Encapsulation of *Stevia rebaudiana* Bertoni aqueous crude extracts by ionic gelation – Effects of alginate blends and gelling solutions on the polyphenolic profile

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Abstract

We formulated and characterised two alginate blends for the encapsulation of stevia extract (SE) via ionic gelation through an extrusion technique. Calcium chloride in SE and calcium chloride solutions were assessed as crosslinkers to overcome phenolic losses by diffusion and increase encapsulation efficiency (EE). Regardless of the blend, all stevia-loaded beads exhibited high EE (62.7-101.0%). The size of the beads decreased as EE increased. Fourier transform infrared analysis showed increased hydrogen bonding between SE and alginates, confirming the successful incorporation of SE within the matrix. Untargeted metabolomics profiling identified 479 free and encapsulated polyphenolic compounds. Flavonoids (catechin and luteolin equivalents) were predominant in SE whereas tyrosols and 5-pentadecylresorcinol equivalents were predominant in all bead formulations. Three-common discriminant compounds were exclusive to each blend and were inversely affected by the crosslinking conditions. Both alginate blends have been shown to be feasible as carrier systems of stevia extracts independent of crosslinking conditions.

Keywords: Food metabolomics, Stevia, Phenolic profiling, Encapsulation, Sodium alginate, Ionic gelation.

Chemical compounds studied in this article

Tyrosol (PubChem CID: 10393); 3,5-dicaffeoylquinic acid (PubChem CID: 6474310); Pyrogallol (PubChem CID: 1057); Epirosmanol (PubChem CID: 23243694); Malvidin 3-O-arabinoside (PubChem CID: 91810654); Peonidin 3-O-glucoside (PubChem CID: 14311151); 5-pentacosylresorcinol (PubChem CID: 155463); Matairesinol (PubChem CID: 119205); Cyanidin (PubChem CID: 68247); 5-Pentadecylresorcinol (PubChem CID: 76617).

1. Introduction

Encapsulation of polyphenolic-rich crude extracts constitutes a current research need since it can be incorporated in foods to act as a food additive, preventing or delaying food deterioration, as well as functioning as a nutritional supplement with health benefits, giving rise to innovative functional foods (Kurozawa & Hubinger, 2017). Encapsulation technologies have been extensively applied in the pharmaceutical, nutraceutical and food industries as an important tool to enhance the stability of bioactive compounds during processing and storage as well as to improve their bioavailability (McClements, 2015; Nedovic et al., 2011).

Ionic gelation through extrusion dripping represents a simple, efficient and low-cost encapsulation technique that does not require specialised equipment, high temperature, or organic solvents, making it suitable for both hydrophobic or hydrophilic compounds (Đorđević et al., 2015; Nedovic et al., 2011). Because of their ionic gelation properties and biocompatibility, sodium alginates are considered one of the most versatile, natural anionic polymers widely employed as an encapsulation agent of bioactive compounds, including plant extracts polyphenols (López-Córdoba, Deladino, & Martino, 2014; Lozano-Vazquez et al., 2015; Stojanovic et al., 2012). However, there are limitations commonly associated with hydrophilic bioactive compounds encapsulated with alginates such as burst release mechanisms and loss by diffusion during gel formation and storage (Arriola, Medeiros, Prudencio, Müller, & Amboni, 2016; Gombotz & Wee, 2012).

Therefore, a current challenge in the design of carrier systems for water-soluble compounds, such as plant polyphenols, by ionic gelation is to increase encapsulation efficiency and enhance controlled release properties (Kurozawa & Hubinger, 2017). Current approaches include the use of filler materials within the polymer matrix, internal ionic gelation, coating as a barrier as well as the use of natural polymer mixtures (Kurozawa & Hubinger, 2017; Lozano-Vazquez et al., 2015).

Stevia rebaudiana Bertoni (stevia) is a perennial South American herb, also known as ka'a hee or sweet-leaf, originally native to the north-east region of Paraguay (Gaweł-Bęben et al., 2015; Wölwer-Rieck, 2012). Besides its commercial application due to its foliar steviol glycosides with natural and intense sweetening properties, a growing body of research is pointing to stevia leaf extracts as a multifunctional source of natural bioactive compounds (Ruiz-Ruiz, Moguel-Ordoñez, Matus-Basto, & Segura-Campos, 2015; Shivanna, Naika, Khanum, & Kaul, 2013). Different therapeutic properties have been linked to stevia phytoconstituents that comprise vitamins, phytosterols, triterpenes, essential oil components as well as polyphenols – mostly consisting of flavonoids and phenolic acids – associated with its anti-inflammatory and antioxidant properties (Gaweł-Bęben et al., 2015; Karaköse, Müller, & Kuhnert, 2015; Molina-Calle, Priego-Capote, & Luque de Castro, 2017; Wölwer-Rieck, 2012). Yet, despite the relevance of phenolic compounds, studies involving stevia crude extract characterisation are few and still emerging, especially compared to the extensive work published regarding its glycoside derivatives (Gaweł-Bęben et al., 2015; Wölwer-Rieck, 2012).

In our previous study, the successful encapsulation of crude stevia extracts with alginates through ionic gelation was described for the first time in scientific literature (Arriola et al., 2016). However, both the alginate employed and the free and encapsulated polyphenolic profile of stevia lacked a thorough characterisation analysis. Furthermore, we observed a significant diffusional migration of phenolic compounds from stevia in and out of beads during both the ionic gelation process and during beads storage.

In this study, we hypothesise that the entrapment efficiency of stevia phenolic constituents by the formulated systems will be enhanced not only by employing alginate blends, but also by employing the extract in the crosslinking solution. Therefore, mixtures of sodium alginates with different molecular characteristics were formulated and characterised as carrier blends. The two blends were formed into plain alginate beads (no stevia in either the bead or the collecting solution) and two types of stevia-loaded beads which were either gelled in calcium chloride solution, or in calcium chloride dissolved in stevia extract. The structural and morphological differences between beads of the same blend and beads across blends were investigated using Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM) analyses. Subsequently, secondary metabolites (phenolics and their derivatives) were identified through a comprehensive untargeted metabolomics profiling, an innovative approach in stevia publications.

2. Materials and Methods

2.1. Chemicals

The Folin–Ciocalteu reagent, gallic acid and calcium chloride dehydrate were purchased from Sigma-Aldrich (Poole, UK). Sodium alginates employed in this study were characterised using ^1H nuclear magnetic resonance spectroscopy (ASTM F2259-12) (Chater, Wilcox, Brownlee & Pearson, 2015; Wilcox, Brownlee, Richardson, Dettmar & Pearson, 2014). Alginates employed in the blend preparations included LFR 5/60, a low viscosity and low molecular weight (MW) (40,000) sodium alginate rich in guluronate F_g 0.64; SF120, a high viscosity and high MW (225,000) sodium alginate rich in guluronate F_g 0.69; and LF120L, a high viscosity and high MW (245,000) sodium alginate low in guluronate F_g 0.44. All alginates were a gift from FMC BioPolymer AS, Drammen, Norway and were stored at $4 \pm 2^\circ\text{C}$ in tightly-sealed containers. All reagents were of analytical grade.

2.2. Preparation of aqueous leaf extract of stevia

Dried stevia leaves were harvested in Asunción, Paraguay, in March 2013 and supplied by Stevia Paraguaya Co. (Asunción, Paraguay). Prior to extraction, dried leaves were milled in a low-speed commercial blender (Wallita-Philipps, Varginha, MG, Brazil) operating at a speed of 770 rpm for 60 seconds. Aqueous leaf extraction of stevia was performed based on our previous optimisation study (Arriola et al., 2016). Briefly, aqueous extraction was performed using 1 g of dry stevia powdered leaves with 20 mL of deionised water at 90°C for 10 min. The extract was filtered through a Whatman #1 filter paper and stored in amber flasks at $4 \pm 2^\circ\text{C}$ until required.

