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Sustainable Preparation of Cardanol-Based Nanocarriers with Embedded Natural Phenolic Compounds

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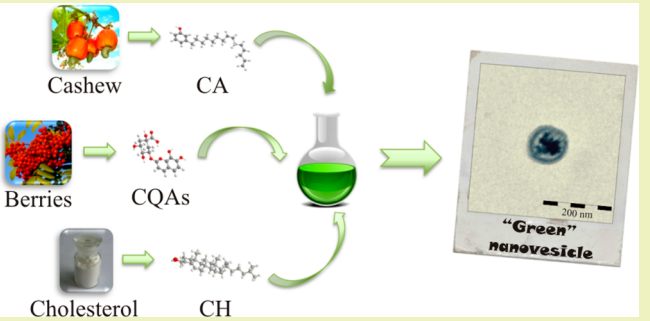
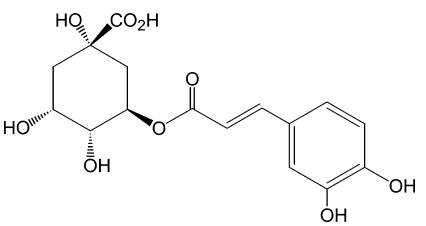
ABSTRACT: In this article, the environmentally friendly preparation of "green nanocarriers" based on the combination of natural renewable materials is described. Cardanol (CA), obtained as the major byproduct of the cashew industry, and cholesterol (CH) have been used to encapsulate chlorogenic acids (CQAs), a class of natural phenolic compounds extracted from two different rowanberries (*Sorbus Americana* and *Vaccinium* sp.). The chlorogenic acid extracts and cardanol-based vesicular nanodispersions have been characterized, respectively, by ultra-high performance liquid chromatography (UHPLC), transmission electron microscopy (TEM), and dynamic light scattering (DLS).



KEYWORDS: *Green nanocarrier, Cardanol, Chlorogenic acid, Cardanol-based vesicles, Natural renewable materials, Natural phenols*

INTRODUCTION

Phenolic compounds are reported to have multiple biological effects including antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory, antiproliferative, and antimicrobial activities. Because of this, numerous plant species have been analyzed for their phenolic content and antioxidant capacity, and among them, berries resulted as the best sources. Chlorogenic acid (CGA, Figure 1) and its derivatives [e.g., 3-O-caffeoylquinic acid (3-CQA) and 5-O-caffeoylquinic acid (5-CQA)] are well known to be among the major compounds in rowanberries and in coffee beverages. Several reports have indicated that a diet rich in CGA-derivative compounds might have a significant action in preventing various diseases associated with oxidative stresses such as cancer, cardiovascular afflictions, aging, neurodegenerative disease,[1] and inflammation reactions.[2,3]



CGA

Figure 1. Molecular structure of chlorogenic acid.

The microencapsulation of CGA into yeast cell-based compounds permitted improvement of their stability, preventing the damage produced from oxidizing agents and light.[4]

Food and medical industries are increasingly interested in natural materials with high contents of bioactive compounds as sources of biologically active non-nutrient compounds. The extraction and purification of these compounds from natural sources are desired because such bioactive substances are often used in the preparation of dietary supplements, nutraceuticals, pharmaceuticals, and cosmetic products. Many analytical procedures have been proposed within the past decade for the extraction of phenolic compounds from berries[5−7] aimed at using the phenolic extract in the preparation of supplements.

Cardanol (CA, Figure 2) represents another class of phenol type compounds obtained as the main fraction from the distillation of cashew nut shell liquid (CNSL)

The presence of a C15-long alkyl chain attached to the meta position of the phenolic ring confers exclusive properties to CA derivatives, such as high solubility in nonpolar environments

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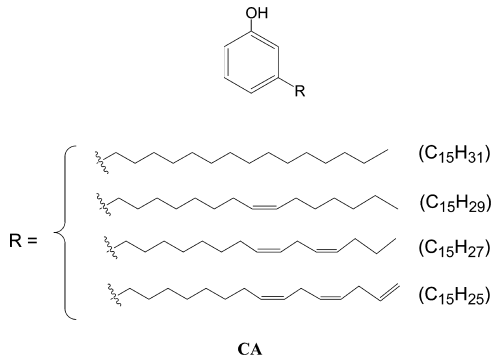

Figure 2. Molecular structure and composition of cardanol.

