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SC-CO_2 Extraction of guayule biomass (*Parthenium argentatum*) – yield and selectivity towards valuable co-products, lipids and terpenics

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ABSTRACT

Guayule (*Parthenium argentatum*) a shrub native to Mexico, is now being acclimated in Europe as a potential crop. The main extractible fractions are polyisoprene (similar to *Hevea* rubber) for producing tires and allergy-free gloves and resin (GRe). This work deals with valorising this last fraction, after water-based extraction of the latex. To date GRe has not been extensively investigated, in spite of containing useful compounds, and there is a need for a green and selective extraction process, which is the aim of present work.

First, resin -a mixture of lipids and terpenics- was analysed with focus on fatty acids and other carboxylic acid containing classes. Then the influence of extraction conditions was investigated, under "soft" conditions with supercritical carbon dioxide (SC-CO_2). Investigated parameters act on extraction efficiency (yield, selectivity) towards valuable fractions. Our results do show that SC-CO_2 alone cannot extract substantial amounts of resin. As co-solvent, ethanol –a benign one- and acetone, this last being the most common solvent for that purpose, here also used alone as reference, under pressurized extractions conditions (ASE). Under SC-CO_2, ethanol allows the highest resin yield (12.1%-dw biomass dry weight) compared to acetone (7.0%-dw) and to the reference ASE-acetone method (5.2%-dw). Found suitable conditions with SC-CO_2-ethanol are affordable: flow rate 34 gCO_2/min and 3.0mL/min of co-solvent, temperature 45°C, pressure 300 bar. Saponification was used for separating resins components before analysis. Aromatic carboxylic acids (cinnamic, *p*-anisic) initially linked to a sesquiterpene, are the major components found in G-Re from the biomass (branches) using the reference ASE method. But these are even more prominent in SC-CO_2-ethanol extracts from bagasse (68.7%), compared to SC-CO_2-acetone (51.1%) which allows a lower selectivity. The unsaponifiable fraction shows a low dependency on extraction temperature with SC-CO_2-ethanol, playing in favor of preserving extract value, guayulins (sesquiterpenes) and argentatins (triperpenes) being known anti-insect and fungistatic agents for wood preservation. Minor resin components are fatty acids containing lipids C18:2, C18:3, C16:0. SC-CO_2 and ethanol, which can be easily recycled, are highly suitable for extracting high-value natural compounds, given their low environmental impact. This work provides the first detailed insight for selecting appropriate parameters and their variation range, in view of further optimizing a "green" process for extracting above compounds.

Keywords: *guayule, Parthenium argentatum, resin, supercritical extraction, terpenics, guayulin, argentatin.*

1. INTRODUCTION

The main industrial source of natural rubber (NR), hevea, is facing an increasing demand [1]. Guayule (***Parthenium argentatum***), a perennial crop under semi-arid climate, synthesizes polyisoprene (PI) extractible as a latex or as rubber (GRu), and also resin (GRe) [2]. GRu, similar to hevea's, is the most probable alternative source of NR [3]. Recently, guayule was successfully grown in France and in Spain, for producing tires and allergy-free gloves [4]. A water-based process for extracting latex was set [5], and the resulting solid or bagasse left after latex extraction needs to be valorised. Indeed economic computations from our group [6, 7] and others [8-11] concluded that multiple-product extraction would be necessary to reach financial viability.

There has been a substantial amount of research work about guayule over last century, mainly performed in Mexico and in the United States of America [12,13]. To date efforts aimed at investigating GRu production and properties, as the prominent product to be marketed from this crop –if not the sole-, whereas the bagasse left after solvent or water-based rubber extraction attracted less interest, in spite of representing about 90%-dw (dry weight of starting biomass) [14,15]. Efforts had been made to find useful properties and applications for the bagasse; for example [16] analyzed the major classes of lignocellulosic components in the bagasse left after GRu extraction and [17] investigated the heating power of an oil obtained after pyrolysis as a possible outlet. Also potential high value resin extracted from the bagasse is an obvious candidate, although not being extensively investigated in comparison to GRu.

In addition to laboratory methods for quantifying GRe in the starting biomass and in the bagasse, a few published works also provide partial information about its chemical composition, based on solvent extraction and subsequent fractionation through various options, down to molecular structural identification of some components [18, 23]. GRe comprises triterpenes keto alcohols [19, 24], diterpenes ketoalchols [25]; sesquiterpenes esters [26-27] polyphenolics, alcaloids, phytosterols and other lipids [20, 28]. A patent was issued for a process aimed at partitioning GRe [22]. All this shows the potential interest of guayule biomass as a source of bio-based chemicals [13, 14], for replacing synthetic compounds whose uses are being restricted or forbidden in many countries. This is the case within the European Union through the application of the Registration, Evaluation,

Authorization and Restriction of Chemicals Rule (REACH), entered in force on June 1st 2007 [29].

Regarding the extraction of resin for industrial production, which is the aim of the present preliminary work, two main ways were reported over the last century: (i) simultaneous extraction of GRe and PI with solvents, followed by subsequent fractionation and washing steps with solvents; (ii) water-based alternatives: parboiling biomass to yield coagulated rubber and resin; grinding with water followed by concentration steps to produce latex or by acid coagulation to recover GRu. At the end the main difference deals with the form under which PI is recovered, i.e. solid rubber dedicated to manufacturing tires, or water-based emulsion (latex). Worth noting that this last case only allows manufacturing both dipped goods like gloves, and tires.

The former way brings safety problems linked to handling hexane in a plant and for eliminating it from GRu and GRe. It was tested at large pilot scale [30] but apparently not used for industrial production. In fact authors also proposed two-step options: extraction of resin by a polar solvent (acetone), prior to separating PI with hexane [31-34]. However these processes do not extract selectively, and low molecular weight PI was found in the acetonic extract, while resin was also found in the hexanic one [35]. Need to cite a water-based alternative comprising parboiling biomass, followed by milling in caustic solution to release GRu worms. These, representing ~95% of the initial PI, sink to the bottom, while GRe forms a layer at the surface [36]. This process uses quite harsh conditions that are not suitable when operating a plant, neither for preserving high molecular weight PI chains. The other water-based option includes biomass grinding in a dilute ammonia solution in order to stabilize the latex dispersion under basic pH during storage; it may also face safety problems. It was tested at large pilot scale in Arizona and there are plans to develop it at larger scale [37]. This water-based process yields a co-product, the bagasse left after separating the liquid phase, still containing the main part of GRe together with residual PI, GRe not being extractable under above conditions [33, 38]. Therefore several teams have investigated methods for recovering GRe from this bagasse. These include extraction processes (i) similar to above mentioned options using an organic solvent under atmospheric pressure, and (ii) involving pressurized fluids.