2.3. Encapsulation of stevia extract

Two alginate blend formulations were prepared by the following alginate mixtures: LFR5/60 (2.0 % w/v) plus LF120L (1.0 % w/v), denominated LF blend, and LFR5/60 (2.0 % w/v) plus SF120 (1.0 % w/v), denominated SF blend. Briefly, both the LF (Fig.1A) and SF (Fig.1B) blends were dissolved in 20 mL of either aqueous leaf extract of stevia or deionised water to obtain plain alginate beads (A-PL) or stevia-loaded (A-SE) alginate beads, respectively. Once homogenised, the solution was drawn into a 20 ml syringe (BD Plastipak, Drogheda, Ireland) attached with a blunt tip 22 gauge metal needle. The solution was extruded (20 ± 2 °C) at a constant flow rate of 2 mm per min, being dropped into 60 ml of crosslinking solution. The hardening solution consisted of calcium chloride (2 mol l^{-1}) dissolved in either aqueous leaf extract of stevia (H-SE) or in deionised water (H-PL), to obtain the stevia-loaded beads denominated A-SE/H-SE/LF, A-SE/H-SE/SF and A-SE/H-PL/LF, A-SE/H-PL/SF, respectively. Plain alginate beads, namely A-PL/H-PL/LF and A-PL/H-PL/SF (no stevia in either the bead or the collecting solution), were obtained solely in the calcium chloride hardening solution (Table A.1). The beads were maintained in the gelling bath to harden for 30 min and then filtered and washed with distilled water. Finally, they were weighed, freeze-dried and stored in amber flasks. The lyophilised beads were stored at 4 ± 2 °C until required.

2.4. Determination of total phenolic content (TPC) of free and encapsulated aqueous leaf extract of stevia

TPC of aqueous leaf extract of stevia once encapsulated was determined after bead (~ 0.1 g) dissolution in an aqueous sodium citrate solution (5 g 100 mL $^{-1}$) at 37 ± 2 °C as proposed by Deladino, Anbinder, Navarro, & Martino (2008). TPC

of free and encapsulated stevia extract was determined in a 96-well microplate following the Folin–Ciocalteu procedure (Singleton & Rossi, 1965) with minor modifications (Zhang et al., 2006). A stock solution of 1 mg ml⁻¹ gallic acid was used as a standard (1.0 - 0.008 mg ml⁻¹). The absorbance was read at 750 nm on a microplate reader (ELx808 BioTek, Bedfordshire, UK), and results expressed as mg of gallic acid equivalents (GAE) of the dry weight of extract or lyophilised alginate beads, respectively.

2.5. Characterisation of the beads

2.5.1. Encapsulation efficiency (EE)

The amount of TPC encapsulated in alginate beads was determined as previously described in Section 2.4. The EE was calculated using the following expression:

$$EE (\%) = \frac{L}{L_o} \times 100 \quad (1)$$

where

L = TPC in the sodium citrate solution of dissolved alginate beads.

L_o = TPC in the feed stevia extract and alginate solution (theoretical load).

2.5.2 Size and Morphology

Alginate beads microstructure and morphology were characterised using a scanning electron microscope (SEM) (TESCAN VEGA LMU, Cambridge, UK) at accelerating voltage of 8.0 kV. The diameter of at least 50 lyophilised alginate beads was measured from the SEM micrographs in their original magnification using Image J (version 1.47; <http://rsb.info.nih.gov/ij/>).

2.5.3 Fourier transform infrared analysis (FT-IR)

Fourier Transform Infrared (FT-IR) measurements were taken on sodium alginates involved in the blends (LFR5/60, SF120 and LF120L); lyophilised stevia extract (LSE); plain (A-PL/H-PL/LF and A-PL/H-PL/SF) and stevia-loaded alginate beads (A-SE/H-SE/LF, A-SE/H-PL/LF and A-SE/H-SE/SF, A-SE/H-PL/SF). Samples were reduced to powder and analysed in KBr discs using an FT-IR spectrometer (Varian 800 FTIR, Scimitar Series). Spectra of samples were operated in transmission mode with 32 scans and a resolution of 4 cm^{-1} covering a wavenumber range of $600\text{--}4000\text{ cm}^{-1}$ at $18 \pm 2^\circ\text{C}$.

2.6. Screening of free and encapsulated polyphenols of stevia extract

Free and encapsulated phenolics in the stevia extract were investigated through an untargeted profiling approach based on a 1290 ultra-high-pressure liquid chromatography (UHPLC) coupled to a quadrupole-time-of-flight G6550 iFunnel mass spectrometry detector via a JetStream dual electrospray ionization source (UHPLC-ESI/QTOF-MS) (Agilent technologies, Santa Clara, CA, USA). UHPLC-ESI/QTOF-MS chromatographic, mass spectrometric and ESI source conditions were optimised in previous experiments (Lucini, Rocchetti, Kane, & Trevisan, 2016). Briefly, the mass spectrometer worked in MS-only scan to acquire the range of $90\text{--}1000\text{ m/z}$, under positive polarity mode. Chromatographic separation was achieved using a gradient of water (proteomic grade, VWR, Milan, Italy) and methanol (LCMS grade, VWR, Milan, Italy) as mobile phases, and a Knauer BlueOrchid ($100 \times 2\text{ mm i.d.}$, $1.8\text{ }\mu\text{m}$) C18 column.

Formic acid 0.1% (v/v) and ammonium formate (5 mM) (both from Sigma-Aldrich) were added to the mobile phases. The gradient was designed from 5% methanol to 90% methanol within 35 min, the flow rate was 220 $\mu\text{l min}^{-1}$ and the injection volume was 3 μl .

Raw data were then processed using the 'find-by-formula' algorithm in the Agilent Profinder B.06 software. Monoisotopic accurate mass, isotopic spacing and isotopic ratios (i.e., the whole isotope pattern) were used for phenolic compound identification, to ensure a higher confidence in profiling. The database exported from Phenol-Explorer 3.0 (Rothwell et al., 2013) was used as reference for identification, adopting a 5 ppm tolerance for mass accuracy. Based on the strategy adopted, identification was carried out according to Level 2 (i.e., putatively annotated compounds) as set out by the COSMOS Metabolomics Standards Initiative (<http://cosmos-fp7-eu/msi>). After mass and retention time alignment, compounds were then filtered by abundance and by frequency (only those compounds having an area of > 5000 counts and appearing in 100% of samples in at least one condition were retained).

The phenolic compounds putatively annotated in different extracts were next quantified according to their class and sub-class, using calibration curves built from pure reference standard compounds. With this regard, a representative compound per class was used, namely: sesamin (furan and furofuran lignans), matairesinol (dibenzylbutyrolactone and dihydroxydibenzylbutane lignans), ferulic acid (hydroxycinnamic acids and other phenolic acids), cyanidin (anthocyanins), catechin (flavanols and flavonols), luteolin (flavones and other remaining flavonoids), resveratrol (stilbenes), 5-pentadecylresorcinol (or cardol, for alkylphenols) and tyrosol (tyrosols and other compounds). To this purpose, a

linear fitting (not weighed and not force to axis origin) was built and used for the quantification step. The results were finally expressed as mg kg⁻¹ phenolic equivalents.

2.7. Statistical analysis

All data was analysed using GraphPad Prism 4 statistical software. The data were expressed as means $\pm$ standard deviation (SD) of replicates, followed by the analysis of variance (one-way ANOVA) and Tukey's studentized range test (5% significance) to verify significant differences.

The data on phenolic profiling was interpreted using Agilent Mass Profiler Professional B.12.06. Compounds were normalized at the 75th percentile and baselined to the median. Unsupervised hierarchical cluster analysis for both the compounds and the treatments was carried out based on a fold-change derived heat map (setting similarity measure as 'Euclidean' and 'Wards' as linkage rule). Then, ANOVA (unpaired t-test, $p < 0.001$) and fold-change analysis (fold-change threshold = 5) were combined into Volcano plots to investigate differences between all pairwise comparisons.

