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Enzymatic production of bioactive docosahexaenoic acid phenolic ester

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ABSTRACT

Docosahexaenoic acid (DHA) is increasingly considered for its health benefits. However, its use as functional food ingredient is still limited by its instability. In this work, we developed an efficient and solvent-free bioprocess for the synthesis of a phenolic ester of DHA. A fed-batch process catalyzed by *Candida antarctica* lipase B was optimised, leading to the production of 440 g/L vanillyl ester (DHA-VE). Structural characterisation of the purified product indicated acylation of the primary OH group of vanillyl alcohol. DHA-VE exhibited a high radical scavenging activity in acellular systems. *In vivo* experiments showed increased DHA levels in erythrocytes and brain tissues of mice fed DHA-VE-supplemented diet. Moreover, *in vitro* neuroprotective properties of DHA-VE were demonstrated in rat primary neurons exposed to amyloid-β oligomers. In conclusion, DHA-VE synergized the main beneficial effects of two common natural biomolecules and therefore appears a promising functional ingredient for food applications.

1. Introduction

Omega-3 polyunsaturated fatty acids from fish oils promote well-established health and anti-aging benefits that justify their use as functional ingredients in dietary supplements, healthy foods and nutraceutical products (Swanson, Block, & Mousa, 2012). Among them, eicosapentaenoic (EPA, C20:5) and docosahexaenoic acids (DHA, C22:6) continue to receive particular attention because of their numerous biological properties and positive effects on human health (Lorente-Cebrian et al., 2013). In addition to recognised benefits for the prevention of cardiovascular diseases, EPA and DHA were reported to protect from inflammation-mediated disorders including obesity and diabetes, Alzheimer's and related neurodegenerative diseases (Calder, 2013; Farooqui, 2012).

Human metabolism exhibits limited ability to synthesise ω3 PUFAs. Dietary supply of preformed compounds therefore appears an essential alternative. However, the practical use of such lipids as food ingredients is often limited by their high susceptibility to oxidation, which is responsible for the undesirable off-flavour and odour of rancid oils, associated with a loss of nutritional value (Albert, Cameron-Smith, Hofman, & Cutfield, 2013). Various solutions can be implemented to minimise these degradation pathways and the most commonly used by manufacturers is addition of antioxidants (Wang et al., 2011). Intensive research has been pursued on natural phenolic antioxidants issued from plants. Many studies reported their high efficiency to protect ω3-enriched food products from oxidation (Huber, Rupasinghe, & Shahidi, 2009; Sekhon-Loodu, Warnakulasuriya, Rupasinghe, & Shahidi, 2013). In addition, some works pointed out the interest of formulations mixing ω3 PUFAs and phenolic compounds for the prevention of

Abbreviations: Aβ, amyloid-β; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); BHT, butylated hydroxytoluene; CALB, *Candida antarctica* lipase B; DHA, docosahexaenoic acid; DHA-EE, DHA-ethyl ester; DHA-VE, DHA-vanillyl ester; DPPH, 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl; EPA, eicosapentaenoic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TEAC, trolox equivalent antioxidant capacity; VA, vanillyl alcohol.

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Alzheimer's disease and the treatment of obesity through lipid-lowering effects (Cole et al., 2005; Radler et al., 2011). Another approach consists in bringing together ω3 lipids and phenolic compounds into a single entity as described by few authors (Mbatia, Kaki, Mattiasson, Mulaa, & Adlercreutz, 2011; Torres de Pinedo, Penalver, Rondon, & Morales, 2005; Ying Zhong & Shahidi, 2011). Such an association was shown to improve the stability of highly oxidizable fatty acids while facilitating the solubilisation of phenolic compounds in lipid phases. Additional effects could be an increased bioavailability of phenols as well as cumulative and even synergistic biological activities (Tan, Le, Moghadasian, & Shahidi, 2012; Wahle, Brown, Rotondo, & Heys, 2010). In addition, PUFA phenolic esters were reported to exhibit higher antimicrobial, antioxidant and anti-inflammatory activities than native phenols (Mellou et al., 2005; Zhong, Chiou, Pan, & Shahidi, 2012).

Fatty-acid phenolic esters are preferentially produced by enzymatic bioprocesses that exhibit high selectivity towards polyfunctional substrates and mild reaction conditions comparing with chemical synthesis pathways. Most often, esterification reactions were carried out in dry organic solvent, but few studies reported the possibility to process without any solvent (Hong, Ma, Kim, Seo, & Kim, 2012; Weitkamp, Weber, & Vosmann, 2008). Moreover, most of these studies only intended to make the proof of concept, but ignored upscale potential.

The objective of the present work was to develop an efficient and environment-friendly solvent-free bioprocess for the synthesis of DHA vanillyl ester (DHA-VE) in sufficient quantities to allow further *in vitro* and *in vivo* experiments as well as potential applications. DHA was chosen because of its major protective potential, while vanillyl alcohol (VA) is a phenolic compound commonly found as antioxidant in foodstuffs. DHA-VE bioavailability was evaluated in mice, as well as its neuroprotective properties *in vitro*, in comparison with those of DHA and VA used alone or in combination.

