Strategy of experimental design for intensification of solvent extraction of natural antioxidant flavonoids and phenols from buckthorn textured leaves

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Abstract: Prior to solvent extraction of plant-based active molecules, adequate texturing by Détente Instantanée Contrôlée (DIC; French for “Instant controlled pressure drop”) results in overcoming the slow diffusion of the solvent/solute through the solid matrix. This work aimed at determining the impact of DIC pretreatments on buckthorn (*Rhamnus alaternus* L.) morphology. DIC-operating parameters were selected as the saturated steam pressure, the thermal treatment time, and the number of cycles. A three-parameter five-level response surface method was used to optimize DIC processing parameters. Response factors were the overall and individual yields of flavonol aglycone extraction, and antioxidant activity of both expanded dried material (swell dried leaves) and extracts. The yield of flavonol aglycones was 18.23 mg Kaemp eq/g dry basis (mg Kaemp eq/g db) in 3 min for DIC-treated buckthorn, against 12.24 mg Kaemp eq/g db in 150 min for untreated natural buckthorn raw material. Furthermore, the antioxidant activity of DIC-treated material was exceptionally higher and the effectiveness of reducing power of DPPH radical was 68 times more than the untreated plant material.

ABOUT THE AUTHOR

Karim Allaf, responsible of research unit of “Intensification of Industrial Eco-Processes”, at LaSIE-UMR-CNRS 7356 (University of La Rochelle; France), was graduated in Master in Physics in 1973 from Lebanese University; PhDs in Physics of Plasmas in 1976 from University of Paris-XI, and in Thermodynamics and Chemical Engineering in 1981 from Polytechnic Institute of Toulouse. Since 1988, Allaf has developed specific activities on the technologies of Instantaneous Thermodynamics and intermittent unit operations, and defined innovative processes of drying (swell-drying, three-stage expanded granule spray-drying, intermittent Multi Flash and Air-Flow MFD-IAF drying Drying), extraction (essential oils, antioxidant extraction, vegetal oil extraction ...), decontamination, etc.

Allaf has got 12 Patents and 30 extensions, 310 international papers, books, and international conferences, and was the mentor of 38 PhD and HDR works; the coordinator of 8 European projects, and participated in activities of transferring technologies toward the industry.

PUBLIC INTEREST STATEMENT

DIC texturing was optimized to greatly increase the availability of flavonol.

DIC treatment was optimized to increase extraction of flavonoid yields and diffusivity.

DIC swell-drying of buckthorn leaves was optimized to get the highest antioxidant activity.

Swell-dried buckthorn leaves can be used as natural sustainable antioxidant ingredients.

The operation allows using buckthorn leaves as sustainable source of natural antioxidants.

Economical impact of the operation encourages to scale-up this well-controlled process.
1. Introduction

Very often, different classes of active molecules are the means the plant uses as adaptation and defense against its environment. Such molecules can be used as potential sources for new drugs or precursors for the synthesis of certain complex molecules. The buckthorn (*Rhamnus alaternus* L.) belongs to the family of Rhamnaceae, which contains over 900 species (Punt, Marks, & Hoen, 2003). This is a woody, shrubby, usually small shrub although it can reach 5–6 m high. It is present in the rocky and calcareous Mediterranean area regions. This shrub requires little in terms of the nature of soil and tolerates drought and harsh winters.

In Algeria, it grows wild throughout the mound, often accompanied by Holm and mastic. Buckthorn yellow or greenish flower is followed by 3–6-mm spherical berry. These berries are first green in color, then red, and turn black when they mature. Commonly called “m’lilesse” or “oud el Kheir”, buckthorn has a great reputation in Algerian folk medicine. It is mainly used against jaundice and viral hepatitis, hypertension, and even as a diuretic and depurative (Abou-Chaar, Kabbara, & Shamlian, 1982; Coşkun, 1992). In recent years, it has received increasing attention, and numerous publications have focused on its flavonoid antioxidant characters and pharmacological properties (Ammar et al., 2009). Indeed, quercetin and kaempferol derivatives are widely present in the family Rhamnaceae. In addition, numerous investigations have shown that buckthorn leaves and bark contained glycosides and aglycones flavonoids, anthracene, and tannins (Abou-Chaar et al., 1982; Ammar et al., 2009; Faugeras & Paris, 1962; Touati & Fkih-Tetouani, 1995; Wei, Lu, Tsao, Wang, & Lin, 2001). The very low presence of essential oils (EO) not exceeding 0.02% db (dry basis) (Berka, Hassani, Allaf, & Chemat, 2008) would allow its direct use without needing any deodorization stage.

Recently, the development of flavonoids has gained new impetus by highlighting their many therapeutic activities. Some flavonoids may prevent atherosclerosis and thereby reduce the risk of cardiovascular disease (Hertog, Feskens, Kromhout, Hertog, & Hollman, 1993). Some studies even reported their power to reduce the intracellular replication of herpes simplex virus and human immunodeficiency virus (Asres, Seyoum, Veeresham, Bucar, & Gibbons, 2005; Goncalves et al., 2001). Studies have shown that they can have a higher antioxidant potential than Butylated hydroxytoluene (BHT) and vitamins C and E (Sokół-Łętowska, Oszmiański, & Wojdyło, 2007; Suhaj, 2006).

In food industry, great interest is granted to prevent fat oxidation by atmospheric oxygen. This inevitably leads to food spoilage because of alteration of taste, smell, color, and storage stability. To avoid this degradation, manufacturers have added antioxidants in formulations, privileging the use of synthetic antioxidants such as BHT and butylated hydroxyanisole. However, recently, consumers have manifested reluctance toward these synthetic additives because of their toxicity, instability, and volatility. They are suspected of being carcinogenic and causing many diseases (Valentão et al., 2002). This has implied a growing interest in replacing them by probably free of toxicity natural antioxidant molecules, which usually have consumer confidence. Such natural ingredients can also act as flavoring and antimicrobial (Burt, 2004).

Flavonoids are the most biologically active compounds among polyphenols. They have a common biosynthetic origin with the same basic structure \( (C_6-C_3-C_6) \). This 15-carbon-atom backbone consists of two \( C_6 \) aromatic rings linked by an intermediate \( C_3 \) chain, corresponding to the 2-phenyl-benzopyrone structure or the 2-phenyl-chromone structure. Flavonoids represent thousands of molecules divided into several classes (Dinelli et al., 2006; Rocha-Guzmán et al., 2007).
In nature, flavonoids are found almost exclusively in the form of glycosides. Their kinetics depends not only on temperature, extractant solvent properties (polarity, affinity with molecules to be extracted ...), and processing conditions (agitation, recuperation of extracts ...), but also on the material morphology. Because of the natural structure of plant materials, traditional extraction methods require large amounts of solvent, and usually are time and energy consuming (Li, Chen, & Yao, 2005).

