A bioactive formulation based on *Fragaria vesca* L. vegetative parts: chemical characterization and application in *k*-carrageenan gelatin

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Abstract

A bioactive formulation based on the vegetative parts of the wild strawberry, *Fragaria vesca* L., was developed by using a microencapsulated extract (lyophilized infusion form). For that purpose, a process based on an atomization/coagulation technique with alginate as the wall material was applied. Among the tested hydromethanolic and aqueous extracts, both obtained from wild and commercial samples, the infusion of a wild species emerged as the most antioxidative one. The higher amounts of flavonols and flavan-3-ols found in the aqueous extracts seem to be responsible for this greater antioxidant activity. Furthermore, the developed bioactive formulation was applied in *k*-carrageenan gelatin, being observed that the antioxidant properties of the extract were preserved, as compared with the free form. Thus, the antioxidant activity of the *Fragaria vesca* L. vegetative parts was demonstrated, as well as the advantages of using microencapsulation to produce effective bioactive formulations.

Keywords: Fragaria vesca L.; Vegetative parts; Hydromethanolic/Aqueous extracts; Microencapsulation; Alginate; *k*-Carrageen

1. Introduction

Wild strawberry, *Fragaria vesca* L., is an herbaceous perennial plant from the Rosaceae family. It is widely spread across Europe and North America and it can be found in roadsides and slopes, as also in forests (Castroviejo et al., 1998). The antioxidant properties of *F. vesca* fruits and leaves (Raudonis, Raudone, Jakstas & Janulis 2012; Nuñez-Mancilla, Pérez-Won, Uribe, Vega-Gálvez & Scala 2013; Žugić et al., 2014), pulp (Özşen & Erge, 2013), achenes, thalamus (Cheel, Theoduloz, Rodríguez, Caligari & Schmeda-Hirschmann 2007) and roots (Dias, Barros, Oliveira, Santos-Buelga & Ferreira 2015a) have been described. Although being mostly known by the sweat small fruits, their vegetative parts are also consumed as decoctions for hypertension treatment due their detoxifying, diuretic, stimulant and dermatological protective properties (Neves, Matos, Moutinho, Queiroz & Gomes 2009; Camejo-Rodrigues, Ascensão, Bonet & Vallès, 2012).

The bioactive properties of different strawberry parts (fruits, leaves and roots) have been related to the presence of various phenolic compounds, such as hydroxycinnamic acid and ellagic acid derivatives (e.g., ellagitannins), and flavonols (Clifford & Scalbert, 2000; Zheng, Wang, Wang & Zheng 2007; Pinto, Lajolo & Genovese 2008; Simirgiotis & Schmeda-Hirschmann, 2010; Bubba, Checchini, Chiuminatto, Doumett, Fibbi & Giordani 2012; Gasperotti et al., 2013; Dias et al., 2014; Sun, Liu, Yang, Slovin & Chen 2014). The presence of these bioactive compounds makes this plant very appealing, not only for consumers, but also for food and pharmaceutical industries. However, after ingestion, phenolic compounds can undergo transformation to methylate, glucuronate and sulphate metabolites (Heleno, Martins, Queiroz & Ferreira, 2015). In fact, the stability and functionality of this type of compounds within the human body, and consequently their bioavailability, is highly influenced by the ingested amount, structure and chemical form, molecular interactions and the organism itself (Holst & Williamson, 2008; Leong & Oey, 2012). A major problem of phenolic compounds is the poor solubility in water and the

low permeability due the absence of specific receptors in the small intestinal epithelial cells surface (Li, Jiang, Xu & Gu, 2015).

To overcome these problems microencapsulation emerges as a reliable response to protect and stabilize bioactive compounds/extracts, also offering a controlled or targeted delivery (Dias, Ferreira & Barreiro, 2015b). The microcapsules can present sizes ranging from 1 to 1000 micrometers and two main types of morphology: reservoir and matrix type. In the first case a wall/shell protects a core (bioactive) and in the second one the bioactive is dispersed along a continuous polymeric matrix. The controlled release of the bioactives, that should be tailored according to the final application of the microencapsulated product, can be achieved by several mechanisms, for example, mechanical action, heat gradients, diffusion, pH modification, biodegradation and dissolution. Water-soluble polymers are the most used wall materials (Dias et al., 2015b), being alginate the most common one; its physiochemical properties have been intensively studied proving to have good stability, biocompatibility, exudate-retaining ability and some antimicrobial activity (Goh, Heng & Chan, 2012). Furthermore, enzymes presented in the gastrointestinal tract do not affect alginate structure, being the encapsulated bioactive extracts or compounds released in the intestine at pH 7.2 (Zhang, Guo, Peng & Jin, 2004).

Microencapsulation technique could find many applications in different fields such as the pharmaceutical, food, agriculture, biomedical and even electronics (Martins, Barreiro, Coelho & Rodrigues, 2014a; Martins et al., 2014b). As far as we know there are no studies using *Fragaria* species, namely in what concerts the microencapsulation of *F. vesca* extracts and their subsequent use to enrich food matrices such as *k*-carrageenan gelatin.

k-Carrageenan is a linear anionic heteropolyshaccharide extracted from red algae and composed by galactose and anhydrogalactose units containing ester sulfate groups, (Baeza, Carp, Pérez & Pilosof, 2002). It is widely used in the food industry as gelling, stabilizing and thickening agents. The gelling process occurs upon solution cooling, being affected by factors such as salt

concentration, temperature, and pH, forming generally very firm gels (Bartkowiak & Hunkeler, 2001; Grenha et al., 2010).

