Screening for pancreatic lipase natural modulators by capillary electrophoresis hyphenated to spectrophotometric and conductometric dual detection

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Keywords

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Abstract

Orlistat has been since its approval the only pancreatic lipase (PL) inhibitor used for the management of obesity. Nonetheless, it manifests several unpleasant side-effects shifting the balance between its efficiency and undesirability towards the latter. The search for novel PL inhibitors has gained increasing attention in recent years. Capillary electrophoresis (CE) present interesting advantages over other techniques such as economy in consumption, superior separation efficiency and the availability of several detection and separation modes. A dual detection CE-based homogeneous enzymatic assay was developed employing both the offline and online reaction modes. Tris/MOPS (10 mM, pH 6.6) was used as the background electrolyte for CE analyses as well as the incubation buffer for enzymatic reactions. The effect of dipalmitoylphosphatidylcholine vesicles on PL reaction kinetics was found to be minimal showing only a 4-5 fold increase in maximum velocity V_{max} and 3 times increase in the Michaelis constant K_m. Once validated, the method was applied to screen aqueous extracts of *Crataegus oxyacantha* (hawthorn), *Ribes nigrum* (blackcurrant) and *Chrysanthellum americanum* as well as **11** novel PL inhibitors and activators purified from natural extracts of oak wood, grapes and wine. The promising potential of using hawthorn leaves as herbal tea infusions for PL inhibition was demonstrated (37 ± 3 % at 1 mg mL^{-1}). Two triterpenoids purified from extracts of oak wood used in wine aging were identified for the first time as potent PL inhibitors demonstrating 51 and 57 % inhibition at 1 mg mL^{-1}.

Introduction

Pancreatic lipase (PL) (EC: 3.1.1.3) is a hydrolytic enzyme that catalyzes the digestion of the ester linkages of triglycerides (TGs) hydrolyzing them into monoglycerides (MGs) and free fatty acids in the small intestine thus playing an important role in facilitating the absorption of dietary fats through the intestinal wall. TG digestion by PL contributes to 50-70% of dietary fat digestion.[1] This digestion incorporates contributors such as bile salts and the pancreatic protein co-lipase both of which enhance PL activity in physiological conditions and thus ensure complete TG digestion and absorption.[2,3] Its contribution to TGs digestion and absorption has made PL and its inhibition a trending topic for the last century as a way to combat obesity and its ensuing diseases. The community's interest in the topic also arises from the scarcity of approved PL inhibitors in pharmaceutical markets. Xenical, also known as orlistat, is the only approved PL inhibitor in Europe and the USA used for long term weight management.[4] It is a chemically synthesized derivative of lipstatin, produced by the gram positive bacteria *Streptomyces toxytricini*.[3,5] Orlistat covalently binds to the active serine residue of PL hindering its TGs hydrolyzing faculty.[6,7] Despite orlistat's ability of depleting 30 % of TGs absorption,[8,9] several unpleasant secondary effects such as flatulence, oily stool, diarrhea[10,11] in addition to more grave but rare reports of hepatic and renal damage have been reported amongst patients.[12–14] As a result, there has been a large scale search for new natural and synthetic molecules capable of inhibiting PL but with more tolerable side effects. The extracts of many plants and fruits are considered as rich sources of biomolecules that serve as potential orlistat substitutes. For instance, epigallocatechin gallate (EGCG), a flavonoid abundant in green tea demonstrated promising PL inhibition with a half maximal inhibitory concentration (IC_{50}) of 0.8 ± 0.1 µM, lower than that determined for orlistat (IC_{50}= 32 ± 8.5 µM).[15] Saponins, a class of triterpenoids isolated from *Acanthopanax senticosus,* had an IC_{50} ranged between 220-290 µM compared to 40 µM for orlistat.[16] On the other hand, some synthetic molecules have been shown to induce PL inhibition. Recent examples include rhodanine- 3- acetic acid derivatives (IC_{50} = 5.16 ± 0.31 µM)[17] or indole glyoxylamide analogues (IC_{50} = 4.92 ± 0.29 µM) *vs* 0.99 ± 0.11 µM for orlistat.[18]

Several techniques have been described for assaying the activities of enzymes such as PL. These biocatalysis assays can be divided into two categories namely, homogeneous and heterogeneous assays. Homogeneous biocatalysis involves the use of free enzymes in a homogeneous phase with the other reactants. In heterogeneous biocatalysis, the enzymes are often immobilized onto a phase distinct from that of the other reactants.[19–23] It has been reported that in comparison to free

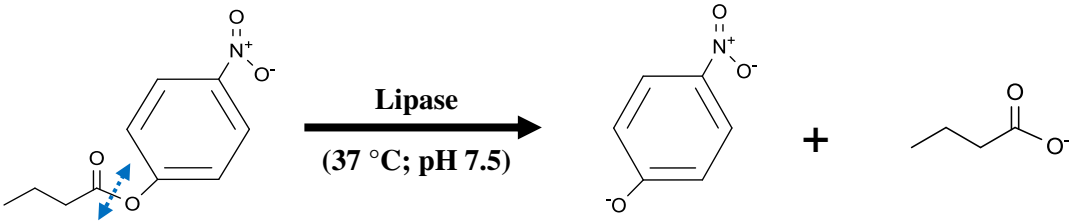
enzymes, immobilized enzymes maintain better storage stabilities and are more tolerant to pH and temperature changes.[24] Nonetheless, immobilization may bring about modifications in the enzymes' structure that may reduce its catalytic activity.[25] The fact that several substrates besides TG can be used for assaying PL activity permits the use of a variety of detection techniques.[26–28] A commonly used technique is spectrophotometry using either fluorescence or ultra-violet/visible (UV-Vis) detectors. Microtiter plates are commonly used in conjugation with the latter technique for assaying PL activity or screening for novel modulators[29]. Moreover, spectrophotometric techniques have also been hyphenated to analytical separative techniques such as capillary electrophoresis (CE).

CE relies on ions' charge to size ratios for their separation in an electric field applied across a capillary. The technique excels in separation efficiency, economy of reagents and samples, versatility of operating modes and availability of multiple hyphenation options.[30,31] Assaying enzymatic activity and screening for modulators using CE has been widely reported in the literature.[32–37] Two common operating modes of CE enzymatic assays are the offline (pre-capillary)[38,39] and online (in-capillary)[40] modes. With the offline mode, the enzymatic reaction takes place in a microvial and the reaction mixture is thereafter injected into the capillary.[41–43] The offline reactions are rather easy to control and optimize. The online mode involves the capillary acting as a nanoreactor into which few nanoliters of the different reactants are injected and mixed. Several modes are available for in-capillary mixing of analytes. Electromigration microanalysis (EMMA)[44] and pressure mediated microanalysis (PMMA)[45] are used to mix analytes according to their electrophoretic mobilities and diffusion coefficients, respectively. In this case, the mixing step can be inefficient when more than two reactant plugs are injected.[34,46] Transverse diffusion of laminar flow profiles (TDLFP)[47] is a more suitable approach to mix multiple reactant plugs. In this mode, the reactants are injected sequentially using high injection pressures and short injection times giving rise to parabolic plugs. The diffusion and mixing of the different plugs is almost exclusively transversal with limited longitudinal diffusion. TDLFP can be easily used to mix more than two plugs. However, dilution of reactants inside the capillary is a significant drawback of this approach. Compared to offline ones, online reactions consume even less reactants (few tens of nanoliters) and it is possible to automate the enzymatic assay minimizing the need for manual intervention.[48,49]

As mentioned previously, several types of detectors can be hyphenated to CE. The default detection technique is typically spectrophotometric with UV/PDA detectors usually supplied by the vendor as the built-in detector with the CE instrument. Other examples of CE-hyphenated detection techniques

include laser-induced fluorescence (LIF),[50] mass spectrometry (MS)[51,52] and capacitively coupled contactless conductivity detection (C^4D).[53] C^4D was first described as a CE hyphenated detector in 1998.[54,55] Since then, the CE/C^4D couple have seen further development and their fields of application ranged from the analysis of various inorganic[56] and organic[57] molecules to their adaptation onto miniaturized CE microchips for spatial exploration.[58,59] C^4D is excellent in detecting ions with low or no molar extinction coefficient. Furthermore, its use in duality with spectrophotometric detection have been proven to be a powerful way to enhance CE selectivity towards various analytes. Our team has recently developed a CE-UV-C^4D method for monitoring gold nanoparticle functionalization after conducting enzymatic reaction.[60] Spectrophotometric detection was used to monitor the hydrolysis of the thiol glucosinate catalyzed by myrosinase in addition to the evaluation of the stability of nanoparticles in the capillary. On the other hand, C^4D was used as a complementary detector to monitor the increase of inorganic sulfate, another product of the thiol glucosinate hydrolysis, in order to confirm the results obtained by UV detection.

Recently, online and offline CE PL assays have been described. An offline CE-C^4D assay was developed by A. Schuchert-Shi *et al.*[61] to demonstrate the superior selectivity of PL towards the methyl ester substrate of L-threonine compared to L-serine. Y. Tang *et al.*[22] screened PL inhibition potential of ten natural extracts using a heterogeneous, online CE enzymatic assay with PL immobilized onto the capillary walls. Using 4-nitrophenyl acetate (4-NPA) as the substrate, the reaction was monitored by detecting the 4-nitrophenol (4-NP) hydrolysis product using spectrophotometric detection at a wavelength of 400 nm. Six of the screened extracts showed inhibition of immobilized PL with the ethanolic *Fructus Crataegi* extract at 10 mg mL^{-1} demonstrating the most relevant inhibition (~ 70 %). In this study, we describe a homogeneous CE-UV/C^4D dual detection method for assaying PL activity. Both offline and online homogeneous CE-PL assays were developed. 4-nitrophenyl butyrate was chosen as a substrate of the PL which leads to the production of both 4-nitrophenolate (4-NP) and butyrate, detected by spectrometry and by C^4D respectively (Fig. 1).



4-Nitrophenyl butyrate (4-NPB)

4-Nitrophenolate (4-NP) → **Spectrometry** detection at 400 nm

Butyrate → **C^4D** detection (no absorption at 400 nm)

Figure 1: Hydrolysis of 4-NPB into 4-NP and butyrate catalyzed by pancreatic lipase (PL). The dashed double-sided arrow indicates the enzyme cleavage site.

