order to prevent the formation of ethyl carbamates in the process.

Furthermore, Fig. 2 shows some similarities in the behavior of LA-ALA and GLA-SDA, respectively, in the urea adducts formation. These similarities could be explained by the resemblance on the chemical structure of LA with ALA, and GLA with SDA, respectively (see Fig. 3).

Urea crystallizes in a hexagonal structure of 8-12Å diameter channels, which are enough to accommodate long chain linear molecules. The number and position of double bonds affect the geometry of the molecule and thus, the stability of the urea-FA adducts. As shown in Fig. 3, the position of the first double bond from the carboxyl terminal group in fatty acids makes LA and ALA have a linear region of eight carbons, whereas that of GLA and SDA have five. This suggests that LA and ALA, which have a longer linear region, can better enter into the urea channels and, therefore, the urea complexation can be more effective with these compounds. On the other hand, GLA and SDA structures have a shorter linear region, which produces weaker adducts, so they preferably tend to remain in the liquid phase. In addition, this hypothesis suggests that, the entry by the fatty acid into the urea channels occurs from the terminal group, i.e., the ester bond in case of FAEE or the carboxyl terminal group in case of free fatty acids.

Hence, the curvature on the chemical structure caused by the position of the double bonds in the molecule can affect to urea complexation and other methodologies, that involves the crystallization point of fatty acids (winterization) or its entry into de active site of different lipases (enzymatic separation).

3.4 Volume of hexane

Subsequently, the volume of hexane was evaluated. As shown in Fig. 4, the lowest volume of hexane (2.67 mL) gave the highest GLA and SDA concentration, with a decreasing in LA and ALA. Lower under 2.67 mL were not investigated because they did not provide an acceptable mixture of the components and an homogeneous reaction medium. When low amounts of hexane were used, saturated and monounsaturated FAEE were completely removed, ~85% of ALA and LA, and ~40% of GLA and SDA (data not shown on Fig. 4). One possible reason may be that hexane is capable to enter into the urea channels and, accordingly, compete with FAEE to form adducts.

Therefore, low amounts of hexane provide greater amounts of urea available to form adducts in the medium, which enhanced the enrichment of polyunsaturated (GLA and SDA). Hence, considering SDA concentration achieved

Fig. 3 SDA (A), GLA (B), LA (C), ALA (D) tridimensional chemical structures, in free fatty acids form. Oxygen (●), carbon (●), hydrogen (○).
(39%), 2.67 mL of hexane were selected as optimal volume, although SDA yield was reduced to 63%.

3.5 Amount of FAEE

At this point, augmented loads of FAEE were investigated in order to improve the volumetric productivity of the process (see Fig. 5).

As with hexane, higher amounts of FAEE caused a reduced selectivity in the fractionation, since lower amounts of urea were available to form adducts. Hence, higher amounts of FAEE resulted in less removal of LA and ALA, which increased the concentration and yield of these compounds in the liquid fraction, decreasing GLA and SDA concentration. We concluded that an intermediate amount of 800 mg of FAEE per 2.67 mL of hexane (300 mg/mL) can be a reasonable optimum for a good production in a scaling-up, providing a good relationship between SDA concentration (28%) and yield (50%).

3.6 Volume of ethanol (second study)

The ratio hexane/ethanol is crucial, because this parameter can modulate the capacity to dissolve urea, putting it in contact with the FAEE for the adducts formation. Thus, a good ratio between solvents largely ensures an efficient formation of adducts. For that reason, a second study of ethanol was performed to achieve the best enrichment according to the optimal amount of hexane tested (2.67 mL). The least amount of ethanol was intended in order to prevent the formation of ethyl carbamates.

As shown in Fig. 6, when reducing the volume of ethanol, SDA concentration decreased and SDA yield was greater. Under 0.4 mL of ethanol, SDA concentration was excessively low, so 0.4 mL of ethanol for 2.67 mL of hexane and 800 mg of FAEE provided a good ratio for the two responses (28% SDA concentration and 66% yield).

3.7 Physical appearance of the product

It was observed that the product obtained after reaction at optimal conditions at laboratory scale, was a hard solid with appearance of crystal. So, it was necessary to mechanically disaggregate it for the subsequent steps. For that reason, the volume of the solvents was slightly increased, maintaining the hexane/ethanol ratio constant, in order to better handle the product in a further pilot plant.

Fig. 4 FAEE concentration for different volumes of hexane. 3 g of urea, 0.8 mL of ethanol and 200 mg of FAEE were used.

Fig. 5 FAEE concentration (5A) and yield (5B) for different loads of FAEE. 3 g of urea, 2.67 mL of hexane and 0.8 mL of ethanol were used.