The emerging extraction methods try to ensure rapid kinetics, better selectivity, and greater efficiency (Wang & Weller, 2006). From kinetic point of view, since the limiting factor is the diffusion of the solute within the plant, one can assume that the best for intensifying would be to insert an adequate expansion pretreatment. It would improve the raw material (RM) porosity and has to reduce its own resistance against the specific mass transfer. Lebovka et al. (2011) used fundamental approaches, modeling, and experimental studies to prove all aspects of this hypothesis (Table 1).

The use of Instant controlled pressure drop Détente Instantanée Contrôlée texturing (DIC, in French) offers major advantages of direct extraction of the volatile molecules (essential oils ...), and improvement of the extraction of non-volatile molecules (Ben Amor & Allaf, 2009). DIC pretreatment is not only causing an acceleration of both kinetics and yields of solvent extraction but it also implies better availability, higher performance, greater control of the finished product quality and functional properties compared with raw untreated material (Ben Amor & Allaf, 2009).

### Table 1. Main fundamental equations for solvent extraction (Allaf, 1982; Allaf & Allaf, 2014; Allaf et al., 2012; Crank, 1975; Lebovka et al., 2011).

| Equation                                                                                   |
|--------------------------------------------------------------------------------------------|
| First-Fick’s law—Allaf’s formulation                                                     |
| \[
\frac{\partial (\rho_s \mathbf{v}_s)}{\partial t} = -D_{eff} \nabla \frac{\rho_s}{\rho_d} \frac{\partial \rho_s}{\partial t} \] (1) |
| Absence of expansion or shrinkage (\( \mathbf{v}_d = 0 \) and \( \rho_d = \text{constant} \)) |
| \[
\rho_s \mathbf{v}_s = -D_{eff} \nabla \rho_s \] (2)                                    |
| Crank’s solution Infinite plate                                                           |
| \[
\frac{x - x_0}{x_\infty - x_0} = \sum_{i=1}^{m} \frac{\delta}{\rho \pi d_i^2} \exp\left(\frac{-i^2 \delta^2 \Delta \rho_{eff}}{2 \rho \pi d_i^2} \right) \left(\frac{r^2 \Delta \rho_{eff}}{\delta^2} \right) \] (3) |
| Crank’s Sphere solution (\( d_i = \text{radius} \))                                        |
| \[
\frac{x - x_0}{x_\infty - x_0} = \sum_{i=1}^{m} \frac{\delta}{\rho \pi d_i^2} \exp\left(\frac{-i^2 \delta^2 \Delta \rho_{eff}}{2 \rho \pi d_i^2} \right) \left(\frac{r^2 \Delta \rho_{eff}}{\delta^2} \right) \] (4) |
| Crank calculation to determine \( D_{eff} \): (1st term approach; \( A = 1 \))               |
| \[
\frac{x - x_0}{x_\infty - x_0} = A \exp\left(-k(t - t_b)\right) \] (5)                   |
| 1st Term approach                                                                           |
| \[
\ln\left(\frac{x - x_0}{x_\infty - x_0}\right) = -k(t - t_b) \] (6)                      |
| Infinite plate                                                                              |
| \[
D_{eff} = k \frac{\delta^2}{\pi} \] (7)                                                  |
| Sphere (\textbf{Deff}: Effective diffusivity)                                                 |
| \[
D_{eff} = k \frac{\delta^2}{\pi} \] (8)                                                  |
| Starting accessibility (the amount of solute directly extracted by superficial interaction) is calculated by extrapolating diffusion model to \( t = 0 \) |
| \[
X_0 - X_t = X_t = 4X_0 \] (9)                                                            |
2. Materials and methods

2.1. Plant material
The leaves of *R. alaternus* L. were collected in April 2010, in a mountainous region in the hills of Tablat at 980-m altitude and 60 km from Algiers. This site was chosen because of the wide availability of this plant, due to favorable environmental conditions and climate, adequate rainfall, and its ease of access. The plant was identified by the manager of the herbarium of the National Institute of Agronomy of Algiers (Algeria).

2.2. Chemicals
2,2-Diphenyl-1-picrylhydrazyl free radical (97%), vitamins C (≥99.0%) and E (≥99.5%), and other flavonoid standards; (+)-catechin (≥99.0%), quercetin (≥98%), kaempferol (≥99%), rhamnetin (≥99%), and isorhamnetin (≥99%) analytical grade were provided from Fluka Chemicals and Sigma-Aldrich (St. Quentin Fallavier, France). Rhamnocitrin (grade analytical standard) was supplied from Apin Chemicals Limited (UK). BHT (≥99%), sodium sulfate anhydrous (≥99.0%), Gallic acid (≥99%), Folin and Ciocalteu’s phenol reagent (2 N), sodium carbonate (≥99.5%), aluminum chloride hexahydrate (≥99.%), sodium nitrite (≥99%), sodium hydroxide (≥97%), potassium chloride (≥99%), hydrochloric acid (37%), trifluoroacetic acid (≥99%, HPLC), and sodium acetate trihydrate (≥99%) were ordered from Fluka and Sigma-Aldrich (St. Quentin Fallavier, France). All solutions were prepared with analytical grade methanol from Carlo Erba (Val de Reuil, France).

2.3. Measurement of the moisture content
Three grams of samples of buckthorn leaves coarsely chopped were arranged in small cups with a thin layer and left in an oven at 105°C for 24 h until a constant weight is obtained. The moisture content was expressed as %db (dry basis).

2.4. Drying
Buckthorn leaves of 87.69% db as initial moisture dry basis were sorted, separated, and hot air-dried. The drying temperature was set at 29 ± 1°C, airflow velocity was about 1.2 m/s, and its relative humidity was HR = 6%. Samples devoted to DIC treatment were dried toward a final moisture content of 16.28% db.

2.5. DIC treatment

2.5.1. DIC reactor
The laboratory scale DIC unit used in this work was described in various studies (Besombes, Berka-Zougali, & Allaf, 2010). It consists mainly of three parts: (1) the treatment vessel, which can operate up to 1 MPa and 200°C; (2) a double-jacket vacuum tank, usually cooled by a cold water stream to condensate vapor; (3) an instant opening valve, which interconnects the treatment vessel and vacuum tank.

2.5.2. DIC processing cycle
A DIC cycle is a two-successive step treatment, which takes place in the processing vessel closed and perfectly sealed after having placed the material in an adequate tray.

It is a thermal treatment step, using high-pressure saturated steam for a short time (~5 to 30 s); followed by a second stage operated once the homogeneity of both temperature and water content inside the product is reached. The instant opening (<200 ms) of the main valve results in an abrupt pressure drop toward a vacuum (5 kPa), which implies a pressure-drop rate of ΔP/Δt > 0.5 MPa/s (Figure 1).

This swift drop in pressure results in an autovaporization of volatile compounds and a rushed cooling of the residual solid, thus stopping any thermal degradation. Finally, vacuum pressure is kept...
constant during this step by maintaining the expansion valve open between the treatment vessel and the vacuum tank.

2.6. DIC assisted extraction processing

Most often DIC processing implies to carry out several DIC cycles, possibly at the same steam pressure level and the same periodicity. The protocol of the whole DIC-assisted extraction process is shown in Figure 2.