3. Results and Discussion

3.1 Characterisation of alginate beads

In preliminary tests, all sodium alginates used as ingredients in the blends were assessed for their individual entrapment efficiency of total phenolic content of stevia extracts (data not shown). The alginate LFR5/60 (2 % w/v) was selected

as the main ingredient of both the blends since it showed the best encapsulation performance (data not shown).

3.1.1 Total phenolic content (TPC) and Encapsulation Efficiency (EE)

Table 1 shows the TPC and EE of alginate beads encapsulating aqueous leaf extract of stevia. Plain alginate solutions (A-PL), plain hardening solutions (H-PL) and the plain alginate beads of both the blends, namely A-PL/H-PL/SF and A-PL/H-PL/LF, were factored into the EE calculation (Eq. 1) but showed negligible TPC and, therefore, were not included in Table 1. Both the stevia-loaded formulations of the blend SF, namely A-SE/H-SE/SF and A-SE/H-PL/SF, showed higher TPC ($p < 0.01$) compared to the matching beads (same gelling conditions) of the blend LF (i.e., A-SE/H-SE/LF and A-SE/H-PL/LF, respectively). This result was as expected since both the SF blend constituents were High-G alginates ($Gg \geq 0.64$). The gel-forming properties of alginates are strongly correlated with the proportion and lengths of blocks of contiguous L-guluronic acid residues (G-blocks) in the polymeric chains. High-G alginates are characterised by their tighter packing and smaller pore sizes, although only to a certain point since a high porosity can be achieved with alginates with over 70% guluronate content and block length longer than 15 (Martinsen, Skjåk-Braek, & Smidsrød, 1989). Therefore, the higher TPC entrapment by the SF blend was attributed to the greater availability of active calcium-binding sites in the SF polymeric chains and, consequently, the greater degree of crosslinking, thus yielding a higher TPC entrapment in relation to the beads of the LF blend.

Considering the impact of the crosslinking conditions on the TPC entrapped, beads gelled in calcium chloride solutions (H-PL) showed significantly

lower ($p < 0.05$) TPC in relation to beads gelled in solutions containing the extract, independent of the blend. The TPC decrease can be explained by the significant migration of phenolic compounds into the calcium chloride solution, determined as the fraction of non-encapsulated phenolic compounds found in the gelling bath after the beads were removed (Table 1). This finding showed that, during encapsulation, stevia extract leaked into the aqueous calcium chloride solution and that the extent of leakage depended on the alginate blend formulation. The LF blend retained higher TPC in A-SE/H-PL/LF beads (71%) compared to that retained in A-SE/H-PL/SF beads (54%) in relation to the TPC of A-SE/H-SE/LF and A-SE/H-SE/SF beads, respectively. A similar diffusional trend was reported in our previous work (Arriola et al., 2016) and elsewhere (Deladino et al., 2008; Stojanovic et al., 2012), and corresponds to a major setback in the encapsulation of low molecular weight and hydrophilic compounds with alginates (Deladino et al., 2008; Kurozawa & Hubinger, 2017). Nevertheless, this finding reinforces the idea that the bead's TPC can be significantly increased when employing stevia extract as collecting solution, also causing a significant impact in the encapsulation efficiency of the system.

Regardless of the blend, the four formulated stevia-loaded beads showed high encapsulation efficiency (EE) of total phenolic constituents ($> 60\%$). The beads gelled in solutions containing stevia extract reached very high EE ($> 96\%$) in relation to similar literature data on plant phenolics encapsulation with alginates via ionic gelation (Pasukamonset, Kwon, & Adisakwattana, 2016; Stojanovic et al., 2012). The high EE of H-SE beads achieved in both formulations was attributed to the reduction of the concentration gradient for diffusion, overcoming phenolic compounds losses during encapsulation. Accordingly, EE of A-SE/H-

SE/SF beads was increased by 28% in relation to A-SE/H-PL/SF beads. The same was noted for the LF group of beads, resulting in an increase of 54% in the EE of A-SE/H-SE/LF beads compared to A-SE/H-PL/LF beads.

Thus, our data indicated that not only the blends formulations, but also the gelling conditions significantly influenced the TPC entrapment efficiency of aqueous leaf extract of stevia in alginate blends by ionic gelation.

3.1.2. Size and Morphology

In general, both alginate blends showed similar size distribution between their matching plain and stevia-loaded alginate beads formulations. The LF beads were slightly larger in relation to the beads originating from the SF blend, however, there was no significant difference ($p > 0.05$) in terms of size comparing matching beads across blends. Accordingly, average diameters of beads decreased in the following order: A-PL/H-PL/LF > A-PL/H-PL/SF > A-SE/H-PL/LF > A-SE/H-PL/SF > A-SE/H-SE/LF > A-SE/H-SE/SF.

The mean diameters of the plain alginate beads A-PL/H-PL/LF and A-PL/H-PL/SF were 2.4 ± 0.33 and 2.1 ± 0.28 mm, respectively, and were significantly larger ($p < 0.001$) when compared to the stevia-loaded beads of their respective blend. Likewise, mean diameters of A-SE/H-PL/LF and A-SE/H-PL/SF beads were of 1.8 ± 0.20 and 1.8 ± 0.15 mm, respectively, and were significantly larger ($p < 0.01$) than A-SE/H-SE/LF and A-SE/H-SE/SF beads. The average diameters of A-SE/H-SE/LF and A-SE/H-SE/SF beads were 1.5 ± 0.18 and 1.4 ± 0.28 mm, respectively. The size of calcium alginate beads obtained in our study was consistent with previous studies that reported beads size larger than 1 mm when employing the extrusion technique (López-Córdoba et al., 2014; Pasukamonset, Kwon, & Adisakwattana 2016). The size and shape of beads are

important parameters that should be considered in the formulation of pharmaceutical and food products, as spherical beads with uniform size distribution are desired for the delivery of active functional ingredients (Đorđević et al., 2015).

Furthermore, results showed that the size of beads decreased at higher TPC entrapment independent of the blend (Table 1). The difference in bead dimensions may be explained by differences in the gel forming and shrinking properties of alginates during ionic gelation. By employing stevia extract in the hardening solution, the calcium-alginate bonding was possibly affected by interactions with stevia active compounds through hydrogen bonding (shown in FTIR spectra, Fig. 3A and 3B), causing a deeper penetration of the calcium into the alginate bead and therefore smaller beads (Pasukamonset, Kwon, & Adisakwattana 2016). Similar results were obtained by Pasukamonset et al. (2016), who also found that plain alginate beads were significantly larger than alginate beads encapsulating *Clitoria ternatea* petal flower extracts. The authors attributed this behaviour to the extensive hydrogen bonding (involving hydrophilic phenolic hydroxyl groups) between the extracts polyphenols and the alginate polymeric chains determined through FT-IR analysis.

Figure 2 displays the scanning electron micrographs of lyophilised plain and stevia-loaded calcium alginate beads originating from the SF (Fig. 2 A-C) and LF (Fig. 2 D-F) alginate blends. All beads exhibited heterogeneous surface morphologies and a wrinkled spongy shape, due to partly collapsing the polymeric gel network during freeze drying (Fig.2 A-F). Similar morphological observations of lyophilised alginate beads have been reported previously and can be usually overcome by employing polysaccharides such as starch, inulin and

pectin, as filler-ingredients (Belscak-Cvitanovic et al., 2015; López-Córdoba et al., 2014). Moreover, the incorporation of stevia extract into the polymer matrix led to bead structure changes in shape and surface characteristics.

