Chemical composition and antimicrobial activity of *Satureja montana* byproducts essential oils

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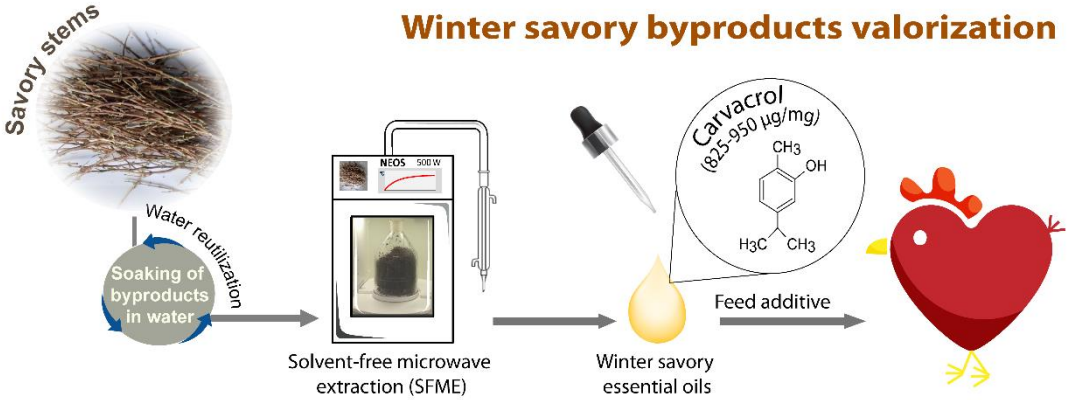

Keywords

Winter savory; aromatic plants; solvent-free microwave extraction; hydrodistillation; carvacrol; minimal inhibitory concentration

Abstract

Satureja montana is a worldwide consumed aromatic plant whose essential oils (EOs) are used as spice and preservative by food industry. Only the leaves are marketed, generating a large amount of stems as byproducts. It is possible that the EOs present in these byproducts represent a source of compounds with antimicrobial activity, as observed for the leaves. In this work, dried *S. montana* byproducts were used to extract EOs. Although differences were observed for the harvest year (0.42 ± 0.11 mg/g in 2016 and 0.14±0.07 mg/g in 2017), monoterpenoids were the major components, accounting for 84.4 to 97.6%, being carvacrol the most abundant component (825 – 950 µg/mg). Sesquiterpenoids accounted for 0.3 to 0.5%. Similar EOs yield and composition were obtained using solvent-free microwave extraction (16 min using 100 g of *S. montana* stems, previously, soaked in water and drained) or hydrodistillation (120 min using 1000 mL of water/ 100 g of dried *S. montana stems*). The *in vitro* minimal inhibitory concentrations (MICs) of *S. montana* EOs against *Escherichia coli* ATCC 25922, *Salmonella enterica* sv Anatum SF2, and *Staphylococcus aureus* ATCC 6538, which belong to pathogenic species with economic impact in poultry industry, were 225 µg/mL, 250 µg/mL, and 150 µg/mL, respectively. As these MICs are similar to the ones reported for carvacrol against the same or related strains, it can be concluded that carvacrol is the active compound in *S. montana* byproducts.

1. Introduction

Satureja montana (Winter savory), botanically included in the *Lamiaceae* family, is an aromatic plant frequently used as spice and as traditional medicinal herb (Jafari et al., 2016; Oliveira et al., 2011). The medicinal properties of *Satureja* plants, such as anti-inflammatory, analgesic, antidiabetic, anti-hypercholesterolemic and fertility effects, have been demonstrated, providing the plant an important role in the pharmaceutical industry (Jafari et al., 2016; Tepe and Cilkiz, 2016). These properties are due to several compounds, mainly those present in *Satureja* essential oils (EOs) derived from commercial parts of the plants (Skocibusic and Bezic, 2004). The EOs of *S. montana* possess strong terpenic, usually phenolic, character. *S. montana* EOs have a remarkable chemical composition variability that can be due to several factors such as plant development stage, ecologically different locations and genetic characteristics (Ćavar et al., 2008; Kustrak et al., 1996; Mastelic and Jerkovic, 2003; Milos et al., 2001). For example, carvacrol ranges from 84% (Kustrak et al., 1996) to only 0.4% (Slavkovska et al., 2001) and thymol follows the same trend, from 46.0% (Mastelic and Jerkovic, 2003) to trace amounts (Prieto et al., 2007). Depending on the plant, *p*-cymene (traces to 41.4%), α-pinene (0.2 - 20.7%), linalool (traces – 24.0%), borneol (1.75 – 12.2%), geraniol (0.1 – 22.3%) and ϒ-terpinene (0.2 – 15.9 %) also present a large variation (Ćavar et al., 2008; Djenane et al., 2011; Kustrak et al., 1996; Mastelic and Jerkovic, 2003; Oliveira et al., 2011; Slavkovska et al., 2001). Despite the variability of the chemical composition of *Satureja* EOs, these are generally recognized for their antibacterial, fungicidal, antiviral and antioxidant proprieties (Skocibusic and Bezic, 2004).