Several parameters were investigated such as the nature of the separation and reaction buffers, preparation of the 4-NPB substrate solution and the effect on PL kinetics of dipalmitoylphosphatidylcholine (DPPC), a zwitterionic surfactant with a critical micellar concentration of 0.46 nM in water. [62] In fact, PL is well known to be an enzyme with an interfacial activation mechanism. It is active at the lipid-water interface such as that present in the intestine where TGs are hydrolyzed. This happens due to the presence of a lid over the enzyme's active site. This lid is opened upon increasing the hydrophobicity of the media or in the interface between aqueous and organic media facilitating access to the enzyme's active site.[63] Orlistat was used as the reference inhibitor to validate the inhibition assays. Furthermore, the developed enzymatic assays were carried out to test the modulatory potential of 11 compounds purified from oak wood, grapes and wine extracts in addition to the aqueous extracts of three different plants, Hawthorn (*Crataegus oxyacantha*), blackcurrant (*Ribes nigrum*) and *Chrysanthellum americanum* extracted by infusion in water to be eventually consumed as herbal tea. We have recently[64] demonstrated promising angiotensin-converting enzyme (ACE) inhibition by *C. americanum* extracts (> 90 %), hyaluronidase inhibition by hawthorn extracts (≈ 100 % relative to reference) as well as antioxidant capacities of blackcurrant extracts (≈ 100 % relative to reference). The molecular features of the extracts were also characterized and compared to identify the molecules or families of molecules responsible for the observed bioactivities. The extracts were thus screened for PL modulation given the promising biological and antioxidant activities they have previously demonstrated.

Experimental

Chemicals

Tris(hydroxymethyl)aminomethane (Tris) ($C_4H_{11}NO_3$, ≥ 99.8 %), N-cyclohexyl-2-aminoethanesulfonic acid (CHES) ($C_8H_{17}NO_3S$, ≥ 99 %), 3-(N-morpholino)propanesulfonic acid (MOPS) ($C_7H_{15}NO_4S$, ≥ 99.5 %), sodium butyrate ($C_4H_7NaO_2$, ≥ 98.5 %), 4-nitrophenol (4-NP) ($C_6H_5NO_3$, spectrophotometric grade), 4-nitrophenyl Butyrate (4-NPB) ($C_{10}H_{11}NO_4$, purity ≥ 98 %), lipase from porcine pancreas (EC 3.1.1.3, Type II), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) ($C_{40}H_{80}NO_8P$, ≥ 99 %), orlistat ($C_{29}H_{53}NO_5$, ≥ 98 %) and lithium hydroxide monohydrate ($LiOH.H_2O$, ≥ 98 %) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Hydrochloric acid (HCl, 37 %) was purchased from Fisher Scientific (Loughborough, UK). HPLC

grade methanol (MeOH) was purchased from VWR International (Fontenay-sous-Bois, France). HPLC grade acetonitrile (ACN) and N,N-dimethylformamide (DMF, ≥ 99.8 %) were purchased from Carlo Erba (Val de Reuil, France). Water used throughout this study was ultra-pure (18 MΩ-cm) produced by an Elga apparatus (Elga, Villeurbanne, France). Different parts of hawthorn (*Crataegus oxyacantha*, origin France), *i.e.* dry flowering tops (2 lots, number 20335 and number CB58120) and dry hawthorn flowers (lot number: 20334), dry *Chrysanthellum americanum* (origin Ivory Coast, lot number: 559980), and dry blackcurrant leaves (*Ribes nigrum*, origin Poland, lot number: 55870) raw materials were purchased from France Herboristerie (Noidans-Lès-Vesoul, France). Fresh hawthorn leaves, flowers and flowering tops were harvested on April 24, 2020 on a wild isolated tree on Montpellier (France).

Instrumentation

Experiments were carried out using a Beckman-Coulter P/ACE MDQ CE instrument (Fullerton, CA, USA) equipped with a photodiode array (PDA) detection system and an on-capillary TraceDec C^4D detection cell (Innovative Sensor Technologies GmbH, Strasshof, Austria). The C^4D parameters were fixed as follows: frequency medium, voltage 0 dB, gain 100 %, offset 010, filter: frequency 1/3 and cut-off 0.02. CE control and the analysis of electropherograms was performed using the 32 Karat software (Beckman Coulter). C^4D signals were acquired by the Tracemon software (Istech, version 0.07a). Uncoated fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). The capillaries had a total length of 61 cm, an internal diameter of 50 µm and effective length of 37 cm to the C^4D detection cell and 51 cm to the optical detection window. The capillary was contained in a system of connecting tubings and reservoirs used for circulating a coolant liquid in a closed circuit within the capillary cartridge. A lab-made 3D printed scaffold was constructed using an Ender 3 printer (Creality 3D, Shenzhen, China) and adapted to fix the various components of the C^4D detection cell and to avoid coolant liquid leakage (supplementary Fig. S.1). New capillaries were conditioned with 5 mM LiOH, H_2O and BGE for at least 15 min each followed by the application of +30 kV for 15 min. Between analyses, the capillary was rinsed with 5 mM LiOH (5 min), H_2O (1 min), 5 mM HCl (3 min), H_2O (1 min) and BGE (5 min). At the end of each working-day, the capillary was rinsed with 5 mM LiOH and H_2O for atleast 15 min each before storing its ends overnight dipped in H_2O vials. All rinse cycles were carried out at 50 psi. The electrophoretic separations were conducted under +30 kV and at 37 °C. The volumes of the injected plugs were estimated using CE expert Lite from Sciex (https://sciex.com/ce-features-and-benefits/ce-expert-lite).

UHPLC-DAD system was composed of a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS equipment with WPS-3000TBRS auto sampler, TCC-3000RS column compartment set at 35°C and Chromeleon 7 software, (Thermofisher Scientific, Waltham MA, USA). UHPLC-ESI-MS system was composed of a Synapt G2-S equipment with ESI operating in resolution mode and MassLynx 4.1 software (Waters Corp., Milford MA, USA). For both methods, a Kinetex C18 100A 100×2.1 mm, 2.6 µm column in association with a security guard ultra-cartridge was used (from Phenomenex Inc., Torrance CA, USA). A binary solvent system was used, consisting of water/formic acid (1 ‰, v/v) mixture as solvent A and acetonitrile/formic acid (1 ‰, v/v) mixture as solvent B. The gradient program started with 95 % A, then A was progressively decreased to 0 % in 30 min with a convex increase (curve 5 in Chromeleon 7). The flow rate of the mobile phase was set to 0.4 mL.min-1 and the injection volume was 20 µL. For the analysis by UHPLC-DAD, the peaks were monitored at 280, 320 and 360 nm and the UV-Vis spectra of the various compounds were recorded between 200 and 500 nm. For the analysis by UHPLC-ESI-MS, positive and negative ionization modes and fast DDA MS methods with automatic MS/MS intensity-based switching parameters, were used. The cone voltage, the extractor voltage and the capillary voltage were set to 30 V, 3 V and 2.4 kV, respectively. The source temperature and the desolvation temperature were 140°C and 450°C, respectively. Ions were scanned between m/z = 50 and m/z = 1200 to obtain MS spectra.

Solutions

Unless otherwise stated, all solutions were prepared in H_2O and filtered through polyvinylidene fluoride (PVDF) syringe filters of 0.2 µm pore size purchased from Agilent Technologies (Waldbronn, Germany).

Background electrolyte (BGE) and incubation buffer (IB): Tris/MOPS (ionic strength = 10 mM, pH 6.6) was used as the BGE for CE analyses as well as the IB for enzymatic reactions. The pH of solutions was measured using a MeterLab PHM201 Portable pH-Meter (Radiometer Analytical, Villeurbanne, France) and their properties obtained by Phoebus software (Analis, Namur, Belgium) and PeakMaster 5.3.[65]

Reactants: PL stock solutions were prepared daily by suspending the lyophilized enzyme powder in the IB at 10 mg mL^{-1} followed by 15 min agitation before storage at 0 °C. 4-NPB was chosen as the PL substrate due to its similarity to the natural TG substrate in addition to the possibility of following its hydrolysis products using dual detection. The 4-NPB solution was prepared in 100 % acetonitrile at a concentration of 1 mg mL^{-1}. Unless otherwise stated, orlistat stock solution was prepared in 70 % DMSO at 3 mg mL^{-1} and was later diluted to 1.5 mg mL^{-1} in H_2O to prepare the working solution.

The PL modulation by **11** compounds (Table 1) purified from oak wood, grapes and wine extracts was investigated. These compounds were prepared at different percentages of organic solvents taking into account their solubilities. The preparation of the different compounds was as follows: compounds **1** to **7** in 70 % methanol, compound **8** in 50 % ethanol and compounds **9** to **11** in 80 % ethanol. All of the purified molecules were prepared at a stock concentration of 40 mg mL^{-1} and were then diluted to 5 mg mL^{-1} in H_2O to prepare the working solutions. The modulation of different water extracts from three plants was also investigated. All extracts were prepared before use at 5 mg mL^{-1} in H_2O and centrifuged at 2000 × g for 10 min before isolating the supernatant. The different modulators (purified molecules + aqueous extracts) were assayed at 1 mg mL^{-1}.

Extraction by infusion of different plant parts: The extracts of different parts of *Crataegus oxyacantha* (hawthorn), *Ribes nigrum* (blackcurrant) and *Chrysenthallum americanum* plants were obtained as previously described by P. Cao-Ngoc *et al.*[66]. Unless otherwise stated, plant material was grinded before extraction using a commercial MKM6003 Bosch grinder.

Fresh hawthorn: 20 g of fresh plant material (leaves, flowers or flowering tops) were infused in 1 L of boiling water using a 'French press' Bodum® (Bistro model, Triengen, Switzerland) under 500 rpm magnetic stirring. After 30 min, the herbal tea solution was filtered first with the Bodum® cover to remove the largest particles, then with Whatman filter paper placed on a Büchner funnel using a vacuum pump (KNF Model N820FT.18, Freiburg, Germany) to remove any residual solid plant particles. Finally, the herbal tea solution was concentrated using a rotary evaporator (down to 20 mL volume) and finally freeze-dried (Cryotec Model CRIOS-80, Saint-Gély-du-Fesc, France) to obtain the dry extract. Lyophilized dry extracts were stored at 4 °C.

Dry hawthorn: 2.5 g plant material (flowers only: lot No 20334, used as is without grinding, or flowering tops) were infused in 250 mL boiling water for 10 min using the same equipment as above. Two lots of flowering tops were used for comparison: lot No 20335 (sample A) and lot No CB58120 (sample B). The herbal tea solution was then filtered, concentrated, freeze-dried and stored in the same way described above.