and good processability. The relatively low cost of CA as well as the wide world availability make this renewable material an important precursor for the preparation of eco-friendly fine chemicals, composites, and hybrid materials,[8,9] and new amphiphilic nanomaterials.[10,11]

Biological activities of CA itself, such as acetylcholinesterase inhibition and antioxidant properties, have also been reported.[12,13]

Recent progresses in chemotherapy treatments have been related to novel findings in the fields of nanodispersion technology using nontoxic surfactants-based delivery systems.[14,15] Formulations of nanoemulsions from CNSL have shown antitumor efficacy with respect to breast cancer.[16] In addition, current studies about the antiproliferative/cytotoxic activity on cancer cell lines of extracts of Thai *Apis mellifera* propolis containing cardanol and cardol as main bioactive components have established potential anticancer bioactivity.[17]

CNSL-deriving components like resorcinolic lipids or CA itself mixed with cholesterol (CH) can form stable vesicular dispersions.[18,19]

In this work, an efficient and environmentally friendly extraction process of phenolic compounds from two different berries (*Sorbus Americana* and *Vaccinium* sp.) have been developed. Such extracts have been used for the preparation of entirely natural CA-based vesicular nanodispersions working as vectors of bioactive compounds. In particular, the antioxidant properties that the CA component confers to the nanocarrier[12] can be useful in order to enhance the stability of such novel delivery systems reducing the undesired side effects related to the oxidative stress typical of the conventional therapies currently used.

EXPERIMENTAL SECTION

Materials and Methods. Cardanol was kindly furnished by Oltremare S.r.l. Cholesterol, KCl, H_3BO_4, and NaOH were purchased from Sigma-Aldrich (Steinheim, Germany) and used as received. Chlorogenic acid (commercial standard, 95%) was purchased from Sigma-Aldrich (Steinheim, Germany). To prepare the stock standard solution of CGA (with concentration of 1000 μg/mL), the appropriate amount of the solid reagent was weighed and dissolved in 10 mL of Milli-Q water. This solution was stored at 4 °C in the dark and passed through a 0.22 μm nylon filter before injection into the high performance liquid chromatography (HPLC) and ultra-high performance liquid chromatography (UHPLC) system.

The berries *S. Americana* and *Vaccinium* sp. were hand harvested in North America and Spain, respectively. HPLC-grade acetonitrile, glacial acetic acid, and methanol were supplied by Panreac. The ultrapure water was obtained from an in-house Milli-Q Type I purification system (18.2 MΩ cm and TOC < 10 ppb).

Phenolic Compounds Extraction Procedure. Several procedures and different solvents (ethanol, methanol, methanol/water mixture, and water) have been previously used for extracting chlorogenic acids.[3,20,21] However, it can be emphasized that the use of water as an extracting solvent is more clean, economical, and safe than other organic solvents. For this reason, in this work, water has been chosen as the eco-friendly extracting solvent. Thus, the lyophilized plant material (500 mg) was placed into a polyethylene centrifuge tube (50 mL, conical bottom) and extracted with 500 μL of water. After vigorous shaking for 2 min using a vortex (Heidolph relax top, JP Selecta, Barcelona), the sample was centrifuged (Thermo Electron Corp., ALC Multipeed Centrifuge PK121) at 8000 rpm for 10 min at room temperature. Aqueous phase was collected.

For analyzing it in UHPLC-ESI-MS/MS, aqueous phase was filtered through a 0.22 μm pore size and 13 mm diameter nylon filter.

UHPLC-ESI-MS/MS Analysis. Chromatographic analyses were performed on an Agilent 1200 series UHPLC system (Agilent, U.S.A.) with a quaternary pump system, vacuum degasser, cooling autosampler, and thermostated column compartment.

Separation was carried out using a Zorbax SB-C18 (2.1 mm × 50 mm, 1.8 μm) analytical column (Agilent). Optimum separation was achieved with a binary mobile phase gradient at a flow rate of 0.4 mL/min. The column temperature was kept at 30 °C, and the injection volume was 10 μL. Solvents were (A) a mixture of water/acetic acid (0.2% v/v) pH 3.10 and (B) pure acetonitrile. The gradient elution program was as follows: 0−3 min, 5% B; 3−15 min, 5−40% B; 15−15.5 min, 40−100% B; finally returning to the initial conditions in 5 min.