The wide range of bioactive properties of *Satureja* EOs makes them appealing to several applications such as: i) flavor agents in the food processing and cosmetic industry (Lerebour et al., 2016, 2014; Oliveira et al., 2011); ii) replacers of synthetic insecticides, fungicides, bactericides and nematicides for plant protection and against household pests (Chantraine et al., 1998; Kotan et al., 2010; Maedeh et al., 2011; Picard et al., 2012; Sarkhosh et al., 2018; Tozlu et al., 2011), iii) disinfection agents of microbial-contaminated industrial surfaces (Chorianopoulos et al., 2008; Lebert et al., 2007), iv) antibiofilm agents in dentifrice or mouthwashes formulations (Gursoy et al., 2009), v) natural preservative ingredients in food products (Alexa et al., 2018; Hashemi et al., 2012; Oliveira et al., 2011; Ozkan et al., 2007), vi) additives in films and coatings for active food packaging (Atarés and Chiralt, 2016; Choulitoudi et al., 2016; Kfoury et al., 2015; Nasiri et al., 2018), and vii) feed ingredients, since these EOs have recognized phytobiotic effects by reducing plasma cholesterol and triglycerides concentration and the levels of pathogenic bacteria, enhancing the immune status when used in poultry diets (Hashemipour and Kermanshahi, 2013; Masouri et al., 2017; Sadeghi et al., 2014).

Hydrodistillation is the extraction method most commonly used for commercial production of *Satureja* EOs. In recent years, new extraction techniques have been developed to shorten extraction time, reduce solvent consumption, improve extraction yield, enhance extract quality, prevent pollution, and reduce sample preparation costs (Wang et al., 2010). Within these new techniques is included solvent-free microwave extraction of EOs. This technique was applied to aromatic plants to obtain EOs (Bendahou et al., 2008; Lucchesi et al., 2004) and to industrial byproducts in general (Passos and Coimbra, 2017),

but never to the aromatic plant dried byproducts which is the form provided by the aromatic plant industry for this byproduct.

Satureja byproducts, constituted mainly by stems, represent 50-60% of the plant dry weight (Ferreira et al., 2012). Although they are a possible source of compounds with antimicrobial activity, as observed for the leaves, they are not yet exploited, being in general totally discarded as waste. In addition, environmental rules for industrial waste disposal have become stricter, thus emerging the concept of industrial ecology where wastes and byproducts are used as raw material for new products and applications within an industrial symbiosis (Cruz et al., 2018; Ferreira et al., 2014, 2018).

To evaluate the possibility of using *S. montana* stems as a source of compounds with antimicrobial activity, the present work studied the EOs volatile composition of these industrial crop byproducts and their potential as antimicrobial agent against three strains representative of pathogenic species (*Salmonella enterica*, *Escherichia coli* and *Staphylococcus aureus)* with economic impact in poultry industry. To fulfill this objective, the byproducts' EOs were extracted introducing the solvent-free microwave extraction (SFME) of the soaked in water and drained byproducts and comparing with hydrodistillation (HD) using a heating mantle and abundant water. Also for comparison of SFME, the stems EOs yield of *Thymus mastichina*, *T. vulgaris* and *T. fragantissimus* byproducts were also studied using this methodology. These agroindustry byproducts were selected as they are plants of the same botanic family of *S. montana,* but more extensively studied, presenting essential oils with potential antimicrobial activity. In addition, it was also taking into account the availability of the byproducts from the aromatic plant industry.

2. Materials and Methods

2.1. Plant material

Dried agroindustrial byproducts of *Satureja montana, Thymus mastichina*, *T. vulgaris* and *T. fragantissimus* were provided by Ervital - Infusões e Condimentos Biológicos, harvest in 2016 and 2017 from Serra do Montemuro (40º 58' N 07º 58' W and 1000 m of altitude), Portugal. These samples were transported in bags and stored in dark at room temperature.

2.2. Essential oils extraction

A NEOS apparatus from Milestone was used for solvent-free microwave extraction of EOs from moistened plant byproducts. This is a 2.45 GHz multimode microwave reactor with a maximum power of 900 Watts. *S. montana* dried samples (100 g of each individual harvest byproduct) were cut into small pieces and moistened by overnight soaking in 1 L of distilled water, at room temperature (Mellouk et al., 2016). The stems were drained and weighed, and the percentage of water that was stored intrinsically in the plant material was measured gravimetrically, assuring the minimum of 50% of moisture required for a solvent-free microwave extraction approach. The water not incorporated in the tissue was collected for further analysis. The soaked tissues were placed in the Pyrex extraction vessel and irradiated with different irradiation power (300 W, 500 W, and 700 W) under atmospheric pressure. For comparison, *Thymus mastichina*, *T. vulgaris* and *T. fragantissimus* byproducts were also moistened and EOs microwave extracted using the same procedures. The hydrodistillates were continuously collected and, for each fraction of 10 mL, were registered the experiment time and the vessel temperature (monitored by an infrared sensor).

The process was stopped when i) a decrease in the collection flow was denoted, or ii) 85% of the samples water was collected, or iii) it reached 102 ° C in the extraction vessel, avoiding to reach the burning point.

A hydrodistillation was also performed from *S. montana* byproducts (100 g of each individual harvest, cut into small pieces of 2 cm), using 1 L of distilled water. The distillate was collected when 250 mL were obtained, approximately after 2 h.