Plain alginate beads, i.e. A-PL/H-PL/LF (Fig. 2D) and A-PL/H-PL/SF (Fig. 2A), were mostly an oval shape and exhibited a higher number of cavities throughout the rough surface compared with the stevia-loaded beads of both blends. The fewer occurrences of cavities in stevia-loaded beads indicated the possible incorporation of stevia extract within the polymer matrix. Both A-SE/H-PL/LF (Fig. 2E) and A-SE/H-PL/SF (Fig. 2B) beads showed a convoluted shape upon lyophilisation, with surface fissures evidenced in A-SE/H-PL/SF beads. A-SE/H-SE/LF (Fig. 2F) and A-SE/H-SE/SF (Fig. 2C) beads had a spherical shape with differing surface morphologies, with A-SE/H-SE/LF showing a rough surface with fissures and A-SE/H-SE/SF beads showing a porous surface.

There are several factors that can affect or even control the size and shape of ionically crosslinked alginate beads. According to Klok and Melvik (2002), the size and shape of alginate beads can be affected by the flow rate of polymer solution, needle diameter, gelling conditions, as well as alginate solution viscosity, alginate composition and molecular weight distribution. Based on our findings, the gelling conditions provided the main differences obtained in terms of size between beads, as both alginate blends showed similar mean diameters ($p > 0.05$) between their matching plain and stevia-loaded alginate beads. Morphological characteristics, on the other hand, were attributed both to the blend formulation and the crosslinking conditions.

3.1.3 Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR was used to characterise the ingredients used in this study as well as to evaluate the potential molecular interactions between the carrier constituents, the encapsulated stevia extract and the coating blends used.

Figure 3 displays the FT-IR spectra of plain sodium alginates involved in the blends (LFR5/60, SF120, LF120L), plain alginate beads (A-PL/H-PL/LF and A-PL/H-PL/SF) and stevia-loaded calcium alginate beads (A-SE/H-SE/LF, A-SE/H-PL/LF and A-SE/H-SE/SF, A-SE/H-PL/SF). Lyophilised stevia extract (LSE) was employed to reduce spectral interference from water and the LSE spectra is shown in Figure 3A and 3B. Among the main bands, a broad band formed at 3295 cm^{-1} (3600 and 2500 cm^{-1}) was observed. This band refers to the O-H stretching vibration of hydroxyl groups and is associated with the functional groups of alcohols and phenolic compounds involved in inter- and intramolecular hydrogen bond (Silverstein, Bassler, & Morrill, 2005). The band at 2928 cm^{-1} (3000–2800 cm^{-1}) is assigned to the vibration of aliphatic C-H bonds (alkane), which include CH, CH₂, and CH₃ stretching and bending vibrations. The band recorded at 1684 cm^{-1} is due to the stretching vibration of the C=O bond and corresponds to the steviol glycosides characteristic band, as recently reported by Chranioti, Chanioti, & Tzia (2016). The clear peak at 1599 cm^{-1} (1400 – 1600 cm^{-1}) is related to carbon double bonds C=C that is in aromatic and stretching mode or attributed to stretching vibration of C=O of flavonoids (Mot, Silaghi-Dumitrescu, & Sârbu, 2011), while distinct bands in the region of 1300-1000 cm^{-1} were attributed to the stretching vibrations of carboxylic acids, alcohols, ethers, esters and amino functional groups. Other marked bands were detected at 1153, 1080 and 1032 cm^{-1} and attributed to vibrations of C–O–C and C–C of glycosidic bonds, in line with that previously reported in similar publications (Chranioti et al., 2016;

Perumal, Manickam, Bang, Velmurugan, & Oh, 2016). Finally, the band at 856 cm^{-1} is attributed to bending vibration of the C=C bond, related to aromatic ring vibration (Perumal et al., 2016).

The FT-IR spectra of the three individual sodium alginates (Fig. 3C) showed characteristic bands of functional groups with varying intensities, including bands within the range of 3200–3550 cm^{-1} (stretching vibration of the O-H bond), and peaks at 1597 and 1409 cm^{-1} which are assigned to asymmetric and symmetric stretching peaks of carboxylate groups, respectively. Bands at 1084 and 1028 cm^{-1} , also seen in the LSE spectra, are attributed to its saccharide structure, while bands in the region 950–815 cm^{-1} (i.e. 948 cm^{-1} , 904 cm^{-1} and 813 cm^{-1}) are associated with polyguluronic and polymannuronic acid sequences in the alginate backbone. According to Mathlouthi & Koenig (1986), bands at around 948 cm^{-1} are assigned to α 1→4 linkage, and bands at 904 cm^{-1} are attributed to the α -L-gulopyranuronic asymmetric ring vibration.

After bead formation, both the A-PL/H-PL/LF and A-PL/H-PL/SF calcium alginate beads showed all characteristic bands of the alginate backbone (Fig. 3C). A slight up-shift in the –OH peak positions (3550- 3200 cm^{-1}) and in peaks assigned to asymmetric and symmetric stretching of carboxylate groups were observed in both A-PL/H-PL/LF and A-PL/H-PL/SF spectra, in comparison to the spectra of their sodium alginate ingredients. This shift was previously reported in similar studies involving ionic gelation (Belscak-Cvitanovic et al., 2015; López-Córdoba et al., 2014) and is assigned to the crosslinking process, as the alginate C=O bond strength increases due to the exchange of Na^+ with Ca^{2+} (Williams & Fleming, 2008). Furthermore, alginate's polymeric chain interactions through H-bonding can be shown by the widening of the OH bond and differing functional

peak intensities between blends (Fig 3C). The increase in the intensity and narrowing of the absorption band in the 3392 cm^{-1} region in A-PL/H-PL/LF spectra was due to the OH breaking of some inter- and intra-molecular hydrogen bonding. This finding was attributed to the high molecular weight of the LF blend ingredient (alginate LF120, MW: 245,000), which could have provoked steric hindrance between the polymeric chains, and, therefore, affected the intermolecular hydrogen bonding during the bead formation. On the other hand, for the SF blend, the broader OH signals in A-PL/H-PL/SF spectra imply that the interaction between alginates LFR5/60 and SF120 forms new intermolecular H-bonds with lower strength.

Figure 3A and 3B shows the spectra of stevia-loaded beads of both blends. Regardless of the blend, all beads showed various characteristic peaks not only of the stevia extract but also of the alginate blend backbone, confirming the successful entrapment of stevia extract. Beads from the SF blend (Fig. 3A) showed a broad OH band of moderate intensity among the plain and stevia-loaded beads spectra, being broader in A-SE/H-SE/SF beads suggesting increased hydrogen bonding. Moreover, for both the stevia-loaded beads, a slight down-shift in the OH peak position was observed in relation to the plain beads spectra, suggesting that a molecular interaction between alginates and stevia was formed via hydrogen bonding, in agreement with previous studies involving alginate hydrogels encapsulating plants extracts (Pasukamonset et al., 2016). Comparing the fingerprint region in the beads spectra ($1500 - 900\text{ cm}^{-1}$), the SF blend (Fig. 3A) showed similar bands between A-PL/H-PL/SF and A-SE/H-PL/SF spectra, whereas A-SE/H-SE/SF spectra was similar to LSE ($1256, 1153, 1077\text{ cm}^{-1}$). This feature suggests that the incorporation of stevia extract within the

polymer matrix was enhanced by employing the extract in the crosslinking bath. On the other hand, for the LF blend (Fig 3B), the -OH sharp peak was similar among plain and stevia-loaded beads in addition to similar absorption bands with no significant displacements in the fingerprint region, implying that the crosslinking conditions showed no significant difference in the bonding interactions of stevia active compounds and the alginate blend.

Considering that there were no new absorption bands in the stevia-loaded beads spectra of both blends as well as no significant shifts in the peaks observed, we confirm the feasibility and compatibility of alginate matrices as carriers of stevia extracts. This finding is in agreement with similar studies that reported the presence of hydrogen bonding and absence of chemical interactions between alginate matrices and phytochemicals of plants extracts during ionic gelation (Pasukamonset et al., 2016; Stojanovic et al., 2012).

Thus, FT-IR analysis showed that interactions between the carrier ingredients forming the blends led to interactions mainly between polar active compounds together with ionic and hydrogen bonding. The hydrogen bonding was also the main interaction between stevia constituents and alginate blends.