Identification and quantification of phenolic compounds were obtained using a 6410 Triple Quad LC/MS system equipped with an electrospray ionization source (ESI) operating in negative mode and controlled by MassHunter Workstation Software (Agilent, U.S.A.). Source working conditions were capillary voltage 4000 V, gas flow rate 10 L/min, gas temperature 300 °C, and nebulizer pressure 35 psi.

Quantification of 3-CQA and 5-CQA was performed using commercial standards. A calibration curve in the range from 0.1 to 100 mg/L was developed.

Preparation and Characterization of Cardanol-Based Vesicles. Lyophilized phenol extract was mixed with CA, CH, and glass beads (5 g, diameter = 4 mm) by mechanical stirring at 90 °C for 1 h to form a lipid film on the flask's wall. The resulting film was hydrated with 20 mL of a pH 9.0 borate buffer preheated at 50 °C under mechanical stirring (700 rpm) and finally heated at 90 °C for 1 h. The accumulation of a residue of lipid onto the mechanical stirrer indicated that not all the components (CA, CH, and phenol extracts) had been hydrated to form the vesicle dispersion.

The as-obtained vesicle dispersion was submitted to a sonication step (45 min at 70 °C) and then centrifuged (7000 rpm for 30 min), thus collecting the supernatant as the sample. The sediment residue was maintained at 60 °C until constant weight. The difference in weight provides the yield of the supernatant sample.

A sample without phenols has been prepared with the same procedure as the blank reference.

The phenol-entrapped vesicles were separated from the non-entrapped (free) phenols by exhaustive dialysis. The supernatant (5 mL) dispersion was transferred into a dialysis tubing cellulose membrane (12 K_d), which had been treated according to the Fenton method before use,[22] and then sealed at both ends with clips and dialyzed against 150 mL of ultrapure water for 24 h. Monitoring of the free phenols was carried out through optical absorbance measurements of the dialysate until a constant concentration of phenols was reached.

The morphology of the vesicular nanodispersion was examined using transmission electron microscopy (TEM). Low-magnification TEM analyses were performed on a Jeol JEM-1011 electron microscope operating at 100 kV, equipped with a CCD camera ORIUS 831 from Gatan. TEM samples were prepared by initially mixing dilute vesicle dispersions with a few microliters of osmium tetroxide aqueous solution (1% w/v) and then drop-casting them onto carbon-coated copper grids. Hence, each grid is twice rinsed in pure

water, and afterward, the deposited samples are completely dried at 60 °C for one night before examination.

Measurement of dynamic light scattering and electrophoretic light scattering were both carried out on a Malvern Zetasizer Nano ZS90 on diluted samples to establish the size and zeta potential of vesicles. The hydrodynamic diameter (d) of vesicle dispersion has been determined at 25 °C measuring the autocorrelation function at a 90° scattering angle. Cells have been filled with 400 μL of sample solution and diluted to 4 mL with ultra pure water. Each d value is the average of five separate measurements. Values of zeta−potential (ZP) of vesicle dispersions have been determined at 25 °C filling the Zeta meter cell, equipped with gold-coated electrodes, with 400 μL of sample solution and diluted to 4 mL with ultra pure water. The voltage ramps were performed according to the indications given by the purveyor.

The presence of phenols encapsulated into a vesicle dispersion has been evidenced by UV−vis measurements using a Jasco V-660 spectrophotometer. The measurements were carried out by analysis of the free molecular components after lysis of vesicles obtained by dissolving 1 mL of colloidal solution in 5 mL of methanol and then allowing the solvents to evaporate at 70 °C under vacuum and finally dissolving the residue in 2 mL of methanol.

Efficiency of phenol encapsulation was determined using the dialysis for separating the free phenols from those engaged in vesicles. The encapsulation efficiency, E(%), was expressed as the percentage of the phenols trapped in dialyzed vesicular formulations toward the not-dialyzed ones (eq 1) and determined by dissolving 1 mL of dialyzed and 1 mL of not-dialyzed vesicle suspension in 5 mL of methanol, which was dried and finally dissolved in 1 mL of ultra pure water. After filtration through a 0.22 nylon filter, the clear solutions were analyzed by UHPLC-ESI-MS/MS to quantify the phenol conten,t and the results were used to calculate E(%). The equation used follows

$$\mathrm{E}(\%) = \mathrm{D}/\mathrm{ND} \times 100 \quad (1)$$

where ND and D are the phenol concentrations before and after the dialysis, respectively.