As the condensed vapors obtained from both hydrodistillation methodologies form an emulsion, the hydrodistillates were subjected to liquid-liquid extraction with 10 mL dichloromethane, three times, and dried over anhydrous sodium sulphate. After evaporation of the dichloromethane in a stream of nitrogen, the samples were stored in sealed vials at 4 °C. The extraction yield was determined by gravimetry. The water not incorporated in the tissue was also subjected to a liquid-liquid extraction, allowing to obtain the extract non recovered EO (NREO), in order to evaluate if some of the compounds found in the essential oil would be released during the samples hydration process.

2.3. Essential oils volatile characterization

The EOs obtained and NREOs were analyzed on an Agilent Technologies 6890N Network gas chromatograph (from Agilent Technologies, Inc., USA), equipped with a 30 m length, 0.32 mm of internal diameter and 0.25 μm of film thickness DB-FFAP mass spectrometry (MS) column from Agilent Technologies. Helium was used as carrier gas (1.7 mL/min) with the column pressure at 3.52 psi. The temperature program used was as follow: initial temperature was 60 ºC, with a linear increase of 2 ºC/min up to 180 ºC, followed by linear increase of 5

°C/min until 220 °C, remaining thus until the end of the run (70 min.). The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range 33–300 *m/z* in a 1 s cycle in a full scan mode acquisition. The injection was performed in splitless mode. Each EO was injected directly after dilution in dichloromethane (1:200), for quantification 0.496 mg of internal standard 2-undecanol were added prior analysis. The NREO was diluted in dichloromethane (1:30) and filtered with a 0.45 µm filter (after 0.991 mg of internal standard addition) prior to injection. Identification of volatile compounds was achieved by comparison of the GC retention times and mass spectra with those, when available, of the pure standard compounds. All GC mass spectra were also compared with the library data system of the GC–MS equipment (*Wiley 275*) and according to the compounds previously described for respective aerial plant EO. The quantification of the identified compounds were calculated based on GC peak areas, taking into account the concentration of 2-undecanol. The calculated response factor correlation between internal standard and eugenol (1.2033, R^2=0.9802) was applied to quantify the monoterpenoids. All other groups of compounds were quantified with the application of response factor of 1.0.

2.4. Antimicrobial activity

2.4.1. Microorganisms

The antimicrobial activity of EOs was evaluated using two Gram-negative bacterial strains (*Salmonella enterica* sv Anatum SF2, *Escherichia coli* ATCC 25922) and one Gram-positive bacterial strain (*Staphylococcus aureus* ATCC 6538). *S. enterica* sv Anatum SF2 was isolated from seagull feces on the island

of Berlengas (Peniche, Portugal) (Araújo et al., 2014) while *E. coli* ATCC 25922 and *S. aureus* ATCC 6538 were obtained from the American Type Culture Collection. These strains were cryopreserved in 20% glycerol at -80 °C and, during experiments, maintained on Luria-Bertani (LB) agar (NZYTech, Portugal) at 4 °C. Cultivation was on LB, LB agar media overnight at 37 °C.

2.4.2. Disc diffusion assays

Cell suspensions were prepared using fresh bacterial colonies and adjusted to turbidity of 0.5 McFarland. Mueller–Hinton agar (MHA; Oxoid, England) plates were inoculated with the aid of a sterile swab and the plates were allowed to dry. Sterile 6 mm filter paper discs (Liofilchem, Italy) were placed on the plates and immediately sterile water and EO was added (Table 1). Discs with sterile water or dimethyl sulfoxide (DMSO) were used as negative controls and commercial discs containing antibiotics (Oxoid, England) were used as positive controls, namely discs impregnated with chloramphenicol (30 µg), sulfamethoxazole/trimethoprim (25 µg), ciprofloxacin (5 µg) and gentamicin (10 µg). After 15 min at room temperature, the plates were incubated at 37 °C for 18-24 h. The diameter of the inhibition zone was measured in millimeters and the assay was carried out three times for each strain. The amount of EO added to each test disc was adjusted over several previous tests (successively lower quantities) for each microorganism. Table 1 describes in detail which quantities of EO were tested in each assay for each strain.

Table 1 – Amount of EO and controls used in each test (assay 1 and assay 2).

Microorganism	*Test discs*	*Negative control disc*	*Positive control discs*
Assay 1			
S. aureus ATCC 6538	0.44 mg EO	Sterile water	Ciprofloxacin Gentamicin
S. enterica sv Anatum SF2 / *E. coli* ATCC 25922	1.75 mg EO	Sterile water	Ciprofloxacin Gentamicin
Assay 2			
S. aureus ATCC 6538	1) 0.44 mg EO[a] 2) 0.33 mg EO[a] 3) 0.22 mg EO[a]	DMSO	Ciprofloxacin Gentamicin
S. enterica sv Anatum SF2 / *E. coli* ATCC 25922	1) 1.75 mg EO 2) 1.31 mg EO 3) 0.88 mg EO	Sterile water	Ciprofloxacin Gentamicin

[a]The EO solution was prepared in DMSO (43.8 µg EO/µL solution)