2. Materials and methods

2.1. Chemicals and enzyme

Candida antarctica lipase B (CALB) immobilized on a macroporous acrylic resin (Novozym 435®, Novo Industry) was used to catalyze acylation reactions. Docosahexaenoic-acid ethyl ester (DHA-EE, 95% pure) was supplied by KD-Pharma (Bexbach, Germany). Solvents of analytic or HPLC grade were from Merck. Vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol, VA) and all other chemicals were purchased from Sigma–Aldrich Chemicals.

2.2. Enzymatic synthesis of DHA vanillyl ester (DHA-VE)

2.2.1. Enzymatic synthesis of DHA-VE in organic medium

Enzymatic acylation reactions were achieved in organic solvent, under atmospheric pressure. Reaction media were prepared by solubilising VA (100 mM, 15.4 g/L) and DHA-EE (200 mM) in water-free acetonitrile (water activity below 0.1). Reactions were performed under orbital shaking and initiated by adding 20 g/L of Novozym 435®. This protocol was further referred as solvent system.

2.2.2. Synthesis of DHA-VE in molten media

Reactions were performed under either atmospheric or reduced pressure. For syntheses achieved under atmospheric pressure, reaction media were prepared by solubilising VA (200 mM, 30.8 g/L, which corresponds to the maximal solubility of the substrate at 50 °C) in a large excess of DHA-EE as acyl donor (2 mL), at 50 °C. Reactions were initiated by adding 20 g/L of Novozym 435®. After 72 h of reaction, the supernatant was taken after enzyme particle decantation. This protocol led to reaction system A.

Syntheses achieved under reduced pressure were performed at 37 °C and 500 mbar in a rotary evaporator, so that the by-product of the reaction, *i.e.* ethanol, could be continuously eliminated while avoiding VA evaporation. Reaction media were prepared by solubilising VA (162 mM, 25 g/L that corresponds to the substrate solubility at 37 °C) in 10 mL of DHA-EE. Reactions were started by adding 20 g/L of Novozym 435®. After 72 h, the enzyme was eliminated by filtration. This protocol led to reaction system B.

2.2.3. Process intensification

The production of DHA-VE was intensified by increasing VA intakes. VA (50 g/L) was introduced under a nitrogen stream (fed batch process) in 10 mL of DHA-EE. Syntheses were performed as described in Section 2.2.2. After the first 4 h of synthesis, the reaction medium was filtered and transferred to another flask. A new supply of VA (50 g/L) was then carried out. Once the phenolic compound was totally solubilised, the reaction was restarted by adding fresh enzymatic preparation, thereby maintaining optimal activity. This protocol was repeated twice. This protocol led to reaction system C.

2.2.4. Kinetic following of the syntheses

Kinetic following of the reactions was performed by HPLC, using a Shimadzu Class-VP system equipped with a computer-controlled system (Class-VP 6.1 software). Separations were carried out on a reversed-phase Altima C18 column (150 × 2.1 mm, 5 μm, Grace-Alltech). VA and DHA-VE were detected at 280 nm on a multichannel photodiode-array detector (SPD-M10A VP). DHA-EE and free DHA were followed by evaporative light scattering detection (ELSD), using nebulizer and evaporator temperatures of 35 °C and 45 °C, respectively. Analyses were carried out with compressed air as ELSD gas at a pressure of 1.5 bars. Elution was performed using a gradient of solvent A [methanol/water 70/30 (v/v)] and B [methanol (100%)], at a flow rate of 0.2 mL/min. Elution protocol was as follows: 0–5 min: 100–0% A, 5–25 min: 100% B, 25–35 min: 100–0% B, 35–45 min: 100% A. Calibrations were made using analytical standard compounds. During synthesis, aliquots were withdrawn from each reactor at predetermined times and then diluted 100 times in solvent A. All samples were filtered through a 0.2-μm membrane before injection.

2.3. Purification of DHA-VE by flash chromatography

DHA-VE was purified on glass columns KONTES CHROMAFLEX packed with silica gel 60 (particle size of 40–63 μm). Gradient system was generated by 2 pumps Gilson model 306. Cyclohexane and ethyl acetate were used as mobile phase. The elution gradient was as follows: 0–40 min: 5–15% ethyl acetate, 40–60 min: 15–40% ethyl acetate. The flow rate was 20 mL/min. A sample of 20 mL was collected every minute for proper identification. The presence of the ester was detected by thin-layer chromatography on precoated silica gel $60F_{254}$ TLC plates, referring to the pure molecule as standard. The plates were visualised under UV light at 254 nm and then sprayed with a solution of sulphuric acid 20% in methanol. Fractions containing the ester were combined and the solvent was evaporated.

2.4. Structural analyses

2.4.1. Liquid chromatography–mass spectrometry (LC–MS)

The structure of the product was determined by HPLC-MS. The mass spectra were obtained using a binary solvent delivery pump and a linear ion trap mass spectrometer (LTQ-MS, Thermo

Scientific, San Jose, CA, USA) equipped with an atmospheric pressure ionisation interface operating in ESI mode. Control of equipment and data processing were realised using Xcalibur software (version 2.1). The operational parameters were as follows: the spray voltage was 4.5 kV and the temperature of heated capillary was set at 200 °C. Flow rates of sheath gas, auxiliary gas, and sweep gas were set to 20.5 and 4 (in arbitrary units/min), respectively. Capillary voltage was −18 V, tube lens was −80 V, split lens was 11 V, and the front lens was 6.25 V. MS parameters were optimised by infusing a standard solution of DHA-VE in mobile phase (methanol/water: 70/30) at a flow rate of 5 μL/min.