In the present study, the main objective was to develop a bioactive formulation based on *Fragaria vesca* L. vegetative parts for application in functional foods. Wild and commercial samples were used to obtain hydromethanolic and aqueous extracts. After evaluation of their antioxidant activity and establishment of the individual phenolic profile, the most active extract was protected by microencapsulation through the atomization/coagulation technique using alginate as the wall material. An applicability assay was developed using *k*-carrageenan gelatin as food matrix, as a way to explore new bioactive formulations for food applications.

2. Materials and methods

2.1. Samples

The commercial samples of *Fragaria vesca* L. vegetative parts (leaves and stems) were purchased in a local supermarket. The wild vegetative parts of *F. vesca* were collected in Serra da Nogueira, Bragança, North-eastern Portugal, in July 2013. Morphological key characters from the Flora Iberica (Castroviejo et al., 1998) were used for plant identification. Voucher specimens (nº 9687) are deposited in the School of Agriculture Herbarium (BRESA). All the samples were lyophilized (FreeZone 4.5, Labconco, Kansas, MO, USA) and powdered (20 mesh).

2.2. Standards and Reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acid was purchased from Prolabo (WWR International, France). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was acquired from Sigma (St. Louis, MO, USA. Phenolic standards (catechin, ellagic acid, gallic acid, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, kaempherol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside and *p*-coumaric acid) were from Extrasynthèse (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from

Alfa Aesar (Ward Hill, MA, USA). Sodium alginate was obtained from Fluka Chemie (Steinheim, Switzerland) and calcium chloride dihydrate was purchased from Panreac (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Preparation of the hydromethanolic and aqueous extracts

Hydromethanolic extraction was performed by stirring the powdered sample (1 g) with 30 mL of a methanol/water mixture (80:20, *v/v*) at 25 ºC and 150 rpm during 1 h, followed by filtration through a Whatman filter paper No. 4. The residue was then extracted with one additional 30 mL portion of the hydromethanolic mixture. For each sample, the combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and further lyophilized.

For infusions preparation, each sample (1 g) was added to 200 mL of boiling distilled water (pH 6.6) at 100 ºC, left to stand at room temperature for 5 min, and then filtered under reduced pressure (0.22 μm, through Whatman No. 4 paper).

For decoctions preparation, each sample (1 g) was added to 200 mL of distilled water (pH 6.6), heated (heating plate, VELP scientific, Keyland Court, NY, USA) and le to boil during 5 min at 100 ^{o}C, in a closed recipient to prevent evaporation. The mixture was left to stand for 5 min and then filtered under reduced pressure (0.22 μm, through Whatman No. 4 paper). The obtained infusions and decoctions were frozen and lyophilized.

2.4. Phenolic compounds analysis

The lyophilized extracts were re-dissolved in a water/methanol mixture (80:20, *v/v*) or in pure water to determine the phenolic profiles by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA), as previously described elsewhere (Barros et al., 2013). For the separation, a Waters Spherisorb S3 ODS-2 C_{18}, 3 μm (4.6 mm × 150 mm) column thermostatted at 35 °C was

used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% B for 5 min, 15% B to 20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between m/z 100 and 1800.

The phenolic compounds were identified by comparing their retention times, UV-vis and mass spectra with those obtained from standard compounds, if existing. Otherwise, peaks were tentatively identified by comparing the obtained information with available data reported in the literature. For quantitative analysis, an estimation was performed by a manual integration using a baseline to valley integration mode with baseline projection. The individual standards calibration curves were constructed based on the UV signal: catechin (y=158.42x+11.38, R^2=0.999); ellagic acid (y=32.748x+77.8, R^2=0.999); gallic acid (y=421.11x+546.14, R^2=0.996); quercetin-3-*O*-glucoside (y=253.52x-11.615, R^2=0.999); quercetin-3-*O*-rutinoside (y=281.98x-0.3459, R^2=1);

kaempherol-3-*O*-glucoside (y=288.55x-4.0503, R^2=1); kaempferol-3-*O*-rutinoside (y=239.16x-10.587, R^2=1) and *p*-coumaric acid (y=884.6x+184.49, R^2=0.999). For the identified phenolic compounds with no available commercial standard, an estimation was performed based on the calibration curve of a similar compound belonging to the same phenolic group. The results were expressed in mg per g of extract.

2.5. Antioxidant activity evaluation

The lyophilized extracts were re-dissolved in the methanol/water (80:20, *v/v*) or water to obtain stock solutions of 2.5 mg/mL, which were further diluted to obtain a range of concentrations for antioxidant activity evaluation.

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated through the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Barros et al., 2013; Dias et al., 2015a). The final results were expressed as EC_{50} values (μg/mL), sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

2.6. Encapsulation of the most antioxidant extracts

Microspheres containing the lyophilized infusion of wild vegetative parts of *F. vesca*, were prepared by using an atomization/coagulation technique as previously described by the authors (Martins et al., 2014b). Briefly, sodium alginate was used as the matrix material and calcium chloride ($CaCl_2$) as the coagulation agent. The atomizing solution was prepared by firstly dissolve 50 mg of the lyophilized extract in 10 mL of distilled water under stirring followed by filtration to remove eventual non-soluble trace residues. Thereafter 400 mg of sodium alginate were added and the solution kept under stirring until complete dissolution was achieved. The obtained alginate solution containing the extract was then atomized using a NISCO Var J30 system (Zurich, Switzerland) at a feed rate of 0.3 mL/min and a nitrogen pressure of 0.1 bar. The generated microspheres were immediately coagulated by contacting with the $CaCl_2$ aqueous solution (250 mL at a concentration of 4%, *w/v*), for a period of 4 hours. The resulting microspheres were collected by filtration under reduced pressure, washed twice with distilled water, and further lyophilized and stored under dark conditions at 4 ^{o}C.