2.4.3. Microdilution assays

The minimal inhibitory concentration (MIC) values were determined using a broth microdilution assay (Djenane et al., 2011). Microorganism suspensions were prepared as described above. Aliquots of the EO under investigation, dissolved in 1% of DMSO, were first diluted to the highest concentration (500 µg/mL) to be tested and then serial two-fold dilutions were made in order to obtain the concentration range from 5 to 500 µg/mL in Mueller–Hinton broth (MH broth; Merck). The MIC was defined as the lowest concentration of the respective compound able to inhibit the growth of microorganisms. 96-well plates were prepared by dispensing 95 µL of nutrient broth and 5 µL of the microorganism suspensions into each well. Further 100 µL of the different concentrations of EO solutions were added into the wells. The last well contained 195 µL of DMSO:nutrient broth (1:194) in addition to 5 µL of the microorganism

suspensions, being used as negative control. Consequently, each well had a final volume of 200 µL. The plates were incubated at 37 °C for 18–24 h with a stirring of 200 rpm. Microbial growth in each medium was determined by measuring the optical density at 600 nm using the ELx 800 universal microplate reader (Biotek Instrument Inc, USA). Essential oils were tested three times against each organism.

2.5. Statistical analyses

Statistical analyses were performed in order to i) evaluate the effect of the harvest and extraction methodologies on the extraction yield and on each compound content of *S. montana* EOs and ii) compare the antimicrobial effects of *S. montana* byproducts EOs, being considered statistically significant when $p<0.05$. One-way analysis of variance (ANOVA) was performed followed by a multiple comparison test (Tukey`s HSD), using the GraphPad Prism version 5.00 for windows (trial version, GraphPad Software, San Diego California, USA).

3. Results and discussion

3.1. Solvent-free microwave extraction of EOs (SFME) from *S. montana* byproducts

As the studied byproducts are dried matrices, it was necessary their moistening before microwave extraction procedure. The moistening capacity of each dried byproduct is a requirement for the extraction efficiency. As can be seen in Figure 1a, *S. montana* byproducts have an average water absorption capacity of 153%, corresponding to a final moisture content of 60%. A very similar moistening behavior was observed for *Thymus* byproducts.

In order to investigate the effects of applied microwave irradiation power on hydrodistillation flow, extraction time and essential oil yield, three irradiation powers (300 W, 500 W, and 700 W) were evaluated. Using the same amount of moistened *S. montana* byproducts (253 g) in all experiments, a correspondent power density of 1.2, 2.0 and 2.8 $W.g^{-1}$ can be associated. Fig. 1b shows that microwave extraction flow strongly depends on the power applied: higher power increases the hydrodistillate flow both for *S. montana* and *Thymus* plants. The higher energy applied, the higher the hydrodistillate flow and, consequently, the volume collected per unit of time. Also, the extraction time increased inversely with the power applied, 37-62 min for 300 W, 16-30 min for 500 W, and 12-18 min for 700 W. Fig.1c shows that the irradiation at 500 W allowed to obtain a highest EO extraction yield then the irradiation at 300 W and 700 W, observed for all plant byproducts. When submitted to low irradiation power, *in situ* water molecules are less stimulated to rotate, generating a lower increase of pressure and temperature inside the cells, resulting in a smaller number of broken cells. As a consequence, part of the target molecules are not released into the extracellular medium, not being carried and recovered in the process (Chemat et al., 2017). On the other hand, when submitted to high irradiation power, a shorter contact time between the target molecules and the water molecules occurs since the distillation time is lower when compared with lower irradiation powers (Filly et al., 2014). For instance, for the same amount of byproducts with 60% of moisture, the 500 W power extraction was due in 27±2 min. Although the 700 W power extraction was shorter (17±1min), the 10 min less contact between the solvent and the sample, resulted in a decrease of 60-75% in EO yield. The intermediate power of 500 W is thus the more promising irradiation condition for recovery of

267 phytochemicals from *S. montana,* as well as *Thymus* spp. byproducts as
268 observed in Fig. 1c with the highest yields.

269 Figure 1c also shows that for the same power applied, *S. montana* was
270 the plant byproduct whose EOs had higher extraction yields in relation with initial
271 material (0.20 mg/g at 500 W, 25 min). Within the *Thymus* byproducts, *T.*
272 *mastichina* had higher extraction yield (0.11 mg/g at 500 W, 30 min) which is half
273 of that obtained for *S. montana* under the same conditions. Although the amount
274 of EOs of *S. montana* stems is much higher than *Thymus* spp., this is not
275 observed for the commercial parts, where 4 – 22 mg/g were reported for *S.*
276 *montana* (Ćavar et al., 2008; Dunkic et al., 2010) and 2 – 20 mg/g for *T.*
277 *frangantissimus* (Horváth et al., 2006; Toncer et al., 2017). These results justify
278 the use of *S. montana* byproducts for the recovery of valuable EOs, even if the
279 amount of EOs recovery from byproducts is 20 to 100 times less than that
280 obtained from commercial parts.