3.2. Screening of free and encapsulated polyphenols of stevia extract

Untargeted profiling of phenolic compounds extracted as described in the Section 2.6 led to the identification of a total number of 479 compounds, confirming the potential of this untargeted approach to provide a high number of metabolites. The complete dataset of phenolic compounds identified in the free and encapsulated stevia extract is provided as Supplementary Material.

Figure 4 depicts the abundance of phenolic compounds, annotated as cumulative mg kg⁻¹ equivalents, per each class considered during the quantification step. Flavonoids were clearly the predominant class present in Stevia extracts (42%), including flavanols and flavonols (expressed as catechin equivalents), flavones (luteolin equivalents) and anthocyanins (cyanidin equivalents). The second most abundant class was found to be the phenolic acids (31%), comprising hydroxyphenylacetic and hydroxycinnamic acids (quantified as ferulic acid equivalents). The sum of other polyphenols represented 25% of the total content and comprised tyrosols (tyrosols equivalents) and alkylphenols (5-pentadecylresorcinol equivalents), followed by less abundant compounds such as lignans (0.6 %) (expressed as sesamin and matairesinol equivalents) and stilbenes (0.1 %) (resveratrol equivalents). The results are in line with recently reported data by similar studies involving the characterisation of phenolic constituents in stevia extracts (Karaköse et al., 2015; Molina-Calle et al., 2017). However, considering the untargeted nature of the approach applied, the number of polyphenols identified in this study was remarkably higher when compared to previous literature (Karaköse, Jaiswal, & Kuhnert, 2011; Karaköse et al., 2015; Molina-Calle et al., 2017). Karaköse et al. (2011) analysed the polyphenolic profile of stevia methanolic extracts and identified 24 hydroxycinnamic acid derivatives employing LC-MS and LC-TOF techniques. In a subsequent publication, 15 flavonoids were identified in stevia methanolic extracts through LC-MS analysis with an ion-trap spectrometer (Karaköse et al., 2015). More recently, Molina-Calle, Priego-Capote, & Luque de Castro (2017) applied an untargeted screening analysis of the main compounds present in polar and non-polar extracts of stevia leaves using LC-MS. Twenty-one phenolic compounds

were tentatively identified in the polar extracts (ethanol-water), which were classified into two main families: flavonoids, and quinic and caffeic acids and derivatives.

The increasing number of works focusing on the identification of plant phenolics reflects a current trend in food analysis. Most of the studies involving the qualitative and quantitative analysis of compositional profiles and fingerprints of polyphenols from plant extracts are focusing on the characterisation and identification of new compounds (Lucci, Saurina, & Núñez, 2017). However, polyphenols identification in food matrices is a relatively complex task due to the plethora of compounds that can be present with diverse polarities and molecular sizes. For this reason, liquid chromatography coupled with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) are currently the most effective techniques for the structural characterisation and determination of both low and high molecular weight polyphenols in food samples (Lucci et al., 2017). Hence, the untargeted metabolomic approach on the phenolic screening allowed us to perform a comprehensive analysis of the whole phenolic profile of free and encapsulated aqueous leaf extract of stevia.

Moreover, while the stevia extract was mainly composed of flavonoids, all calcium alginate beads showed the highest abundance in less common phenolic compounds such as tyrosols and alkylphenols (quantified as tyrosol and 5-pentadecylresorcinol equivalents, respectively; Fig.4B). The abundance of these latter two subclasses was similar between all the stevia-loaded beads, representing about 44% of A-SE/H-SE/SF and A-SE/H-PL/SF and 43% of A-SE/H-SE/LF and A-SE/H-PL/LF, respectively. Another noteworthy finding was that all four stevia-loaded systems showed higher abundance of stilbenes

(expressed as resveratrol equivalents) in relation to the stevia extract. Furthermore, by comparing matching beads across blends, we observed a close similarity in the phenolic abundance profile between A-SE/H-PL/SF and A-SE/H-PL/LF as well as between A-SE/H-SE/SF and A-SE/H-SE/LF beads. This feature depicts the influence exerted by the collecting solution on the phenolics entrapped in each type of bead suggesting a selective uptake. Similarities between matching beads across blends was further supported by the output of the unsupervised hierarchical cluster analysis (supplementary material).

The unsupervised hierarchical cluster analysis (HCA) originating from the fold-change heat map is illustrated in the supplementary material. Each formulation was placed in defined dendrograms composed by unaveraged replicates of free aqueous leaf extract of stevia and of stevia-loaded calcium alginate beads of both blends. The clustering of analysed samples was based on the similarities in the phenolic abundance values. Stevia extract was included in a separate cluster, therefore, indicating dissimilar compound abundance in relation to the four beads formulations. Moreover, the heat maps confirmed the close relationship between the abundance intensities of matching beads, i.e. between both the A-SE/H-PL/LF and A-SE/H-PL/SF beads and between both the A-SE/H-SE/LF and A-SE/H-SE/SF beads. Consequently, we performed pair-wise comparisons between the phenolic profile of stevia extract versus A-SE/H-SE/SF and A-SE/H-SE/LF as well as versus A-SE/H-PL/SF and A-SE/H-PL/LF beads.

Discriminant phenolic compounds exhibiting the most significant variation between bead formulations were selected through Volcano analysis, based on the combination of analysis of variance and fold-change (using a fold-change cut off = 3 and a p-value of 0.01). The Venn diagrams (supplementary material) show

that there were over a hundred discriminant metabolites resulting from the Volcano pairwise analysis of Stevia extract vs. A-SE/H-SE/SF and A-SE/H-SE/LF (supplementary material) and Stevia extract vs. A-SE/H-PL/SF and A-SE/H-PL/LF (supplementary material), respectively.

Among the discriminant compounds, there were also several phenolic compounds exclusively found in each bead formulation, corroborating their selective uptake. Table 2 shows the discriminant phenolic compounds that were exclusively detected in A-SE/H-SE/LF and A-SE/H-SE/SF beads, respectively, grouped by phenolic class. A-SE/H-SE/LF beads showed the highest number of exclusive discriminant compounds including mostly phenolic acids (9), other phenolics (8), flavonoids (7) and lignans (3). Fewer exclusive compounds were identified in A-SE/H-SE/SF beads, comprising mostly flavonoids (9) and other phenolic compounds (6). Table 3 shows the exclusive discriminant phenolic compounds present in H-PL beads. Flavonoids (5) and phenolic acids (5) were the predominant class found in A-SE/H-PL/LF beads, followed by other phenolics (3) and lignans (1). On the other hand, A-SE/H-PL/SF showed mostly flavonoids (10) as discriminant compounds, followed by phenolic acids (3), other phenolics (3) and lignans (1).

Considering the high number of discriminant metabolites identified in each pair-wise comparison and, by comparing discriminant compounds between beads of the same blend, there were only three common discriminant compounds exclusive of each blend. Pyrogallol, epirosmanol and 3,5-dicaffeoylquinic acid were common between beads of the LF blend, and malvidin 3-O-arabinoside, peonidin 3-O-glucoside and 5-pentacosylresorcinol between beads of the SF blend (Tables 2 and 3). The opposite effects observed among the three-common

compounds between the stevia-loaded beads of the same blend confirmed the significant effect exerted by the crosslinking conditions towards the phenolic profile of each bead formulation. Hence, these exclusive phenolic compounds represent the phenolic markers that were inversely affected by the gelling conditions applied in our study. Therefore, considering the whole metabolomic profile of stevia-loaded beads, specifically the discriminant compounds found in each bead formulation, we postulate that differences in the polyphenol profiles between formulations across blends were mainly attributed to both the polymers blends and the collecting conditions.