Microspheres were analysed by optical microscopy (OM) using a Nikon Eclipse 50i microscope (Tokyo, Japan) equipped with a Nikon Digital Sight camera and NIS Elements software for data acquisition and by SEM using a Phenom ProX desktop microscope (Eindhoven, The Netherlands). OM analysis was applied to assess the size and morphology of the microspheres after the atomization and coagulation stages, as well as after rehydration. SEM analysis was used to inspect final morphology of the lyophilized samples. The effective extract incorporation into the alginate matrix was investigated by FTIR analysis. For that purpose, spectra of pure alginate, free extract of *F. vesca* and the corresponding microspheres were collected on a FTIR Bomen (model MB 104) by preparing KBr pellets at a sample concentration of 1% (w/w). Spectra were recorded at a resolution of 4 cm^{-1} between 650 and 4000 cm^{-1} by co-adding 48 scans. The dry residue (DR) was calculated as the ratio between the dry (lyophilized) form and the

corresponding wet microsphere weight (%, *w/w*). The evaluation of the encapsulation efficiency (EE) was performed through the quantification of the non-encapsulated extract. The encapsulation efficiency was calculated according to the following expression:

$$EE = [(M_{e\text{-}t} - M_{e\text{-}ne})/(M_{e\text{-}t})] \times 100$$

in which $M_{e\text{-}t}$ represents the theoretical amount of extract, i.e. the amount of extract used in the microencapsulation process. $M_{e\text{-}ne}$ corresponds to the non-encapsulated extract remaining after the encapsulation process. Since the extract corresponds to a complex mixture of several components, the major compound (quercetin *O*-glucuronide) was selected for EE evaluation. The quercetin *O*-glucuronide quantification was performed by HPLC based on the analysis of the coagulation and first washing solutions since in the second washing solution no extract components were detected.

2.7. Incorporation of free and microencapsulated F. vesca extracts in k*-carrageenan gelatin*

For the incorporation assay, the chosen food matrix was the most common gelling agent found in commercial gelatine, *k*-carrageenan. This strategy of using the gelling agent instead of a commercial gelatin was chosen to avoid the presence of additional antioxidant compounds, e.g ascorbic acid, typical of these formulations, which could mask the results.

The protocol for preparing the gelatin was based on the procedure described by Miyazaki, Ishitani, Takahashi, Shimoyama, Itoh and Attwood (2011), while the used assay volume (125 mL) was based on existing commercial gelatins forms. The used extract amount (and corresponding microspheres) was defined considering the DPPH scavenging activity EC_{50} of the free extract (EC_{50} = 86.17 μg/mL). Therefore, the gelatin was prepared at a concentration of 1% (1.25 g of *k*-carrageenan per 125 mL of distilled water) by heating up to 90°C until complete dissolution. The following samples have been prepared: (i) two samples without adding the extract (control samples); (ii) two samples with free extract (non-encapsulated extract, considering the EC_{50}) and (iii) two samples with lyophilized microspheres (corresponding to the

same amount of free extract). The free extracts and the lyophilized microspheres were added to the gelatin at 90°C. The final products were frozen and lyophilized, for further evaluation of DPPH scavenging activity and reducing power, as previously described. An OM analysis was also performed to assess the integrity of the microspheres after gelatin preparation and lyophilisation.

2.8. Statistical analysis

In the phenolic compounds analysis and antioxidant activity evaluation, three samples of each plant material were used, while for the incorporation assays, two samples were prepared. All the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD), being analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 22.0 (IBM Corp., Armonk, NY, USA) program.

3. Results and discussion

3.1. Phenolic compounds in F. vesca hydromethanolic and aqueous extracts

Thirty individual phenolic compounds were detected and tentatively identified in the hydromethanolic and aqueous extracts prepared from commercial and wild samples of *F. vesca* vegetative parts (**Table 1**): twelve gallic/ellagic acid/HHDP derivatives, nine flavonols (*i.e.* quercetin and kampferol derivatives), eight flavan-3-ols (*i.e.*, catechins and proanthocyanidins) and one hydroxycinnamoyl derivative (*p*-coumaric acid derivative). The phenolic profiles of commercial and wild samples are very similar in terms of compound families, but with differences in individual compounds. Peaks 1, 3, 5, 8, 15, 20, 21, 24, 28 and 29 are common in both samples. An exemplificative phenolic profile of the infusion extract prepared from wild *F. vesca* is shown in **Figure 1**.

3.1.1. Ellagic and gallic acid derivatives

Ellagic acid derivatives represent the largest group of compounds found in the hydromethanolic extracts of commercial and wild samples of *F. vesca* vegetative parts. The total content of these compounds was higher than the one observed in the plant roots (Dias et al., 2015a), which confirms their differential accumulation in certain tissues (Clifford & Scalbert 2000).

Peak 28 was identified as ellagic acid according to its retention, mass and UV characteristics by comparison with a commercial standard. The rest of compounds of this group were tentatively identified based on their mass spectra and comparison with data reported in the literature. Peaks 22 ($[M-H]^-$ at *m/z* 447) and 30 ($[M-H]^-$ at *m/z* 461) showed UV spectra similar to ellagic acid and major MS^2 fragment ions at *m/z* 301 (ellagic acid) and 315, respectively, from the loss of 146 mu (deoxyhexosyl moiety); in the case of compound 30 a second fragment ion was observed at *m/z* 301, pointing to the further loss of a methyl group. These characteristics allowed their tentative identification as ellagic acid deoxyhexose and methyl ellagic acid deoxyhexose. Compounds with similar mass characteristics have been reported in fruits (Bubba et al., 2012; Gasperotti et al., 2013; Sun et al., 2014) and roots (Dias et al., 2015) of *F. vesca*, as well as in fruits of other *Fragaria* species (peak 22; Seeram, Lee, Scheuller & Heber, 2006; Aaby, Ekeberg & Skrede, 2007; Simirgiotis & Schmeda-Hirschmann, 2010).