Once DIC processing was complete, and after reinstating the atmospheric pressure, the DIC-treated buckthorn leaves were collected and placed in sterile bags before undergoing solvent extraction. Moisture content was measured at various stages of the operation (initial raw-material stage, just before and just after DIC treatment, after final drying stage). Microstructure was observed using an environmental-type Scanning Electronic Microscope (JEOL 5410LV FEI Quanta 200F, Philips; Croissy-sur-Seine; France) to define the state of the structure, for raw-material and for DIC treatment samples.
2.7. Experimental strategy and design

Regarding the DIC processing, several operating parameters may contribute to design experimental trials. Special attention was accorded to the sample water content, which can define the glass transition temperature. In our case, the three independent variables that seemed to have a significant influence in the treatment were the saturated steam pressure, the heat processing time, and the number of cycles. An experimental three-factor/five-level design was used. It implied 22 random trials: $2^3 = 8$ factorial points, $2 \times 3 = 6$ star points, and 8 repetitions of the center point (Table 2).

In the case we considered, the main response factor was the yields of extracted flavonoids. The solvent extraction kinetics was modeled and its specific parameters (effective diffusivity, starting accessibility, and extraction time) were also used as relevant responses possibly capable to identify DIC expansion impacts.

The value of $\alpha$ (axial distance) is statistically defined as $\alpha = (2^n)^{1/4}$ depending on the number of independent variables ($n$). For $n = 3$, $\alpha = 1.681793$.

2.8. Extraction flavonoids

Solvent extraction focused on DIC-treated samples and untreated RM. Four grams of ground-dried leaves (3 mm) were introduced into a 200-ml aqueous–alcoholic solution acidified with 1.6 M HCl in a 250-ml balloon heated to reflux in a water bath. Preliminary experiments and bibliography studies allowed optimizing certain parameters such as the water/methanol ratio (80% MeOH–H$_2$O, v/v), the acidity of the medium (HCl at 1.6 M), and temperature (85 ± 1°C). The cooling water was maintained at 5°C throughout the extraction.

| Level | $\alpha$ | $-1$ | $0$ | $1$ | $\alpha$ |
|-------|--------|------|-----|-----|--------|
| $\chi_1 = P$: Saturated steam pressure (MPa) | 0.10  | 0.20 | 0.35 | 0.50 | 0.60   |
| $\chi_2$: Number of cycles | Calculated | 2.3  | 3   | 4   | 5      | 5.7    |
|          | Realized   | 2    | 3   | 4   | 5      | 6      |
| $\chi_3 = t$: Total heating treatment time (s) | 19    | 60   | 120  | 180  | 221    |
2.8.1. Extraction kinetics
Monitoring the kinetics of extraction was carried out by regular sampling of about 3 ml of extract from the first minute of extraction up to 240 min. These samples were filtered (0.45 μm PTFE membrane, Sartorius, (Germany)) before removing the methanol with nitrogen.

2.8.2. Final solutions
After extraction and cooling, solution methanol was evaporated under vacuum at 40°C. Aqueous samples are then filtered (0.45 mm PTFE filter) before being SPE purified.

2.8.3. SPE purification of extracts
Purification of the extract samples was carried out by SPE columns type (ODS-5 Octadecyl; 18%; EC 500 mg/6 ml, Whatman Inc. (USA)), which are a hydrophobic support of fused silica C18 capable to remove sugars and impurities. This procedure was done in four steps:

(1) The first step was a conditioning of the extraction cartridge adsorbent with pure methanol then with purified water to solvate functional groups present on the surface.

(2) The second step was a percolation of 0.5 ml of extract on the support; interferents, which have no affinity with the solid phase, were not retained by it.

(3) The next step was the washing, which was carried out to eliminate the compounds weakly retained by the support. The solvent should have low strength eluent to elute the interfering while maintaining and preserving the target compounds and extracts. Washing liquids were the purified water and then an aqueous methanol (80% methanol–water v/v).

(4) The final step was to extract the elution of impurity-free extract by slowly percolating 3 ml of pure methanol to break the interactions involved between the extract compounds and the solid support.

The extracts obtained were placed in amber bottles and stored in the freezer before being analyzed.

2.9. Dosage and analysis of buckthorn phenolic and flavonoid extracts

2.9.1. Determination of buckthorn total phenols
The determination of total phenolic extracts obtained from DIC-treated and-untreated buckthorn leaves was performed using the reagent molybdate-heteropolyporphotungstates commonly called Folin–Ciocalteu’s phenol reagent (FC) (Singleton & Rossi, 1965). It consists of a mixture of phosphotungstic acid and phosphomolybdic acid. In alkaline medium, this reagent is reduced by phenols in a mixture of blue oxides of tungsten and molybdenum.

This coloration, with maximal absorption at 760 nm, is proportional to the concentration of phenol in the extract. The mass concentration of the constituents was optimized to obtain an almost linear response between the reactive and the total phenols (TP) (absorbance between 0.5 and 1). All spectrophotometric analyzes were performed with a UV–vis spectrophotometer model Helios Omega brand Thermo Fisher Scientific (Saint Herblain; France).

To 0.5 ml of four-time diluted extract, we added 2.5 ml FC (10-time diluted). After a 2-min waiting, we added 2 ml of Na2CO3 at 20%. The whole was vigorously stirred with a vortex and left at room temperature for 90 min. In alkali medium, extracts treated with Folin–Ciocalteu’s phenol reagent acquire a blue color whose intensity depends on the phenol concentration. After this incubation time, quartz vials were filled with solutions and absorbance was measured at 760 nm in the presence of a blank, which was done under the same conditions, but free from extracts.

Measurements of TP are usually made from a compound used as a reference standard; in this case, we used the Gallic acid (GA). A calibration curve was achieved under the same conditions, with
a concentration range from 0.5 to 90 mg GA/ml. The absorbance was measured at 760 nm in the presence of a blank, which is performed under the same conditions as the standard, but free thereof.

2.9.2. Determination of total flavonoids
The total flavonoids (TF) were assayed with the extracts obtained from DIC-treated and-untreated buckthorn leaves using aluminum trichloride hexahydrate as a reagent, following Zhishen, Mengcheng, and Jianming (1999) method with minor modifications.

The flavonoids in the extract react with aluminum trichloride hexahydrate in the presence of sodium nitrite; in an alkaline medium, it gives a pink color, which absorbs in the visible at 510 nm.

In a 5-ml vial, we introduced successively 0.5 ml of extract, 2.5 ml of distilled water, and 0.15 ml of 5% NaNO₂ solution. After 5 min, we added 0.3 ml of a solution of 10% of AlCl₃, 6H₂O, and the mixture was kept on hold for 6 min. We then added 1 ml of concentration 1 M NaOH. We completed up to the mark with distilled water. After stirring the mixture vigorously with a vortex, we immediately measured the absorbance at 510 nm in the presence of a blank. This is performed under the same conditions as the extracts, but free from them.

The calibration curve was performed with catechin (Cat) using concentrations ranged from 10 to 450 μg cat/ml. The preparation of these samples was performed under the same conditions as the buckthorn extracts. The absorbance measurements were also performed at 510 nm in the presence of a blank.