2.4.2. Nuclear magnetic resonance (NMR)

The pure product was identified and characterised by NMR analyses. NMR experiments were performed in $CDCl_3$ with a Bruker Avance DRX-400 instrument operating at a proton frequency of 400.13 MHz and equipped with a 5 mm broadband inverse detection z-gradient probe tuned to ^{13}C (100.61 MHz). For all 1D and 2D NMR experiments, pulse sequences provided by the spectrometer manufacturer were used.

2.5. Evaluation of antioxidant activity

2.5.1. Inhibition of DNA scission

Inhibition activity of biomolecules towards DNA scission was determined by using H_2O_2 (0.2 mM) and $FeSO_4$ (0.1 mM) to generate hydroxyl radicals that induced strand breaks in plasmid DNA (10 μg/mL) (Hiramoto, Ojima, Sako, & Kikugawa, 1996). DNA scission was monitored after 1 h by agarose gel electrophoresis. Negative (DNA only) and positive controls (DNA + hydroxyl radicals) were compared to reactions carried out in the presence of DHA and VA used separately or together. The bands were visualised under UV light and intensity of the band corresponding to the native supercoiled plasmid was measured by densitometry.

2.5.2. Radical scavenging activity

The capacity of the compounds to scavenge radical species was determined by DPPH and ABTS methods, using VA and DHA-VE concentration range of 0.5–2 mM. Free radical scavenging activity was expressed as trolox equivalent antioxidant capacity or TEAC (Brand-Williams, Cuvelier, & Berset, 1995; Re et al., 1999).

2.6. Biological activities and bioavailability

2.6.1. Primary cell cultures and treatments

Cell culture media and materials were from Invitrogen. Brain cortical neurons were taken from Wistar rat fetuses at embryonic day 16–17 and cultured in serum-free medium. Neurons were treated with 1 μM of amyloid-β (Aβ) oligomers for 24 h prior monitoring cell viability using the MTT reduction assay (Florent et al., 2006). Alternatively, indicated concentrations of DHA or DHA-VE were mixed with fatty acid-free BSA (0.1%, w/v) and added to the medium 24 h before Aβ treatment.

2.6.2. Animals and diets

Male C57BL/6J mice (10-week old) came from Janvier (Le Genest-St-Isle, France) and accustomed for 2 weeks before the study. Animals were housed in individual cages, in a controlled environment (22 ± 1 °C, 50 ± 5% humidity) with a 12-h light/dark cycle, and with access to food and water *ad libitum*. The animal facilities and all procedures were approved (# DDSV/54/04/ENV/065) by the Animal Care and Veterinary Committee of Meurthe-et-Moselle (Nancy, France). Mice were randomly assigned to three groups of 6 mice each and fed diets of comparable caloric density. Control mice received standard food (Harlan 2080S), while DHA-supplemented mice were fed specific diet consisting of standard chow containing either 0.3% (w/w) DHA-EE or DHA-VE.

2.6.3. Fatty-acid analysis

Retro-orbital blood samples (100 μL) were collected after 3-h fasting period in lightly anesthetized mice and the cellular fractions were immediately frozen at −20 °C until extraction and analysis. Hippocampal and cortical tissues were taken from anesthetized mice shortly after sacrifice and immediately frozen. FA were extracted from all samples in a methanol/dichloromethane (3/1) mix prior to transesterification, purification of methylated derivatives and separation by GC (Lepage & Roy, 1986).

2.6.4. Statistical analysis

Stat View software was used for the statistical analysis. Data *in vitro* were obtained from three to four separate experiments with four determinations each. Normal data distribution was verified using Kolmogorov–Smirnov's test. Differences between groups and effects of treatments were analysed using parametrical statistic tests (paired and non-paired Student's *t*-tests and ANOVA). Differences were considered significant for $p \leqslant 0.05$.

3. Results and discussion

3.1. Enzymatic synthesis of DHA-VE

The present work intended to develop an efficient and environment-friendly process to synthesise DHA-VE without any solvent. Various operating conditions were applied to produce high concentrations of ester while limiting oxidative degradation. An alcoholysis reaction between DHA-EE and VA was applied, leading to DHA-VE and ethanol as by-product. The reaction feasibility was verified in an organic solvent. The synthesis was then intensified in solvent-free media by shifting the thermodynamic equilibrium of the reaction.

3.1.1. Synthesis of DHA-VE in organic solvent

Lipase-catalyzed acylation reactions are classically carried out in dry organic solvents that allow a good substrate solubilisation, while avoiding the denaturation of the enzyme. In the present study, experiments were achieved in acetonitrile where VA solubility was 15.8 g/L. The DHA-EE/VA molar ratio was adjusted to 2. HPLC analyses showed the synthesis of two products, along with VA and DHA-EE (respective retention times of 2.7 and 17.8 min) consumption. The first product (retention time of 16.5 min) was detected by DEDL and UV equipment, and identified as a monoester of DHA and VA by LC–MS analyses ($M+H^+ = 465.08$ g/mol) (Fig. 1A). The second product (retention time of 15.4 min) was detected by DEDL only, and identified as free DHA resulting from the competitive hydrolysis of DHA-EE. At the end of the reaction, the ester production was 25 g/L, corresponding to conversion yields of 54% and 27% for VA and DHA-EE, respectively.