RESULTS AND DISCUSSION

Identification and Quantification of Phenolic Compounds. Several detection systems have been explored for characterizing phenolic compounds in complex matrices via HPLC. However, the new generation of UHPLC coupled to ESI and tandem MS (MS/MS)[6] has provided significant improvements in the method sensibility (identifying phenolic compounds at low-concentration levels) and a reduction of the analysis time (this takes between 1 and 15 min per sample). This technology allows for elucidating the structure of the ionized sample molecules, ensuring correct identification and quantification of these. Furthermore, the rate of separation in the UHPLC system results three times faster than via conventional HPLC as well as being associated with a 80% less consumption of organic solvents.

Detection in UHPLC-ESI-MS/MS was performed in multiple reaction monitoring (MRM) mode (Agilent 6400 Series triple quad instruments). The two most sensitive transitions were analytically used to identify the different compounds. Additionally, the most sensitive transition was needed for quantification purposes, whereas the second one is employed for confirmation purposes. The MRM transitions and the corresponding acquisition parameters (cone voltage and collision energy) were optimized according to the results obtained from infusing approximately 100 μg/mL of CGA into the mobile phase. The excellent stability of the retention times increased the sensibility of the analysis. Table 1 shows the retention times and MS/MS parameters of the phenolic compounds studied.

Table 1. Retention Times and MS/MS Parameters of Phenolic Compounds

phenolic compounds		3-CQA	5-CQA
precursor ion (m/z)		353.1	353.1
MRM 1 (quantification)	product ion	191.2	191.2
	CE[a] (V)	12	12
MRM 2 (confirmation)	product ion	85.2	85.2
	CE (V)	40	40
fragmentor (V)		200	200
retention time (min)		1.9	4.3

[a]CE: collision energy.

The qualitative and quantitative analyses of these compounds were achieved by comparing their retention times and MS/MS fragmentation patterns to commercial standards. Using this methodology, phenolic compound concentrations (mg/L) obtained in berry extracts are exhibited in Table 2. This shows the presence of phenolic compounds of both isomers 3-CQA and 5-CQA in *S. Americana* extract, whereas in *Vaccinium* sp. extract, only the 5-CQA isomer was detected.

Table 2. Phenolic Compound Concentrations (mg/L) in Berry Extracts

	phenolic compounds (mg/L)	
berry extracts	3-CQA	5-CQA
S. Americana	21.63	80.51
Vaccinium sp.	n.d.[a]	63.16

[a]n.d.: not detected.

Cardanol-Based Vesicles Loaded with Phenolic Extracts. Unlike the standard thin film hydration method[23,24] that primarily involves volatile organic solvents to dissolve lipid components, we have employed a more sustainable organic solvent-free method for producing a completely natural lipid-based system. In our case, CA itself acts as the main component of the system, which when combined with opportune amounts of CH is able to produce stable vesicles dispersions under alkaline conditions.

In a typical preparation, a solution of CA, CH, and phenols was mechanically stirred at 90 °C in a round-bottomed flask until a homogeneous lipid film is formed. The successive hydration step with warm buffer solution allows the formation of vesicles whose size distribution is improved by sonication of the suspension. Centrifugation procedure favors the separation of smallest vesicles in supernatant slightly colored in green and red for *S. Americana* and *Vaccinium* sp. samples, respectively. The sample prepared by using only CA and CH at the same molar ratio was considered as a blank sample. The composition data of tested samples is reported in Table 3.

Transmission electron micrographs confirm the formation of vesicular-shaped structures (Figure 3). The structures of the blank sample were found to show irregular shapes in contrast to those loaded with phenols that tended to display spherical and regular architectures.

Hydrodynamic particle diameters (d) and zeta potentials (ZP) were determined by dynamic light scattering measurements (Table 4).