The rest of the compounds of this group corresponded to hydrolysable tannins. Peaks 1 and 3 showed the same $[M-H]^-$ ion at *m/z* 783 and were identified as bis-HHDP-glucose isomers. The daughter ions at *m/z* 481 and 301 are commonly observed in the fragmentation pattern of ellagitannins and come respectively from the loss of a hexahydroxydiphenoyl unit (HHDP) followed by proton transfer, and the internal rearrangement of the HHDP itself (Gasperotti et al., 2013). Similar compounds were previously reported in fruits of *Fragaria vesca* (Sun et al., 2014) and other *Fragaria* species (Seeram et al., 2006; Aaby et al., 2007; Simirgiotis & Schmeda-Hirschmann, 2010; Gasperotti et al., 2013), being usually associated to pedunculagin.

Peak 11 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 933 yielding main fragment ions at *m/z* 915, 631, 451 and 301, consistent with those described for castalagin/vescalagin isomers previously reported in roots (Dias et al., 2015a) and fruits (Bubba et al., 2012; Gasperotti et al., 2013) of *F. vesca*, as also in the leaves of *F. chiloensis* (Simirgiotis & Schmeda-Hirschmann, 2010). Peak 12 had a pseudomolecular ion $[M-H]^-$ at *m/z* 635 and MS^2 fragments ions at *m/z* 465 (loss of gallic acid, 170 mu), *m/z* 313 (further loss of a galloyl residue, 152 mu) and *m/z* 169 (gallic acid); based on this fragmentation pattern the compound was tentatively identified as trigalloylglucose, previously found in fruits of *F. vesca* by Sun et al. (2014).

Mass characteristics of peak 15 ($[M-H]^-$ at *m/z* 935 yielding fragments at *m/z* 633 and *m/z* 301) coincided with a galloyl-bis-HHDP-glucose isomer, previously reported in the roots (Dias et al., 2015a) and fruits of *F. vesca* (Bubba et al., 2012; Gasperotti et al., 2013; Sun et al., 2014) and associated to galloylpedunculagin or casuarictin/potentillin, one of the monomers frequently found as constituents of the oligomeric ellagitannins (Gasperotti et al., 2013). Peaks 16, 17 and 21 were assigned as Sanguiin h10 isomers, presenting a pseudomolecular ion $[M-H]^-$ at *m/z* 1567 and a characteristic fragmentation pattern at *m/z* 935, 633 and 301, which is in agreement with the identification made by Bubba et al. (2012), Gasperotti et al. (2013) and Dias et al. (2015a) in the fruits and roots of *F. vesca*. Peak 21 was the major compound found in both samples, with the exception of the aqueous extracts prepared from wild *Fragaria vesca*.

Peak 19, only observed in the commercial sample, showed a pseudomolecular ion $[M-H]^-$ at *m/z* 1235, with a subsequent loss of two HHDP units $[M-H-302-302]^-$ giving rise to fragments at *m/z* 933 and *m/z* 631, and then the loss of a glucose-galloyl unit $[M-H-330]^-$ yielding the fragment at *m/z* 301. A compound with similar characteristics was reported in strawberry fruits (*Fragaria x ananassa* Duch.) (Hanhineva et al. 2008; Aaby, Mazur, Nes & Skrede, 2012; Gasperotti et al., 2013) and tentatively associated di-HHDP-glucose-galloyl-ellagic acid, also designed as dauvriicin M1, a hydrolysable tannin previously identified in the roots *Rosa davurica* (Yoshida, Jin & Okuda, 1989).

It is noticeable the difference observed between hydromethanolic and aqueous extracts (infusion/decoctions), probably due to the high temperatures applied to obtain the last preparations. The differences found in the phenolic profile were mainly observed in the hydrolysable tannins, revealing the aqueous extracts lower concentration of this type of compounds and, in some cases, the absence of certain hydrolysable tannins (peaks 11, 12, 15, 16, 19 and 22). These compounds are known for being easily degraded with high extraction temperatures (Theocharis and Andlauer, 2013) and even high storage temperatures (Talcott & Lee, 2002).

3.1.2. Flavonols

Flavonols represent the second largest group of phenolic compounds found in the hydromethanolic extracts, but the largest group in the aqueous extracts obtained from both commercial and wild samples. Quercetin (peaks 7, 18, 20, 24 and 25), kampferol (peaks 23, 27 and 29) and methylquercetin (peak 26) derivatives were the main flavonols found. Peaks 7, 18, 23, 25 and 26 were only found in the wild sample, while peak 27 was only detected in the commercial one.

Peaks 20 (quercetin 3-*O*-rutinoside), 26 (quercetin 3-*O*-glucoside) and 27 (kaempferol 3-*O*-rutinoside) were positively identified by comparison of their retention, mass and UV-vis characteristics with commercial standards. The presence of quercetin 3-*O*-glucoside was described in roots (Dias et al., 2015a) and fruits (Sun et al., 2014) of *F. vesca*. A peak with the same pseudomolecular ion as peak 27 ($[M-H]^-$ at *m/z* 593) was also reported in *F. vesca* fruits (Bubba et al., 2012; Sun et al., 2014) and in other *Fragaria* species (Seeram et al., 2006; Simirgiotis & Schmeda-Hirschmann, 2010; Aaby et al., 2012), but identified as kaempferol-coumaroylhexoside, identity that was discarded in our case once the compound was compared with a standard of kaempferol 3-*O*-rutinoside and lacked in its UV spectrum the characteristic

shoulder of the *p*-coumaroyl substituent expected around 310 nm. As far as we know, the presence of kaempferol 3-*O*-rutinoside has not been cited in *F. vesca.*