This conversion yield appeared somewhat worse than that obtained for the enzymatic synthesis of vanillyl nonanoate, also known as capsinoid, in dioxane (Kobata, Kawaguchi, & Watanabe, 2002). However, this result was supported by other studies on VA acylation with long-chain acyl donors, showing that the longer the chain, the smaller the conversion yield (Reddy, Ravinder, Prasad, & Kanjilal, 2011). This tendency is even more pronounced in CALB-catalyzed acylation reactions between di-*ortho*-phenolic compounds and DHA-EE (Torres de Pinedo et al., 2005). Competitive DHA-EE hydrolysis was held responsible for these results.

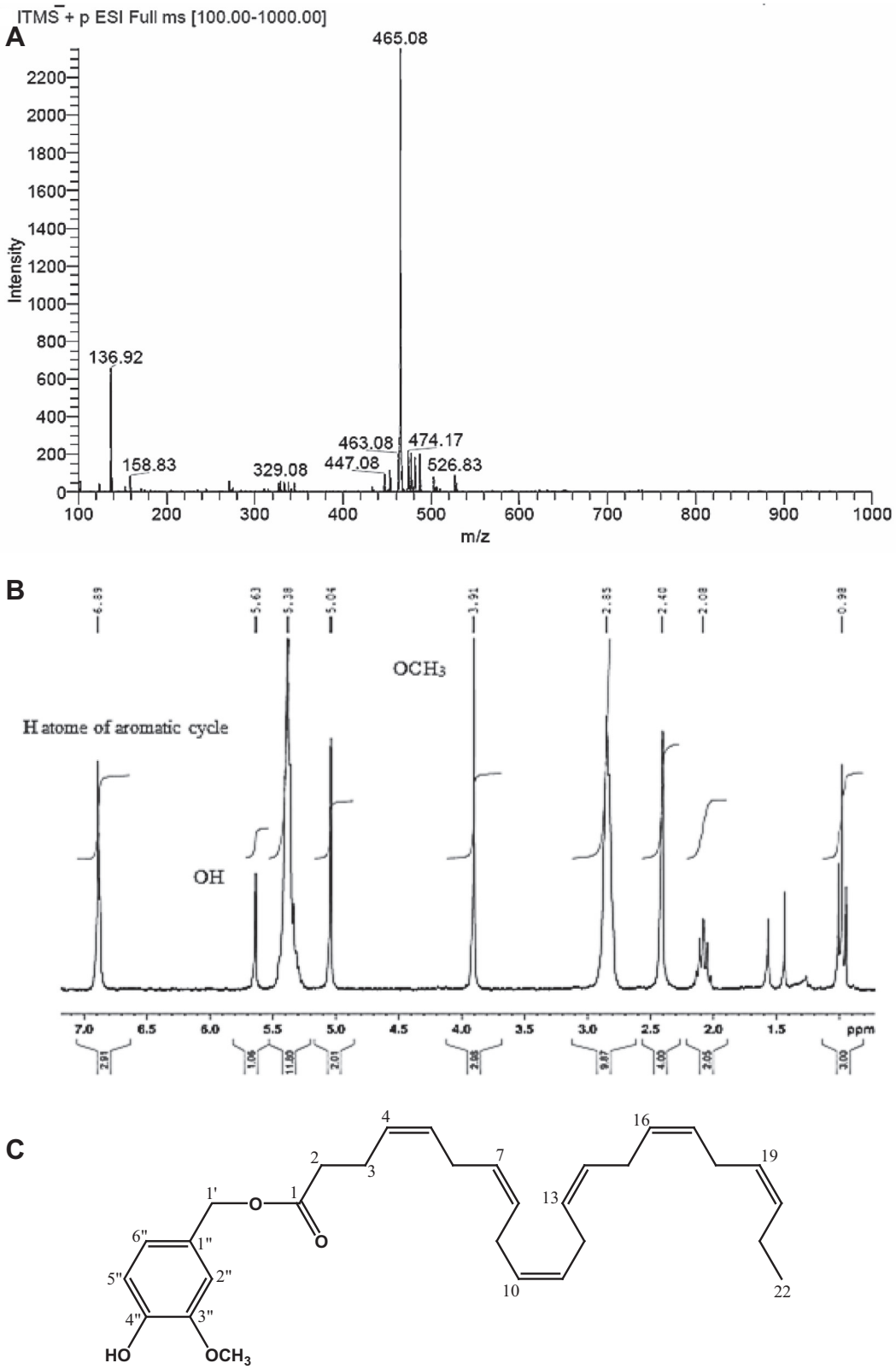

Fig. 1. Structural characterisation of DHA-VE. (A) MS analysis. (B) ^{1}H NMR analysis. (C) Deduced chemical structure.

3.1.2. Purification and structural characterisation of DHA-VE

VA structure exhibits two hydroxyl groups corresponding to one primary alcohol and one phenolic function. According to the literature on CALB specificity properties, the reaction was likely to occur exclusively on the primary hydroxyl group of the substrate (Parmar et al., 1999). This was verified by elucidating the structure of the purified ester by NMR (Fig. 1B and C). The polyunsaturated chain was grafted on the VA primary hydroxyl group, whereas the phenolic hydroxyl group remained unaffected, consistently with CALB selectivity towards polyhydroxylated phenolic

Table 1
Effects of enzymatic reaction conditions on substrate conversion yields and ester production. Reaction systems A–C are described in the Section 2.