4. Conclusions

Encapsulation of stevia crude extract within two alginate blends by ionic gelation has been assessed. The impact of carrier formulations and crosslinking conditions on the overall encapsulation performance was investigated aiming to overcome phenolic compound losses by diffusion and, thus, increase encapsulation efficiency. All the formulated stevia-loaded beads reached high encapsulation efficiency values (>60%), independent of the carrier combination or the crosslinking conditions. Stevia-loaded beads that crosslinked in solutions containing stevia extract reached very high encapsulation efficiency (>96%), clearly avoiding phenolic compounds diffusion during ionic gelation. Furthermore, crosslinking conditions mainly impacted EE, size and polyphenolic abundance profile. We demonstrated that the size of all formulated stevia-loaded beads decreased as the polyphenols entrapment increased. Also, SEM micrographs and FTIR analysis confirmed the successful incorporation of stevia extracts in all

stevia-loaded beads, demonstrating the compatibility of alginates as carriers of natural bioactive compounds. The untargeted metabolomics profiling allowed us to perform a comprehensive analysis of the whole phenolic profile of free and encapsulated stevia crude extracts. Each bead formulation showed unique phenolic profile comprising over a hundred discriminant metabolites. The outcome of the unsupervised cluster analysis evidenced similarities in the phenolic profile between beads that were crosslinked in the same conditions, when comparing beads across blends. In addition, each blend comprised three exclusive phenolic compounds identified as the phenolic markers inversely affected by the gelling conditions, which suggested that the crosslinking conditions provided a selective uptake.

Therefore, considering the overall encapsulation performance, we conclude that not only the crosslinking conditions but also the carrier formulations have shown significant impact in the selective entrapment of polyphenols within each bead formulation. Subsequent work will focus on the untargeted release profiles of encapsulated polyphenols of stevia-loaded beads during *in vitro* digestion in a model gut.

Conflict of interest

The authors declare that they have no conflict of interest in this stud

Aknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

637 **Figure 1.**

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642 Figure 2.

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Figure 3.

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Figure captions

Figure 1. Schematic illustration of the ionic gelation process of aqueous leaf extract of stevia employing alginate blends denominated LF (A) and SF (B). Each blend comprised plain alginate beads (A-PL/H-PL/LF and A-PL/H-PL/SF) and two types of stevia-loaded beads coded based on their gelling solution: calcium chloride (2mol l^{-1}) dissolved in stevia extract (A-SE/H-SE/LF and A-SE/H-SE/SF beads) and aqueous calcium chloride solution (A-SE/H-PL/LF and A-SE/H-PL/SF beads).

Figure 2. Micrographs of lyophilised calcium alginate beads originating from the SF (A-C) and LF (D-F) alginate blends encapsulating aqueous leaf extract of stevia. Each blend comprised plain alginate beads (A and D) and two stevia-loaded beads based on their gelling solution: calcium chloride (2mol l^{-1}) dissolved in stevia extract (C and F) and aqueous calcium chloride solution (B and E). Magnification ($100\times$) – surface ($3,00\times$).

Figure 3. FT-IR spectra of lyophilised plain alginate (A-PL/H-PL/LF and A-PL/H-PL/SF) and stevia-loaded calcium alginate beads (A-SE/H-SE/LF, A-SE/H-PL/LF and A-SE/H-SE/SF, A-SE/H-PL/SF) formed from the blends SF (A) and LF (B) derived from the sodium alginates LFR5/60, SF120, LF120L (C).

Figure 4. Abundance (cumulative intensities) and phenolic classification as identified in the free aqueous leaf extract of stevia (A) and in the lyophilised stevia-loaded calcium alginate beads originating from the SF and LF alginate blends coded based on their gelling solution: calcium chloride (2mol l^{-1}) dissolved in stevia extract (A-SE/H-SE/LF and A-SE/H-SE/SF beads) and aqueous calcium chloride solution (A-SE/H-PL/LF and A-SE/H-PL/SF beads) (B). The data are a result of at least $n=5$ provided from UHPLC/QTOF-MS and reported as mg kg^{-1} equivalents.

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LF Blend

3%(w/v)
Sodium Alginates
LFR5/60+LF120L
+
Stevia Extract

Calcium Chloride +
Stevia Extract (SE)

A-SE/H-SE/LF beads

3%(w/v)
Sodium Alginates
LFR5/60+LF120L
+
Deionized water

Calcium Chloride Solution

A-SE/H-PL/LF beads

3%(w/v)
Sodium Alginates
LFR5/60+LF120L
+
Deionized water

A-PL/H-PL/LF beads

Stevia rebaudiana Bertoni

SF Blend

3%(w/v)
Sodium Alginates
LFR5/60+SF120
+
Stevia Extract

Calcium Chloride +
Stevia Extract (SE)

A-SE/H-SE/SF beads

3%(w/v)
Sodium Alginates
LFR5/60+SF120
+
Deionized water

Calcium Chloride Solution

A-SE/H-PL/SF beads

A-PL/H-PL/SF beads

Pyrogallol

Epirosmanol

3,5-dicaffeoylquinic acid

Malvidin 3-O-arabinoside

Peonidin 3-O-glucoside

5-pentacosylresorcinol

Figure A1 Not averaged unsupervised cluster analysis on the phenolic profile of free aqueous leaf extract of stevia and lyophilised stevia-loaded calcium alginate beads originating from the SF and LF alginate blends based on their gelling solution: calcium chloride (2mol l^{-1}) dissolved in stevia extract (A-SE/H-SE/LF and A-SE/H-SE/SF beads) and aqueous calcium chloride solution (A-SE/H-PL/LF and A-SE/H-PL/SF beads) (similarity: Euclidean; linkage rule: Ward). Compound intensity was used to build up heat maps, based on which the clusters were generated. Each cluster represents a sample replicate (n=5).

Figure A2. Venn diagrams of discriminant phenolic metabolites found in both or solely in one of the two pairs: A-SE/H-SE/SF and A-SE/H-SE/LF (A) and A-SE/H-PL/SF and A-SE/H-PL/LF (B) calcium alginate beads encapsulating stevia extract, based on their gelling solution: calcium chloride (2mol l^{-1}) dissolved in stevia extract (A-SE/H-SE/SF and A-SE/H-SE/LF beads) and aqueous calcium chloride solution (A-SE/H-PL/SF and A-SE/H-PL/LF beads). Data resulted from Volcano analysis ($p < 0.01$, fold-change cut-off = 3).

A

B

Table 1. Total phenolic content (TPC) and Encapsulation Efficiency (EE%) of lyophilised calcium alginate beads from the SF and LF alginate blends encapsulating aqueous leaf extract of stevia based on their gelling solution: calcium chloride (2mol l^{-1}) dissolved in stevia extract (A-SE/H-SE/LF and A-SE/H-SE/SF beads) and aqueous calcium chloride solution (A-SE/H-PL/LF and A-SE/H-PL/SF beads). TPC of non-encapsulated fraction of stevia extract from A-SE/H-PL/LF and A-SE/H-PL/SF beads is shown as gelling solution. The data represent mean values $\pm$ SD of at least $n=4$. Mean values were analysed using a one-way ANOVA with Tukey's post hoc test.

Table 2. Exclusive compounds discriminating between A-SE/H-SE/LF and A-SE/H-SE/SF calcium alginates beads from the LF and SF alginate blends, respectively, grouped in phenolic chemical classes. Results are in relation to the stevia extract phenolic profile as resulted from Volcano analysis ($p < 0.01$, fold-change cut-off = 3).

Table 3. Exclusive compounds discriminating between A-SE/H-PL/LF and A-SE/H-PL/SF calcium alginates beads from the LF and SF alginate blends, respectively, grouped in phenolic chemical classes. Results are in relation to the stevia extract phenolic profile as resulted from Volcano analysis ($p < 0.01$, fold-change cut-off = 3).

Table A1. Coding specifications of alginate blends, dissolving and hardening solutions of the ionic gelation process of aqueous leaf extract of *Stevia rebaudiana* Bertoni.