2.9.3. Quantification of flavonol aglycones by HPLC individual and aggregate
Liquid chromatographic of flavonol aglycones HPLC analysis were produced by a HPLC Agilent 1100 (Massy; France), equipped with a quaternary pump, an autoinjector, a degasser, a column thermostat, and an autosampler; as well as with a UV–vis diode array (DAD). Control of the whole system, the acquisition, and the treatment of data were done using a computer with ChemStation software preinstalled. The column used was an RP Phenomenex Luna C18 (2) brand size: 250 mm long × 4.6 mm internal diameter and having a particle size of 5 μm.

The column temperature was maintained at 28°C, the mobile phase flow rate was 0.5 ml/min, and the maximum wavelength was set at λ = 370 nm. The injection volume was 20 μl. Elution of compounds was carried out in gradient mode. The binary mobile phase consisted of purified water-methanol 70% v/v acidified with 0.05% TFA (total flavonol aglycones, solution A) and pure methanol acidified with 0.05% TFA (solution B). The elution gradient was 24% B between 0 and 6 min, 24–85% B between 6 and 27 min, 85% between 27 and 32 min, 85–24% B between 32 and 35 min, 24% B for 35–38 min, 15% B. A 2-min post time is programmed after each injection.

A standard solution of kaempferol (Kaemp) was prepared with a concentration range from 10 to 250 μg Kaemp/ml from the initial solution. All solutions were analyzed under the same conditions as the extracts.

During this work, we determined the antioxidant activity of the buckthorn and all the extracts obtained with and without DIC treatment.

The radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) of formula C₁₅H₁₁N₅O₅ is widely used for evaluating the antioxidant activity of flavonoids. In methanol solution, this radical has a very intense violet color; its reduction is accompanied by discoloration to pale yellow.

The antioxidant activity of flavonoids extracts of the buckthorn leaves was determined using the method described by Brand-Williams, Cuvelier, and Berset (1995) with some modifications. The
absorbance measurements flavonic extracts were carried out by spectrophotometry at a wavelength maximum of 516 nm.

Series of solutions of different concentrations were performed for treated and untreated material from buckthorn extracts diluted 20 times. A solution of 0.3 mM DPPH was also prepared. All dilutions were made with methanol.

The preliminary tests, which measured the absorption of DPPH radical versus the wavelength, have shown that the maximum absorption corresponded to 516 nm. Other preliminary tests, which studied the reaction kinetics of DPPH-flavonoid extract redox, optimized the incubation time at 30 min.

To 3 ml of 0.3 mM DPPH, we added 1 ml of buckthorn flavonoid extract. The reaction mixture was stirred vigorously and then left for 30 min at room temperature in a dark place away from any light source. Temperature and reaction time affected the color development. A quartz cuvette was carefully filled with the reaction mixture and the absorbance was measured against a blank consisting of a mixture of 1 ml of the same flavonoid extract and 3 ml of methanol. Several dilutions were performed until stabilization of the absorbance.

Our extracts were compared with several commercial antioxidants used as references, subjecting them to the same procedure for evaluating the antioxidant capacity. They were BHT (1–80 μg/ml), quercetin (1–50 μg/ml), vitamins C and E (1–60 μg/ml), and Gallic acid (0.25–20 μg/ml). The different dilutions were obtained from the stock solutions of 200 μg/ml for BHT, 100 μg/ml for quercetin and vitamins C and E, and 50 μg/ml for Gallic acid. The measurements were repeated in triplicates.

2.9.4. Design of experiments
There are many experimental designs that can adapt to different situations for optimization. In our case, we opted a five-level rotatable central composite design. It allows to identify the direct, interaction and quadratic effects of each parameter, usually through a second-order polynomial equation of the response \( Y \) as dependent variable versus different operating parameters as independent variables \( X_i \) (Benoist, Tourbier, & Germain-Tourbier, 1994); using a software Statgraphics Plus version 5.1.

\[
Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{j=1}^{n} \sum_{i=1}^{n} \beta_{ij} X_i X_j + \varepsilon
\]  

(1)

where \( Y \), the response parameter, is the extraction yield expressed in equivalent milligrams of kaempferol per gram dry matter (mg Kaemp eq/g dm). \( \beta_0, \beta_i, \beta_{ii} \) and \( \beta_{ij} \) are the regression coefficients of the model; \( X_i \) and \( X_j \) are the DIC-operating parameters, whose number is \( n \) (in our case \( n = 3 \)). They summarize the five levels of various operating parameters in their ranges. \( \varepsilon \) is the random error.

The number of trials was, in our case (\( n = 3 \)), 22 comporting \( 2^3 = 8 \) factorial points, and \( 2 \times 3 = 6 \) star points. The \( n_o = 8 \) repetitions of central point was evaluate the repeatability.

2.9.5. Fundamental and analysis of kinetics
From kinetic point of view, the limiting factor of solvent extraction process normally is the interaction between solvent/solid. This is the diffusion of the solvent/solute within the plant structure. The gradient concentration of each compound within the solid matrix is then considered as the driving force. This is also a higher value of the superficial interaction, which is usually revealed by the starting accessibility.

Normally, solvent extraction is a solid/liquid interaction process. Since it is usually perfectly intensified at external process point of view (agitation, temperature) and through an adequate choice of
adequate solvent in terms of solubilization; the operation is achieved with the surface interaction and/or the penetration processes (capillarity, molecular diffusion ...) of the solvent within the solid plant as porous material. Within the holes, the solute in the solvent is transported by molecular diffusion; only when US (Ultra-Sound) is used, a possible great intensification by intra-hole convection can take place (Allaf et al., 2013). Whatever with or without US, the driving force of the solvent-solute penetration is the gradient of concentration. It is then possible to adopt a similar-to-1st-Fick’s-Law model with a global effective diffusivity $D_{eff}$ as the process coefficient (Equation 1). However, during a “starting period” ($0 < t < t_o$) parallel to this similar-Fick’s-diffusion, solvent extraction normally starts with an interaction between solvent and the exchange surface. To study the similar diffusion process, one has to identify the time $t_o$ from which the only process to be considered is the similar diffusion process.

In the kinetic model from which $D_{eff}$ has to be computed, the only experimental data to be used have to be limited to the only experimental data for $t > t_o$. By extrapolating this diffusion model till $t = 0$, one can calculate the theoretical value $X_0 = X_{t=0}$ normally distinct from the initial value of $X_i = 0$; $\delta X_i = X_{t=0}$ is defined as the starting accessibility. $X_{\infty}$ is the maximum solute extraction and $X_{t_o}$ the solute extracted after $t_o = 5$ min of extraction.

3. Results and discussion

3.1. Determination of buckthorn flavonic compounds by HPLC

3.1.1. Flavonoid extraction from buckthorn after DIC treatment

We opted for a gradual drying of the buckthorn leaves to reach final moisture content of 16.28 g H$_2$O/100 g db before storing them for the different treatments. The extraction and HPLC analysis of aglycones are very important since they present greater therapeutic effects than glycosides and are the way of bioavailability of flavonoids. The aglycones were identified using acid hydrolysis of flavonoids as a first step to possibly determine the structure of heterosides, whose identification is usually arduous mainly because of non-availability of the standard (Hertog, Hollman, & Venema, 1992).