Reaction system	DHA-EE conversion yield (%)	VA conversion yield (%)	DHA-VE production (g/L)
System A	7.5	90	84
System B	6	100	75
System C	12	100	150
Solvent system	27	54	25

compounds (Chebil, Humeau, Falcimaigne, Engasser, & Ghoul, 2006). For such substrates, this lipase was shown to catalyze primary or secondary hydroxyl group acylation only. Most significant IR bands were observed in the regions 3600–3100 cm^{-1}, 3013 cm^{-1} and 1737 cm^{-1}, corresponding to phenol, *cis* double-bond and ester carbonyl stretching vibrations, respectively. Moreover, the purity of the ester was 98%, referring to the integration of ^{1}H-NMR signals of both terminal methyl (0.98 ppm) and methoxy-phenyl (3.91 ppm) groups.

3.1.3. Synthesis intensification in melted media

This study also intended to develop a bioprocess able to produce DHA-VE in sufficient quantities to allow *in vitro* and *in vivo* experiments. This was achieved without any solvent in melted media, using acyl-donor substrate in large excess, allowing VA solubilisation. According to Le Chatelier's thermodynamic principle, this system tended to reduce the excess of acyl donor by shifting the reaction equilibrium in favour of ester synthesis. Syntheses were performed at 50 °C under atmospheric pressure (reaction system A) or at 37 °C under reduced pressure in either diluted (reaction system B) or saturated (reaction system C) VA solutions. These conditions led to ethanol elimination without VA distillation. VA solubility at 37 °C and 50 °C was 25 g/L and 30.8 g/L, respectively. Corresponding DHA-EE/VA molar ratios were 16.5 and 13.4.

Molten media increased VA conversion yields as compared with solvent medium (Table 1). The performance of the process was further improved by applying reduced pressure that allowed continuous elimination of ethanol throughout the reaction in systems B and C while limiting exposure to oxygen. VA conversion was completed after 2 h, independently of its initial concentration. 150 g/L of ester were obtained starting from VA-saturated media (reaction system C). Yellowing of the reaction medium was observed in syntheses achieved at 50 °C under atmospheric pressure, indicating an oxidative degradation. In contrast, no oxidation was noticed at 37 °C under reduced pressure. DHA-EE conversion yields were 7.5%, 6%, and 12% for reaction systems A–C, respectively.

These results are consistent with data on lipase-catalyzed reactions combining vacuum and molten media, showing total VA conversion with conjugated linoleic acid in the presence of *Rhizomucor miehei* lipase (Hong et al., 2012). However, for a given phenolic alcohol, the conversion yield seems to depend on the acyl-donor substrate, ethyl eicosapentaenoate and docosahexaenoate leading to lower performances than shorter acylating agents (Torres de Pinedo et al., 2005).

3.1.4. Synthesis of DHA-VE in fed batch mode

We developed a fed-batch bioprocess where the reactor was fed with dry VA, aimed to improve ester synthesis and concomitant DHA-EE consumption. Reaction kinetics was defined by subtracting the time required for VA dissolution from the total reaction time.

Fig. 2 illustrates the production of DHA-VE along the reaction when feeding the reactor with VA (50 g/L) three times. VA was totally converted at the end of each step, except for the last feeding that led to a conversion yield of 91%. The reaction rate was

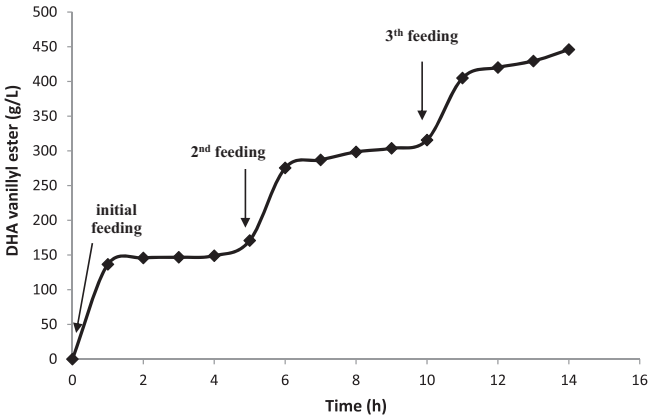

Fig. 2. Kinetic curve relative to enzymatic acylation of VA with DHA-EE. The CALB-catalyzed reaction was performed at 37 °C, under a 500-mbar pressure. The reaction medium was fed three times (arrows) with VA.

observed to decrease progressively with time. 440 g/L of DHA-VE were ultimately produced, corresponding to VA and DHA-EE conversion yields of 97% and 31%, respectively. Additional feedings were attempted to further improve the process, but both reaction