The vesicles loaded with phenols showed mean size as large as ∼300 nm and a monodisperse size distribution as confirmed by polydispersity index (PDI) values and by corresponding

Table 3. Composition and Yield of Supernatant % of Vesicular Nanodispersions Prepared

	blank	CA−CH−*S. Americana*	CA−CH−*Vaccinium* sp.
buffer pH 9 (ml)	20	20	20
CA (mg)	50	50	50
CH (mg)	43	43	43
S. Americana (mg)	–	176	–
Vaccinium sp. (mg)	–	–	190
molar ratio CA−CH−phenols	1/0.6/0	1/0.6/3	1/0.6/3.2
yield supernatant %	73.9	38.2	31.4

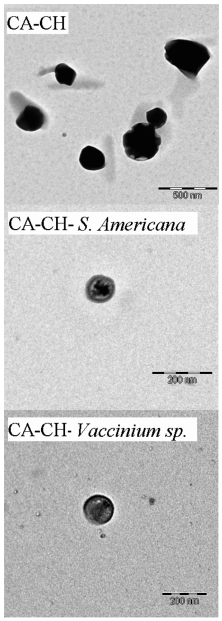

Figure 3. Transmission electron micrographs, respectively, of CA−CH, CA−CH−*S. Americana* and CA−CH−*Vaccinium* sp. dialyzed vesicular-shaped structures.

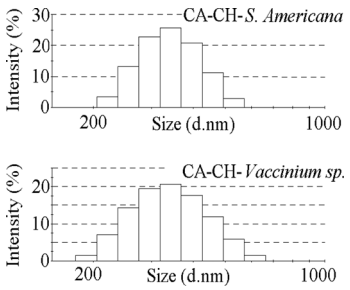

Figure 4. Size distribution of *S. Americana* and *Vaccinium* sp. dialyzed vesicular nanodispersions.

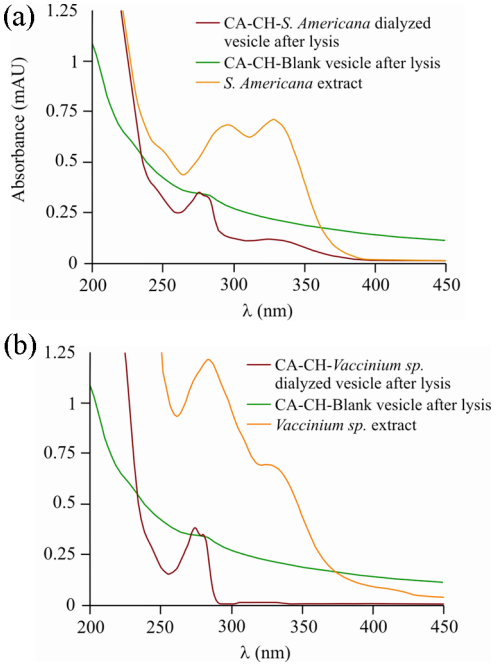

Figure 5. Comparison of UV−vis absorption for (a) CA−CH−*S. Americana* and (b) CA−CH−*Vaccinium* sp. lysed vesicles (brown lines) with CA−CH blank sample (green lines) and berries extract samples (orange lines) in methanol.

f4 histograms (Figure 4). Moreover, the negative values of ZPs are large to ensure a significant stability to all vesicles dispersions.

The encapsulation of chlorogenic acids in CA-based vesicles gave as a result an increase in the hydrodynamic diameter (d) with respect to the d value of the blank sample. Therefore, we may reasonably assume that the phenols are not simply entrapped in the vesicles but that they also interact to a certain extent with the bilayer structure of the vesicles. This suggestion is in accord with the evidence that encapsulated molecules can have an influence on the mean diameter as well as the size distribution of a vesicular system due to a chemical interaction with the bilayer structure and positioning within it.[25,26]

f5 The UV−vis spectra reported in Figure 5 confirm the presence of chlorogenic acids in the vesicular structures. This shows that the blank sample CA−CH does not exhibit any characteristic absorption throughout the investigated spectral region. On the other hand, the spectra of the phenol-loaded vesicles, after their lysis and solubilization in methanol, display a similar absorption to the berries methanolic extract.

The ability of the vesicles to entrap the phenols was quantified also by UHPLC-ESI-MS/MS analysis and expressed as a percent of E(%). In Table 5, a comparison of phenol t5

Table 4. Hydrodynamic Diameter, Zeta Potential, and Polydispersity Index of Phenol-Loaded and Blank CA-Based Vesicles

	CA−CH (blank)	CA−CH−*S. Americana*	CA−CH−*Vaccinium* sp.
d (nm)	253 ± 62	377 ± 71	322 ± 90
PDI	0.21	0.26	0.11
ZP (mV)	−70.7 ± 5.7	−61.2 ± 7.8	−58.1 ± 6.0

Table 5. Phenolic Compound Concentrations (mg/L) in Lysed Vesicles Measured before and after Dialysis Process and E(%) of CA-based vesicles

samples		phenolic compounds (mg/L) 3-CQA	5-CQA
CA−CH−*S. Americana*	ND vesicles	19.96	25.97
	D vesicles	1.77	2.53
	E(%)	8.88%	7.42%
CA−CH−*Vaccinium* sp.	ND vesicles	23.61	25.19
	D vesicles	0.32	0.79
	E(%)	1.37%	3.14%

concentrations in the nanodispersions before and after the dialysis process and the E(%) of CA-based vesicles loaded with phenolic compounds were reported.