Among the last options, a method based on AFEX (ammonia fibre expansion) was reported aiming at pre-treating guayule bagasse prior to converting it into sugars and then ethanol [39]. However, although one may expect resin to be extracted (at least partly) under these conditions under which (T 70°C-130°C) some components are either under liquid or vapour state, AFEX does not act much on resin extraction; in fact this work did not report specifically on the fate of this. Another high pressure option was investigated [38, 40, 41] for the pre-treatment of guayule biomass with the aim of loosening the lignocellulosic structure and eliminating enzyme inhibitors prior to hydrolysing cellulose into glucose and producing ethanol. In fact these works –as above AFEX- were not aimed at extracting GRe, therefore this fraction was not analysed as were sugars and structural polymers (cellulose, lignin), although one could see by difference the decreasing percentage of the so-called "others" (possibly resin) after processing with supercritical carbon dioxide (SC-CO_2). Nonetheless these authors showed the effect of combined SC-CO_2 and water, followed by a quick pressure. They selected the following conditions: pressure (P) 100-260 bar, temperature (T) 175-200 °C, duration ~30 min, bagasse moisture content 60-80%. A patent [33] claims a process for extracting biopolymers from guayule, using SC and/or subcritical solvents. Regarding GRe, the patent concludes that (i) SC-CO_2 alone is convenient for partly extracting it with some selectively, but the yield is quite low, and (ii) polar solvents like ethanol or acetone are suitable co-solvents for enhancing the selectivity. However, none of the disclosed examples show a high selectivity i.e. extracting PI or resin alone; they lead at best to a resin/PI ratio of ~10/1 (about 10% by weight of PI in GRe), thus still requiring a purification step.

Therefore, from above literature review, there is still a need for further investigating selective extraction methods for recovering the resin, and following above discussed -scarce but encouraging- results- SC-CO_2 looks suitable, given its low environmental impact. Therefore, here reported results deal with investigating the extraction of GRe with SC-CO_2 through the influence of operating parameters (solvent, P, T, duration) on yield and selectivity, and results are compared to our reference method based on extraction with acetone alone. Because of the complex composition of resin the chemical analysis of extracts was characterized trough the unsaponifiable and saponifiable fractions, and the last one was further investigated for carboxylic acid composition; sesquiterpenes esters were also considered.

2. EXPERIMENTAL SECTION

2.1. Reagents and solvents. Solvents and reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA): acetonitrile, HPLC ≥ 99.9%; acetone, GC ≥ 99.5%; diethyl ether, GC ≥ 99.8%; hexane, HPLC ≥ 97.0%; methanol, GC ≥ 99.8%; acetic acid, GC ≥ 99.8%, except potassium hydroxide: 85% pellets (Panreac, Barcelona, Spain).

2.2. Samples used for extraction. The solid samples subjected to extraction were biomass of AZ2 lines harvested from experimental fields in France (Montpellier) and in Spain (Cartagena), and bagasse obtained after water-based PI extraction of the same biomass. Each sample is named so as to specify biomass (Bi) or bagasse (Bg), harvest place (Fr or Sp), harvest date (day/month/year) and plant age (number of months). Leaves were separated manually from harvested biomass, and the branches were hand cut in 1-2 cm pieces with a pruning shear. Then the samples were vacuum-dried in an oven (Fisher Bioblock Scientific, Illkirch, France) for 3 days (<0.01 bar, 40 °C). Residual moisture was kept below 10% (5-9%). Finally samples were ground in a Waring Blender BB90E (20,000 rpm). Bagasse samples obtained after water-based extraction of PI following [5],

already under coarse powder form, were oven dried as above specified, then ground in a coffee grinder (<0.50 mm mesh). Moisture content of dried samples was assessed by gravimetric analysis at 105 °C in an oven (Gefran 800, Chopin, Boulogne, France) for 15 hours.

2.3. Accelerated solvent extraction (ASE-acetone). A sample of 5±0.005 g of guayule was weighed (±0.001 g) and loaded into a 22 mL stainless steel cell of an ASE Model 350 (Dionex, Sunnyvale, CA, USA) equipped with an auto-sampler carousel, a solvent controller and a collection tray that allowed up to twenty four samples to be extracted sequentially, and connected to a nitrogen tank. A cellulose micro-filter (diameter 27 mm, Dionex, Sunnyvale, CA, USA) was placed at the bottom of each cell prior to the sample. Glass collecting vials (250 mL) were used. Extraction was performed under the following conditions: empty volume in cells filled with acetone until pressure reached 10.3 MPa, heating time 6 min, static extraction period 20 min, purge time 60 s, flush volume 85%, extraction temperature 40 °C. The extraction of each sample was performed in triplicate. The extracts were joined into a pre-weighed flask. Evaporation of the solvent was done in a rotary evaporator at 40°C under reduced pressure and then taken to dryness under vacuum in an oven at 40 °C for 15 h, then kept 30 min in a desiccator before weighing. After each step operated under reduced pressure, nitrogen was used until having reached atmospheric pressure. Dry resin extracts were dissolved in acetone at 10 mg/mL concentration and stored at 4 °C until analysis.

2.4. SC-CO_2 extraction. The SCl unit, custom built by Separex (Champigneules, France), comprised the following main parts: high pressure CO_2 pump, 50 mL extraction vessel connected to a pressure control valve, extract collector vessel; all parts were set with devices for adjusting and controlling the temperature, including a cooling system. A co-solvent pump was also connected to the extraction vessel. The extractor was then loaded with a sample of biomass or bagasse of about 10 g, heated to the suitable extraction temperature, closed and the pressure increased to the chosen value and set constant, while allowing a flow of CO_2 (34.4 or 51.6 g/min) and of solvent (acetone or ethanol) of 0.0, 1.5 or 3.0 mL/min to pass through the extractor. After the desired contact time, the pump was stopped, and the pressure released, the biomass was withdrawn from the contactor. Then this last was closed again and the system cleaned by passing pressurized CO_2 and solvent for one hour under conditions similar to those used for the extraction step. After pressure release, the condenser was opened and the extract recovered by dissolving it in the co-solvent (acetone or ethanol). Evaporation of the solvent was done in a rotary evaporator at 40 °C, in a pre-weighed flask, taken to dryness under vacuum in an oven at 40 °C for 15 h, and then kept for 30 min in a desiccator before weighting. After each step operated under reduced pressure, nitrogen was used until having reached atmospheric pressure. The total extract mass was determined as the total extract obtained during the extraction and the extract recovered in the cleaning process. The extraction yield was calculated as the ratio between the total extract mass and the feed mass of raw material loaded into the extraction vessel. Extracts in tight-screw closed flasks were kept at 4 °C until analysis. Each extraction was done in triplicate to allow statistical analysis of results. Dry resin extracts were dissolved (10 mg/mL) in the co-solvent used for the extraction step, flushed with nitrogen, stored at 4°C until analysis.