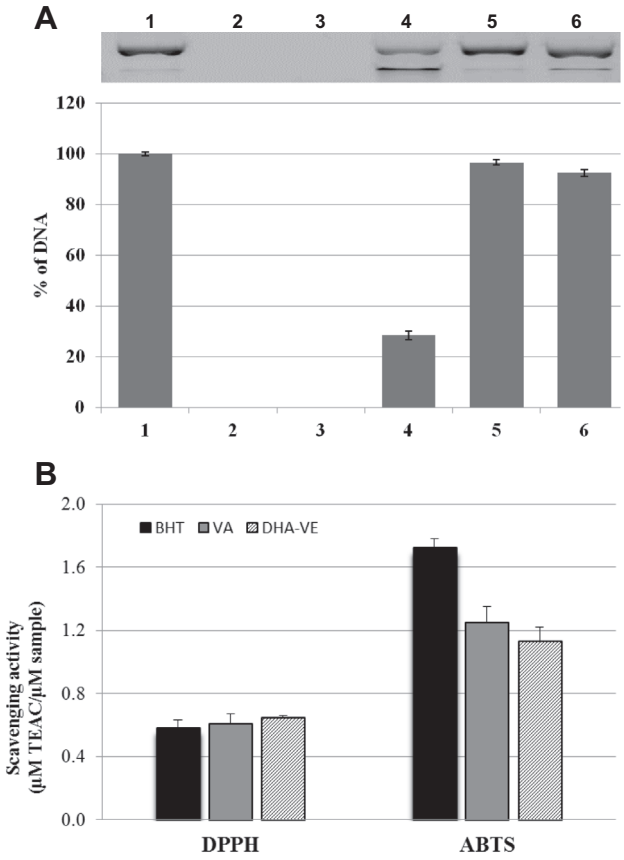

Fig. 3. *In vitro* antioxidant activity of DHA-VE. (A) DNA scission by hydroxyl radicals. Reactions were incubated at 37 °C for 1 h prior analysis in agarose gel electrophoresis. After estimation of band intensity, proportion of native supercoiled DNA was calculated and expressed in % of total DNA in each lane. Lane 1, native plasmid DNA; 2, plasmid DNA + [H_2O_2 + $FeSO_4$]; 3, plasmid DNA + [H_2O_2 + $FeSO_4$] + free DHA; 4, plasmid DNA + [H_2O_2 + $FeSO_4$] + DHA-EE; 5, plasmid DNA + [H_2O_2 + $FeSO_4$] + DHA-VE; 6, plasmid DNA + [H_2O_2 + $FeSO_4$] + VA. (B) Scavenging capacity towards DPPH· and $ABTS^{\cdot+}$ radicals. Activities were compared to that of BHT as a reference molecule. Data were expressed in TEAC values, as mean ± SEM, $n = 3$.

rate and VA conversion yield were observed to decrease along with oxidative degradation (data not shown). The duration of the reaction due to repeated feeding steps could appear as a limiting parameter for industrial scale-up and additional experiments should be investigated to improve the protocol. Nevertheless, this fed batch bioprocess seems to offer several advantages (maximal yields due to total VA consumption, absence of solvent) that should minimise production costs and meet some of the major industrial concerns.

3.2. Biological properties of DHA-VE

3.2.1. Antioxidant activity

Numerous studies showed that antioxidant activities of biomolecules are closely related to their chemical structure. Here, the antioxidant properties of VA and DHA-VE were determined and compared by using different *in vitro* assays.

3.2.1.1. Evaluation by DPPH· method. DPPH· is a stable lipophilic radical characterised by a deep violet colour. The capacity of compounds to quench the DPPH· radical can be evaluated from decrease in absorption at 517 nm (Xu, Yeung, Chang, Huang, & Chen, 2004). The scavenging activity of DHA-VE and VA was compared with that of butylated hydroxytoluene (BHT) as a reference molecule (Fig. 3B). BHT, VA and DHA-VE exhibited similar scavenging potential towards DPPH· radicals, suggesting that acylation did not affect this activity, as supported by other authors (Grasso, Siracusa, Spatafora, Renis, & Tringali, 2007). Acylation with DHA was even shown to increase the DPPH· scavenging capacity of epigallocatechin gallate (Zhong & Shahidi, 2011). Conversely, long-chain phenolic esters were reported to be less active towards DPPH· radicals than short-chain esters or than native phenols, probably due to higher hydrophobicity (Mbatia et al., 2011; Reddy et al., 2011; Salem et al., 2010).

3.2.1.2. Evaluation by ABTS·+ method. ABTS·+ is a blue/green-coloured radical that exhibits maximal absorbance at 734 nm and allows measuring antioxidant activity of both hydrophilic and lipophilic biomolecules (Re et al., 1999). BHT exhibited the highest activity (Fig. 3B). VA acylation did not affect its capacity to inhibit ABTS·+ radicals. A similar observation was reported when acylating isoquercitrin with fatty acids of various chain length, showing no effect of acylation for acyl chain of more than 10 carbon atoms (Salem et al., 2010).

3.2.1.3. Inhibition of DNA scission. Many studies reported the deleterious effect of oxygen radicals on DNA. The capacity of DHA-VE to protect plasmid DNA against oxidative damages induced by hydroxyl radicals was evaluated and compared to that of VA, DHA-EE and free DHA (Fig. 3A). DNA scission appeared in absence of any antioxidant compound as well as with free DHA. Addition of DHA-VE or VA led to retention rates of 96% and 92% of native supercoiled DNA, respectively. Since DHA-EE only retained 29%, the antioxidant properties of DHA-VE clearly pointed out the crucial role of the phenolic moiety. The ester appeared even more protective than VA, suggesting that VA monoacylation with DHA improved the antioxidant protection against DNA scission, based on hydroxyl radical scavenging activity and capacity to bind to iron (Perron, Hodges, Jenkins, & Brumaghim, 2008). Such an effect was also observed for epigallocatechin gallate DHA tetraester (Zhong & Shahidi, 2012), whereas negative outcome of acylation was reported with hydroxytyrosol, homovanillyl alcohol and their saturated esters (Grasso et al., 2007). These results suggest a significant influence of the fatty acid chain on the intrinsic antioxidant properties of phenolic esters.