3.1.1.1. HPLC analysis of the extracts. UV spectrophotometry absorption characterizes and differentiates individually the flavonoid aglycones. With the buckthorn extracts, the maximum of absorption was identified at a wavelength of 370 nm.

The results from HPLC analysis (Table 3) shown for both RM and DIC-treated samples revealed four major compounds of flavonol aglycones determined as quercetin, kaempferol, rhamnetin, rhamnocitrin, and a minor 5th compound identified as isorhamnetin. Thereafter, this last was not quantified because negligible in comparison to the other major compounds. Elution orders are done according to their polarity and their structures. These five flavonol aglycones were identified based on HPLC commercial grade standards.

HPLC analysis of buckthorn extracts showed kaempferol ($C_{15}H_{10}O_6$) as the major compound (peak 2). Consequently, the yield and kinetics are expressed in milligrams of kaempferol equivalent per gram of dry basis (mgKaemp eq/g db). This performance was assessed from a calibration curve of the kaempferol standard obtained under the same analytical conditions as the extracts. For the untreated material, quercetin ($C_{15}H_{10}O_7$) is the third major compound. The other two compounds were methoxyflavonols derived from quercetin and kaempferol. They are rhamnatin ($C_{16}H_{12}O_7$) and rhamnocitrin ($C_{16}H_{12}O_6$).

In the Rhamnaceae family, these compounds O-methylated flavonols (as quercetin and kaempferol derivatives) are widely present. Rhamnatin (7-methyl quercetin or 7-O-methyl quercetin) and rhamnocitrin (7-methyl kaempferol or 7-O-methyl kaempferol) are also naturally present in many plants as glycosides, including xanthorhamnin or rhamnetin-3-O-rhamnoside (for rhamnetin) and catharticin or rhamnocitrin-3-O-rhamnoside (for rhamnocitrin), in which they both play the role of the aglycone.
Table 3. Extraction and antioxidant activity of buckthorn: with 150-min HPLC, the flavonol aglycones were extracted and their amount quantified

| Trial number | RM | DIC1 | DIC2 | DIC3 | DIC5 | DIC7 | DIC8 | DIC12 | DIC13 | DIC14 | DIC16 | DIC19 | DIC20 | DIC22 | DIC10 |
|--------------|----|------|------|------|------|------|------|-------|-------|-------|-------|-------|-------|-------|-------|
| P | 0.2 | 0.1 | 0.35 | 0.35 | 0.5 | 0.2 | 0.2 | 0.2 | 0.35 | 0.6 | 0.5 | 0.35 | 0.5 | 0.35 |
| t | 180 | 120 | 19 | 120 | 60 | 180 | 180 | 60 | 60 | 120 | 120 | 180 | 221 | 60 | 120 |
| C | 5 | 4 | 4 | 4 | 5 | 3 | 3 | 5 | 3 | 6 | 4 | 5 | 4 | 3 | 2 |

Total flavonol aglycone

| X∞ | 12.24 | 14.09 | 11.4 | 13.24 | 15.83 ± 0.18 | 12.32 | 16.22 | 14.95 | 13.16 | 13.92 | 14.71 | 15.38 | 18.72 | 13.28 | 15.68 | 17.86 |
| IR (%) | 100 | 115 | 93 | 129 ± 1 | 13.02 | 11.02 | 11.02 | 11.02 | 11.02 | 11.02 | 11.02 | 11.02 | 11.02 | 11.02 |

Total flavonol aglycone extraction kinetics

| 8°Central DIC | αX | 12.22 | 11.66 | 12.3 ± 0.19 | 12.35 | 12.96 | 13.66 | 10.23 | 8.17 | 12.1 | 11.02 | 11.66 | 13.33 | 13.33 |
| Dtotal | 7.96 | 9.82 | 15.63 ± 1.64 | 21.42 | 9.79 | 12.91 | 11.95 | 16.51 | 10.19 | 7.66 | 14.21 | 12.35 | 7.71 | 19.95 |
| R2 | 0.958 | 0.954 | 0.961 | 0.912 | 0.97 | 0.934 | 0.951 | 0.878 | 0.961 | 0.999 | 0.962 | 0.946 | 0.953 | 0.913 |

Quercetin extraction kinetics

| αX | 2 | 2.63 | 1.76 | 1.64 | 2 ± 0.12 | 2.04 | 2.07 | 2.3 | 2.12 | 1.79 | 2.66 | 2.66 | 2.72 | 2.74 | 2.65 | 2.69 |
| Dtotal | 2.79 | 10.75 | 13.04 | 10.04 | 17.8 ± 2.1 | 21.55 | 14.61 | 12.11 | 10.38 | 19.84 | 10.51 | 8.36 | 11.11 | 10.76 | 14.12 | 15.53 |
| R2 | 0.995 | 0.999 | 0.992 | 0.892 | 0.98 | 0.95 | 0.978 | 0.913 | 0.947 | 0.988 | 0.995 | 0.884 | 0.92 | 0.896 | 0.968 | 0.851 |

Kaempferol extraction kinetics

| αX | 2.49 | 4.81 | 3.45 | 4.11 | 4.31 ± 0.07 | 4.22 | 4.51 | 4.61 | 4.03 | 4.15 | 4.65 | 4.92 | 4.36 | 4.18 | 4.48 | 4.65 |
| Dtotal | 3.16 | 4.84 | 27.9 | 13.99 | 15.45 ± 3.8 | 27.88 | 9.14 | 15.2 | 13.12 | 18.31 | 10.41 | 7.47 | 15.31 | 15.84 | 8.35 | 17.88 |
| R2 | 0.999 | 0.819 | 0.914 | 0.909 | 0.92 | 0.922 | 0.929 | 0.877 | 0.844 | 0.951 | 0.963 | 0.922 | 0.939 | 0.881 | 0.996 | 0.911 |

Rhamnetin extraction kinetics

| αX | 2.04 | 2.2 | 1.66 | 2.37 | 2.71 ± 0.05 | 2.12 | 2.81 | 2.9 | 1.93 | 1.15 | 2.3 | 2.37 | 2.9 | 2.04 | 2.48 | 3.56 |
| Dtotal | 2.62 | 10.98 | 17.74 | 7.4 | 10.87 ± 1.6 | 25.56 | 7.51 | 13.19 | 12.49 | 13.83 | 10.11 | 7.42 | 17.52 | 12.92 | 5.81 | 23.4 |
| R2 | 0.994 | 0.976 | 0.973 | 0.891 | 0.977 | 0.917 | 0.928 | 0.81 | 0.906 | 0.931 | 0.973 | 0.936 | 0.994 | 0.999 |