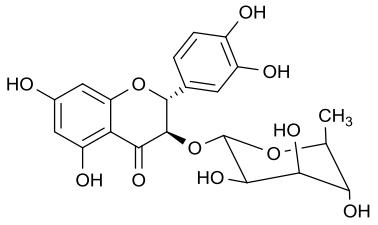
Blackcurrant and *Chrysanthellum americanum*: the extraction protocol was similar to that of dry hawthorn.

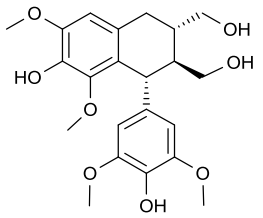
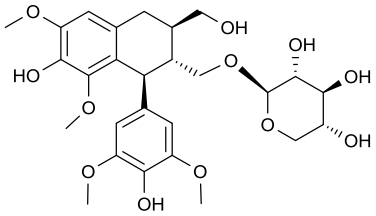
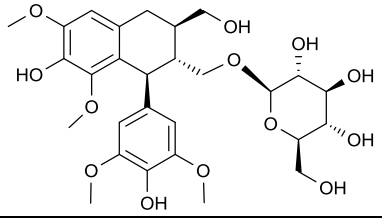
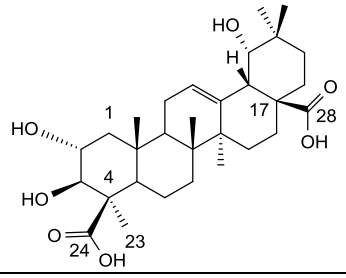
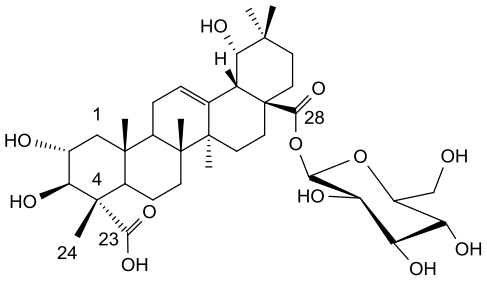
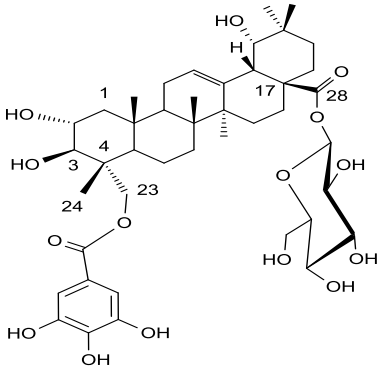
Chemical composition of the plant extracts: The total polyphenols content (TPC), flavonoids content (TFC) and proanthocyanidin oligomers content (OPC) in the various plant extracts were determined by classical colorimetric methods, as previously described.[64,66] UHPLC-DAD and UHPLC-ESI-MS/MS analyses were performed according to the protocol described in our previous paper.[64]

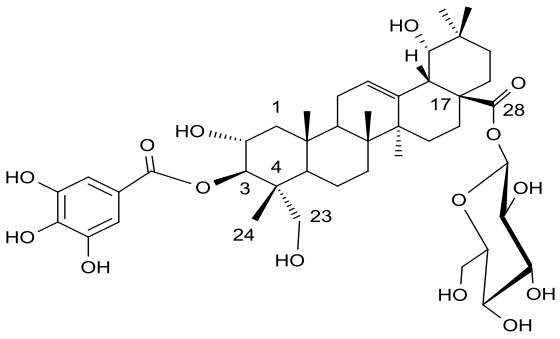
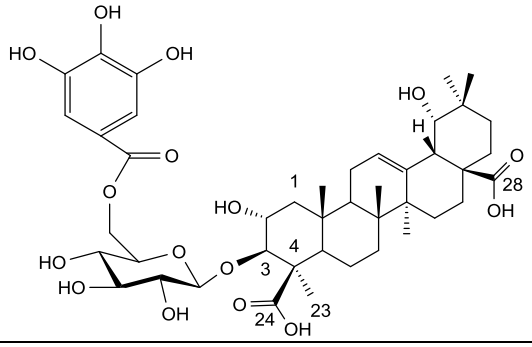
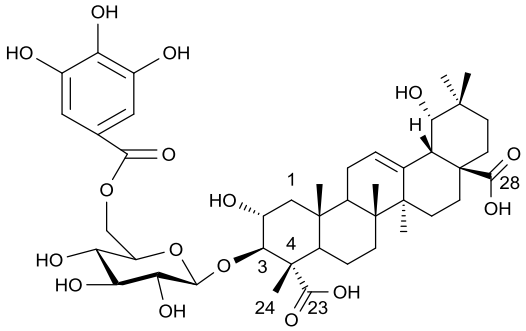
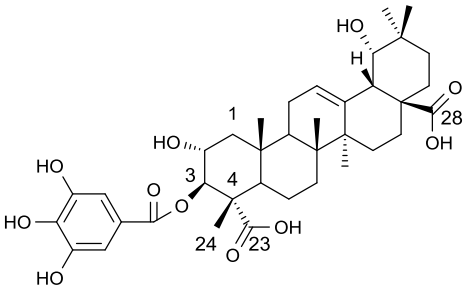
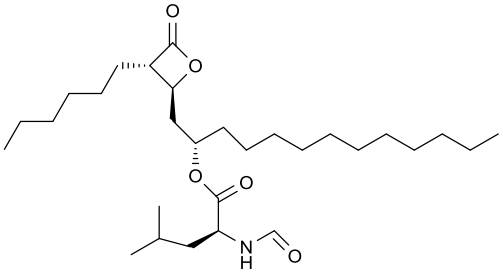
Briefly, 20 mg of plant extract was dissolved in 1 mL water, then strongly vortexed for 2 min. The resulting solution was diluted five times with water, vortexed again for 2 min, and finally analyzed.

Extraction and purification of molecules from oak wood, grapes and wine: 11 natural molecules (Table 1) were extracted from oak wood, grapes and wine using centrifugal partition chromatography fractionation and HPLC purification[67–71]: **1.** Astilbin ($C_{21}H_{22}O_{11}$, 450 g mol^{-1}), **2.** (-)-Lyoniresinol ($C_{22}H_{28}O_8$, 420 g mol^{-1}), **3.** (+)-Lyoniside ($C_{27}H_{36}O_{12}$, 552 g mol^{-1}), **4.** (+)-lyoniresinol 9′-O-β-glucopyranoside ($C_{28}H_{38}O_{13}$, 582 g mol^{-1}), **5.** Bartogenic acid ($C_{30}H_{46}O_7$, 518 g mol^{-1}) and its glucosyl derivative **6.** 28-*O*-*β*-D-glucopyranosylbartogenic acid (Glu-BA) ($C_{36}H_{56}O_{12,}$ 680 g mol^{-1}), **7.** 23-*O*-galloylarjunglucoside (Quercotriterpenoside-I) ($C_{43}H_{62}O_{15}$, 818 g mol^{-1}) and its isomer **8.** (Quercotriterpenoside-III) ($C_{43}H_{62}O_{15}$, 818 g mol^{-1}), **9.** 3-*O*-[(6-*O*galloyl)-*β*-D-glucopyranosyl]bartogenic acid ($C_{43}H_{60}O_{16}$, 832 g mol^{-1}), **10.** 3-*O*-galloylrobural A (C_{43} $H_{60}O_{16}$, 832 g mol^{-1}) and **11.** 3-*O*-galloylbarrinic acid ($C_{37}H_{50}O_{11}$, 670 g mol^{-1}).

Table 1: Structures of **11** compounds purified from oakwook and wine extracts and screened for PL modulation potential extracted. The reference PL inhibitor used in this study is orlistat (compound **12**).

Number	Name	Structure	M_w (g mol^{-1})
1	Astilbin		450

2	(-)-Lyoniresinol		420
3	(+)-Lyoniside		552
4	(+)-lyoniresinol 9′-O-β-glucopyranoside		582
5	Bartogenic acid		518
6	28-O-β-D-glucopyranosylbartogenic acid		680
7	Quercotriterpenoside-I		818

8	Quercotriterpenoside-III		818
9	3-*O*-[(6-*O*galloyl)-*β*-D-glucopyranosyl]bartogenic acid		832
10	3-*O*-galloylrobural A		832
11	3-*O*-galloylbarrinic acid		670
12 (Reference inhibitor)	Orlistat		495

CE-enzymatic assays

All reactions were performed in triplicates (n = 3) at 37 °C. The corrected peak areas (CPA) of the reaction products represented the peak areas corrected for migration time. Blank reactions were carried out in the absence of the enzyme, which was substituted with the IB, to estimate the non-enzymatic spontaneous hydrolysis of the 4-NPB substrate. Control reactions were conducted to set the baseline activity of PL which corresponds to 0 % PL modulation. The modulatory molecules in control reactions were substituted with their respective solvent. The modulation of PL activity was then calculated relative to the activity of PL observed in control reactions after correction for spontaneous hydrolysis observed in the blank assays. The modulation of PL activity (activation and inhibition) was calculated as follows (Eq.1):

$$\% \text{ Modulation} = \left| \left(1 - \left(\frac{A_M - A_B}{A_C - A_B} \right) \right) \times 100 \right| \quad (1)$$

Where A_M and A_C are the CPA of the hydrolysis products in presence and absence of the modulator molecules, respectively. A_B is the CPA of the hydrolysis products in the blank assays. This calculation is conducted for UV as well as for C^4D detection.

The residual PL activity is calculated after incubation with inhibitors as follows (Eq.2):

$$\text{Residual activity } \% = \left(1 - \frac{I}{100} \right) \times 100 \quad (2)$$

Where I is the percentage of PL inhibition, calculated using the equation (1).

Offline CE-enzymatic assay: 6 µL of PL (1.2 mg mL^{-1}) was pre-incubated with 10 µL of the investigated compounds *i.e.* orlistat (0.3 mg mL^{-1}), natural purified molecules or plant extracts (1 mg mL^{-1}) in 29 µL of Tris/MOPS IB for 5 min. Addition of 5 µL of 4-NPB (0.1 mg mL^{-1}) initiated the enzymatic reaction *i.e.* the incubation step. The reaction mixture was incubated for 5 min to avoid excess substrate hydrolysis (≤ 10 %). At the end of the incubation period, the reaction mixture was moved to a boiling water bath (95 °C) for 5 min to terminate the reaction. Then, a bench-centrifuge (model number 250711) (Quirumed, Valencia, Spain) was used to centrifuge the reaction mixture tube at 2000 × g for 5 min. Hydrodynamic injections were carried out at 0.7 psi for 5 sec (9 nL).