3.2.2. Bioavailability of DHA-VE in mice

DHA is the major PUFA in neurons, where it is expected to provide protection in various stress conditions. We compared the bioavailability of dietary DHA in three groups of 3-month old mice

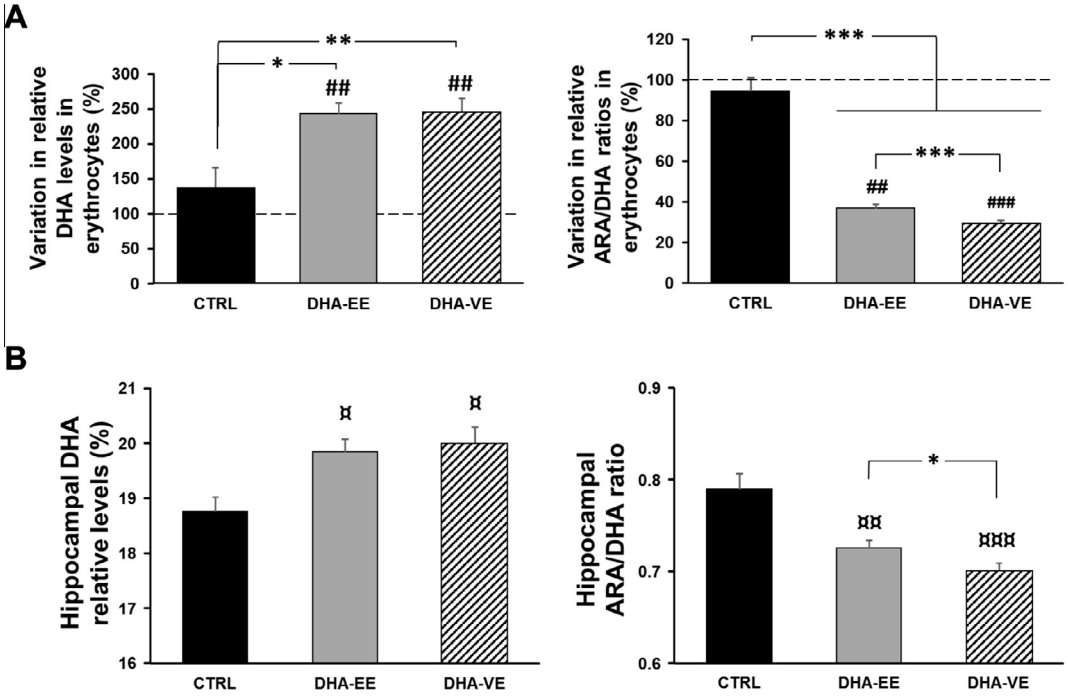

Fig. 4. Effects of DHA-VE-supplemented diet on FA relative contents. Mice (n = 6 per group) were fed specific diet for 30 days. (A) FA relative content in mice erythrocytes. FA concentrations after diet administration were compared for each individual and normalised to levels at baseline. (B) FA relative levels in hippocampus. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$: significant difference between indicated groups. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$: significant difference after 30 days *vs.* baseline on indicated diet. $^{¤}p < 0.05$, $^{¤¤}p < 0.01$, $^{¤¤¤}p < 0.001$: significant difference *vs.* CTRL diet.

fed control, DHA-EE or DHA-VE diet during 4 weeks. Body weight remained unchanged in these groups, although DHA-VE food intake was increased by 2.2% ($p < 0.05$) and 6.0% ($p < 0.001$) as compared to control and DHA-EE, respectively (data not shown). This suggested that VA could render food more appetizing to mice.

The FA profiles were then measured in brain and in blood cell membranes, mainly erythrocytes, known to store and transport DHA in the periphery and to the central nervous system (Hashimoto et al., 2005; Spector, 2001). After the 4-week study, a significant increase in relative DHA content accompanied with decreased ARA levels was observed in blood cell membranes of mice from DHA-EE and DHA-VE groups as compared to control mice as well as to levels at baseline (Fig. 4A). These findings agreed with previous studies on DHA-EE (Arterburn, Hall, & Oken, 2006; Hashimoto et al., 2002, 2005).

Similar enrichment in DHA was measured in hippocampal membranes in DHA supplemented mice *vs.* control group, along with decreased ARA levels and ARA/DHA ratio (Fig. 4B). These antagonistic effects upon DHA supplementation likely resulted from competitive incorporation of DHA and ARA at the *sn*-2 position of membrane phospholipids, as already suggested (Smink, Gerrits, Gloaguen, Ruiter, & van Baal, 2012). Such changes could provide benefits to DHA-enriched cells in preventing inflammation. Interestingly, ARA/DHA ratio was even significantly lower in both tissues of DHA-VE mice *vs.* DHA-EE mice. Altogether, our results clearly indicated that the phenolic moiety of DHA-VE tended to improve DHA bioavailability for target tissues. This also suggests that these esters were comparably metabolized by lipase/esterase activities and none of them induced apparent hepatotoxicity in supplemented mice as deduced from unchanged procaspase-3 profiles (data not shown).