Rhamnocitrin extraction kinetics

| αX | 2.04 | 2.53 | 2.23 | 3.55 | 3.13 ± 0.16 | 2.27 | 3.54 | 3.8 | 2.17 | 2.12 | 1.2 | 1.28 | 3.07 | 3.61 | 2.35 | 3.71 |
| Dtotal | 2.96 | 12.28 | 11.07 | 8.8 | 19.36 ± 1.47 | 14.19 | 8.97 | 11.16 | 12.07 | 14.94 | 9.53 | 7.29 | 12.13 | 10.04 | 6.69 | 22.61 |
| R2 | 0.993 | 0.929 | 0.73 | 0.936 | 0.98 | 0.877 | 0.931 | 0.786 | 0.989 | 0.992 | 0.961 | 0.996 | 0.949 | 0.933 | 0.849 | 0.935 |

Antioxidant activity

| IC50 | 8.22 | 1.51 | 3.3 | 3.34 | 3.96 ± 0.17 | 1.93 | 7.38 | 10.39 | 0.96 | 5.15 | 1.09 | 4.34 | 0.12 | 11.74 | 6.24 | 7.38 |
| Ratio vs. BHT | 5.86 | 31.87 | 14.58 | 14.41 | 12.17 ± 0.52 | 24.94 | 6.52 | 4.63 | 50.14 | 9.35 | 44.16 | 11.09 | 40.11 | 4.10 | 7.71 |
| IF | 1 | 5.44 | 2.46 | 2.46 | 2.1 ± 0.1 | 4.26 | 1.11 | 0.79 | 8.56 | 1.60 | 7.54 | 1.89 | 68.5 | 0.70 | 1.32 | 1.11 |

Notes: P: saturated steam pressure (MPa); t: total processing time (s); C: number of cycles; IF: improvement factor of antioxidant activity, RM referenced; TFA: total flavonol aglycone; X∞: yields (mg Kaemp eq/g db); IR (%): improvement ratio %; δX: starting accessibility; δXs: (mg Kaemp eq/g db); Dtotal: diffusivity Dtotal (10−10 m²/s); R2: coefficient correlation.
In one of the first studies on buckthorn flavonoids, Faugeras and Paris (1962) highlighted the presence of quercetin, possibly kaempferol, and O-methylated flavonols (7-O-methyl derivative of quercetin and kaempferol). In 1995, Touati and Fkih-Tétouani (1995) reported the presence of seven flavonols in the extracts from Morocco buckthorn leaves. They were quercetin, kaempferol, rhamnocitrin, rhamnetin, rhamnazin and, possibly 6-methyl-kaempferol and 8-methyl-kaempferol. Other studies did not mention the last four flavonols. Note also that 6-methyl-kaempferol had never yet been reported in any plant.

More recently, Ammar et al. (2009) studied Tunisian buckthorn. They used two dimensional NMR to extract, isolate, and identify three triglycosides; kaempferol 3-O-β-isorhamninoside, rhamnocitrin 3-O-β-isorhamninoside and rhamnetin-3-O-β-isorhamninoside. They also highlighted three aglycones, which were apigenin, kaempferol, and quercetin.

The extracts obtained in 150 min of extraction from the untreated RM and DIC-treated materials were analyzed by HPLC-DAD. The results were used to optimize the DIC treatment versus the yields of buckthorn TFA. The yields reached up to 18.72 mg Kaemp eq/g db with DIC-treated buckthorn, while it was 12.24 mg Kaemp eq/g db for RM. The buckthorn leaves treated under DIC conditions of 0.5 MPa, 180 s and five cycles gave the best performance with, as increasing value of 53%. Only one exception was highlighted with lower yields (11.40 mg Kaemp eq/g db); it was four cycles at p = 0.10 MPa and t = 120 s. Here too, it would be possible to assume the hypothesis of an important forcing out of non-volatile molecules toward the vacuum tank.

Statistical analysis of the effects of DIC-operating parameters on the extraction yields of TF was carried out using the software Statgraphics Plus 5.1. Saturated steam pressure $P$ and treatment time $t$ of DIC treatment had the main positive effects on HPLC yields of buckthorn TFA. The relatively negative impact of number of cycles should be correlated with a forcing out process, which would reveal some expulsion of non-volatile compounds toward the vacuum tank during each pressure drop. The higher the saturated steam pressure $P$, the higher the yields of TFA. Similar but lower effect was observed with the treatment time $t$. The negative coefficients of both $t^2$ and $P^2$ reveal the presence of a maximum value of such yields depending on these parameters.

An empirical model was evaluated with $R^2 = 72\%$; and adjusted $R^2 = 51\%$ expressing the TFA in kaempferol equivalent milligrams per gram of dry basis (mg Kaemp eq/g db) extracted in 150-min HPLC, vs DIC-operating parameters; the saturated steam pressure ($P$ in MPa), the processing time ($t$ in s), and the number of cycles $C$:

$$
TFA \text{ Yield}_{\text{HPLC}} = 23.68 + 12.85P + 0.003t - 5.36C - 32.22P^2 + 0.03Pt + 275PC - 0.0002t^2 + 0.01tC + 0.30C^2
$$

(2)

3.1.1.2. Impact of DIC treatment on flavonoid composition. HPLC analyses of extracts from both untreated (RM) and DIC-treated buckthorn leaves revealed the presence of four major compounds including quercetin, kaempferol, rhamnetin, and rhamnocitrin, and a minor compound, which was isorhamnetin (Table 4). Since quercetin is the most polar compounds, its elution takes firstly place, followed by kaempferol. The three other compounds (such as rhamnocitrin) are less polarity because of their methoxyl group.

For optimized DIC-treated buckthorn, a drastic change was observed (Figure 3). Quercetin became the fourth major compound while rhamnocitrin ranks second and quantitatively competes with kaempferol remaining majority. Rhamnetin remains the second major compound. Both 7-O-methylated flavonols reached up to 174.26% for the rhamnetin and 208.54% for rhamnocitrin, both compared with the untreated material as a reference. The chromatograms show the evolution and changes incurred in treating DIC compared to the untreated material.
We also note that for the DIC-treated buckthorn extracts were rich in rhamnocitrin with a highly saturated yellow color. This correlates (Tschirch & Polacco, 1900) work, which noted an association between the yellow color of buckthorn berries and the presence of rhamnocitrin.

3.1.1.3. Kinetics of extraction of flavonoids from untreated and DIC-treated buckthorn leaves. Preliminary studies allowed us to limit the kinetic measurements to the first 150 min. Kinetic data of total flavonoid extraction were analyzed, for RM (untreated sample; with triplicates) and for DIC-treated samples.

| Table 4. HPLC extraction yields of total flavonol aglycone (TFA), flavonoids (TF) and phenols (TP) from buckthorn leaves for 150 min: RM and DIC: 0.5 MPa, 180 s and 5 cycles |
|---------------------------------|-----------------|-----------------|
| Flavonol aglycones | Raw material (RM) | DIC-treated sample | DIC Increasing value (%) |
| Total (TFA) | 12.2 | 18.7 | 53 |
| Quercetin | 02.6 | 03.4 | 31 |
| Kaempferol | 04.5 | 05.4 | 20 |
| Rhamnetin | 02.7 | 04.7 | 74 |
| Rhamnocitrin | 02.5 | 05.1 | 104 |
| Flavonoids | Total (TF) | 03.9 | 06.5 | 67 |
| Phenols | Amount (mg Cat eq/g db dry basis) | 23.2 | 41.0 | 77 |

Figure 3. (a) Structure of flavonic compounds of *Rhamnus alaternus* L. (b) Chromatogram analysis of extracts from untreated and DIC-treated buckthorn leaves: quercetin (pic 1, tr = 21.15 min), kaempferol (pic 2, tr = 24.26 min), isorhamnetin (pic 5 tr = 24.58 min), rhamnetin (pic 3, tr = 26.81 min), and rhamnocitrin (pic 4, tr = 29.55 min).