Influence of DPPC vesicles on PL kinetics: The effect of DPPC vesicles on the kinetics of 4-NPB hydrolysis by PL was investigated using the offline CE-based assay. DPPC vesicles were prepared at 1 mg mL^{-1} in the IB and then added to the enzymatic reaction. The enzymatic reactions were carried

out using a range of 4-NPB substrate concentrations (0.25–2 mM). The initial reaction rate V_i (mM min^{-1}) was calculated as the ratio of products formed *per* time interval in the presence or absence of DPPC vesicles. The nonlinear curve fitting program PRISM® 5.04 (GraphPad, San Diego, CA, USA) was used to determine V_{max} and K_m according to following equation (Eq.3):

$$V_i = \frac{V_{max} \times [S]}{K_m + [S]} \quad (3)$$

where V_i is the reaction rate, K_m is the Michaelis constant, V_{max} is the maximal reaction velocity and [S] is the substrate concentration.

Online CE-enzymatic assay: In the optimized conditions, the investigated modulatory compounds (represented by M for online assays) and the enzyme (PL) were mixed together and injected as a single plug. Simulations of plug overlapping by TDLFP and their position in the capillary were done using a software developed by S. Krylov's group[72]. Injection sequence was carried out from the long capillary end (furthest from the optical detection window) as follows: PL + M (0.5 psi × 3 sec, 4 nL), 4-NPB substrate (S) (0.5 psi × 6 sec, 8 nL), PL + M (0.5 psi × 3 sec, 4 nL) and IB (0.5 psi × 90 sec, 120 nL) (Fig. 2). Between each injection, the capillary inlet was dipped into the IB to minimize sample carry over.[73] The reactant plugs were mixed inside the capillary and incubated for 5 min. Then, the separation voltage was applied to separate the reaction components and terminate the enzymatic reaction by separating the enzyme from the substrate.

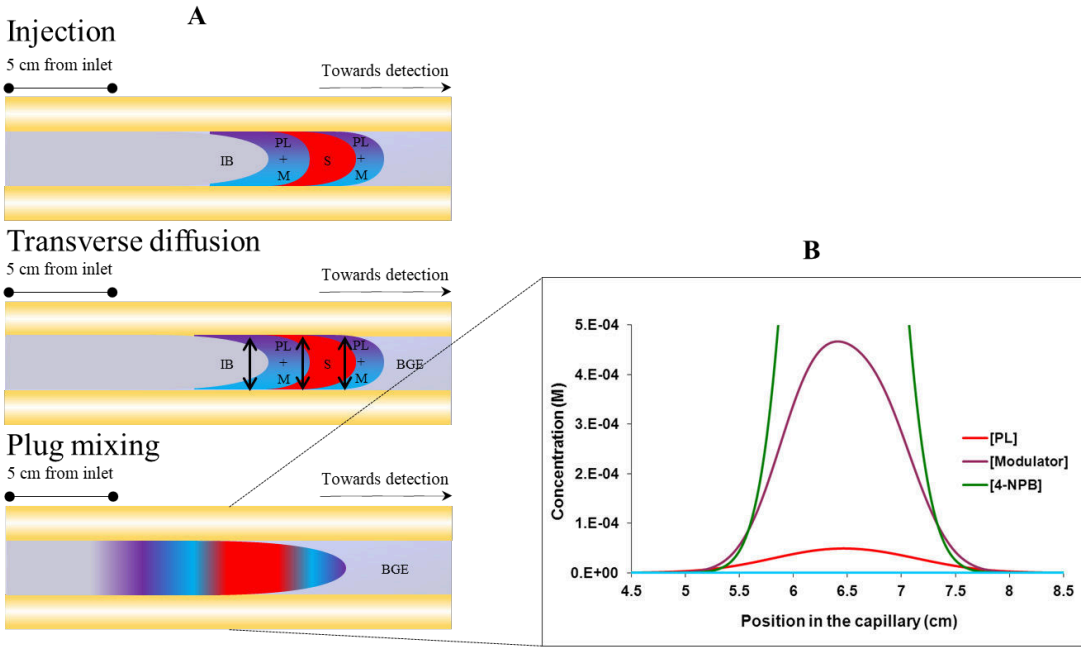


Figure 2. Schematic representation of the different steps of the online enzymatic reaction **A**). *Injection* of the different reactants sequentially into the capillary filled with the BGE as follows; 8 mg mL^{-1} PL + 1 mg mL^{-1} of the

modulator molecule (**M**) (0.5 psi × 3 sec, 4 nL), 1 mg mL^{-1} 4-NPB (S) (0.5 psi × 6 sec, 8 nL), PL + **M** (0.5 × 3 sec, 4 nL) and IB (0.5 psi × 90 sec, 120 nL). *Transverse diffusion* of the different reactant plugs into each other. *Plug mixing* by incubation of the plugs for 5 min at 37 °C allowing them to mix to initiate the enzymatic reaction. Simulation of the concentrations of the different reactants relative to their position in the capillary in addition to the overlapping of their plugs **B)**.

Results and discussion

Before conducting CE-based enzymatic assays, plant extracts were prepared and characterized. The molecular features of *Chrysanthellum americanum*, blackcurrant and dry hawthorn were thoroughly studied in our previous papers.[64,66] In this study, fresh hawthorn was characterized and its biological activity against lipase was evaluated. Moreover, the characterization of all tested extracts was conducted.

Molecular characterization of plant extracts

Global extraction yields, TPC, TFC and OPC results are presented in Table 2. Concerning hawthorn, the extraction yield was similar for fresh leaves, fresh flowers, and dry and fresh flowering tops (20-24 %). The values of TPC and TFC from fresh leaves and fresh flowering tops were much lower (107 mg eq. gallic acid/g dry plant extract for TPC and 10 mg eq. quercetin/g dry plant extract for TFC) than those from fresh or dry flowers (121-171 mg eq. gallic acid/g dry plant extract for TPC and 16 mg eq. quercetin/g dry plant extract for TFC) and those from dry flowering tops (96-150 mg eq. gallic acid/g dry plant extract for TPC and 14-15 mg eq. quercetin/g dry plant extract for TFC). However, the value of OPC from dry plant (8-20 mg eq. cyanidin/g dry plant extract) was much higher than that from fresh plant (4.5-5 mg eq. cyanidin/g dry plant extract). Those findings are in good agreement with the literature.[74,75] The global extraction yield of *Chrysanthellum americanum* (26 %) and of blackcurrant leaves (28.5 %) is higher, the former containing much less phenolic compounds (60 mg eq. gallic acid/g dry plant extract for TPC) and proanthocyanidin oligomers (1.6 mg eq. cyanidin/g dry plant extract for OPC) than the two other plants.

Table 2. Extraction yield, TPC, TFC, and OPC values in various plant extracts issued from the infusion of ground plant materials (see experimental part for the details). [a]: calculated on 1 experiment. [b]: data extracted from [66]. [c]: in mg eq. gallic acid/g dry extract, ±1 standard deviation calculated on *n*=3 repetitions. [d]: in mg eq. quercetin/g dry extract, ±1 standard deviation calculated on *n*=3 repetitions. [e]: in mg eq. cyanidin/g dry extract, ± 1 standard deviation calculated on *n*=3 repetitions. [f]: data extracted from [64].

Nature	Lot number	Extraction yield (%)	TPC[c]	TFC[d]	OPC[e]

Hawthorn fresh leaves	Harvested 2020	19.8[a]	107.7 ±4.1	9.8 ±0.5	5.0 ±0.3
Hawthorn fresh flowers		24.0[a]	121.3 ±6.0	15.7 ±0.1	4.8 ±0.1
Hawthorn fresh flowering tops		20.5[a]	107.1 ±3.9	10.3 ±0.2	4.5 ±0.1
Hawthorn dry flowering tops - Sample A -	20335	23.2±0.6[b]	149.5 ±4.0	15.4 ±0.3	20.0 ±1.7
Hawthorn dry flowering tops - Sample B -	CB58120	22.5 ±0.3[b]	96.0 ±1.8	13.9 ±0.4	8.0 ±0.4
Hawthorn dry flowers	20334	21.7±0.6[b]	171.4 ±3.2	15.9 ±0.4	9.0 ±0.2
Chrysanthellum americanum[f]	559980	25.9 ±0.9	59.9 ±0.2	12.8 ±0.2	1.6 ±0. 1
Blackcurrant leaves[f]	55870	28.6 ±0.5	138.5 ±1.7	15.5 ±0.6	5.8 ±0.3

UHPLC-DAD and UHPLC-ESI-MS/MS analyses showed that the main compounds found in hawthorn are phenolic acids such as chlorogenic acid and flavonoids such as epicatechin, vitexin 2-O-rhamnoside, pinnatifinoside A, hyperoside, isoquercetin and apigenin-C-hexoside (see Fig. 3, Fig S.2 and [66]).

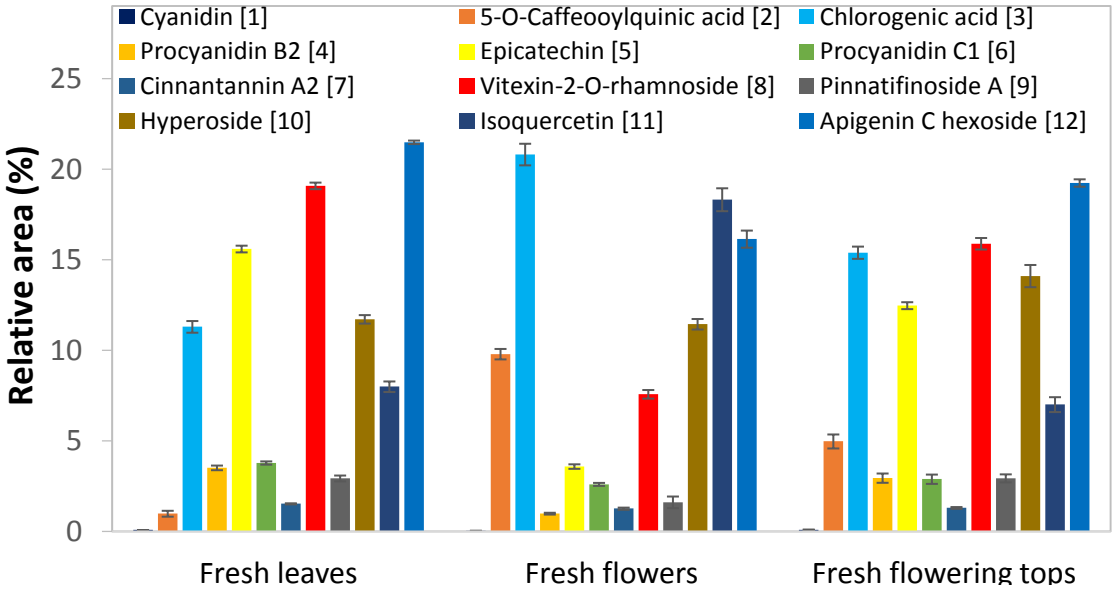


Figure 3. Relative peak area distributions for the main identified chromatographic peaks according to the nature of hawthorn plant part (grinded fresh leaves, flowers and flowering tops). Same experimental conditions as in Figure S.2. The relative area was calculated by dividing the peak area of each component by the sum of the peak area of the 12 identified components. Error bars: ± 1 standard deviation calculated on *n*=3

repetitions. 1=Cyanidin, 2=5-*O*-caffeoylquinic acid, 3=chlorogenic acid, 4=procyanidin B2, 5=epicatechin, 6=procyanidin C1, 7= cinnamtanin A2, 8=vitexin-2-*O*-rhamnoside, 9=pinnatifinose A, 10=hyperoside, 11=isoquercetin, 12=apigenin C-hexoside.