2.5. PI in resin extracts by FTIR. The method from Suchat [35] was applied. Spectra of films prepared from GRe deposited on KBr were recorded with a Perkin Elmer 6200 FTIR spectrometer. Extracts were dissolved in CHCl3 (1% weight) and four drop were deposited on a KBr lab-made cell (pressure 10 psi), dried in air for 15 minutes. Films were prepared in triplicate and the used spectrum was averaged. The base line was recorded with a KBr cell and substracted to the average spectrum. The ratio of area of bands representative of PI (C=C, 878-780 cm^{-1}) and resin (C=O, 1800-1690 cm^{-1}) (wave number limits used for integration) was computed and fed to the following equation $y = 0.87 x + 0.18$, where y is the PI/resin ratio of areas of above selected bands, and x is the weight percent of PI in the acetone extract.

2.6. Saponification of resin prior to analysis. Saponification was used as a way for separating resin components prior to analysis according to [20]. A sample from the 10 mg/mL solution containing 500 mg of resin extract was poured into a 100 mL flask, and the solvent was evaporated under reduced pressure at 40 °C. The sample was refluxed for 2 h in 25 mL of a 0.15M solution of potassium hydroxide in 80/20 <v/v> methanol-water mixture. Methanol was then distilled off with a rotary evaporator and the saponification product was cooled, and diluted with 25 mL of distilled water. The resulting emulsion was extracted with 20 mL of diethyl ether. The extraction was repeated twice and the extracts were joined to form the unsaponifiable fraction named F-Un. This fraction was dried with sodium sulfate, diethyl ether was evaporated in a pre-weighed flask at 40°C with a rotary evaporator, then taken to dryness under vacuum in an oven at 40°C for 15h, and finally kept 30 min in a desiccator before weighing. The aqueous solution containing the saponifiable products was acidified to ~pH 3 with dilute hydrochloric acid and then extracted with 20 mL of diethyl ether. The extraction was repeated three times and extracts were joined to form the saponifiable fraction called F-Sap. Diethyl ether was evaporated following the protocol used for F-Un. The resulting unsaponifiable products formed a reddish viscous oil. After each step operated under reduced pressure, nitrogen was used when filling it up until atmospheric pressure. F-Un was dissolved in acetone (10 mg/mL) and stored at 4°C until analysis. The yields were calculated as the ratio between the mass of the considered fraction and the mass of raw material loaded into the extraction vessel. The percentage of F-Sap and F-Un within the resin was computed from the ratio of the fraction mass to the mass of the resin sample; the balance between the sum of the mass of F-Sap and F-Un and the mass of starting resin sample was taken as representing the water-soluble fraction. This last also contains salts in addition to soluble organic compounds and therefore the water was not evaporated for accessing to the dry weight.

2.7. Fatty acid composition by GC. Fatty acid methyl esters were obtained from about 50 mg of the F-Sap fraction (obtained after saponification) through esterification with 3 mL of HCl methanol solution prepared from 50mL of acetyl chloride dissolved in 625 mL of methanol (reflux time 15 min). 8mL of hexane and 10 mL of water were used to extract the methyl esters. One µL of the hexane solution was injected in a capillary gas chromatography (GC) (Agilent model 7890, Santa Clara, CA, US) equipped with

an injector (split-less mode), a capillary column (DB-Wax, J&W Scientific; 30 m length, 0.25 mm i.d. and 0.25 µm film thickness, a flame-ionization detector (FID). Flow rate of the carrier gas helium was 0.452 mL/min (pressure 8.62 psi). The oven temperature was held at 130 °C for 40 min, increased at 1 °C/min to 160 °C and kept at this temperature for 10 min, then increased at 2 °C/min to 180 °C and kept for 20 min, increased to 220 °C at 2 °C/ min and maintained for 10 min. The temperature of injector and FID was 270 °C and 280 °C, respectively. Identification of the peaks was made by comparison with retention times of methyl esters of known oils and of pure esters as reference for aromatic and dibasic acids.

2.8. Resin composition by HPLC. Resin extract and F-Un fraction were injected as 10 mg/mL solutions in acetone into an HPLC Ultimate 3000 (Thermofisher Scientific, Waltham, MA, USA) comprising: Rheodyne injection valve (20 µL loop), Luna C8 column (Phenomenex, France, pore size 3 µm, length 150 mm, diameter 4.6 mm), UV-DAD and CAD detectors. The solvent was acetonitrile-water from 40:60 for 60 min, increased to 75:25, kept for 15 min, then increased to 100:0 and kept for 35 min (flow rate 1.0 mL/min). Guayulins and argentatins peaks were identified according to [20] and quantified as percentage of uncorrected peak area based on total chromatogram area.

2.9. Statistical analysis. All assays were performed in triplicate and the results are expressed as mean values ± standard deviation (SD). The effect of the extraction method on yields was analyzed statistically using SPSS software. Data were tested by analyses of variance ANOVA and evaluate significant difference by LSD at the P=0.05 level. In Tables, values within the same row having the same or without superscript are not statistically different ($p>0.05$).

3. RESULTS SECTION

The main objective was to investigate a way for extracting resin from guayule biomass or bagasse according to the scheme in Fig. 1 that could later become a "green" and efficient process for extracting resin components above laboratory scale. According to [42], leaves were manually separated from freshly harvested shrubs, yielding biomass mainly containing branches. This biomass was processed with an aqueous ammoniated phase for extracting PI under latex form [42, 43]. After this step the solid named bagasse was dried to a water content lower than 10% which allows long storage time. The biomass and bagasse were powders passing the 0.5 mm mesh. Prior to SC-CO_2 extraction, a portion of each sample was submitted to our reference method, based on multiple steps extraction with acetone by ASE (accelerated solvent extraction) under pressurized conditions, for accessing to the resin content by gravimetry [44] as described in the Experimental Section. According to this method, the content of crude GRe ranged between 5.2 and 8.7%-dw (based on dry weight of starting biomass or bagasse).The remaining part of the sample was submitted to SC-CO_2 extraction under various conditions, yielding a resin extract, also named GRe although the composition may differ from the one obtained through the reference ASE-acetone method. The extract was weighed after complete solvent removal and analyzed. This allowed to evaluate the efficiency of a given set of experimental conditions in terms of resin yield. In addition, these extracts were also analyzed in order to evaluate the selectivity towards some classes of resin components.

As already stated, the chemical composition of resin that can be extracted from guayule biomass and bagasse is rather complex. Among the reported methods for investigating the chemical composition of GRe, the simplest one is based on direct HPLC analysis on a reverse phase column, as detailed by [21]. However, chromatograms were quite complex; this is why, like Schloman et al. (1983) [20], we also followed another way, based on splitting carboxylic acid esters, said to include fatty acid esters like triacylglycerols -but probably phospholipids or non-glycerol esters like waxes- and also aromatic acid esters of germacrene - sesquiterpene alcohol- called guayulins (a family of compounds, including isomers). This procedure which involves strong basic conditions yields a mixture of soaps soluble in water or in alcohol-water called saponifiable fraction (F-Sap) and of compounds soluble in diethyl ether called unsaponifiable fraction (F-Un), like alcohols freed by the saponification reaction, and other compounds already present in the initial GRe. Extraction of F-Un was applied to GRe according to [20, 45]. This procedure then allowed investigating separately each fraction, towards -to our knowledge- the first insight on the selectivity of SC-CO_2 and ASE-acetone extraction methods regarding guayule resin components.