We also note that for the DIC-treated buckthorn extracts were rich in rhamnocitrin with a highly saturated yellow color. This correlates (Tschirch & Polacco, 1900) work, which noted an association between the yellow color of buckthorn berries and the presence of rhamnocitrin.

3.1.1.3. Kinetics of extraction of flavonoids from untreated and DIC-treated buckthorn leaves. Preliminary studies allowed us to limit the kinetic measurements to the first 150 min. Kinetic data of total flavonoid extraction were analyzed, for RM (untreated sample; with triplicates) and for DIC-treated samples.
The kinetics of total and individual flavonol aglycone extraction was identified versus the extraction time of DIC-treated (at optimal conditions of 0.5 MPa, 180 s, and 5 cycles) and untreated buckthorn. For buckthorn untreated, the maximum yield is obtained after 150 min. It is 18.72 mg Kaemp eq/g db instead of 12.24 mg Kaemp eq/g db for RM, which is an increase in 52.94%. It is interesting to note that the yield obtained after 150 min for untreated buckthorn is obtained within 3 min of extraction for the plant treated with the optimal conditions. Similar results with the individual flavonol aglycones were obtained.

From different trials, one noted that after treatment with DIC, the leaf morphology differ greatly than the untreated leaves (a). DIC-treated leaves were “puffy” appearance with an expanded and alveolated internal structure. Consequently, the solvent/solid interaction normally was easier than the case of the compact structure untreated leaves, at both superficial and deep levels.

3.1.1.4. Kinetics of flavonol aglycones. Monitoring the kinetics of extraction of the treated and untreated buckthorn leaves (Figure 4) allowed to adopt the superficial-interaction/similar-diffusion model. The values of the kinetic parameters calculated from this model are the starting accessibility $\delta X_s$ and the effective diffusivity $D_{eff}$ for the total and individual compounds of TFA from the DIC-treated and the untreated buckthorn leaves. $R^2$ values related to these different situations were systematically high, up to 0.999, proving that the model was adequate for the various situations studied in our case (Table 5).

The statistical analysis of experimental data issued from the kinetic model of the extraction of total and individual compounds of TFA, from the DIC-treated and the untreated buckthorn leaves, can better visualize the effects of DIC treatment.

3.2. Spectrophotometry quantification of TF and TP

3.2.1. Total flavonoids
DIC treatment was studied using spectrophotometry to quantify the total buckthorn flavonoids (TF). The same optimized conditions as HPLC quantification were established; such as a saturated steam pressure $p = 0.5$ MPa, a heating treatment time $t = 180$ s, and $C = 5$ cycles. Yields, expressed as equivalent mg of Catechin per g of dry basis (mg Cat eq/g db) were determined via a calibration curve with absorption measured at 510 nm.

The best estimated yield for DIC-buckthorn leaves was 6.50 against 3.95 ± 0.14 mg Cat eq/g db for untreated RM.

3.2.2. Total phenols
For all the buckthorn extracts, the quantification of TP, measured by the Folin–Ciocalteu method, was done by spectrophotometry at 760 nm. A calibration curve obtained with Gallic acid (GA), under the same conditions, allowed us to estimate the amount of TP contained in each extract. This quantity is expressed in mg of Gallic acid equivalent per g of dry basis (mg AG eq/g db). The best yield of TP extracted from the buckthorn was 40.97 against 23.18 ± 0.74 mg AG eq /g db for the untreated RM. It was obtained for DIC treatment conditions of $p = 0.35$ MPa, for 120 s, and 6 cycles. These DIC conditions were different than that allowing the maximum yields of TF and TFA.

Buckthorn leaf phenolic compounds and especially flavonols are generally located in the outer cell tissues of plant organs with high levels especially in the skins of fruits and leaves. Moreover, some simple flavonoids (such as apolar methyl derivatives of flavonoids) are often accumulated in exudates at the surface. Structural changes incurred on buckthorn DIC treated leaves compared with untreated material were identified by SEM (Figure 5). It is worth noting that DIC treatment implied opening stomata, increasing pore size. Furthermore, DIC-treated surface was almost free of cellulose. This allowed the solvent to more easily move from the outside to the inside of the plant and vice versa. Consequently, this provided better extraction kinetics, causing a valued time saving.
3.2.3. Determination of buckthorn antioxidant activity

Generally, hydrogen transfer is the basis of polyphenol antioxidant reactivity (Wright, Johnson, & DiLabio, 2001). In the present study, this powerful reactivity should probably be due to a synergy between the different compounds increased by the addition of a large ionization potential of...
Table 5. Starting accessibility $\delta X_s$ and effective diffusivity $D_{\text{eff}}$ of kinetic model of the extraction of total and individual flavonol aglycones from the DIC-treated and the untreated buckthorn leaves