The quantification of several representative compounds for each subfamily such as chlorogenic acid (peak 3 in Fig. S.3), epicatechin (peak 5), vitexin-2-O-rhamnoside (peak 8), isoquercetin (peak 11) was determined by external calibration, using commercially available standards as presented in Fig. S.3. Interestingly, for fresh hawthorn leaves, the quantity of epicatechin, vitexin-2-O-rhamnoside in the leaves is 103.2 ± 3.2 mg *per* g fresh plant extract and 17.6 ± 0.5 mg *per* g fresh plant extract, respectively, and higher than that in the flowers and flowering tops, while chlorogenic acid and isoquercetin are major peaks in the flowers, with the quantity of 32.7 ± 0.4 mg *per* g fresh plant extract and 53.6 ±1.5 mg *per* g fresh plant extract, respectively.

Nine major peaks were detected in blackcurrant leaves, two of them (chlorogenic acid and quercetin 3-O-glucoside (isoquercetin)) were unambiguously identified, while the seven others were tentatively assigned to quercetin 3-rutinoside, quercetin 3-O-galactoside, quercetin-3-6-malonyl-glucoside, kaempferol-3-O-rutinoside, kaempferol-3-O-hexoside, kaempferol-malonylglucoside, and kaempferol-malonylglucoside isomer.[64] Concerning *Chrysanthellum americanum* fibers and blackcurrant leaves, the main compounds have been listed in [64]. Ten major peaks were detected in *Chrysanthellum americanum*, one of them (chlorogenic acid) was unambiguously identified and the nine others were tentatively assigned to eriodicyol-7-O-glucoside, 6,8-C,C-diglucosylapigenin, isookanin-7-O-glucoside (flavanomarein), maritimetin-6-O-glucoside (maritimein), luteolin-7-O-glucuronide, di-caffeoylquinic acid, apigenin-7-glucuronide, and di-caffeoylquinic acid isomers.

Optimization of the electrophoretic separation buffer (BGE)

Before conducting CE-based enzymatic reactions, it is important to ensure that the reaction can be monitored and quantified using suitable separation and detection conditions of both products, 4-NP and butyrate. The choice of the BGE should be carried out carefully considering its compatibility with the technique of detection as well as the enzymatic reaction. BGE is influential to the sensitivity of analyte detection, peak shape and symmetry and the duration of the analysis.[76] For direct detection using dual conductometric and spectrophotometry and in order to limit noise, the buffer should have low background conductivities and minimal absorption at the wavelength of analyte detection. With conductometric detection, the analytical response is proportional to the difference between the analyte's and the BGE's co-ion mobilities.[77] However, this difference results in the decline of peak symmetry which relies on the equivalence of these mobilities.[78]

The properties of two BGE, 100 mM Tris/50 mM CHES (12 mM, alkaline pH 9.0) and 10 mM Tris/40 mM MOPS (10 mM, slightly acidic pH 6.6) were compared using PeakMaster 5.3 (Table S.1). Firstly, both buffers had low conductivities and thus were suitable for direct conductometric detection. Additionally, they did not absorb at the wavelength of interest (λ = 400 nm).

The limits of detection (LOD) of the products were evaluated by injecting different concentrations of standard solutions. The 4-NP peak detected at 400 nm was symmetrical with Tris/MOPS (peak asymmetry of 0.9) and LOD was found to be 30 µM. Peak symmetry in this case could be explained by the equivalence of the 4-NP mobility and the BGE co-ion's (MOPS) mobility. 4-NP peak was completely distorted with Tris/CHES. In addition, C^4D was a lot more sensitive to butyrate with Tris/MOPS as BGE (LOD = 0.5 µM *vs* 10 µM). Comparing the mobilities of the butyrate ion to the co-ions mobilities shows that the difference between both mobilities is almost identical in both buffers (Table S.1). The higher sensitivity with Tris/MOPS is thus probably due to the lower conductivity of this BGE couple compared to Tris/CHES. It is worthy to note that the effective mobility of 4-NP (μ_{eff} = -7.9 m^2 V^{-1} s^{-1}) is very close to that of MOPS (μ_{eff} = -5.7 m^2 V^{-1} s^{-1}) which explains that the Tris/MOPS is not suitable to the conductometric detection of the 4-NP.

Additionally, the velocities of the 4-NP and butyrate ions, calculated by dividing the distance from the capillary inlet to the respective detector of the ion over the migration time of each ion, were 0.93 ± 0.004 cm s^{-1} and 0.57 ± 0.002 cm s^{-1} using Tris/MOPS and 0.03 ± 1.66 ×10^{-5} cm s^{-1} and 0.15 ± 0.003 cm s^{-1} when using Tris/CHES as BGE, respectively. The greater velocities with Tris/MOPS are reflected by shorter migration times and therefore permit rapid analyses (less than 7 min, Fig. 4). Finally, non-enzymatic hydrolysis of nitrophenyl esters is common especially at alkaline pH due to their high reactivities.[79] The extent of spontaneous hydrolysis of the 4-NPB substrate was acceptable with Tris/MOPS at pH 6.6 (< 10 %) compared to Tris/CHES at pH 9 where it was about 30 %.

For all these reasons, the remainder of the study was carried out using Tris/MOPS (10 mM, pH 6.6).

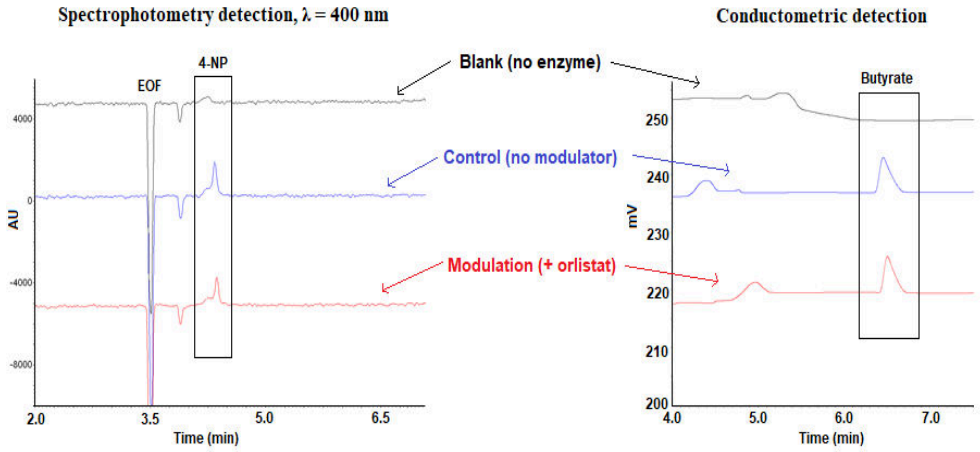


Figure 4. Electropherograms obtained after monitoring 4-NPB hydrolysis by PL using online CE-UV/C^4D assay. The injection sequence was carried out as follows: PL + Orlistat (0.5 psi × 3 sec, 4 nL), 4-NPB (0.5 psi × 6 sec, 8 nL), PL + Orlistat (0.5 psi × 3 sec, 4 nL) and IB (0.5 psi × 90 sec, 120 nL). The reactants were incubated for 5 min at 37 °C before the application of + 30 kV. BGE/IB: Tris/MOPS (10 mM, pH 6.6). For more details: see "Experimental section". EOF: electroosmotic flow, AU: absorbance units and mV: millivolts.

Optimization of the enzymatic reaction conditions

After having chosen the suitable conditions to monitor the enzymatic reaction, the preparation of the 4-NPB substrate was optimized and a suitable IB was chosen. We mainly focused on the stability of the substrate in the stock solution as well as in the final incubation mixture.

Choice of the substrate solvent and of the IB: In Fig. 5.A, the spontaneous non-enzymatic degradation of 4-NPB prepared in either water or ACN and then diluted in Tris/MOPS buffer at pH 7.5 and pH 6.6 (which corresponds to the BGE tested in the previous section) was observed. The incubation of the different 4-NPB solutions correspond to the offline enzymatic reaction steps (see section "CE-enzymatic assays") for comparison between spontaneous and enzymatic hydrolysis of 4-NPB. When working at pH 7.5, the degree of spontaneous hydrolysis of the 4-NPB, dissolved in water or ACN, was significant as concluded by comparing the intensities of the yellow colored solutions. Conventionally, Tris adjusted to pH 7.5 is used as the IB to assay PL activity since this conforms to the optimal pH of PL activity.[80,81] At lower pH 6.6 no spontaneous degradation was visually observed with the stock solution prepared in ACN. Owing to its chemical nature, it is a common practice to prepare 4-NPB in organic solvents such as ACN. It is worthy to note that significant hydrolysis of 4-NPB was observed when prepared in DMF, even at 1 % (v/v) (results not shown). To further investigate the spontaneous degradation, the 4-NPB solution in ACN diluted in Tris/MOPS pH 6.6 was analyzed by CE at 400 nm.