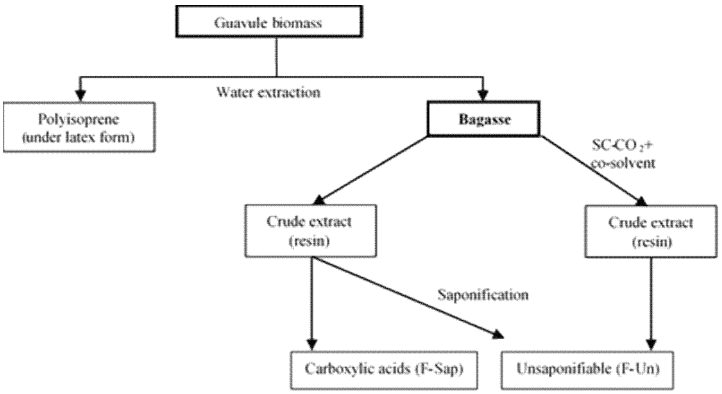

Figure 1. Processing flow chart of guayule samples.

3.1. Effect of reaction conditions on yield of extracted resin under SC-CO_2 conditions.

3.1.1. Extraction with SC-CO_2 without co-solvent. From the bibliography about the extraction of guayule resin summarized in the Introduction section, one concludes that SC-CO_2 alone has not been widely investigated as a selective solvent for PI or resin, in comparison to organic solvents in usual methods like ASE and Soxhlet. Nonetheless SC-CO_2 alone was claimed as a solvent of PI and resin in a patent [33].

When starting the present study, trials were done under softer conditions assumed to be acceptable for operating at larger scale: a moderate CO_2 flow rate 34.4 g /min; T 33 °C, close to ambient (still above SC point); P 250 bar, close to the limit of available extraction equipment. A relatively long extraction time of 60 min was chosen in an attempt to recover almost all extractible matter from the solid. Using SC-CO_2 alone under these experimental conditions, only traces of resin (0.003±0.001%) were obtained.

Need to mention that [33] who obtained a substantial amount of extract, operated for a shorter time (30 min) but under quite harsh conditions i.e. high CO_2 flow rate (3 L/min) and

pressure (345 bar), which would be quite costly for large scale production. In addition T was set at 60 °C, possibly contributing to degrading known unstable resin components [20, 21]. The yield of resin (~3%) was said to be much lower than the one obtained at higher T and P, i.e. under even stronger conditions. Last, this extract also containing an equivalent amount of PI would require further separation and purification steps. In addition to above differing operating conditions, differences between the starting solids may also play a role (particle size, moisture content, pretreatment of the starting biomass, in addition to genotype, plant age and harvest season), and thus explain this apparent discrepancy between results. Anyhow, our result did not play in favor of further investigating pure SC-CO_2. Therefore co-solvents were chosen and investigated: acetone (the current solvent in our reference ASE method) and ethanol which is a safe and biobased industrial feedstuff; both were also tested by [38, 40, 41] for pretreating guayule bagasse prior to ethanol production.

3.1.2. Extraction time with co-solvent. Extraction time was investigated with acetone over the 30 – 120 min range (Fig. 2); the yield at 30 min was somewhat lower than the one found at 60 min, but by far not half the value; this means that after 30 min the extraction has already entered the stage in which it is limited by diffusion of the solutes within the vegetal matrix [46], and this is why the extraction rate is quite slow; indeed above this 60 min threshold, further increasing the flow-rate of co-solvent does not result in any visible gain. Therefore the extraction time was then kept constant at 60 minutes for all trials in this study.

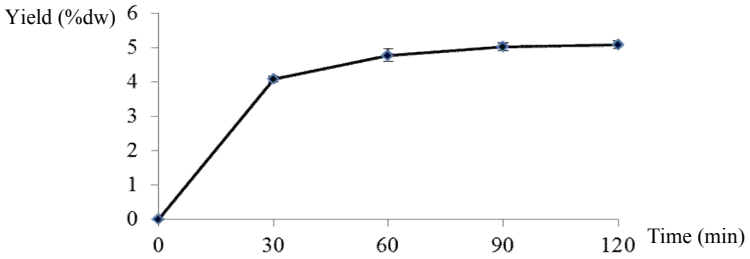

Figure 2. Influence of extraction time on yield of resin extracted under SC-CO_2 conditions**.**

Bi-Fr 07/2012-35m; SC-CO_2-acetone: co-solvent 3 mL/min, CO_2 34.4 g/min, P 250 bar, T 40 °C, extraction time 1 h. ASE-acetone: GRe 5.2+0.7%.

Figure 3 shows the effect of co-solvent flow rate under conditions same as those used when checking with pure CO_2 (CO_2 flow rate 34.4 g /min, T 33 °C, P 250 bar, extraction time 60 min). The extraction yield is still low when the co-solvents are injected at flow rate 0.32 L/min, but it increases until flow rate reaches 3.0 mL/min and then stays constant within experimental error. Ethanol clearly displays a better co-solvent power than acetone, with yields respectively of 9.6 and 6.3% based on dry extract weight and dry biomass weight. These results do not match exactly those reported by [33]. These authors worked in the presence of acetone and ethanol but under differing conditions as above discussed (CO_2 flow rate 3 L/min, P 5000-7500 psi, T 60-80 °C, extraction time 45 min); they concluded to higher yield and selectivity for resin with the former solvent (4.8% and 3.8% of feedstock respectively). Need to mention that under conditions not far from those applied in Fig.3, our reference ASE-acetone method (T 40°C, P 103 bar, total extraction time 60 min) yielded only 5.2%, thus less than above mentioned 6.3% with SC-CO_2-acetone (T 33 °C, P 250 bar, extraction time 60 min). The main difference between trials lies in the applied pressure 103 and 250 bar respectively, and of course in the presence of CO_2 in the last case.

A first conclusion is that the GRe content in a given guayule biomass which is computed -in this work and others as well- from the ASE-acetone extract is in fact quite dependent on the extraction method even with a given solvent, namely acetone. This is of course acceptable because "resin" is a broad name given to mixtures of a wide panel of classes of extractible compounds in this biomass, and extractability of each class may depend on operating conditions. However Suchat [35] who optimized the extraction of GRe with acetone both with Soxhlet and ASE, did not find a statistically significant difference, although these two methods were not operated exactly at same T and P. Thus the difference found in the present study is likely to come from the presence of SC-CO_2 which enhances the extraction yield compared to pure acetone. Indeed it is well known that: dissolved CO_2 decreases by an order of magnitude the viscosity of the oil to be extracted; CO_2 displaces oil when dissolving, entrains oil when depressurizing, and may rupture cell walls by swelling [47]. These authors used a small amount of CO_2 in a process called gas assisted mechanical expression (GAME) of oilseeds in order to save SC-CO_2, by not using it as a solvent but rather playing with its solubility inside the oil phase. We could also consider that these phenomena contribute to here observed results regarding the positive effect of SC-CO_2.