Mass characteristics of peak 24 ($[M-H]^-$ at *m/z* 477 yielding a unique MS^2 fragment at *m/z* 301) were coherent with quercetin *O*-glucuronide, compound that was previously identified in the fruits of *F. vesca* (Bubba et al., 2012; Sun et al., 2014) and other *Fragaria* species (Simirgiotis & Schmeda-Hirschmann, 2010; Aaby et al., 2012). Peak 24 was the major compound found in infusion and decoction preparations of wild *F. vesca*; this compound has not been reported as the main compound present in this sample. Nevertheless, this could be explained by the heating process used to obtain the aqueous extracts (infusion/decoction), and that could increase the extractability of some compounds. We have also observed this in infusion/decoction extractions from other natural products such as *Salvia officinalis* L. (Martins et al., 2015a), *Thymus vulgare* L. (Martins et al., 2015b) and *Origanum vulgare* L. (Martins et al., 2014c), where the aqueous preparations (infusions/decoctions) increased aglycones linked to glucuronide moieties, such as luteolin *O*-glucuronide. Similar behaviour to peak 24, was found for compound 29 ($[M-H]^-$ at *m/z* 461 yielding an MS^2 fragment at *m/z* 285 from the loss of a glucuronyl residue) that was thus identified as kaempferol *O*-glucuronide, already described in the fruits of *F. vesca* (Sun et al., 2014) and other *Fragaria* species (Seeram et al., 2006; Simirgiotis & Schmeda-Hirschmann, 2010; Aaby et al., 2012).

Peak 7 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 639 with fragments at *m/z* 463 (loss of a glucuronyl group) and *m/z* 301 (further loss of an hexosyl residue), being tentatively identified as quercetin hexose glucuronide. A similar compound was reported in strawberry flowers by Hanhineva et al. (2008). Peak 18 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 623, releasing MS^2 fragment ions at *m/z* 301 ($[M-H-322]^-$), which might correspond to the joint loss of deoxyhexosyl (-146 mu) and glucuronyl (-176 mu) groups, so that the compound was tentatively assigned as quercetin deoxyhexose glucuronide. Similar loss of 322 mu (176+146 mu) was observed for peaks 23 ($[M-H]^-$ at *m/z* 607 yielding an MS^2 fragment at *m/z* 285) and 25 ($[M-H]^-$

at *m/z* 637 releasing a major MS^2 fragment ion at *m/z* 315 and a minor one at *m/z* 300, further loss of a methyl group), which allowed their tentative identification as kaempferol deoxyhexose glucuronide and methylquercetin deoxyhexose glucuronide, respectively. As far as we know, these latter three compounds have been previously reported in *F. vesca* or other *Fragaria* species (Simirgiotis & Schmeda-Hirschmann, 2010; Aaby et al., 2012).

3.1.3. Flavan-3-ols

Peak 8 was positively identified as (+)-catechin according to its retention time, mass and UV-vis characteristics by comparison with a commercial standard. Peak 2 presented a pseudomolecular ion $[M\text{-}H]^-$ at *m/z* 451 releasing an MS^2 fragment at *m/z* 289 ($[M-H\text{-}162]^-$, loss of a hexosyl moiety), corresponding to an (epi)catechin monomer, being tentatively identified as (epi)catechin hexoside. The earlier elution of this compound comparatively to peak 8 (parent aglycone) is in agreement to its higher polarity (presence of a sugar). A compound with similar characteristics was detected in *F. vesca* roots (Dias et al., 2015a) and fruits (Bubba et al., 2012) and given the same tentative identity.

Peaks 4, 5, 6, 9, 10 and 14 were identified as proanthocyanidins (PAC) based on their pseudomolecular analysis and MS^2 fragmentation patterns. The analysis of the produced fragments provides information about the type of elementary units and also about their relative position in the PAC oligomer; however, mass spectrometry does not provide the enough information to establish the position between flavonol units (i.e. C4-C8 or C4-C6) and does not differentiate between isomeric catechins. Peaks 5 and 10 were identified as procyanidin dimers, presenting the same pseudomolecular ion $[M\text{-}H]^-$ at *m/z* 577 and MS^2 fragmentation patterns coherent with B-type (epi)catechin dimers. Characteristic product ions were observed at *m/z* 451 (-126 mu), 425 (-152 mu) and 407 (-152 to 18 mu), attributed to the HRF (heterocyclic ring fissions), RDA (retro-Diels-Alder) and further loss of water from an (epi)catechin unit, and at *m/z* 289 and 287, that could be associated to the fragments corresponding to the lower and upper

(epi)catechin unit, respectively. Peaks 4 and 6 were identified as B-type (epi)catechin trimers with pseudomolecular ions $[M-H]^-$ at *m/z* 865, producing characteristic MS^2 fragmentation ions at *m/z* 289 and 287. Additional fragments were observed at *m/z* 713, 695, 577 and 575, corresponding to the alternative HRF, RDA and interflavan bonds cleavages. Peaks 9 and 14 were tentatively assigned as B-type (epi)afzelechin-(epi)catechin, presenting a pseudomolecular ion $[M-H]^-$ at *m/z* 561 and characteristic fragment ions at *m/z* 435, 407 and 289.

Similar proanthocyanidins to the mentioned above have been previously reported in commercial and wild samples of *F. vesca* roots (Dias et al., 2015a) and fruits (Simirgiotis & Schmeda-Hirschmann, 2010; Bubba et al., 2012; Sun et al., 2014), as well as in other *Fragaria* species (Määttä-Riihinen et al., 2004; Seeram et al., 2006; Hanhineva et al., 2008; Simirgiotis & Schmeda-Hirschmann, 2010; Aaby et al., 2007, 2012).

As observed for total flavonols, the aqueous extracts showed higher quantities of total flavan 3-ols than the hydromethanolic extracts.