281 In order to study the potential of *S. montana* EOs recovered from its
282 byproducts and the feasibility of the use of SFME with the moistened byproducts,
283 a new harvest was used. Table 2 shows that the extraction yield of *S. montana*
284 EOs in 2017 was 0.10 ± 0.01 mg/g, which was half of the yield obtained in 2016
285 harvest (Fig. 1) using the same methodology. Nevertheless, HD showed also an
286 extraction yield of 0.14 ± 0.07 mg/g, which is not significantly different (p>0.05)
287 from the one obtained with SFME for the same harvest year. Accordingly, the
288 extraction yield by the HD from 2016 harvest was 0.42 ± 0.11 mg/g, significantly
289 higher (p<0.05) than that observed for 2017 harvest. These results show that the
290 harvest year, reflecting intrinsic byproduct variability (Figueiredo et al., 2008;

Sangwan et al., 2001), seems to be more relevant for *S. montana* byproducts EOs than the methodology of extraction.

Despite similar extraction yields, the time consumed to recover the EOs by HD and SFME was clearly different, as SFME presents a shorter extraction time, 7.5 times smaller than the time required in HD, in accordance with literature for other aromatic herbs (Bendahou et al., 2008; Lucchesi et al., 2004). SFME is reported to cause glandular walls to crumble or rupture more rapidly and more efficiently than HD (Golmakani and Rezaei, 2008). The extraction time required in this study for *S. montana* byproducts (16±1 min) is well below the microwave assisted hydrodistillation (MHD) extraction time (90 min) of *S. montana* commercial parts, which used a higher amount of water/plant ratio (20:1, w/w) and a lower power density (0.52 $W.g^{-1}$) (Rezvanpanah et al., 2008). In addition to the lower extraction time, SFME methodology used in present study, where the dried parts of the plant are soaked in water and drained, presents also the advantage of providing a dehydrated plant material stable to be stored for later use, as demonstrated also by Ferreira et al. (2018) for broccoli byproducts.

3.2. Volatile composition of *S. montana* essential oils

A total of 13 compounds were identified in EOs obtained by SFME from 2017 harvest, accounting for 84.9% of the total oil content (Table 2). Monoterpenoids account for about 84.4% of these EOs and sesquiterpenoids represent only 0.5%. All monoterpenoids and sesquiterpenoids identified were oxygenated compounds. A similar qualitative and quantitative chemical composition is visible from EOs obtained by HD from the same harvest, where monoterpenoids account for 89.6% and sesquiterpenoids represent 0.8%

316 (p>0.05). For mango (*Mangifera indica* L.) flowers, the predominance of
317 oxygenated monoterpenoids over hydrocarbons monoterpenes using solvent-
318 free microwave extraction was observed (Wang et al., 2010), however, in the
319 essential EOs of *S. montana* the terpenoids present are all oxygenated, which
320 did not allow to observe differences between the two methods.

321 A high carvacrol content (82 to 95%) was observed in all EOs,
322 independently of the harvest year and hydrodistillation methodology used
323 (p>0.05) (Table 2). The high level of this phenolic monoterpenoid in *S. montana*
324 byproducts EO is in accordance with the high value (84%) reported by Kustrak et
325 al. (1996) for the commercial parts of *S. montana* EO. All other reported values
326 are below 65% (Dunkic et al., 2010; Miladi et al., 2013; Trifan et al., 2015) and
327 some authors report carvacrol amounts below 7% (Mastelic and Jerkovic, 2003;
328 Radonic and Milos, 2003; Slavkovska et al., 2001). Carvacrol is synthesized via
329 γ-terpinene by aromatization, forming *p*-cymene, and hydroxylation (Friedman,
330 2014). In this study, there is clear propensity for the formation of carvacrol in
331 relation to its isomer, thymol (0.3 – 0.4%). Carvacrol levels in *S. montana* are
332 reported to be higher in the early vegetative stages (Kustrak et al., 1996). In
333 addition to plant development, the crop cultivation site and environmental
334 conditions are also factors which affect carvacrol levels (Ćavar et al., 2008;
335 Kustrak et al., 1996; Mastelic and Jerkovic, 2003; Milos et al., 2001).

336 In order to evaluate the possible migration of carvacrol and the other EO
337 compounds during the moistening process, the volatile compounds present in the
338 non recovered EOs (NREOs) were analysed. After the moistening process of the
339 100 g of stems, 860 mL of water was not incorporated in the plant tissue. Liquid-
340 liquid dichloromethane extraction allowed to recover 0.17 mg/g of stems (Table

341 2). A total of 12 terpenoids were identified, accounting for 10.1% of the volatile
342 compounds present. Taking in account the EOs terpenoids content, this
343 represents a loss of 17% due to drainage process. With the exception of
344 dihydroactinidiolide and spathulenol, all other terpenenoids found in NREOs are
345 also found in the *S. montana* byproducts EOs. As the moistening process of the
346 stems was simultaneously an extractive process, aliphatic carboxylic acids and
347 aromatic compounds, namely, vanillin, acetovanillone, syringaldehyde and
348 syringol, which are lignin degradation blocks were also quantified (Hartley, 1971;
349 Rocha et al., 1996). Over the time, compounds that diffuse from the plant material
350 will be more concentrated in water. These compounds can be also recovered by
351 solvent extraction. The reuse of the hydration water will increase the
352 concentration of the extracted compounds, which are recognized as having
353 antioxidant and antimicrobial properties (Ao et al., 2008; Fitzgerald et al., 2004;
354 Garrote et al., 2004; Zemek et al., 1979), should be considered under a circular
355 economy concept.
356

Table 2- Volatile components identified in essential oils (EOs) and in the drained water recovered after the moistening of *S. montana* stems (NREO) obtained by hydrodistillation (HD) and solvent-free microwave extraction (SFME).