From this set of results we also concluded that neat SC-CO_2 in the region not far from the critical point is not suitable for extracting a substantial amount of resin, and that a co-solvent, like acetone or ethanol, is required.

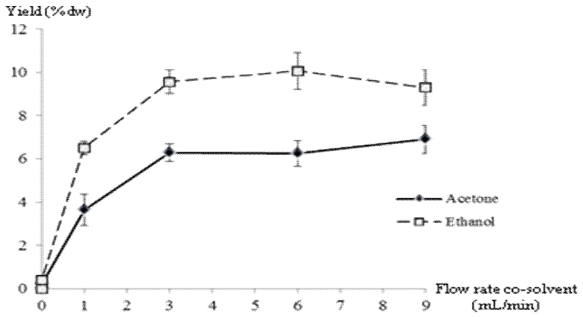

Figure 3. Influence of flow rate of co-solvent on yield of resin extracted under SC-CO_2 conditions.

Bi-Fr 07/2012-35m; SC-CO_2-ethanol and SC-CO_2-acetone: CO_2 34.4 g/min, T 33 °C, P 250 bar, extraction time 1h. ASE-acetone: GRe 5.2±0.7%.

3.1.3. Flow rate of CO_2, pressure, temperature with co-solvent. Regarding operating cost, in addition to handling the co-solvent, CO_2 consumption is also an important parameter together with P, although being recycled, because of the energy consumed by the compression step. Over the quite wide range of tested flow rate of CO_2 with ethanol under above conditions (ethanol flow rate set at 3.0 mL/min), Fig. 4 shows a maximum at 34 g/min (or between 34 and 51 g/min), and no need to work at higher flow rate of CO_2, which is rather advantageous. Regarding the influence of P (Fig, 5), with ethanol as co-solvent there is a flat from 100 to 200 bar and then a substantial and constant increase until 300 bar, i.e. the maximum affordable by the SCF unit, with a fairly high yield (12.1%). Acetone behaves in a different way: when increasing P

there is a slight increase until 200 bar and then the yield remains constant at an average 9.1%. Worth noting that over the whole range of tested P, ethanol allows always the highest yield compared to acetone. From this it looks that working with ethanol should be preferred.

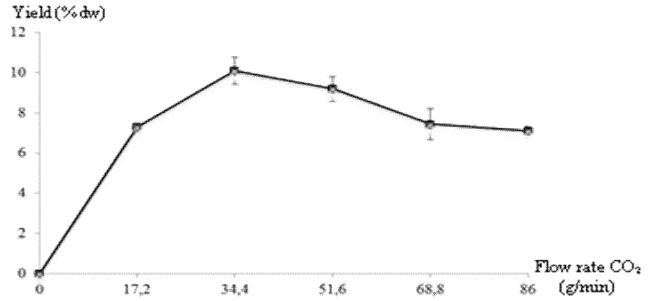

Figure 4. Influence of CO_2 flow rate of co-solvent on yield of resin extracted under SC-CO_2 conditions. Bg-Sp 04/2012-31m; SC-CO_2-ethanol 3 mL/min, P 250 bar, T 33°C, extraction time 1h. ASE-acetone: GRe 6.9±0.5%.

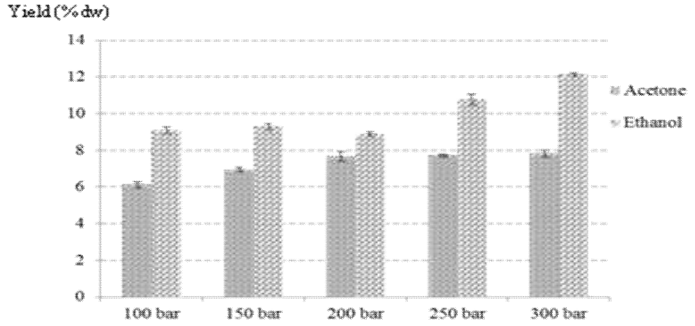

Figure 5. Influence of pressure on yield of resin extracted under SC-CO_2 conditions. Bg-Sp 04/2012-31m; SC-CO_2-ethanol and SC-CO_2-acetone: co-solvent 3 mL/min, CO_2 34.4 g/min, T 33 °C, extraction time 1h. Ase-acetone: GRe 6.9±0.5 %.

The last investigated parameter was extraction T, although we limited the variation between 33 and only 80°C, because of the known low thermal stability of resin components [20]. Here again (Fig. 6) the co-solvents do not act in the same way: with ethanol there is a clear maximum yield of 12.0% between 45 and 60 °C, whereas acetone gives a slight –statistically significant- maximum at 60°C, but in practice the co-solvent power of acetone is almost T independent over the tested range. Here again the maximum yield with the later solvent (7.0%) is much lower than the one given by ethanol (12.1%), and close to the reference ASE-acetone method (6.8%).

From these results, ethanol displays definitely a higher co-solving power than acetone. At present suitable conditions are the following: with ethanol [flow rate 3.0 mL/min, T 45-60 °C, P 300 bar], with acetone [flow rate 3.0 mL/min, T 33°C (close to SC T), P 200 bar], at same CO_2 flow rate (~40 g/min). Worth noting that extraction time was 1h in all experiments; but it can be set at 30 min with very low yield loss, placing the CO_2 consumption at ~1 kg / 0.01 kg of biomass or 1kg/g of extract. Still this consumption could be improved by investigating in detail the influence of time, when optimizing the extraction conditions. These conditions look compatible with an industrial application. But in addition to the yield, before making a choice between both the co-solvents, extract composition should also be taken into account. This is the aim of the following section.

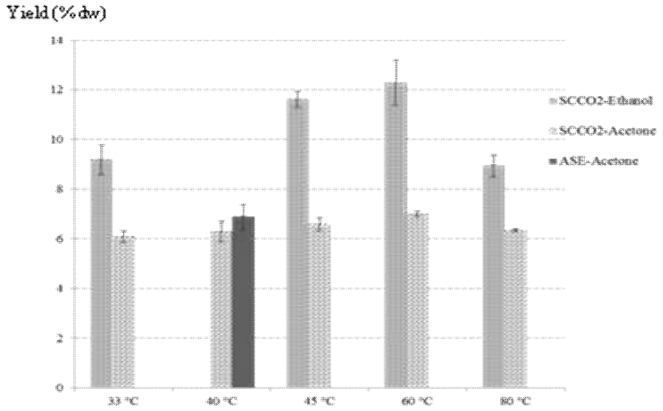

Figure 6. Influence of temperature on resin yield extracted under SC-CO_2 or ASE-acetone. Bg-Sp 04/2012-31 m; $SCCO_2$-ethanol and SC-CO_2-acetone: co-solvent 3 mL/min., CO_2 51.6 /min, P 250 bar, extraction time 1h. ASE-acetone at 40 °C.