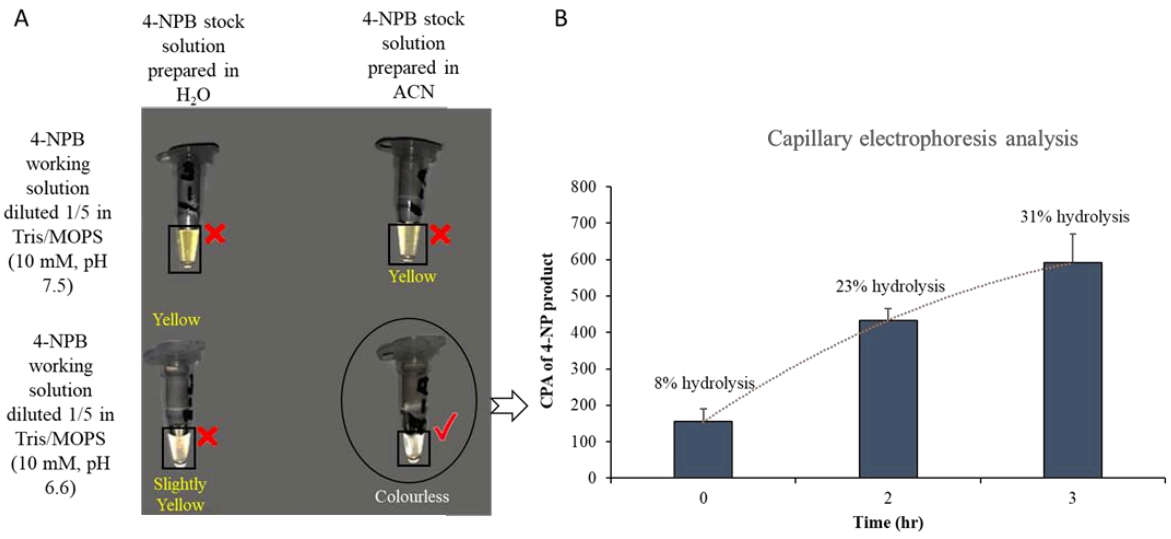


Figure 5. Visual spontaneous hydrolysis of 4-NPB in the absence of PL. **A)** The yellow color represents the release of 4-NP as a result of spontaneous 4-NPB hydrolysis. The substrate was prepared at a stock concentration of 5 mM in either water (left) or ACN (right). 10 µL of 5 mM 4-NPB was then mixed with 40 µL of 10 mM Tris/ 40 mM MOPS (Ionic strength= 10 mM, pH 7.5 (top row) or pH 6.6 (bottom row)) to have a final substrate concentration of 1 mM and a final volume of 50 µL. The mixtures were incubated at 37 °C for 5 min and then at 95 °C for another 5 min before the color of each tube was observed. **B)** CE injection at different time points of 4-NPB solution prepared in ACN and diluted in Tris/MOPS (10 mM, pH 6.6) to estimate time dependent spontaneous hydrolysis.

The results (Fig. 5.B) show a time-dependent increase in 4-NP corrected peak areas (CPA) corresponding to an increase of 4-NPB spontaneous degradation from only 8 % (immediately after incubation) to 31 % over a period of three hours (n=3). This emphasizes the importance of short CE analysis times in order to analyze rapidly the reaction mixtures after their preparation. Accordingly, the 4-NPB substrate was prepared in pure ACN and diluted in the Tris/MOPS (10 mM, pH 6.6) for the remainder of the study. The ACN quantity in the final reaction was estimated to be 10 %. Being enzymes that function superiorly in the aqueous-organic interfaces, lipases show improved stability in relatively highly organic media rather than purely aqueous ones due to the interfacial activation phenomenon.[63,82]

The Tris/MOPS (10 mM, pH 6.6) was also used as the IB. Using 5 min of incubation time confirmed that the activity of the PL in this buffer was satisfactory. Moreover, using the same solution as both the BGE and IB is simpler and of great interest particularly when adapting the online enzymatic assay.

K_m and V_{max} evaluation and influence of DPPC phospholipidic vesicles on PL activity: As before mentioned, PL is active at a lipid-water interface. For this, the offline CE-based assay was conducted using the optimized conditions above presented in order to investigate the effect of DPPC vesicles on 4-NPB hydrolysis by PL in terms of molecular affinity (K_m) and of reaction rate (V_{max}). Both UV results *i.e.* quantification of 4-NP and C^4D results *i.e.* quantification of butyrate were used.

The results obtained in the absence of DPPC confirmed the substrate-enzyme relationship between 4-NPB and PL as confirmed by K_m values obtained using UV (K_m = 0.57 mM) and C^4D (K_m = 0.59 mM) (Table 3). K. Shirai *et al.*[83] found similar K_m value of 0.52 mM for the hydrolysis of 4-NPB by lipoprotein lipase (LPL) when using fluorescence polarization spectroscopy. V_{max} was slightly higher for the hydrolysis of 4-NPB with PL in this study as compared to the hydrolysis with LPL (Table 3).

Table 3: Kinetic constants of 4-NPB hydrolysis by PL in the presence or absence of 1 mg mL^{-1} DPPC. The constants were calculated by following the formation of the hydrolysis products, 4-NP and butyrate, using spectrophotometric and conductometric detection, respectively. The constants were compared to those obtained for 4-NPB hydrolysis by LPL as described by K. Shirai *et al*.[83]

		4-NP	**Butyrate**	**4-NP**
(n=3)		**UV**	**C^4D**	**Lit.**[83]
K_m (mM)	**- DPPC**	0.57±0.03	0.59±0.12	0.52
	+ DPPC	1.54±0.01	1.66±0.01	0.55
V_{max} ($\mu M.s^{-1}$)	**- DPPC**	1.20±0.13	0.71±0.07	0.66
	+ DPPC	4.22±0.01	3.68±0.01	2.93

When DPPC vesicles were added to the incubation mixture, the affinity of PL towards 4-NPB was slightly decreased as represented by the increase of the K_m value (3 fold) and the reaction rate was slightly increased (4-5 fold). In Shirai's work [83], the affinity of 4-NPB to LPL was unchanged and the reaction rate was similarly increased upon addition of DPPC (Table 3).Moreover, the relative standard deviation (RSD) of the migration times and CPA were calculated as 1% and 3% for 4-NP and 2% and 1% for butyrate, respectively. This confirms that the offline method can detect and quantify the PL catalyzed hydrolysis reaction with excellent repeatabilities.

To recapitulate, the influence of DPPC on the PL hydrolysis of 4-NPB remain small. For this reason, no vesicles were used in the remainder of the study, neither for the offline nor for the online assays. Furthermore, the results obtained confirms the choice of the 4-NPB as a substrate for the PL. The

good repeatability (n=3) and the concordance of the results obtained using the dual detection system confirms the reliability of the developed offline CE-UV/C^4D based lipase assay.

PL inhibition using the reference inhibitor orlistat

The influence of the reference inhibitor orlistat (Table 1, entry **12**) on PL using the developed CE-UV/C^4D method was first evaluated to develop and validate the offline and online inhibition assays. Due to its limited solubility, orlistat was tested at a maximum concentration of 0.3 mg mL^{-1} in 7 % DMSO using the offline assay. To assess any non-enzymatic degradation of 4-NPB, blank assays were conducted in the absence of the enzyme, as previously detailed. Control reactions, where no modulatory molecules are involved, were conducted for comparison (Fig. 4).

Offline CE-UV/C^4D assay to evaluate the effect of orlistat on PL activity: The offline assay consists of 4 steps: pre-incubation, incubation, termination and centrifugation. The pre-incubation step involves incubating PL with the orlistat. It was found to be essential to allow the establishment of any enzyme-inhibitor interaction as well as for thermal equilibrium of the reaction mixture at 37 °C.[84] In these conditions, the orlistat at 0.3 mg mL^{-1} was found to inhibit the PL activity by 24 ± 1 % (n = 3). Both UV and C^4D results were obtained with excellent reproducibility. This assay was easy to optimize and the quantification could be assessed in a very straightforward manner.

Online CE-UV/C^4D assay to evaluate the effect of orlistat on PL activity: The online mode combines the enzymatic reaction and the CE analysis into a single automated and very economic process since only few nanoliters of reactants are required. The temperature of the capillary in which the PL reaction takes place was fixed at 37 °C (as for offline assays). A plug of the IB is typically injected first into the BGE filled capillary to avoid any denaturation of the enzyme by the BGE. This is known as partial filling[85]. However, since we chose similar BGE and IB (Tris/MOPS 10 mM, pH 6.6), no such precaution was necessary. Furthermore, an IB plug needs to be injected at the end of the injection sequence to improve plugs' overlapping by increasing the lengths of their longitudinal interfaces. Firstly, an IB plug as long as the sum of the lengths of all the other reactant plugs was used.[47] In these conditions, the injection sequence was as follows: PL (0.5 psi × 3 sec, 4 nL), **M** (0.5 psi × 3 sec, 4 nL), S (0.5 psi × 6 sec, 8 nL), **M** (0.5 psi × 3 sec, 4 nL), PL (0.5 psi × 3 sec, 4 nL) and IB (0.5 psi × 18 sec, 24 nL). In the case of control reactions, **M** plugs were substituted with plugs of the IB. While the simulations using Krylov's program showed good reactant mixing, low detected quantities of 4-NP and of butyrate were indicative of limited PL activity. Thus, the influence of the homogeneity of the temperature along the capillary was addressed. In fact, when conducting online reactions, incubation takes place at the inlet of the capillary, in a non-thermostated part.[86] More

precisely, we measured a drop of 10 °C of the temperature of the solutions in which the entry of the capillary is dipped after 5 min (corresponding to the time used for enzymatic reactions). This means that PL reactions conducted at the capillary inlet take place roughly at 37 °C at the beginning and at 27 °C at the end of the incubation time. This important decrease in temperature during the PL reaction may explain the very low enzymatic activity detected.[83],[86] To overcome this, the length of the IB terminal plug was increased (injection at 0.5 psi for 90 sec, 120 nL) to push all the plugs at least 5 cm into the thermostated region of the capillary (Fig. 2). It is important to mention that the longer the terminal IB plug, the better the reactant mixing but the higher is the dilution of the reactant. This dilution is particularly difficult to measure, which makes quantification and comparison with offline assays challenging.[47,87,88] In these conditions, good enzymatic activity was obtained with the control assay as observed by a 207 % increase in the area and 167 % increase in the height of the 4-NP product peak relative to the blank assay. Additionally, the RSD of the migration time and CPA were determined as 1 % and 5 % for 4-NP and 1 % and 2 % for butyrate, respectively.

Then, the reference inhibitor orlistat was prepared in 20 % (v/v) DMSO and injected at 1 mg mL^{-1}. The exact concentration of orlistat in the capillary is difficult to determine. In fact, the dilution factor would be 18-times assuming complete overlapping of all the injected plugs (8 nL of modulator in a total volume of 144 nL). In these conditions, no significant inhibition of PL neither by UV nor by C^4D was detected. This can be due to the direct contact between the PL's plugs and the DMSO in the inhibitor solution before dilution.[89] It can also be due to the absence of a pre-incubation period between the tested inhibitor and enzyme.[84] Subsequently, the injection sequence was replaced by the one presented in the "experimental section" and in which the PL and the modulator plugs were combined into an individual plug by injecting both reactants from the same solution (Fig. 2.A). In these conditions, good inhibition of PL by orlistat was obtained (32 ± 5 %).