					HD		SFME	Hydration
No.	**RI_{cal}[a]**	**RI_{lit}[b]**	**Compound**	**Reliability of ID[c]**	**% (w/w) Harvest 2016 EO[d]**	**% (w/w) Harvest 2017 EO[d]**	**% (w/w) Harvest 2017 EO[d]**	**% (w/w) NREO[d]**
Monoterpenoids								
1	1520	1526	Linalool	A, B, C	0.48 ± 0.09*	0.33 ± 0.06*	0.62 ± 0.36*	0.08 ± 0.01
2	1537	1576	4-Terpineol	B, C	0.65 ± 0.01*	0.38 ± 0.09*	0.39 ± 0.23*	0.04 ± 0.01
3	1680	1668	Borneol	A, B, C	0.48 ± 0.03*	0.84 ± 0.32*	0.90 ± 0.41*	0.08 ± 0.02
4	1687	1664	α-Terpineol	A, B, C	0.60 ± 0.11*	0.23 ± 0.07#	0.23 ± 0.06#	0.12 ± 0.01
5	1782	-	Carvacrylacetate	B, C	0.25 ± 0.13*	0.92 ± 0.69*	0.38 ± 0.11*	-
10	2063	2048	*p*-Cymen-α-ol	B, C	Tr[e]	Tr[e]	Tr[e]	0.06 ± 0.03
13	2146	-	Thymol isomer	C	0.11 ± 0.02*	0.08 ± 0.01*	0.11 ± 0.03*	Tr[e]
14	2156	2165	Thymol	B, C	0.43 ± 0.01*	0.30 ± 0.07*	0.32 ± 0.11*	0.12 ± 0.09
15	2175	2218	Carvacrol	B, C	94.61 ± 2.43*	86.56 ± 17.82*	81.46 ± 21.33*	9.09 ± 1.22
Sesquiterpenoids								
6	1908	1883	Caryophyllene oxide	B, C	0.26 ± 0.14*	0.80 ± 0.19#	0.52 ± 0.19*#	0.09 ± 0.02
8	1953	1918	Viridiflorol	B, C	Tr[e]	Tr[e]	Tr[e]	-
11	2071	2129	Spathulenol	B, C	Tr[e]	Tr[e]	Tr[e]	Tr[e]
Aliphatic carboxylic acids								
9	2038	2024	Caprylic acid	B	-	-	-	0.15 ± 0.01
18	2249	2276	Capric acid	B	-	-	-	0.18 ± 0.01
20	2442	2471	Lauric acid	B	-	-	-	0.04 ± 0.01
24	>2480	2871	Palmitic acid	B	-	-	-	0.52 ± 0.01
26	>2480	-	Stearic acid	B	-	-	-	0.28 ± 0.10
28	>2480	-	Linoleic acid	B	-	-	-	0.24 ± 0.06
29	>2480	-	7,10,13-hexadecatrienoic acid	B	-	-	-	0.28 ± 0.01

Aromatic compounds								
12	2123	2179	Eugenol	A, B, C	Tr[e]	Tr[e]	Tr[e]	0.08 ± 0.01
17	2231	2273	Syringol	B	-	-	-	0.07 ± 0.01
21	2480	2571	Vanillin	A, B	-	-	-	1.49 ± 0.02
23	>2480	-	Acetovanillone	B	-	-	-	0.29 ± 0.06
25	>2480	-	Syringaldehyde	B	-	-	-	0.79 ± 0.05
27	>2480	-	3-(*p*-hydroxy-*m*-methoxyphenyl)-2-propenal	B	-	-	-	0.33 ± 0.04
Others								
7	1941	-	*trans*-*β*-ionone-5,6-epoxide	B	-	-	-	0.04 ± 0.01
16	2220	-	Methylethylmaleimide	B	-	-	-	0.06 ± 0.01
19	2236	-	Dihydroactinidiolide	B	-	-	-	0.31 ± 0.01
22	>2480	-	2,6-dimethyl-3-(methoxymethyl)-*p*-benzoquinone	B	-	-	-	0.12 ± 0.01
			Total (%):		97.9 ± 2.0	90.4 ± 18.9	84.9 ± 22.8	15.0 ± 1.6
			Yield (mg/g):		0.42 ± 0.11*	0.14 ± 0.07[#]	0.10 ± 0.01[#]	0.17 ± 0.01

Each value in the table is represented as mean ± standard deviation (n = 3); different symbols (*, [#]) in the same line indicate significant difference ($p < 0.05$).
[a]Retention indices relative to C8–C22 *n*-alkanes serie. [b]Retention indices reported in the literature for DB-FFAP columns or equivalent (Cheong et al., 2012; Choi, 2003; Guillard et al., 1997; Jarunrattanasri et al., 2007; Mebazaa et al., 2011, 2009; Munk et al., 2000; Santos, 2010; Zeller and Rychlik, 2006) [c]The reliability of the identification or structural proposal is indicated by the following: A, mass spectrum and retention time consistent with those of an authentic standard; B, structural proposals are given on the basis of mass spectral data (*Wiley 275* Library); C, mass spectrum consistent with spectra found in the literature. [d]Estimated concentrations for all compounds were made by peak area comparisons to the area of a known amount of internal standard (2-undecanol).
[e]Tr, traces, <0.01%.