Alginate Blend		Dissolving Solution		Hardening Solution		Final Code
Code	Details	Code	Details	Code	Details	
LF Blend	LF120L and LFR5/60	A-PL	Plain	Deionised water	(H-PL)	A-PL/H-PL/LF
		A-SE	Stevia Loaded	Aqueous leaf extract of stevia	H-SE	A-SE/H-SE/LF
				Deionised water	(H-PL)	A-SE/H-PL/LF
SF Blend	SF120 and LFR5/60	A-PL	Plain	Deionised water	H-PL	A-PL/H-PL/SF
		A-SE	Stevia Loaded	Aqueous leaf extract of stevia	H-SE	A-SE/H-SE/SF
				Deionised water	H-PL	A-SE/H-PL/SF

Table 1.

	A-SE/H-SE/SF	A-SE/H-PL/SF	A-SE/H-SE/LF	A-SE/H-PL/LF
TPC*	1.82 ± 0.06 ^{aA}	0.99 ± 0.01 ^{bA}	1.28 ± 0.02 ^{cB}	0.91 ± 0.01 ^{dB}
EE (%)	101 ± 0.01 ^a	78.6 ± 0.01 ^b	96.6 ± 0.01 ^c	62.7 ± 0.0 ^d
TPC* Gelling Solution	n.d.	0.99 ± 0.01 ^e	n.d.	1.00 ± 0.03 ^e

n.d. = not determined.

*TPC= total phenolic content expressed as mg/g Gallic Acid Equivalent (GAE) dry weight.

^{a-b}Different superscript lowercase letters in the same line indicate significant differences ($p < 0.05$) between the total phenolic content of the stevia-loaded lyophilised calcium alginate beads from the SF blend.

^{c-d}Different superscript lowercase letters in the same line indicate significant differences ($p < 0.05$) between the total phenolic content of the stevia-loaded lyophilised calcium alginate beads from the LF blend.

^eSame superscript lowercase letters in the same line indicate insignificant differences ($p > 0.05$) between the total phenolic content of the remaining aqueous calcium chloride solution after A-SE/H-PL/SF and A-SE/H-PL/LF beads encapsulation.

^{A-B}Different superscript uppercase letters in the same line indicate significant differences ($p < 0.05$) between the total phenolic content of the stevia-loaded lyophilised calcium alginate beads from different blends (between A-SE/H-SE/SF, A-SE/H-SE/LF and between A-SE/H-PL/LF, A-SE/H-PL/SF).

Table 2.

Sample	Class	Subclass	Compound	Molecular Weight	p (Corr)	FC (abs)	Regulation
A-SE/H-SE/LF	Flavonoids	Chalcones	Butein	272.2528	0	36701,28	down
		Flavonols	Isorhamnetin	316.2623	0	16	down
		Anthocyanins	Malvidin 3-O-(6"-p-coumaroyl-glucoside)	639.5801	0	4658,048	down
		Flavanones	Naringenin	580.5346	0	16	down
		Flavones	Nepetin	316.2623	0	16	down
		Flavonols	Patuletin 3-O-(2"-feruloylglucosyl)(1-6)-[apiosyl(1-2)]-glucoside	964.8262	4,05E-09	9,342045	up
		Flavonols	Rhamnetin	316.2623	0	16	down
	Lignans	Lignans	7-Hydroxysecoisolariciresinol	374.4706	7,68E-04	5,7096176	up
		Lignans	Sesaminol	370.3527	0	3508,909	down
		Lignans	Episesaminol	370.3527	0	3508,909	down
	Other polyphenols	Alkylphenols	3-Methylcatechol	124.1372	2,91E-14	124979,64	down
		Hydroxybenzaldehydes	4-Hydroxybenzaldehyde	122.1213	0,002326482	16740,914	down
		Alkylphenols	4-Methylcatechol	124.1372	2,91E-14	124979,6	down
		Alkylphenols	5-Heneicosylresorcinol	404.6688	0,003164	116755,6	up
		Methoxyphenols	Guaiacol	124.1372	2,91E-14	124979,6	down
		Other polyphenols	Pyrogallol	126.11	4,10E-07	8,734744	down
		Hydroxybenzaldehydes	Syringaldehyde	182.1733	5,26E-13	1032682	up
		Phenolic terpenes	Epirosmanol	346.4174	1,22E-13	2342863,5	up
	Phenolic acids	Hydroxycinnamic acids	3,5-Dicaffeoylquinic acid	516.4509	7,39E-15	1,65E+07	down
		Hydroxycinnamic acids	3-Feruloylquinic acid	368.3353	2,08E-08	8,961844	down
		Hydroxybenzoic acids	3-Hydroxybenzoic acid	138.1207	1,67E-13	860538,4	down
		Hydroxycinnamic acids	4-Feruloylquinic acid	368.3353	2,08E-08	8,961844	down
		Hydroxycinnamic acids	5-Feruloylquinic acid	368.3353	2,08E-08	8,961844	down
		Hydroxybenzoic acids	Benzoic acid	122.1213	0,002326	16740,91	down
		Hydroxyphenylpropanoic acids	Dihydrocaffeic acid	182.1733	5,26E-13	1032682	up
		Hydroxybenzoic acids	Ellagic acid	302.1926	0	16	down
		Hydroxyphenylacetic acids	Homovanillic acid	182.1733	5,26E-13	1032682	up
A-SE/H-SE/SF	Flavonoids	Flavanones	8-Prenylnaringenin	340.3698	1,86E-04	5,269171	up
		Flavones	Apigenin 7-O-glucuronide	446.361	0	6398,84	down
		Flavonols	Kaempferol 3-O-acetyl-glucoside	490.4136	0	16	down
		Flavonols	Kaempferol 7-O-glucoside	447.369	0	6384,914	down

	Anthocyanins	Malvidin 3-O-arabinoside	463.4114	0	16	down
	Anthocyanins	Peonidin 3-O-glucoside	463.4114	0	16	down
	Anthocyanins	Peonidin 3-O-rutinoside	609.5526	2,76E-07	3,153877	down
	Dihydrochalcones	Phloretin 2'-O-xylosyl-glucoside	568.5239	3,91E-07	3,243539	down
	Flavonols	Quercetin 3-O-acetyl-rhamnoside	490.4136	0	16	down
Other polyphenols	Alkylphenols	5-Pentacosylresorcinol	460.7751	0,001969	259052,2	up
	Hydroxyphenylpropenes	Acetyl eugenol	206.2378	0,002108	552605,9	up
	Tyrosols	Hydroxytyrosol 4-O-glucoside	316.3038	1,46E-07	3,316227	up
	Hydroxybenzaldehydes	p-Anisaldehyde	136.1479	0,005084	29660,07	down
	Hydroxycinnamic acids	Avenanthramide 2p	299.2781	1,17E-09	11,3456	up
	Hydroxybenzoic acids	Gallic acid 3-O-gallate	322.2238	0	5,025687	up

For each compound, unpaired t-test p-values, absolute fold-change (FC) and regulation are provided. According to Mass Profiler Professional B.12.06 output, values of p (Corr) = 0 or FC = 16 denote very high significance at t-test or extremely high fold-change, respectively. A-SE/H-SE/LF and A-SE/H-SE/SF beads: lyophilised calcium alginate beads originated from the SF and LF alginate blends, respectively, encapsulating aqueous leaf extract of stevia based on their gelling solution: calcium chloride (2mol l⁻¹) dissolved in stevia extract.

Table 3.