3.1.4. Phenolic acids derivatives

Finally, peak 13, only detected in the commercial sample, was tentatively identified as *p*-coumaric hexose based on its pseudomolecular ion $[M-H]^-$ at *m/z* 325 releasing a daughter ion at *m/z* 163 ([coumaric acid-H]$^-$) from the loss of a hexosyl moiety ([M-H-162]$^-$). A compound with similar characteristics was reported to occur in different strawberry (*Fragaria x ananassa* Duch.) varieties (Määttä-Riihinen et al., 2004; Seeram et al., 2006; Aaby et al., 2007, 2012; Sun et al., 2014).

3.2. Antioxidant activity of F. vesca hydromethanolic and aqueous extracts

The aqueous extracts of both samples (commercial and wild) gave higher antioxidant activity than the corresponding hydromethanolic extracts (**Table 2**). This was observed in all the assays: DPPH scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS

formation inhibition. Nevertheless, in commercial samples the aqueous extract obtained by decoction was the most active, while for the wild samples it was the extract obtained by infusion that gave the highest activity. Therefore, the antioxidant activity seems to be more related with the flavonoids content (flavonols and flavan-3-ols) than with ellagic acid levels, since aqueous extracts gave higher amounts of flavonoids than the hydromethanolic extracts (in both commercial and wild samples) (**Table 1**). This fact could be due to the high temperatures applied to obtain the aqueous extracts; in fact, heat can increase cell walls permeability, solubility and diffusion coefficients and, at the same time, can decrease the viscosity of the solvent used facilitating the phenolic compounds to pass through the cell wall (Santos-Buelga et al., 2012).

It should be noticed that all the extracts prepared from wild samples showed, in all the assays, higher antioxidant activity than the correspondent extracts from commercial vegetative parts (**Table 1**). These results can be related to a higher concentration of phenolic compounds, mainly flavonoids, found in the wild samples. These samples are normally exposed to adverse and non-controlled conditions during their growth, which stimulate the production of secondary metabolites such as flavonoids. In a study with *F. vesca* roots, the authors observed this same behaviour (Dias et al., 2015a).

The antioxidant activity of other Fragaria species and parts was previously reported namely, DPPH scavenging activity of *F. chiloensis ssp. chiloensis f. chiloensis* leaves and roots (Simirgiotis & Schmeda-Hirschmann, 2010), and *F. vesca* leaves (Žugic et al., 2014).

The extract of *F. vesca* vegetative parts showing the highest antioxidant activity (infusion from wild samples) was used in the development of a bioactive formulation for further application in *k*-carrageenan gelatin. This is an attractive approach since aqueous extracts are more suitable for food applications than the hydromethanolic ones.

3.3. Alginate microspheres with F. vesca infusion extract

3.3.1. Microspheres production, morphology and encapsulation efficiency

The atomization/coagulation technique, spray-based process, was used to prepare alginate-based microspheres containing infusion extracts of wild *F. vesca* vegetative parts. Immediately after the atomization and the coagulation steps, the produced microspheres were analysed by OM (**Figure 2A** and **2B**). In the first stage, atomization, the microspheres presented a high degree of teardrop-shaped due to the passage through the equipment nozzle. After 4 hours of coagulation the microspheres' shape becomes spherical. In both stages, the microspheres were perfectly individualized (no agglomerates were detected). Their final estimated size (using a magnification of 400X) ranged between 39 and 202 μm. With the incorporation of the infusion extract the microspheres presented a light brown colour, characteristic of the used extract, which indicates its incorporation and a good distribution inside the microspheres. The encapsulation efficiency (EE) determination, based on quercetin-*O*-glucuronide, was done by HPLC by analysing and conducted to a value close to 97%. A SEM analysis was also performed on the final lyophilized microspheres. As it can be observed in the shown micrographs (Figure 2E), the microspheres have spherical shape and a rough surface. The observed round cavities are due the proximal presence of other particles during the drying process. It was also observed (data not shown) that microspheres containing no extract have the tendency to collapse giving rise to particles with a disc-like morphology. This type of morphology was not noticed for microspheres incorporating the extract.

3.3.2. Microspheres rehydration after lyophilisation

To test the rehydration capacity and, consequently, the initial morphology recovery, the lyophilized microspheres were rehydrated with distilled water for a period of 48 hours. An OM analysis was made for dried and rehydrated forms using the magnifications of 40, 100 and 400X. The rehydrated microspheres practically acquired the same initial shape and size (**Figure 2C** and **2D**), proving to have a good rehydration capacity. The water recovery after 48 hours of rehydration was close to 100%.

3.3.3. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of pure alginate, pure infusion extract and microspheres incorporating the extract, are shown in **Figure 3**. The microsphere's spectrum, as expected, is dominated by the presence of alginate (dotted orange lines). The ratio extract/alginate was 100/800, which explains the alginate preponderance. Nevertheless a noticeable contribution from both carbonyl (C=O) and hydroxyl (OH) groups of the extract (dotted green lines) was observed. Also a widening of the OH and C=O bands can be observed. These facts represent an evidence of effective extract encapsulation.

3.4. Application in k*-carrageenan gelatin*

Figure 4A and **4B** show, respectively, the morphology of the enriched microspheres immediately after incorporation in the *k*-carrageenan gelatin and after subsequent lyophilisation. It can be observed that the temperature used to prepare the gelatin solution (90 ºC) did not affect the microsphere's integrity that shown a perfect round shape as a result of a prompt rehydration. After lyophilisation the spherical structure was maintained. Also it is clearly the presence of dark black dots inside the microspheres representing the encapsulated extract, showing the effective protective effect of the alginate matrix.