366 **3.3 Antimicrobial activity of *S. montana* byproducts essential oil and its**
367 **relation to the volatile composition**

368 The antimicrobial activities of *S. montana* EOs were qualified by inhibition
369 zone diameters using the agar disc diffusion method, and quantified by
370 determining the minimal inhibitory concentration (MIC) using a broth microdilution
371 assay. *S. montana* EOs obtained by SFME and HD were mixed and their
372 antimicrobial potential was evaluated as a single sample, since they presented
373 an identical chemical composition. The results of the disc diffusion testing are
374 listed in Table 3 (assay 1) and in Figure 2 (assay 2). The DMSO or sterile water
375 negative controls showed no inhibition effects.

376 Two different inhibition zones around EO discs were visible in testing
377 plates (Table 3), here designated as total and partial inhibition. The total inhibition
378 zone is the circumference around the disc where no colony was detected, while
379 in the subsequent zone of partial inhibition a growth inhibition is observed but
380 some colonies are visualized. The formation of these growth inhibition zones
381 around *S. montana* EO discs for all tested bacterial strains confirms the EOs
382 antimicrobial activity.

383 The inhibition zones reveal different susceptibility of different
384 microorganisms to the *S. montana* EOs (Table 3). The EOs amount of test disc
385 for *S. aureus* ATCC 6538 (Gram-positive bacteria) in Assay 1 was 0.44 mg, 3.5
386 times lower than the amount tested against the Gram-negative bacterial strains,
387 resulting in an inhibition diameter equal to or greater than the diameters observed
388 for the other strains. *S. enterica* sv Anatum SF2 and *E. coli* ATCC 25922 strains
389 (Gram-negative bacteria) were therefore much less susceptible than *S.*
390 *aureus* ATCC 6538 (Gram-positive bacteria) to *S. montana* EO, in agreement

391 with the results obtained by Chao et al. (2000) that investigated the effects of 45
392 EOs, including *S. montana* EO from commercial parts of the plant. The lower
393 susceptibility of Gram-negative bacteria to essential oils is reported to be due to
394 the presence of hydrophilic lipopolysaccharides (LPS) in outer membrane which
395 create a barrier toward macromolecules and hydrophobic compounds, blocking
396 the penetration of these components through the target cell membrane (Mirjana
397 and Nada, 2004). Gram-positive bacteria, in turn, possess a cell wall more
398 permeable to these molecules since the major component is peptidoglycan
399 covalently linked to teichoic and teichuronic acids (Cristani et al., 2007).

400 The total inhibition zones diameter was shown to have a linear relationship
401 to the amount of *S. montana* EO impregnated in each test disc for each
402 microorganism (Figure 2), with correlation coefficients (R^2) of 0.9627, 0.9552, and
403 0.8258 for *S. aureus*, *E. coli*, and *S. enterica* strains, respectively. The
404 susceptibility of each strain seems to be reflected in the slope value, since the
405 largest slope corresponded to *S. aureus*, followed by *E. coli* and *S. enterica*, with
406 slopes more comparable to each other. The high slope (inhibition diameter /
407 amount of EO) of *S. aureus* means that a small amount of EO resulted in a high
408 inhibition diameter, showing consequently a higher susceptibility to the EO.

409 It is known that the hydrophobicity of EOs components enables them to
410 partition in the lipids of the bacterial cell membrane, disturbing the structures and
411 rendering them more permeable. This loss of differential permeability is
412 considered the cause of cell death of microorganisms since it leads to extensive
413 loss of cell contents or to the exit of critical molecules and ions (Ferreira et al.,
414 2012; Oliveira et al., 2011; Simões et al., 2008). In specific case of the tested
415 EOs, the antibacterial activity is probably due to the presence of carvacrol

because of its high abundance in *S. montana* EOs and its high specific activity as compared to other EO components, considered one of the main active components of EOs (Friedman, 2014; Veldhuizen et al., 2006). This is also in accordance with the results reported for *S. montana* EOs with high carvacrol content (Miladi et al., 2013) that showed higher antimicrobial activity than the EOs with lower carvacrol content (Ćavar et al., 2008). The EOs recovered from *S. montana* byproducts exhibited, with less concentration, a highest inhibition diameter (Table 3) than that reported for the same bacterial species (Ćavar et al., 2008). This effect is probably due to the highest concentration of carvacrol.