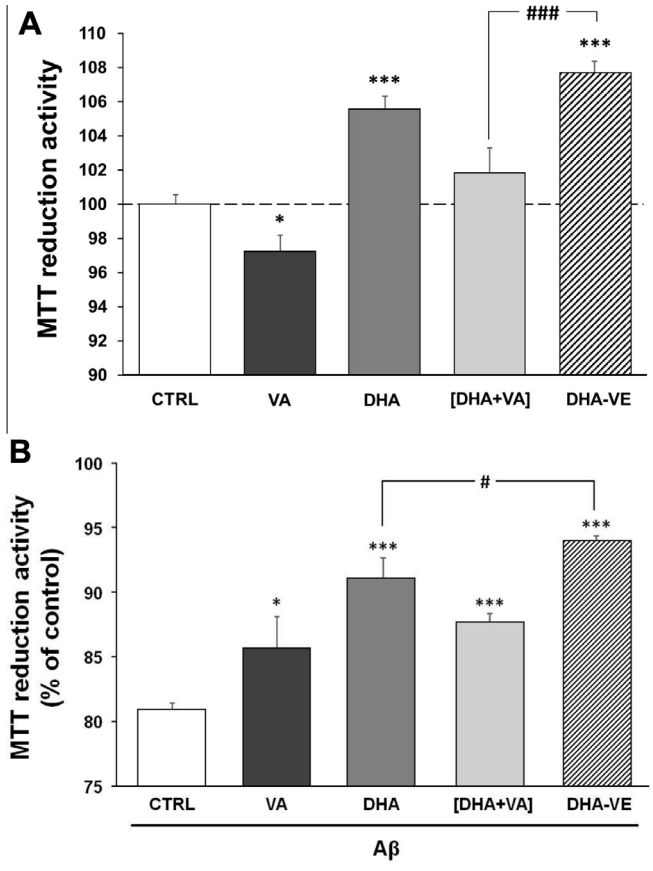

Fig. 5. Effects of DHA-VE on primary neurons *in vitro*. Cell viability was assayed by measuring mitochondrial MTT reduction activity. (A) Beneficial effects of DHA-VE. Neurons were cultured for 24 h in standard medium (CTRL) or supplemented with 1 μM VA, DHA, [DHA+VA] (equimolar mixture), or DHA-VE. (B) Neuroprotective effects of DHA-VE on Aβ-induced neurotoxicity. Neurons were pre-incubated for 24 h with VA, DHA, [DHA+VA] and DHA-VE, prior to exposure to Aβ (1 μM, 24 h). Data were normalised to the activity in CTRL cells exposed to vehicle (no Aβ) designated as 100%. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$: significant difference *vs.* CTRL. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$: significant difference between indicated groups.

3.2.3. Effect of DHA-VE on neuronal cell viability upon Aβ exposure in vitro

Free DHA was reported to protect neurons from amyloid stress *in vitro* (Florent et al., 2006). Using the MTT mitochondrial activity test, we observed that DHA-VE (up to 10 μM) did not induce any deleterious effects on primary neurons, in contrast with DHA-EE whose metabolism led to cytotoxic ethanol release (data not shown). Cell viability was lower when neurons were treated with free VA, but significantly increased with DHA and DHA-VE (Fig. 5A). Interestingly, a higher MTT activity was measured in neurons treated with DHA-VE than with DHA alone or in equimolar [DHA+VA] mixture. This suggests that free VA could be toxic in neuronal cultures, independently of DHA presence in the medium. Conversely, cumulative protective effects with DHA could be provided by VA upon its intracellular release from DHA-VE.

Next, we studied the influence of these treatments on neuronal resistance to Aβ insult known to induce oxidative synaptic impairment and neuronal apoptosis (Sponne et al., 2003). Expectedly, Aβ cytotoxicity led to a residual 80% MTT activity as compared with control cells. In contrast, neurons pretreated with 1 μM VA, DHA, [DHA+VA] or DHA-VE showed significant resistance (Fig. 5B). Upon Aβ exposure, viability of AV- and DHA-pretreated neurons was improved by 30 and 55%, respectively, which is in agreement with our previous data (Florent et al., 2006). DHA-VE offered the highest protection level in cultures as compared with the equimolar [DHA+VA] mixture (60% *vs.* 40% of Aβ-exposed neurons). Additional experiments should elucidate the beneficial pathways specifically activated by DHA-VE. Linkage of DHA to VA might prevent oxidation of DHA and promote its beneficial effects on neuronal membranes, thereby preventing Aβ-induced pro-apoptotic effects as widely demonstrated (Cao et al., 2009; Florent et al., 2006).

4. Conclusions

This paper presented the operating conditions that allowed efficient enzymatic acylation between two common natural food ingredients, *i.e.* vanillyl alcohol and DHA. The solvent-free protocol developed here led to high-level production of an ester that carries interesting potential for food industry and nutrition: (i) improved organoleptic qualities of DHA-VE-supplemented diet; (ii) elevated antioxidant activity that should stabilize DHA as well as various food matrices like oils, fats and emulsions; (iii) increased bioavailability of DHA leading to higher DHA levels in erythrocytes and neurons; (iv) combined beneficial effects of phenols and ω3 PUFAs; (v) increased neuroprotection against amyloid stress; (vi) no visible toxicity. DHA-VE and analogous esters can be considered stable bioactive ingredients that should broaden the scope of nutritional applications of ω3 PUFAs whose health benefits are increasingly sought.

Conflict of interest

The authors have no conflict of interest to declare.

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