Regarding the antioxidant activity of the final product (*k*-carrageenan gelatins with or without the bioactive extract), evaluated by DPPH scavenging activity and reducing power, as expected, only gelatin enriched with the free infusion extract (non-encapsulated) showed antioxidant activity (EC_{50} DPPH scavenging activity = 2.74±0.11 mg/mL; EC_{50} reducing power = 1.23±0.12 mg/mL). Nevertheless, a loss of antioxidant activity, relatively to the extract in its free form, was noticed possibly due to the high temperatures needed to prepare the gelatin, which lead to extract degradation. Neither the control nor the gelatin with microencapsulated extracts showed antioxidant activity. The first result (control) was predictable since no antioxidant additives were

present. In the second case (microencapsulated extract) the result is justified by an efficient protection of the alginate microspheres. In fact, the extract was effectively protected inside the alginate microspheres by the help of a surrounding viscous medium (gelatin) that hinders its easy diffusion. It is therefore expected that this kind of bioactive formulation (gelatin enriched with alginate-based microencapsulated extracts) works well for liberation at pH=7.4 (intestinal preferable absorption) since at this pH the alginate microspheres lose this integrity (disruption of the ionic polymeric network) and liberate the encapsulated extracts.

Overall, wild samples of *F. vesca* vegetative parts showed higher contents in phenolic compounds and higher antioxidant activity than the commercial ones. Aqueous preparations were more active than hydromethanolic extracts due to the higher amounts of flavonols and flavan-3-ols. The microencapsulation technique of atomization/coagulation was effectively applied to produce microspheres enriched with the most antioxidant extract, the infusion from wild *F. vesca* (encapsulation efficiency close to 95%). The incorporation of the microspheres into a gelatin food matrix proved that this system preserves the antioxidant properties of the extract as compared with the free form. This is an innovative study on the development of bioactive formulations based on *F. vesca* extracts. Further studies will be required to establish a controlled release of the bioactive extract within the organism, using an *in vitro* gastrointestinal model.

Competing interests

The authors declare no competing financial interest.

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Figure 1. HPLC phenolic profile of the infusion extract obtained from wild *F. vesca* vegetative parts, obtained at 370 nm (A) and 280 nm (B).

Figure 2. OM analysis with magnifications of 40, 100 and 400× of the microspheres immediately after atomization (A), after 4 hours coagulation period under stirring at 400 rpm (B), lyophilized microspheres (C), after 48 hours rehydration (D); and SEM analysis with magnification of 550, 1000 and 2000x (E).

Figure 3. FTIR spectra of pure alginate, pure infusion extract and microspheres enriched with the infusion extract.

Figure 4. OM analysis with magnification of 40, 100 and 400× of *k*-carrageenan with microencapsulated infusion extract before (A) and after (B) lyophilisation.

Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and phenolic compounds quantification/estimation (mg/g) in the hydromethanolic and aqueous extracts prepared from commercial *F. vesca* vegetative parts.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Commercial			Wild		
						Hydromethanolic	Infusion	Decoction	Hydromethanolic	Infusion	Decoction
1	4.9	258	783	481(8),301(23)	Bis-HHDP-glucose[B]	1.72 ± 0.22	0.77 ± 0.03	1.57 ± 0.23	1.03 ± 0.18	1.72 ± 0.12	0.79 ± 0.21
2	5.6	278	451	289(100)	(Epi)catechin hexoside[A]	-	-	-	1.90 ± 0.02	4.51 ± 0.09	2.02 ± 0.18
3	5.8	260	783	481(10),301(38)	Bis-HHDP-glucose[B]	1.41 ± 0.18	0.47 ± 0.10	0.91 ± 0.17	0.83 ± 0.01	0.63 ± 0.06	0.79 ± 0.09
4	7.0	278	865	713(11),695(10),577(11),575(13),289(10),287(19)	B-type (epi)catechin trimer[A]	1.72 ± 0.14	4.05 ± 0.18	6.38 ± 0.24	-	-	-
5	7.3	280	577	451(23), 425(54),407(93), 289(58), 287(10)	Procyanidin dimer[A]	5.86 ± 0.29	5.01 ± 0.07	3.38 ± 0.08	3.75 ± 0.05	8.47 ± 0.29	5.75 ± 0.08
6	7.1	280	865	713(8),695(17),577(18),575(16),289(5),287(10)	B-type (epi)catechin trimer[A]	-	-	-	2.26 ± 0.09	4.82 ± 0.16	2.85 ± 0.23
7	7.7	356	639	463(69),301(59)	Quercetin hexose glucuronide[E]	-	-	-	2.27 ± 0.05	4.04 ± 0.08	3.35 ± 0.05
8	8.1	280	289	245(80), 203(61), 137(37)	(+)-Catechin	2.01 ± 0.25	2.21 ± 0.22	1.80 ± 0.05	11.76 ± 0.19	21.65 ± 0.01	15.39 ± 0.08
9	9.7	278	561	435(27),407(30),289(80)	B-type (epi)afzelechin-(epi)catechin[A]	-	-	-	2.64 ± 0.00	5.53 ± 0.04	3.58 ± 0.56
10	10.2	280	577	451(21), 425(43), 407(100), 289(72), 287(9)	Procyanidin dimer[A]	-	-	-	3.04 ± 0.05	2.68 ± 0.21	2.42 ± 0.09
11	10.7	276	933	915(2),631(7),451(14)301(4)	Castalagin/Vescalagin[B]	0.34 ± 0.02	-	-	-	-	-
12	11.3	264	635	465(100),313(18),295(2),169(14)	Trigalloylglucose[C]	0.10 ± 0.03	-	-	-	-	-
13	13.5	288	325	163(12),119(100),113(2)	*p*-Coumaroyl hexose[H]	0.39 ± 0.02	0.36 ± 0.01	0.26 ± 0.01	-	-	-
14	14.7	278	561	435(28),407(37),289(80)	B-type (epi)afzelechin-(epi)catechin[A]	-	-	-	2.10 ± 0.06	3.75 ± 0.29	3.84 ± 0.92
15	15.1	268	935	633(25),301(21)	Galloyl-bis-HHDP-glucose[B]	2.43 ± 0.00	-	-	0.94 ± 0.03	-	-
16	15.8	268	1567	935(100),783(39),633(77),613(2),301(19)	Sanguiin h10 isomer[B]	1.75 ± 0.04	-	-	-	-	-
17	16.8	268	1567	935(100),783(87),633(94),613(2),301(47)	Sanguiin h10 isomer[B]	4.65 ± 0.10	1.38 ± 0.12	-	-	-	-
18	17.0	252	623	301(100)	Quercetin deoxyhexose	-	-	-	0.51 ± 0.11	15.21 ± 0.08	13.57 ± 0.01