Sample	Class	Subclass	Compound	Molecular Weight	p (Corr)	FC (abs)	Regulation
A-SE/H-PL/LF	Flavonoids	Anthocyanins	Cyanidin 3-O-galactoside	449,3848	0.008018919	28448,15	up
		Flavonols	Isorhamnetin 3-O-glucoside 7-O-rhamnoside	624,5441	0	129196,375	up
		Anthocyanins	Pelargonidin 3-O-sambubioside	565,5001	0	16	up
		Anthocyanins	Peonidin 3-O-rutinoside	609,5526	0	16	up
		Flavonols	Quercetin 3-O-glucosyl-xyloside	596,4909	0	109911,81	up
	Lignans	Lignans	Syringaresinol	418,437	2.85822E-09	66965,03	up
	Other polyphenols	Phenolic terpenes	Epirosmanol	346,4174	1.01741E-13	2139996	down
		Other polyphenols	Pyrogallol	126,11	0.002411872	113517,02	up
		Phenolic terpenes	Rosmanol	346,4174	1.01741E-13	2139993,2	down
	Phenolic acids	Hydroxyphenylacetic acids	3,4-Dihydroxyphenylacetic acid	168,1467	3.65242E-14	25266944	down
		Hydroxycinnamic acids	3,5-Dicaffeoylquinic acid	516,4509	3.65242E-14	10033528	up
		Hydroxycinnamic acids	Sinapic acid	224,21	2.42239E-06	4,0135565	up
		Hydroxybenzoic acids	Vanillic acid	168,1467	3.65242E-14	25266944	down
		Hydroxycinnamic acids	Verbascoside	624,5871	0.000480977	6,557212	up
A-SE/H-PL/SF	Flavonoids	Flavanones	Didymin	594,5612	1.09983E-06	4,3271585	up
		Isoflavonoids	Glycitin	446,4041	0	7,0401845	down
		Flavonols	Kaempferol 3,7,4'-O-triglucoside	772,6581	1.29633E-13	167201,11	up
		Flavonols	Kaempferol 3-O-sophoroside 7-O-glucoside	772,6581	1.29633E-13	167201,11	up
		Anthocyanins	Malvidin 3-O-arabinoside	463,4114	0	16	up
		Anthocyanins	Peonidin	301,2708	0	462,9642	down
		Anthocyanins	Peonidin 3-O-glucoside	463,4114	0	16	up
		Flavonols	Quercetin 3-O-glucosyl-rhamnosyl-galactoside	772,6581	1.30E-13	167201,11	up
		Flavonols	Quercetin-3-O-glucosyl-rhamnosyl-glucoside	772,6581	1.30E-13	167201,11	up
		Flavanones	Poncirin	594,5612	1.10E-06	4,3271585	up

Lignans	Lignans	7-Hydroxysecoisolariciresinol	374,4706	0.002650058	6,2477264	down
Other Polyphenols	Alkylphenols	5-Nonadecylresorcinol	376,6157	0.002653626	23,409887	down
	Alkylphenols	5-Pentacosylresorcinol	460,7751	3.09373E-10	1376592,1	down
	Phenolic terpenes	Carvacrol	150,2176	3.62514E-05	10,866659	down
Phenolic acids	Hydroxycinnamic acids	24-Methylcholestanol ferulate	578,8647	0.000351492	739120	down
	Hydroxybenzoic acids	5-O-Galloylquinic acid	344,272	0	1263885,1	down
	Hydroxycinnamic acids	Caffeic acid 4-O-glucoside	342,298	0	149,65288	down
	Hydroxycinnamic acids	Caffeoyl glucose	342,298	0	149,65288	down

For each compound, unpaired t-test p-values, absolute fold-change (FC) and regulation are provided. According to Mass Profiler Professional B.12.06 output, values of p (Corr) = 0 or FC = 16 denote very high significance at t-test or extremely high fold-change, respectively. A-SE/H-PL/LF and A-SE/H-PL/SF beads: lyophilised calcium alginate beads originated from the SF and LF alginate blends, respectively, encapsulating aqueous leaf extract of stevia based on their gelling solution: aqueous calcium chloride (2mol l⁻¹) solution.

RESULTS ARE EXPRESSED AS mg/kg Phenolic Class Equivalents (cumulative inte

ID	Replicates	Cyanidin Eq.		Luteolin Eq.		Catechin Eq.
4_L20SE1	1	68.49832	75.98153	54.21833	61.84305	183.1361
4_L20SE2	2	59.08051		52.63563		160.9143
4_L20SE3	3	93.71274		74.35053		248.1508
4_L20SE4	4	75.86509		62.50257		208.2635
4_L20SE5	5	82.75098		65.50819		219.3288
4_L20H201	1	51.10686	50.65897	36.30333	34.15631	126.7031
4_L20H202	2	49.26281		36.74534		132.9348
4_L20H203	3	50.13022		32.02271		117.4314
4_L20H204	4	50.53908		33.86777		120.315
4_L20H205	5	52.2559		31.84238		108.287
2_SF120SE1	1	69.75417	73.67661	56.24967	55.28344	185.6406
2_SF120SE2	2	70.92164		52.52306		183.7571
2_SF120SE3	3	91.82804		63.66415		221.1371
2_SF120SE4	4	75.20091		55.14194		195.3896
2_SF120SE5	5	60.67832		48.83838		167.127
3_SF120H201	1	43.91385	44.11167	33.17386	32.98679	115.6695
3_SF120H202	2	40.02292		30.83525		102.2685
3_SF120H203	3	47.22228		34.60401		122.7849
3_SF120H204	4	38.43958		31.23811		104.1249
3_SF120H205	5	50.95973		35.08272		120.5167
1_SE2	2	1021.628		1056.704		4629.598
1_SE3	3	1250.965		1155.982		4382.217
1_SE4	4	1351.171		1061.627		4439.055
1_SE5	5	1212.212		1193.677		4774.648

nsities transformed in cumulative ppm per phenolic subclasses)

λ.	5-Pentadecylresorcino	Tyrosol Eq.			Ferulic acid Eq.		Sesamin Eq.		Matairesin
203.9587	443.7705	605.8822	165.9114	139.9351	158.0268	229.6027	17.36454	13.93622	7.65176
	412.4413		102.827		224.1237		16.89526		7.135316
	825.2206		157.7202		263.2655		10.32656		7.305565
	713.1361		110.7441		251.4363		10.01972		6.34194
	634.8424		162.4728		251.1614		15.07503		4.491162
121.1343	576.3178	502.1449	48.52652	76.27023	198.0113	171.861	11.04778	7.816372	1.929661
	320.6406		106.2792		181.8993		12.70569		4.564279
	601.26		120.4393		169.6438		2.893023		2.924208
	510.3613		59.37798		164.2102		9.184682		1.922951
	502.1449		46.72819		145.5404		3.250683		0.857109
190.6103	436.8939	503.0926	201.3294	136.3457	183.5913	174.8685	10.87994	13.64734	5.643369
	532.4962		108.4944		133.4707		16.86786		10.47332
	626.55		130.8286		177.6857		19.13869		5.934869
	365.2259		153.0387		173.5177		11.5479		5.550848
	554.2969		88.03755		206.0772		9.802319		4.204198
113.0729	319.0331	378.7138	51.40435	98.64534	179.0413	162.6235	6.789444	7.712665	5.967349
	276.4085		120.5542		149.7648		5.910121		1.619706
	402.4154		101.3418		151.8549		10.88812		3.514928
	567.0969		104.163		155.7066		11.66479		2.61161
	328.6151		115.7633		176.75		3.310844		6.141807
	3268.335		1207.161		3362.75		135.0907		19.8236
	3379.721		1262.081		3470.582		104.9262		19.12798
	3542.52		1235.704		3625.942		120.3918		22.17549
	3256.291		1200.248		3932.596		186.4939		22.11919

ol Eq.	Resveratrol Eq.	
6.585149	73.65863	72.36229
	76.94851	
	68.13738	
	93.58896	
	49.47796	
2.439642	29.12172	31.35513
	37.21011	
	29.22592	
	31.49974	
	29.71817	
6.36132	72.96915	63.04452
	58.48165	
	64.85902	
	70.43205	
	48.48074	
3.97108	31.54565	30.17122
	23.4835	
	31.4447	
	29.36954	
	35.01269	
	30.01049	
	32.38057	
	13.95145	
	22.25946	