3.2 Effect of SC-CO_2 conditions on the selectivity towards resin components

In the previous sections the influence of experimental parameters on extraction was investigated in terms of total yield (GRe %-dw of starting biomass or bagasse). However, the value of these extracts depends on their use and therefore on their chemical composition.

First, GRe extracts were analyzed trough FTIR spectrometry for detecting the presence of PI that could have been extracted together with resin components; this was based on a calibration equation obtained by spiking a resin extract with known amounts of a standard sample of PI [35]. As a representative example, PI content in extracts from one of above samples (Bi-Fr 07/2012-35m) under selected conditions (co-solvent 3 mL/min, CO_2 34.4 g/min, P 250 bar, T 33 °C, time 1 h) ranged from 0.04±0.01% (SC-CO_2-acetone) to 0.07±0.01% (SC-CO_2-ethanol). Therefore it can be concluded that, under investigated conditions, both the co-solvents with SC-CO_2, shows a very low extraction capability for PI, thus enabling a high selectivity for resin. Concerning the reference ASE-acetone method, with same biomass the PI in GRe was 0.19±0.03%; this last result is consistent with Suchat [35] who also found less than 1% of PI. From this it can be considered that extracts from the present study are "true" resin extracts, not polluted by significant portions of PI.

As described in the Experimental Section, a procedure derived from the standard fats and oils analysis [20] was adapted and applied to here obtained GRe. This procedure yields a mixture of soaps soluble in water (saponifiable; F-Sap) and of compounds soluble in diethyl ether (unsaponifiable; F-Un), like alcohols freed by the saponification reaction and other compounds already present in the initial GRe. This then allowed investigating separately each fraction, namely F-Sap and F-Un; the last was derived into methyl ester prior to GC analysis, in addition to direct HPLC analysis of the initial GRe. These analytical procedures allows discussing the influence of process parameters on extraction selectivity, at two levels (i) the ratio of these saponifiable and unsaponifiable fractions, and (ii) the chemical composition of extracts. The comparison is made for extraction trials performed under same conditions for both co-solvents, and within above defined range of partially SC optimized conditions.

3.2.1. Unsaponifiable and saponifable fractions. The selectivity for unsaponifiable and saponifiable compounds was defined as the ratio of %-weight: F-Un / F-Sap. When operating at the lowest temperature (33°C) and CO_2 flow rate (34.4 g/min), Table 1 shows that with both ethanol and acetone as co-solvents, F-Un amounts 53-55%, clearly higher than F-Sap (~40%), resulting in a selectivity (F-Un / F-Sap) of 1.3-1.4, thus in favor of the former fraction.

Table 1. Influence of co-solvent on selectivity towards unsaponifiable and saponifiable fractions in resin extracted under SC-CO_2 conditions and with ASE-acetone reference method

Extraction method	Unsaponifiable		Saponifiable		F-Un/F-Sap	Other (in water phase)
	(%-dw resin)	(%-dw biomass)	(%-dw resin)	(%-dw biomass)		(%-dw resin)
SC-CO_2-ethanol	55.5±2.0	5.31	29.9±0.5[b]	3.81	1.39	5.5±0.9
SC-CO_2-acetone	53.5±2.3	3.36	39.2±1.4[a]	2.54	1.33	5.2±1.9
ASE-acetone	53.0±1.4	2.76	41.6±0.8[a]	2.08	1.33	5.7±1.1

Bi-Fr 07/2012-35 m; SC-CO_2-ethanol and SC-CO_2-acetone: co-solvent 3 mL/min, CO_2 34.4 g/min, P 250 bar, T 33 °C, extraction time 1 h. ASE-acetone: reference method at 40 °C.

The ASE-acetone method gives a similar pattern. But when working under different conditions (Table 2), at higher flow rate (51.6 g/min of CO_2) and higher T (45 and 80 °C), acetone as co-solvent yields an equivalent portion of both the fractions close to 47-48% corresponding to a selectivity of ~1.0. Ethanol on its side keeps a slightly lower content of F-Un (42-45%) but with a marked decrease of F-Sap, resulting in a high selectivity especially at 50 °C (1.3). Need to mention here that with ethanol a substantial portion of the extract (~20%) still remains in the water phase after the two extraction steps with diethyl ether (extraction under basic and then acidic pH). All this marks the strong influence of the co-solvents on extraction selectivity, ethanol enabling to recover more oxygenated compounds in the resin fraction (water soluble products and sesquiterpenes as seen hereafter) than does acetone. Therefore the composition of the carboxylic acids and of the sesquiterpene groups is investigated separately in the following sections.

Table 2. Influence of temperature on selectivity towards F-Sap and F-Un fractions in resin extracted under SC-CO_2 conditions with co-solvents acetone and ethanol and with ASE-acetone reference method

Solvent	T (°C)	Unsaponifiable F-Un		Saponifiable F-Sap		F-Un/F-Sap	Other (in water phase)
		(%-dw resin)	(%-dw bagasse)	(%-dw resin)	(%-dw bagasse)		(%-dw resin)
Ethanol	45	45.5±2.7[ab]	5.29	35.6±1.0[b]	4.12	1.28	18.9±1.4[a]
	80	42.1±2.0[b]	3.77	37.0±1.2[b]	3.31	1.14	20.9±1.1[a]
Acetone	45	48.5±2.3[a]	3.20	45.6±1.7[a]	3.01	1.06	5.9±1.1[b]
	80	47.1±2.2[a]	3.00	47.2±1.1[a]	3.00	1.00	5.7±1.2[b]

Bg-Sp 04/2012-31m; SC-CO_2-ethanol and SC-CO_2-acetone: co-solvent 3 mL/min, CO_2 51.6 g/min, P 250 bar, extraction time 1h.

3.2.2. Composition of carboxylic acids in the saponifiable fraction. From the bibliography, the compounds making F-Sap result from the splitting of carboxylic esters, yielding (i) soaps coming from glycerol-containing lipids (triacylglycerols, phospholipids) and from other fatty acid esters like waxes, then extracted as free fatty acids, and (ii) soaps coming from guayulins, esters between a sesquiterpene and aromatic carboxylic acids. All these diethyl ether-soluble carboxylic acids (F-Sap) were analyzed as methyl esters by GC. When considering only the group of fatty acids in a GRe sample obtained by the reference method (ASE-acetone), Table 3 shows in the upper line the composition for an extract from a sample of biomass harvested in Spain (Bi-Sp 07/2012-34m). The main fatty acid is linolenic acid (C18:3) then comes C15:0 (pentadecanoic), making more than 50%. Quite far away come C16:0 (palmitic) and C18:2 (linoleic). This table allows to compare the influence of growing place (with same genotype AZ-2) on the composition of fatty acids and it appears that these parameters do have a strong effect. Indeed Bi-Fr 07/2012-35m contains mainly heneicosanoic acid (C21:0), also behenic (C22:0), and myristic (C14:0) –all being saturated acids- whereas C18:2 and C18:3 were not detected.