				(6),613(2),301(16)							
22	19.7	250/sh370	447	301(100)	Ellagic acid deoxyhexose[B]	0.91 ± 0.09	-	-	0.25 ± 0.07	-	-
23	19.8	346	607	285(100)	Kaempferol deoxyhexose glucuronide[G]	-	-	-	6.61 ± 0.12	11.96 ± 0.07	9.21 ± 0.05
24	20.6	358	477	301(100)	Quercetin *O*-glucuronide[D]	5.07 ± 0.04	6.23 ± 0.16	6.23 ± 0.04	12.74 ± 0.11	22.10 ± 0.32	16.75 ± 1.20
25	20.4	354	637	315(95),300(26)	Methylquercetin deoxyhexose glucuronide[E]	-	-	-	6.14 ± 0.40	10.43 ± 0.23	7.95 ± 0.11
26	21.1	356	463	301(100)	Quercetin 3-*O*-glucoside	-	-	-	0.59 ± 0.00	1.41 ± 0.06	0.53 ± 0.01
27	21.2	348	593	285(100)	Kaempferol 3-*O*-rutinoside	3.22 ± 0.01	4.97 ± 0.00	5.56 ± 0.10	0.69 ± 0.08	-	0.15 ± 0.04
28	21.7	252/sh370	301	284(16),256(11),229(18), 185(11)	Ellagic acid	1.66 ± 0.06	2.37 ± 0.02	4.08 ± 0.33	1.18 ± 0.02	1.77 ± 0.02	1.40 ± 0.02
29	24.8	350	461	285(100)	Kaempferol *O*-glucuronide[F]	0.79 ± 0.01	1.05 ± 0.01	1.05 ± 0.01	-	-	-
30	26.1	248/sh372	461	315(89),301(38)	Methyl ellagic acid deoxyhexose[B]	-	-	-	1.85 ± 0.01	1.47 ± 0.00	0.54 ± 0.02
					Total Ellagic Acid derivatives	35.31 ± 0.84[a]	13.98 ± 0.29[c]	15.06 ± 0.48[b]	69.49 ± 1.18[a]	11.22 ± 0.06[b]	5.78 ± 0.27[c]
					Total Flavonols	13.35 ± 0.01[b]	18.38 ± 0.11[a]	18.51 ± 0.11[a]	41.42 ± 0.03[c]	72.02 ± 0.40[a]	56.98 ± 1.11[b]
					Total Phenolic Acid derivatives	0.39 ± 0.06[a]	0.36 ± 0.01[b]	0.26 ± 0.01[c]	-	-	-
					Total Flavan 3-ols	9.59 ± 0.09[b]	11.27 ± 0.03[a]	11.56 ± 0.22[a]	27.46 ± 0.01[c]	51.41 ± 0.44[a]	35.83 ± 0.52[b]
					Total Phenolic Compounds	58.73 ± 0.83[a]	43.99 ± 0.37[c]	45.38 ± 0.80[b]	138.37 ± 1.20[a]	134.65 ± 0.09[b]	98.59 ± 0.85[c]

For the total compounds, in each row and for each sample (commercial or wild), different letters mean significant statistical differences between samples ($p<0.05$), where "a" and "c" correspond to the highest and lowest values, respectively.

Calibration curves used to quantify compounds which standards are not available: A- catechin, B- ellagic acid, C- gallic acid, D- quercetin-3-*O*-glucoside, E- quercetin-3-*O*-rutinoside, F- kaempherol-3-*O*-glucoside, G- kaempferol-3-*O*-rutinoside, H- *p*-coumaric acid.

Table 2. Antioxidant activity of the hydromethanolic and aqueous extracts obtained from commercial and wild *F. vesca* vegetative parts.

	Commercial			Wild			Trolox
EC_{50} values (μg/mL)	**Hydromethanolic**	**Infusion**	**Decoction**	**Hydromethanolic**	**Infusion**	**Decoction**	
DPPH scavenging activity	139.33 ± 2.61[a]	121.94 ± 6.40[b]	118.89 ± 1.13[c]	123.67 ± 7.92[a]	86.17 ± 2.42[c]	109.10 ± 1.28[b]	43.03 ± 1.71
Reducing power	324.49 ± 2.20[a]	91.88 ± 1.33[b]	88.20 ± 0.50[c]	81.40 ± 2.43[a]	62.36 ± 1.43[c]	77.28 ± 3.13[b]	29.62 ± 3.15
β-carotene bleaching inhibition	388.90 ± 15.06[a]	76.41 ± 0.66[b]	69.98 ± 2.65[c]	56.71 ± 0.66[a]	12.34 ± 1.62[c]	13.40 ± 1.81[b]	2.63 ± 0.14
TBARS inhibition	24.36 ± 0.68[a]	23.07 ± 0.40[b]	17.52 ± 0.31[c]	12.63 ± 0.77[a]	3.12 ± 0.17[c]	5.03 ± 0.06[b]	3.73 ± 1.9

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. For the total compounds, in each row and for each sample (commercial or wild), different letters mean significant statistical differences between samples ($p<0.05$), where "a" and "c" correspond to the highest and lowest values, respectively.