Fig. 6 SDA concentration and yield in the second study of ethanol. 3 g of urea, 2.67 mL of hexane and 800 mg of FAEE were used.
3.8 Reaction time of urea complexation (kinetic study)

A kinetic study was performed at optimal conditions to evaluate the time needed to form the urea adducts. Figure 7 shows the SDA concentration reached at different reaction time, using powdered and granulated urea for SDA concentration.

It was observed that, when granulated urea was used, adducts formation was inefficient regardless the time. On the other hand, adducts formation occurred immediately when powdered urea was used. Thus, in the first 5 min most of saturated and monounsaturated FAEE formed adducts and SDA concentration reached 20%. From that point on, SDA concentration increased slowly and progressively, whereas SDA yield only showed slight variations (data not shown). For that reason, 20 h was considered appropriate to attain acceptable SDA concentration and yield to scale-up the process at pilot plant.

3.9 Pilot plant scale

When all variables involved were optimized at laboratory scale, a scaling-up of the process was carried out in a pilot plant. The conditions of this study were as follows: 500 g of powdered urea; 600 mL of hexane; 90 mL of absolute ethanol; 133 g of FAEE; at room temperature (25°C), for 20 h. This procedure was performed twice and more than 100 g of final product enriched in polyunsaturated FAEE was obtained (see Table 1).

As shown in Table 1, SDA concentration and yield were ~29% and ~78%, respectively. It should be noted that the process was readily scalable with high reproducibility, and the final product was composed of ~84% of bioactive fatty acids with potential anti-inflammatory properties.

3.10 Analysis of ethyl carbamates

Finally, the products obtained from the optimized process at laboratory and pilot plant scale, were analyzed to elucidate their content in ethyl carbamates. It should be noted that these compounds were not detected with a detection limit of 0.4 ppm.

Table 1 Concentration (%) and yield (%) after concentration by urea complexation from Echium oil, at pilot plant scale. Number after mean values and “±” symbol denotes standard deviation.

| FAEE                  | Original Echium Concentration (%) | Pilot Plant Scale Concentration (%) | Pilot Plant Scale Yield (%) |
|-----------------------|-----------------------------------|-------------------------------------|-----------------------------|
| Palmitic (16:0)       | 6.8                               | 0.1 ± 0.0                           | 0.4 ± 0.1                   |
| Stearic (18:0)        | 3.5                               | 0.0 ± 0.0                           | 0.0 ± 1.5                   |
| Oleic (18:1)          | 15.1                              | 1.8 ± 0.6                           | 4.6 ± 0.4                   |
| Linoleic (18:2 ω-6)   | 15.0                              | 14.1 ± 0.1                          | 36.0 ± 0.1                  |
| γ-linolenic (18:3 ω-6)| 11.4                              | 22.5 ± 0.3                          | 75.2 ± 1.4                  |
| α-linolenic (18: ω-3) | 33.2                              | 32.0 ± 0.1                          | 36.9 ± 0.4                  |
| Stearidonic (18:4 ω-3)| 14.3                              | 29.2 ± 0.3                          | 77.8 ± 1.2                  |

Fig. 7 Kinetic study for SDA concentration. 3 g of urea, 3.6 mL of hexane, 0.54 mL of ethanol, 800 mg of FAEE were used. 72 h was also studied (data not shown), without increase of % SDA in both cases.
4 Conclusions

Concentration of SDA and other bioactive PUFA by urea complexation, from *Echium* oil, is a simple, inexpensive and highly effective process, so its viability for an industrial application can be considered in the future. Thus, optimal conditions at laboratory scale were achieved: 3 g of urea, 3.6 mL of hexane, 0.54 mL of absolute ethanol, 800 mg of FAEE, and 20 h (at 25 °C), to maximize composition (~26%) and yield (~74%) of SDA. In addition, it has been demonstrated that the reduction particle size of urea is essential to accomplish an effective urea complexation at 25 °C. Besides, it was proved that the chemical structure of the FAEE, conditioned by the curvature caused by the number and position of double bonds, lead to parallelisms between LA-ALA and GLA-SDA in the fractionation process. The process was easily scaled to the pilot plant to obtain more than 100 g of a product with ~29% SDA concentration and ~78% yield, and with absence of ethyl carbamates. Hence, at optimal conditions, a blend of about 85% bioactive fatty acids with anti-inflammatory properties was obtained, which is very interesting as high added-value product with great potential for the synthesis of functional lipids and nutraceuticals.

Acknowledgments

This work has been supported by the Comunidad Autónoma de Madrid: ALIBIRD: project number S2013/ABI-2728, and by Ministerio de Economía y Competitividad: project number AGL2016-76736-C3-1-R.

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