Table 5 shows that in both experiments by either using *S. Americana* or *Vaccinium* sp. extracts phenolic compounds are trapped in the vesicles, and the transformation of a part of 5-CQA into 3-CQA in CA−CH−*Vaccinium* sp. vesicle nanodispersion occurs. Furthermore, part of the chlorogenic acid was removed as free phenols dissolved in water after the dialysis step.

The maximum value of E(%) obtained with these formulations is 16.30% for *S. Americana* loaded vesicles, calculated as the sum of the two phenolic compounds 3-CQA and 5-CQA. Hence, it is evident that the preparation methodology must be improved.

MRM chromatograms corresponding to the quantified phenolic compounds in *S. Americana* extract, *Vaccinium* sp. extract, and phenols entrapped by vesicles are illustrated in Figure 6.

The mass spectrum shows the product ions of 5-CQA and 3-CQA obtained using MRM mode. They were exactly the same. Because of this, both were identified and quantified based on retention time.

As previously mentioned, when *Vaccinium* sp. was trapped in the vesicle part, 5-CQA was transformed to 3-CQA (Table 5 and Figure 6). Studies on the influence of pH on 5-CQA thermal stability[27] have shown that CGA can undergo transformations, such as isomerization in 3-CQA, during the heating of its water solution at different pH.

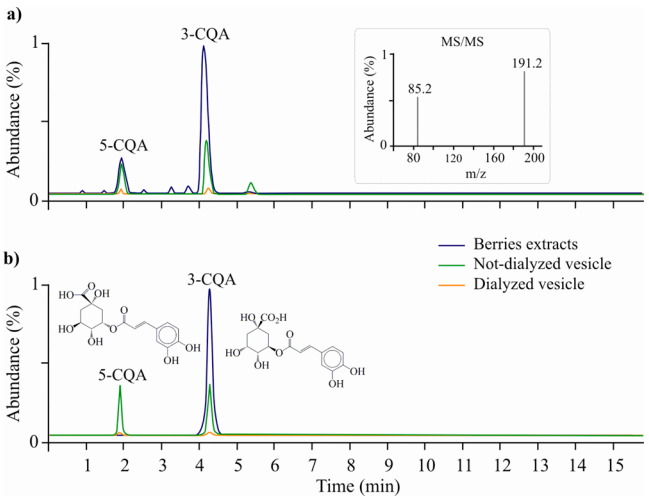

Figure 6. Total ion chromatograms (TIC) in MRM acquisition from the UHPLC-ESI-MS/MS analysis of phenolic compounds in berry extracts, not-dialyzed vesicles, and dialyzed vesicles using *S. Americana* (a) and *Vaccinium* sp. (b). Inset in (a) corresponds to mass spectra in the MRM mode of 5-CQA and 3-CQA.

CONCLUSIONS

The results presented herein demonstrate that for the first time novel "green nanocarriers" can be obtained from natural renewable materials encapsulating chlorogenic acids into cardanol-based vesicular nanodispersions in an aqueous environment.

In particular, two chlorogenic acid derivatives, 3-O-caffeoylquinic acid and 5-caffeoylquinic acid extracted in water from *S. Americana* and *Vaccinium* sp., were identified and quantified by UHPLC-ESI-MS/MS.

Cardanol-based vesicle-shaped nanodispersions were prepared starting from CA-CH without or with the chlorogenic acids extracts. Stable, spherical, and regular vesicles were obtained with mean diameters in the range of 300−380 nm.

Efficiency of phenol-encapsulation, E(%), studied by UHPLC-ESI-MS/MS-MRM analysis have confirmed loaded vesicles of phenolic compounds.

These findings are helpful to promote new pioneering researches such as the release *in vitro* of chlorogenic acid as well as the interaction of the constituents of the green vesicles with selected biomaterials.

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Notes

The authors declare no competing financial interest.

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