Therefore, this method was able to detect modulators of PL activity. It is not trivial to compare this result to the one obtained previously by the offline method due to the difficulty of calculating the exact concentrations of the different reactants after using the long terminal IB plug.

PL modulation potential of aqueous plant extracts

The aqueous extracts of *Crataegus oxyacantha* (hawthorn), *Ribes nigrum* (blackcurrant) and *Chrysanthellum americanum* were screened at 1 mg mL^{-1} using the offline CE-UV/C^4D based assay. The modulation of PL activity by these extracts is graphically represented in Fig. 6.

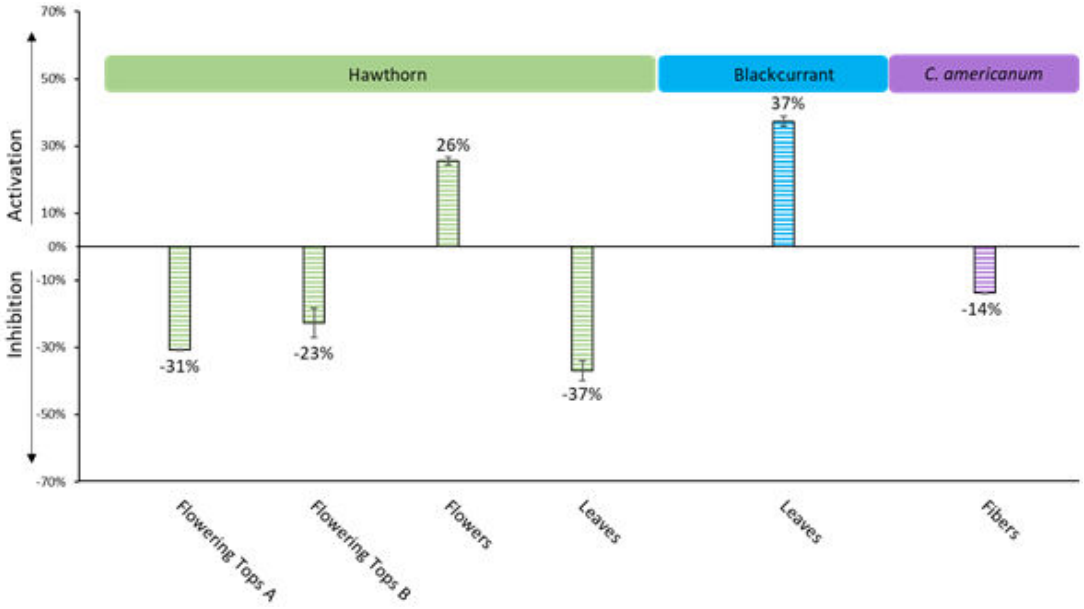


Figure 6. Modulation of PL activity by aqueous extracts of hawthorn flowering tops (flowers and leaves, 2 lots A and B), flowers only and leaves only, blackcurrant leaves and *Chrysanthellum americanum* fibers at 1 mg mL^{-1}. The percentage of modulation is represented relative to a control reactions conducted in the absence of the extracts. Error bars indicate the standard deviation on PL activity for n = 3 experiments.

It can be seen that the highest PL inhibition was obtained by extracts of hawthorn leaves (37 ± 3 %) and of the first lot of hawthorn flowering tops or sample A (31 ± 0.3 %). The second lot of flowering tops (sample B) had lower inhibition effect on PL (23 ± 4 %). Extracts of *Chrysanthellum americanum* fibers show slight inhibition of PL (14 ± 0.1 %). On the contrary, activation of PL (positive activity, superior to the control assay corresponding to 0 %) was noticed in the presence of the extracts of hawthorn flowers as well as of blackcurrant leaves (26 ± 1 % and 37 ± 1.5 %, respectively). Interestingly, inhibition was common between the hawthorn extracts of flowering tops and leaves, but not flowers, which activated PL. This finding suggests that the inhibitory potential of PL by extracts of flowering tops is mainly due to the components of the leaves rather than the flowers. The composition of these extracts not only varied from one plant to another but also in different parts of the same plants and between lots of the same part of a plant (Table 2). This undoubtedly explains the different behavior of PL in the presence of extracts from hawthorn flowers, leaves and flowering tops. In our recent study,[64] we found that almost 16 % of the extracts' molecular features were unique to each of the different plants. Additionally, certain features were unique to certain parts of hawthorn.[66] For instance, flowering tops extracts were mainly abundant in CHO compounds corresponding to carbohydrates and polyphenols followed by CHON corresponding to amino acids and sugars. More precisely, higher TPC and OPC values were found

for the first lot of hawthorn flowering tops (sample A) compared to sample B which could explain the lower inhibition potential of the latter. CHON was observed abundantly in flower extracts of hawthorn. Additionally, flower extracts also contained CHO features that corresponded to lipids and polyphenols. Both hawthorn flowering tops and flowers show contrasting PL modulation and this may be traced to the small but important difference in the extracts' compositions. In other words, the activation may have resulted from the CHON classes abundant in flowers. On the other hand, CHO classes were present at lower levels in flower extracts compared to flowering tops (leaves + flowers). This may suggest that the majority of inhibitory carbohydrates and polyphenols are present in the hawthorn leave extracts. Concerning extracts of blackcurrant leaves which demonstrated PL activation (37 %), the predominant compounds were flavonoids, diterpenes, fatty acids and carbohydrates. Also, lower levels of CHONS features and higher levels of CHOS features were observed with these extracts. *C. americanum* fiber extracts demonstrated mild inhibition of PL (14 %) and similarly to hawthorn flowering tops extracts had high levels of CHONS features. The fiber extracts mainly contained polyphenols, fatty acid-like compounds and terpenoids. Thus, the inhibition or activation can be related to the molecular feature and type of compounds in the extracts.

Moreover, the application of the online PL assay proved to be tricky to optimize when applied to screen the modulatory effect of these plant extracts. The complex nature of the extracts negatively affected the quality and the repeatability of the reactant mixing by TDLFP.

To conclude, the six aqueous extracts have generally shown low to moderate influence on PL activity. Mainly, hawthorn leaves extracts induced 37 % inhibition of PL despite their low content in bioactive compounds. Recently,[22,23] heterogeneous CE-based assay were developed to screen PL modulation by several natural extracts. For instance, at 10 mg mL^{-1} ethanolic extracts of *Fructus Crataegi* demonstrated PL inhibition of 70 %.[22] In another work,[23] methanolic extracts of *Oxytropis falcate* Bunge demonstrated 54 % and 68.6 % PL inhibition at 50 mg mL^{-1} and 100 mg mL^{-1}, respectively. In comparison, only 1 mg mL^{-1} of the aqueous hawthorn leaves extracts was sufficient to demonstrate 37 % inhibition using our developed method. This certainly reflects an interesting inhibitory effect of hawthorn leaves extracts at concentrations 10-100 times lower than that described in the literature for assaying PL inhibition by naturals extracts. This also confirms the sensitivity of the CE-UV/C^4D assay developed in this study. Moreover, it is interesting to specify that the exact same CE-based PL assay was used to monitor both the inhibition and the activation of the enzymatic activity.

PL modulation potential of natural purified compounds

The influence of **11** purified natural compounds on PL activity was evaluated (Table 1) using the CE-UV/C^4D offline based assay. All of these compounds were extracted and purified from oak wood used for wine aging with the exception of compound **1** which was purified from grapes and wine. These compounds can be divided into three groups based on their chemical structure presented in Table 1: flavonoids (compound **1**), lignans (compounds **2** to **4**) and triterpenoids (compounds **5** to **11**). Fig. 7 represents graphically the effects of the assayed compounds at 1 mg mL^{-1} on PL activity. Many of the investigated compounds demonstrated activation of PL reflected by a positive activity. Compounds **6** and **10** demonstrated the highest PL activation percentages (37 ± 1 % and 40 ± 0.5 %, respectively). Compounds **3**, **4** and **7** induced moderate activation of PL (between 20 and 27 %) whilst slight PL activation by compounds **1**, **2** and **8** was observed (between 5 and 13 %). On the other hand, the compounds **5**, **9** and **11**, induced considerable inhibition of PL. Compounds **5** and **11** demonstrated good inhibition of PL activity; 51 ± 1 % and 57 ± 4 %, respectively.

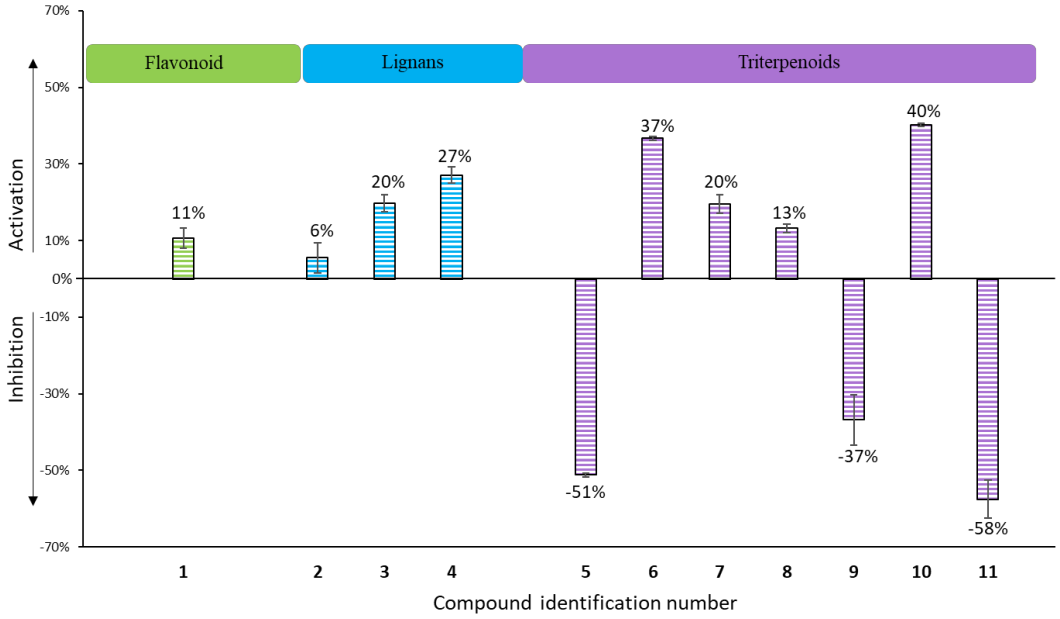


Figure 7. Modulation of PL activity by **11** compounds purified from oak wood and wine extracts (structures in **Table 1**). The compounds were assayed at 1 mg mL^{-1} using the offline assay (**1**-**11**). The influence of these compounds on PL activity was relative to control reactions conducted in the absence of the compounds. Error bars indicate the standard deviation on PL activity for n = 3 experiments.