Considering only components each accounting for more than 1%, worth noting that these lipids cover a wide range of chain length, from C14 up to C22. For comparison, only five components have been reported yet for genotypes grown in the USA and in Mexico, in the C16 – C18 range, instead of here detected nine fatty acids including saturated long chain fatty acids which were not previously reported. Also, in literature data palmitic acid is much lower than linoleic (about one half or one tenth), therefore the composition of these oils was rather close to that of soybean oil, in spite of large variations, data from literature agree on the linoleic type of these lipids –being close to cotton or soybean oils- however with C18:3 being second ranked. But fatty acid profiles obtained under present work are difficult to classify among main common food oils: one is rich in 18:3, but much less than linseed oil, while the other contains primarily long chain acids (>C20). From this one concludes that the fatty acid composition of lipids present in guayule resin, which has not been extensively investigated, is highly variable, and reflects not only genotypes but also growing conditions.

Table 3. Fatty acid composition of guayule resin from various cultivars and vegetable oils

Fatty acids (% total fatty acids)	C14:0	C15:0	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	C21:0
Guayule									
Bi-Sp 07/2012-35m[a]	0.0	27.1	16.9	6.0	6.5	11.7	26.4	2.9	2.5
Bi-Fr 07/2012-35m[b]	16.9	0.0	2.7	2.0	1.6	0.0	0.0	49.9	27.0
593[c]	-	-	21	3	7	51	17	-	-
593[c]	-	-	13	1	5	57	32	-	-
CIQA[c]	-	-	7	6	6	65	17	-	-
CIQA[c]	-	-	10	1	10	64	14	-	-
Vegetable oils									
Cotton	0.7	-	24	3	17	52	0.2	-	-
Soybean	0.1	-	11	4	23	53	8	-	-

[a,b] This work: GRe extracted by ASE-acetone, then saponified for recovering F-Sap ; [c] Guayule cultivars 593 and CIQA [20].

Now checking the influence of co-solvent on the composition of extracted F-Sap (the whole fraction, not only fatty acids), one should remind that in addition to fatty acids this fraction also contains other carboxylic acids, such as aromatic ones derived from the guayulins. In Table 4, with another sample of biomass, ethanol gives an extract having a composition very close to that obtained with ASE-acetone: aromatic acids make 68% of all acids, compared to only 51% in the SC-CO_2-acetone extract. Surprisingly the second ranked aromatic acid –p-anisic acid- is not influenced by the type of co-solvent, and as a consequence the variation of aromatic acids is governed only by, which is lowered with SC-CO_2-acetone compared to SC-CO_2-ethanol (48.9 and 66.4% respectively). Consequently the percentage of fatty acids is higher with SC-CO_2-acetone. In this table (contrary to Table 3, but different biomass samples) the group of saturated fatty acids as a whole is the main component of the fatty acid group, behenic making a noticeable contribution to the difference between both co-solvents. Having in mind that (i) ethanol provides a higher yield of resin, and (ii) it also brings a higher proportion of unsaponifiable, one concludes that SC-CO_2-ethanol enables extracting more sesquiterpenes than does SC-CO_2-acetone. Table 4 shows that under selected conditions, SC-CO_2-acetone and ASE-acetone lead to quite differing compositions, whereas this last solvent and SC-CO_2-ethanol give similar extracts.

Table 4. Influence of SC-CO_2 co-solvent on carboxylic acid composition of F-Sap

Carboxylic acid (% F-Sap)	SCCO$_2$-ethanol	SCCO$_2$-acetone	ASE-acetone
Aromatic	68.6	51.1	68.0
P-Anisic	2.2±0.1	2.2±0.0	2.4±0.8
Cinnamic	66.4±1.2[a]	48.9±1.0[b]	65.6±2.6[a]
Unsaturated	1.0	2.2	0.24
Oleic (C18:1)	1.0±0.2[b]	2.2±0.2[a]	0.24±0.05[c]
Saturated	19.6	27.4	14.6
Myristic (C14:0)	3.8±0.5[a]	2.0±0.2[b]	2.5±0.4[ab]
Palmitic (C16:0)	1.8±0.1[b]	3.6±0.1[a]	0.4±0.0[c]
Stearic (C18:0)	0.7±0.0	0.5±0.0	0.3±0.1
Eicosanoic (C20:0)	7.4±0.1[b]	10.1±0.0[a]	7.4±0.4[b]
Heneicosanoic (C21:0)	6.0±0.0[b]	11.2±0.1[a]	4.0±0.1[c]
Others	10.7	19.2	17.1

values within the same row having the same or without superscript are not significantly different (p>0.05); Bi-Fr 07/2012-35 m; SCCO$_2$-ethanol and SC-CO_2-acetone: co-solvent 3 mL/min., CO_2 34.4 g /min, P 250 bar, T 33 °C, extraction time 1h. ASE-acetone: extraction with acetone at 40 °C.

In Table 5, with a bagasse and at higher extraction T, polyunsaturated fatty acids were extracted. Here the effect of co-solvent is not marked, although at 80 °C the percentage of aromatic acids is again higher with ethanol compared to acetone. Table 5 also shows the influence of T on the content of aromatic acids and also on the balance between saturated and unsaturated fatty acids, the content of unsaturated group with ethanol falling from 26.3 to 6.6% between 45 and 80 °C respectively.

Therefore it is possible to play with co-solvent and temperature to act on the extraction of the fatty and aromatic acids. These last acids are linked to the sesquitepene (partheniol); in other words ethanol at 80°C likely allows increasing the yield of guayulins. The next section deals with these compounds.

3.2.3. Oxygenated terpenes. In regard to the large number of scientific articles about guayule agronomy and rubber, literature data about resin composition are scarce. Indeed there is even no clear definition of the so-called resin, and achieving a detailed knowledge of these complex natural extracts was out of the scope of this process-oriented preliminary work; however the study was limited to the main components of the oxygenated terpenic fraction (guayulins and argentatins), which have attracted most interest from analytical chemists [20, 48], owing to their potential uses. In this work these were analyzed by direct injection in a reverse phase HPLC column, following the method described by [21]. In Table 6, dealing with the influence of P on resin composition, the total yield of these two groups of bioactive compounds (guayulins and argentatins) which is at minimum at 100 bar (4.0%-dw of starting biomass), then increases gradually to 4.8%-dw until 250 bar. Argentatins are the main extracted group, about twice the total extracted guayulins over the whole P range.