| Trial number | Total flavonol aglycones (TFA) | Individual flavonol aglycones | Quercetin | Kaempferol | Rhamnetin | Rhamnocitrin |
|--------------|-------------------------------|-------------------------------|-----------|------------|-----------|--------------|
|              | $\delta X_s$ | $D_{\text{eff}}$ | $\delta X_s$ | $D_{\text{eff}}$ | $\delta X_s$ | $D_{\text{eff}}$ | $\delta X_s$ | $D_{\text{eff}}$ | $\delta X_s$ | $D_{\text{eff}}$ |
| Control      | 8.34 | 3.0 | 2.00 | 2.79 | 2.69 | 3.16 | 2.04 | 2.62 | 1.81 | 2.96 |
| DIC*         | 12.3 ± 0.2 | 16 ± 1.5 | 2.0 ± 0.1 | 18 ± 2 | 4.3 ± 0.1 | 15.4 ± 3.5 | 2.7 ± 0.1 | 10.9 ± 1.5 | 3.1 ± 0.2 | 19.9 ± 2.2 |
| DIC1         | 14.87 | 19.95 | 2.69 | 15.53 | 4.65 | 17.88 | 3.56 | 23.40 | 4.13 | 22.61 |
| DIC2         | 13.66 | 14.21 | 2.72 | 11.11 | 4.36 | 15.53 | 2.90 | 17.52 | 3.61 | 12.13 |
| DIC3         | 8.92 | 19.03 | 1.76 | 13.04 | 3.45 | 27.90 | 1.66 | 17.74 | 2.23 | 11.07 |
| DIC5         | 10.58 | 21.42 | 2.04 | 21.55 | 4.22 | 27.88 | 2.12 | 25.56 | 2.27 | 14.19 |
| DIC6         | 13.60 | 12.91 | 2.30 | 12.11 | 4.61 | 15.20 | 2.90 | 13.19 | 3.80 | 11.16 |
| DIC7         | 8.17 | 16.51 | 1.79 | 19.84 | 4.15 | 18.31 | 1.15 | 13.83 | 1.22 | 14.94 |
| DIC8         | 13.33 | 7.71 | 2.65 | 14.12 | 4.48 | 8.35 | 2.48 | 5.81 | 3.71 | 6.69 |
| DIC10        | 11.66 | 9.82 | 1.64 | 10.04 | 4.11 | 13.99 | 2.37 | 7.40 | 3.55 | 8.80 |
| DIC11        | 12.22 | 7.96 | 2.63 | 10.75 | 4.81 | 4.84 | 2.20 | 10.98 | 2.53 | 12.28 |
| DIC12        | 13.02 | 7.66 | 2.66 | 8.36 | 4.92 | 7.47 | 2.37 | 7.42 | 3.07 | 7.29 |
| DIC15        | 12.96 | 9.79 | 2.07 | 14.61 | 4.51 | 9.14 | 2.81 | 7.51 | 3.54 | 8.97 |
| DIC19        | 11.29 | 12.35 | 2.74 | 10.76 | 4.18 | 15.84 | 2.04 | 12.92 | 2.35 | 10.04 |
| DIC20        | 10.23 | 11.95 | 2.12 | 10.38 | 4.03 | 13.12 | 1.93 | 12.49 | 2.17 | 12.07 |
| DIC21        | 12.10 | 10.19 | 2.66 | 10.51 | 4.65 | 10.41 | 2.30 | 10.11 | 2.68 | 9.53 |

Notes: DIC*: central points (DIC4, 9, 13, 14, 16, 17, 18, 22); $\delta X_s$: starting accessibility (g H$_2$O/g dry matter) or % db; $D_{\text{eff}}$: diffusivity (10$^{-10}$ m$^2$/s); $R^2$: correlation coefficient 0.98–0.99.

Figure 5. Impacts of DIC treatment on morphologic structure of buckthorn leaves: (a) Cross sections of buckthorn leaves: RM; (b) Cross sections of buckthorn leaves DIC-textured at 0.5 MPa, during a total processing time of 180 s, for 5 cycles; (c) Surface micrographs of buckthorn leaves (RM); and (d) Surface micrographs of textured buckthorn leaves by DIC at 0.5 MPa, during a total processing time of 180 s, for 5 cycles.
polyphenols vis-à-vis free radicals. This reflects the ability and ease that polyphenols have to transfer an electron to the radical, allowing the rupture of the O–H bond.

The results listed in Table 6 show that the buckthorn DIC-treated leaves had the greatest redox potential for DPPH radical, revealed by the lowest IC50% value. The treatment by DIC increases even more this radical scavenging activity of polyphenols vis-à-vis the DPPH•. Indeed, while untreated buckthorn extract reduces 50% of free radicals DPPH with a concentration of 8.22 μg/ml; DIC-treated buckthorn antioxidant activity had the same impact with 0.12 μg/ml. DIC-processing conditions which gave this performance were 0.50 MPa, 180 s, and 5 cycles. This is the same point for which we obtained the highest yield of TFA. DIC treatment could dramatically increase the buckthorn antioxidant efficiency, up to 68 times more than the initial untreated buckthorn. It is then about 400 more than BHT, which is one of the most used of commercial antioxidants taken as references.

It is worth noting that the correlation between radical scavenging activity and phenolic content or flavonic was not significant, since the correlation factor $R^2$ did not exceed 36.68%. This result shows in the case of buckthorn that the inhibitory activity appears to possibly be attributed to the molecular structure more than the amount of phenolic content. However, the lack of information of the compounds contained in the latter does not allow us to state the conclusion with certainty.

The antioxidant activity would not be the result of a single molecule but there would be a synergy between the compounds of polyphenol family. Many studies have confirmed that the antioxidant activity was correlated to phenolic content, especially flavonoid extracts obtained from plants. However, other studies converge to the same fact that this relationship is closely related to the number, position, and nature of the substituents grafted on rings B and C of the flavonoid molecules. It is the same for their polarity and their degree of polymerization. The case of flavonols justifies this difference. But these cases are not a general rule and are far from elucidating any differences or irregularities that may occur.

In addition to the phenolic content, several factors can influence the reduction potential of an extract-containing antioxidants, such as the ratio antioxidant/DPPH$, the type of solvent, pH, light, synergy between the compounds (Mimica-Dukic, Bozin, Sokovic, & Simin, 2004; Ozcelik, Lee, & Min, 2003; Rice-Evans, Miller, & Paganga, 1996).

### Table 6. Minimum inhibitory concentrations IC$_{50}$ of buckthorn, and antioxidant commercial references

| Antioxidant Activity of buckthorn leaves and BHT as reference | IC50 (μg/ml) | Ratio vs. BHT as reference | DIC Improvement factor |
|--------------------------------------------------------------|-------------|---------------------------|-----------------------|
| Untreated raw material buckthorn                             | 8.22 ± 0.01 | 5.86                      | 1                     |
| DIC19-treated buckthorn                                      | 0.12        | 401.08                    | 68.5                  |
| BHT as antioxidant reference                                | 48.13 ± 0.13| 1                         | –                     |

4. Conclusion

As relevant response facing the great interest accorded to the natural antioxidant compounds, buckthorn leaves can be envisaged as sustainable source of natural flavonoids, possibly able to insert in various food, cosmetic, and/or pharmaceutical industrial formulations. However, the compact structure of plant materials does not allow the direct use of buckthorn leaves. Moreover, the conventional solvent extraction is difficult because of the highly compact natural structure. Thus, DIC as a texturing way was used to obtain much more porous structure. This resulted in widely higher diffusivity of the solvent and dramatically greater availability of numerous bioactive molecules. This greatly facilitates and intensifies both extraction process and direct use of the plant.
DIC treatment allowed buckthorn leaves to expand and have greater porosity. This intensely increased the TFA yields reaching up to 18.72 mg Kaemp eq/g db with DIC-treated buckthorn, against 12.24 mg Kaemp eq/g db for the untreated RM.

Finally, the study of antioxidant activity showed that the DIC-textured buckthorn leaves compared to unprocessed leaves had 68 times more reducing potential regarding DPPH radical. This activity strongly increased after DIC treatment. It is important to note that all extracts showed greater activity than the commercial synthetic standards as a benchmark. Indeed, the impact of DIC treatment on the extraction time, kinetics, and yields was correlated with highly more significant functional antioxidant characteristics.

These results encourage for a better use of buckthorn as a functional and medicinal plant. Future work will aim at evaluating this antioxidant activity by directly integrating this DIC-powder as protection of food products (lipsids ...) against atmospheric oxidation.

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