The potential of these two compounds was also evaluated by using the online assay previously developed. The results confirm the inhibition potential of both compounds **5** and **11**, however lower inhibition was evaluated (23 ± 4 % and 40 ± 5 %, respectively) due to the dilution phenomena lowering the concentration of the modulators at the time of interaction with the enzyme. The ratios of the residual activities of the online and offline assays were similar, 1.6 for **5** and 1.4 for **11**,

demonstrating a constant effect of dilution in the online assay. These ratios also suggest that the dilution factor is not as high as expected probably due to incomplete mixing between all reactant plugs.

The dissimilarity in the bioactivity of the **11** compounds tested is due to the difference in their structure.[3] Compound **5**, bartogenic acid, one of the most potent tested PL inhibitors, is a penta-cyclic triterpenoid having carboxyl groups at positions 24 and 28 bound respectively to C4 and C17. Compounds **6** to **11** all have a common penta-cyclic backbone structure very similar to that of **5**. Compound **6** is carboxylated at position 23 and has a glucosyl moiety attached to position 28. Compared to the bioactivity of **5** against PL, compound **6** resulted in PL activation. Glycosylation and/or the position of the carboxyl group thus appears to play a role in attenuating any enzyme inhibition induced by the molecules. On a similar note, the structures of compounds **9** and **10** differ solely in the positions of the carboxyl groups (position 24 in **9** and position 23 in **10**). Contrasting modulations of PL activity were observed where **9** was found to be an interesting inhibitor whereas the compound **10** was amongst the most activating compounds. Relating these findings to the position of the carboxyl group shows a similar trend to that described for compounds **5** and **6**. Nonetheless, the position of the carboxyl group in the structure does not seem to be the sole contributor to PL modulation. For instance, compound **11** has the carboxyl group at position 23 yet has demonstrated the highest PL inhibition percentage amongst all screened compounds. Interestingly and in contrast to compounds **9** and **10**, compound **11** is galloylated at C3. The presence of galloyl groups in the structure of the compounds has been found to influence competitive inhibition of PL. However, this inhibition was weaker in non-flavonoid compounds compared to flavonoids.[90] Thus, the inhibitory potential of compound **11** can be due to the absence of a glucosyl moiety and/or the presence of a galloyl moiety at C3. This can be further supported by comparing the structures of compounds **7** and **8** which have respectively demonstrated moderate and limited activation of PL despite both containing a galloyl moiety in their structures at position 23 in **7** and attached to C3 in **8**. Both compounds are however, glycosylated at position 28 thus suggesting that the glycosylation at this position results in decreased inhibition towards PL. In conclusion, glycosylation seems to negate the effects of other substituents such as the galloyl group particularly when this glycosylation is at position 28. Additionally, compounds with glucosyl-galloyl groups on C4 show a modulation of PL activity dependent on the position of the carboxyl groups, demonstrating inhibition when the latter is at position 24 and activation at position 23. In the recent work of J. Liu *et al.*[23] several natural compounds demonstrated interesting PL inhibition using a

heterogeneous CE-based PL inhibition assay. Mainly, kaempferol demonstrated the highest PL inhibition; respectively 92 % and 96 % at 50 mg mL^{-1} and 100 mg mL^{-1}. In our work, compound **11** was shown to inhibit PL by 58 % using only 1 mg mL^{-1} (50 – 100 times lower compared to literature). On a similar notice to that described for the offline assay, the results described here for modulation of PL activity by purified compounds demonstrate interesting PL inhibition potential of the compounds at merely 1 mg mL^{-1}. Additionally, despite the dilution associate with online CE assays, some molecules demonstrated potent PL inhibition at final concentrations inferior to 1 mg mL^{-1} reflecting the sensitivity of the online CE-UV/C^4D assay developed.

Conclusion

In this study, we described the development and optimization of a CE enzymatic assay hyphenated to dual spectrophotometric and conductometric detection (CE-UV/C^4D). A lab-made 3D printed scaffold was constructed to hold the C^4D detection cell while avoiding any coolant liquid leakage. Tris/MOPS (10 mM, pH 6.6) was chosen as the BGE for providing good peak symmetries, low LOD and short analysis times. This buffer was also chosen as the IB as it also proved to be adequate for PL activity. It also limited spontaneous non-enzymatic 4-NPB hydrolysis observed at higher pH. Additionally, having the same buffer as both the BGE and IB facilitated the CE online assay as there was no longer need to implement the partial filling technique. In this study, we describe for the first time, an homogeneous online CE-based PL assay (using non-immobilized PL). The offline CE-UV/C^4D assay was used to screen the aqueous extracts of various parts of different plants for PL activity modulation. The six aqueous extracts tested revealed rather moderate to low influence on PL activity. Hawthorn leaves presented a promising inhibition potential (37 %) whereas the flowers had an opposite effect on PL activity (activation by + 26 %). Furthermore, triterpenoid compounds **5**, **9** and **11**, purified from oak wood used for wine aging, were shown for the first time to have interesting inhibitory effect on PL (up to 57 %), which could be considered as comparable to the one induced by the reference PL inhibitor orlistat. The modulation was related to the structures of these compounds where the presence of a glucosyl, galloyl or carboxyl groups at certain positions of the molecule modulated the activity of PL. The online assay was used to evaluate the inhibition of orlistat (for validation of the assay) and of compounds **5** and **11**. Online results confirmed the inhibition obtained by the offline assay. However, the dilution of the reactants after TDLFP mixing caused an underestimation of the inhibitory effects. All these results are very interesting to further develop our

structure-activity relationships (SAR) studies, helping to understand and then to predict biological activity toward PL from molecular structure of interesting compounds.

Further studies will be conducted in the near future to obtain additional insights into the binding and inhibition mechanisms of the best inhibitors identified. Mainly, microscale thermophoresis will be used as a complementary technique to determine the dissociation constant of inhibition (K_i) of such promising compounds.

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Author contribution

R. Nehmé, G. Al Hamoui Dit Banni, R. Nasreddine and S. Fayad planned and performed CE-based enzymatic experiments and analyzed the corresponding data; S. Fayad and A. Marchal planned and performed purifications and characterization of oak wood and wine extracts; P. Cao-Ngoc, J-C. Rossi, L. Leclercq and H. Cottet performed purifications, characterization and quantification of aqueous extracts of *Crataegus oxyacantha* (hawthorn), *Ribes nigrum* (blackcurrant) and *Chrysanthellum americanum*. All authors discussed the results and contributed to the final manuscript.

Figure list

Figure 1. Hydrolysis of 4-NPB into 4-NP and butyrate catalyzed by pancreatic lipase (PL). The dashed double-sided arrow indicates the enzyme cleavage site.

Figure 2. Schematic representation of the different steps of the online enzymatic reaction **A)**. *Injection* of the different reactants sequentially into the capillary filled with the BGE as follows; 8 mg mL^{-1} PL + 1 mg mL^{-1} of the modulator molecule (**M**) (0.5 psi × 3 sec, 4 nL), 1 mg mL^{-1} 4-NPB (S) (0.5 psi × 6 sec, 8 nL), PL + **M** (0.5 × 3 sec, 4 nL) and IB (0.5 psi × 90 sec, 120 nL). *Transverse diffusion* of the different reactant plugs into each other. *Plug mixing* by incubation of the plugs for 5 min at 37 °C allowing them to mix to initiate the enzymatic reaction. Simulation of the concentrations of the different reactants relative to their position in the capillary in addition to the overlapping of their plugs **B)**.

Figure 3. Relative peak area distributions for the main identified chromatographic peaks according to infusion extraction (grinded fresh leaves, flowers and flowering tops) of hawthorn. Same experimental conditions as in Figure S.2. The relative area was calculated by dividing the peak area of each component by the sum of the peak area of the 12 identified components. Error bars: ± 1 standard deviation calculated on *n*=3 repetitions. 1=Cyanidin, 2=5-*O*-caffeoylquinic acid, 3=chlorogenic acid, 4=procyanidin B2, 5=epicatechin, 6=procyanidin C1, 7= cinnamtanin A2, 8=vitexin-2-*O*-rhamnoside, 9=Pinnatifinose A, 10=hyperoside, 11=isoquercetin, 12=apigenin C-hexoside.

Figure 4. Electropherograms obtained after monitoring 4-NPB hydrolysis by PL using online CE-UV/C^4D assay. The injection sequence was carried out as follows: PL + Orlistat (0.5 psi × 3 sec, 4 nL), 4-NPB (0.5 psi × 6 sec, 8 nL), PL + Orlistat (0.5 psi × 3 sec, 4 nL) and IB (0.5 psi × 90 sec, 120 nL). The reactants were incubated for 5 min at 37 °C before the application of + 30 kV. BGE/IB: Tris/MOPS (10 mM, pH 6.6). For more details: see "Experimental section". EOF: electroosmotic flow, AU: absorbance units and mV: millivolts.

Figure 5: Visual spontaneous hydrolysis of 4-NPB in the absence of PL. A) The yellow color represents the release of 4-NP as a result of spontaneous 4-NPB hydrolysis. The substrate was prepared at a stock concentration of 5 mM in either water (left) or ACN (right). 10 µL of 5 mM 4-NPB was then mixed with 40 µL of 10 mM Tris/ 40 mM MOPS (Ionic strength= 10 mM, pH 7.5 (top row) or pH 6.6 (bottom row) to have a final substrate concentration of 1 mM and a final volume of 50 µL. The mixtures were incubated at 37 °C for 5 min and then at 95 °C for another 5 min before the color of each tube was observed. B) CE injection at different time points of 4-NPB solution prepared in ACN and diluted in Tris/MOPS (10 mM, pH 6.6) to estimate time dependent spontaneous hydrolysis.

Figure 6. Modulation of PL activity by aqueous extracts of hawthorn flowering tops (flowers and leaves, 2 lots A and B), flowers only and leaves only, blackcurrant leaves and *Chrysanthellum americanum* fibers at 1 mg mL^{-1}. The percentage of modulation is represented relative to a control reactions conducted in the absence of the extracts. Error bars indicate the standard deviation on PL activity for n = 3 experiments.

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