Their increase between 100 and 200 bar, from 2.6 up to 3.4%-dw, is the main contribution to the better yield noted for total terpenics; then their yield stays constant until 300 bar. Actually this effect is due to argentatin A which shows an increase of ~40% whereas the yield of argentatin B goes up of less than 20%.

For the other group of terpenics the case is quite different: P does not look to act on total yield of guayulins within experimental error. The ones containing cinnamic acid, A and C, are by far the main guayulins. When the percent of A goes down, showing a marked minimum at 200bar, C goes clearly up, and the total stays constant at 0.9-1.1%-dw. This plays in favor of C resulting from the isomerization of A, if not thermal as shown by [21] owing to the quite low operating T (33°C), here possibly due to the effect of P. This table highlights the influence of P with ethanol as co-solvent, for example the minimum yield of guayulins found at 200 bar, while the yield of argentatins is maximum. Cross checking the results from Tables 2 and 6, corresponding to the same bagasse sample, for trials performed with ethanol, and under same P (250 bar) and close T (45 and 33 °C respectively), shows that (i) F-Un containing the terpenics (argentatins, and partheniol derived from guayulins after having separated the corresponding aromatic acids, being the main fraction; against F-Sap; Table 2) and (ii) argentatins making by far the main part of identified terpenics (Table 6), therefore the resin extract contains mainly argentatins, especially the isomer A.

Thus these results show the influence of SC-CO_2 extraction conditions (P with co-solvent ethanol in present case) on the detailed composition of GRe regarding oxygenated terpenes, and that it should be possible to maximize the yield of argentatins and therefore the purity even in the crude resin extract. This plays in favor of optimizing the selective extraction of these valuable terpenics with SC-CO2, coupled to the detailed chemical composition of the whole fraction which has been shown to be rather complex [20, 49, 50].

Table 5. Influence of temperature and co-solvent on carboxylic acid composition of F-Sap.

Carboxylic acids (% F-Sap)	Ethanol		Acetone	
	45 °C	80 °C	45 °C	80 °C
Aromatic	41.8	56.4	44.6	50.9
P-Anisic	2.4±0.0^{c}	3.8±0.1^{a}	2.0±0.0^{d}	3.3±0.1^{b}
Cinnamic	39.4±0.4^{d}	52.6±0.5^{a}	42.6±0.3^{c}	47.6±0.5^{b}
Unsaturated	26.29	6.6	30.0	22.3
Oleic (C18:1)	2.8±0.1^{a}	1.5±0.0^{c}	2.8±0.1^{a}	2.4±0.2^{b}
Linoleic (C18:2)	16.5±0.2^{b}	3.6±0.1^{d}	19.5±0.3^{a}	14.2±0.1^{c}
Linolenic (C18:3)	6.9±0.3^{b}	1.5±0.0^{d}	7.7±0.3^{a}	5.7±0.4^{c}
Saturated	7.0	6.3	19.9	21.9
Palmitic (C16:0)	6.3±0.1^{b}	4.6±0.1^{c}	6.9±0.1^{a}	7.1±0.1^{a}
Stearic (C18:0)	0.7±0.0^{c}	1.7±0.1^{a}	1.7±0.0^{a}	1.2±0.1^{b}
Eicosanoic (C20:0)	-	-	1.9±0.1	2.0±0.0
Heneicosanoic (C21:0)	9.2±0.2^{a}	7.6±0.1^{b}	6.7±0.0^{c}	7.1±0.2^{bc}
Behenic (C22:0)	6.6±0.1^{b}	11.7±1.3^{a}	2.7±1.1^{d}	4.5±1.0^{c}
Others (~)	9.0	11.0	7.3	7.3

values within the same line having the same or without superscript are not statistically different (p>0.05); Bg-Sp 04/2012-31m; SC-CO2-ethanol and SC-CO2-acetone: co-solvent 3 mL/min, CO_2 51.6 g /min, P 250 bar, extraction time 1h.

Table 6. Influence of pressure on composition of resin extracted with SC-CO_2-ethanol.

% (dw)	Pressure (bar)				
	100	150	200	250	300
Total guayulins	1.3	1.2	1.2	1.3	1.4
A	0.5±0.0^{a}	0.5±0.0^{a}	0.3±0.0^{b}	0.3±0.0^{b}	0.5±0.0^{a}
B	0.1±0.0^{a}	0.1±0.0^{a}	0.0±0.0^{b}	0.1±0.0^{a}	0.1±0.0^{a}
C	0.6±0.0^{a}	0.5±0.0^{b}	0.6±0.0^{a}	0.6±0.0^{a}	0.6±0.0^{a}
D	0.2±0.0^{b}	0.3±0.0^{a}	0.3±0.0^{a}	0.3±0.0^{a}	0.3±0.0^{a}
Total argentatins	2.6	3.2	3.4	3.4	3.2
A	1.6±0.0^{c}	2.0±0.0^{b}	2.2±0.0^{a}	2.1±0.0^{ab}	2.0±0.0^{b}
B	1.1±0.0^{c}	1.2±0.0^{b}	1.3±0.0^{a}	1.3±0.0^{a}	1.3±0.0^{a}
Guayulins+argentatins	4.0	4.4	4.6	4.8	4.7

Values within the same line having the same superscript are not statistically different (p>0.05); standard deviation is 0.05% or lower; Bg-Sp 04/2012-31m; SC-CO2-ethanol: flow rate ethanol 3 mL/min, CO_2 34.4 g /min, T 33 °C, extraction time 1h.

4. CONCLUSIONS

These results provide a deeper insight in the field of resin composition and resin extraction from guayule biomass and bagasse under SC-CO_2 conditions, complementing scarce information from literature. Under investigated experimental conditions a co-solvent is required, and ethanol appears to be an acceptable option compared to acetone taken as co-solvent with SC-CO_2 but also as sole solvent, while it has been widely used in research work during the last 15 years. This work emphasizes on the large influence of extraction conditions (i) on total extraction yield, and (ii) on the resin composition investigated through various ways, all showing the presence of potentially valuable bioactive guayulins and argentatins (total ~35% of crude resin extract). The results show also the suitable working range for main experimental parameters (CO_2 consumption less than 1 kg/g of

extract, ethanol proportion (~1/10), moderate temperature and contact time, affordable pressure), which look compatible with large scale production, especially with ethanol which gave an extract yield higher than acetone in all investigated cases. Incidentally this work has shown the high variability of the lipid fraction in terms of fatty acid composition, widening the panel between C14 and C22 compared to literature data.

This work opens the way for further optimizing a low environmental impact multiple step extraction process for the guayule biomass or bagasse left after water-based rubber extraction as low-allergy latex. The choice of co-solvent should be made after having found conditions bringing not only the yield but also the selectivity high enough for obtaining fractions meeting the required standard in order to bring them on the market of renewable feedstocks. We do believe that understanding how extraction selectivity works with this highly complex vegetal matrix is a key point for setting an efficient process, and that SC fluids, which allows fine tuning extraction conditions, bring strong advantages for achieving this task.

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