Table 3 - Zones of growth inhibition (mm) of *S. montana* byproduct essential oils using agar disc diffusion method (Assay 1)

Microorganisms	***S. montana* L. EO[a]**	**CIP[b] (5 µg/disc)**	**GEN[c] (10 µg/disc)**	**Sterile water**
Total inhibition zone				
E. coli ATCC 25922	25 ± 3* (1.75/1.53 mg)	34 ± 1[#]	21 ± 0*	ND[d]
S. enterica sv Anatum SF2	13 ± 1* (1.75/1.53 mg)	31 ± 1[#]	20 ± 1[$]	ND[d]
S. *aureus* ATCC 6538	23 ± 4* (0.44/0.38 mg)	28 ± 1*	25 ± 1*	ND[d]
Partial inhibition zone				
E. coli ATCC 25922	33 ± 2 (1.75/1.53 mg)	42 ± 1	27 ± 0	ND[d]
S. enterica sv Anatum SF2	20 ± 4 (1.75/1.53 mg)	38 ± 1	25 ± 0	ND[d]
S. aureus ATCC 6538	40 ± 8 (0.44/0.38 mg)	NPI[e]	NPI[e]	ND[d]

Each value in the table is represented as mean ± standard deviation (n = 3); different symbols (*, [#], [$]) in the same line indicate significant difference ($p < 0.05$). [a]inhibition zone in diameter around the discs impregnated with the amount of essential oil / amount of carvacrol in the essential oil described in parenthesis for each microorganism. Inhibition zones values include the disk diameter (6.0 mm). [b]CIP, ciprofloxacin. [c]GEN, gentamicin. [d]ND, no inhibitory effect was detected. [e]NPI, no partial inhibition was detected beyond the total inhibition diameter.

435 The antimicrobial activity of the carvacrol is highly related to the presence
436 of the aliphatic ring substituents and the hydroxyl group (Veldhuizen et al., 2006),
437 which make it more active than other monoterpenoids. The synergism of this
438 compound with the other minor terpenoids present in EO tested is an hypothesis
439 that should not be excluded, namely carvacrol/thymol and carvacrol/eugenol (Pei
440 et al., 2009).

441 The EOs MICs for the different tested strains are listed in Table 4. The
442 lower MIC value for *S. aureus* (150 µg/mL) reflects the highest susceptibility of
443 this strain when compared with the *E. coli* (225 µg/mL) and *S. enterica* (250
444 µg/mL). These values are below those reported for the EOs from the commercial
445 parts of the plant (53% of carvacrol, 14% of ϒ-terpinene and 13% *p*-cymene)
446 against strains of the same bacterial species: 780 µg/mL for *S. aureus* ATCC
447 25923, 780 µg/mL for *E. coli* ATCC 35218, and 390-780 for *Salmonella* spp.
448 strains (Miladi et al., 2013). The MICs of carvacrol for the same tested strains are
449 slightly above the MIC values of the tested EOs (Table 4), reinforcing the
450 hypothesis that carvacrol is the active principal of *S. montana* byproducts EOs.

451

452 **Table 4** - Minimal inhibition concentration (MIC) of byproducts *S. montana* essential oils.
453 The values are expressed as µg/mL of liquid medium.

Microorganisms	***S. montana* L. EO_{cal}[a]**	**Carvacrol MIC_{lit}[b]**
E. coli ATCC 25922	225	250
S. enterica sv Anatum SF2	250	NR[c]
S. *aureus* ATCC 6538	150	175

454 [a] The negative control with 1% of DMSO showed no inhibition effects. [b]Values reported in the
455 literature for the respective strains (Juliano et al., 2000; Lambert et al., 2001) . [c]NR, no reported
456 value for this strain.
457

In higher doses, *S. montana* EOs can generate *in vitro* similar effects to those generated by antibiotics (ciprofloxacin and gentamicin) (Table 3). Although the *in vitro* antimicrobial tests showed an effective antimicrobial activity of *S. montana* byproducts EOs, *in vivo* assays are still required to confirm their applicability as animal health promoters. Other EOs, such as those derived from thyme (Denli et al., 2004), with high levels of carvacrol, have already been tested *in vivo* and are active as antibiotic alternatives. Taking in to account that the supplementation of 60 mg thyme essential oil (67.8% carvacrol, 4.9% γ-terpineol, 4.7% cymene, 3.4% thymol and 3.2% linalool) by kg of chickens` diet resulted in significantly higher body weight gains and better-feed efficiency (Denli et al., 2004), the incorporation of 47 mg of *S. montana* byproducts EOs per kg of diet may produce biological effects equivalent to those reported. These results pave the way to test the incorporation of *S. montana* byproducts in poultry diets.

4. Conclusion

Agroindustrial *S. montana* byproducts can be used to obtain essential oils. Carvacrol is the most abundant component (825 – 950 µg/mg) of *S. montana* byproducts in all EOs obtained. Solvent-free microwave extraction, as a green extraction method for valorization of aromatic plant byproducts, offers important advantages such as short extraction time and reduction of water consumption. Additionally, the method results in the dehydration of the non-extractable remaining material, making it stable for other possible applications.

S. montana byproducts essential oils exhibited antimicrobial activity against Gram-positive and Gram-negative bacteria related with poultry infections, presenting MIC values of 150 µg/mL to *S. aureus*, 225 µg/mL to *E. coli*, and 250

μg/mL to *S. enterica,* pathogenic species with economic impact in poultry industry. This antimicrobial potential could be exploited within the livestock sector towards reducing/abolishing antibiotics, while promoting a circular economy.

Conflict of interest

Authors declare no conflict of interest.

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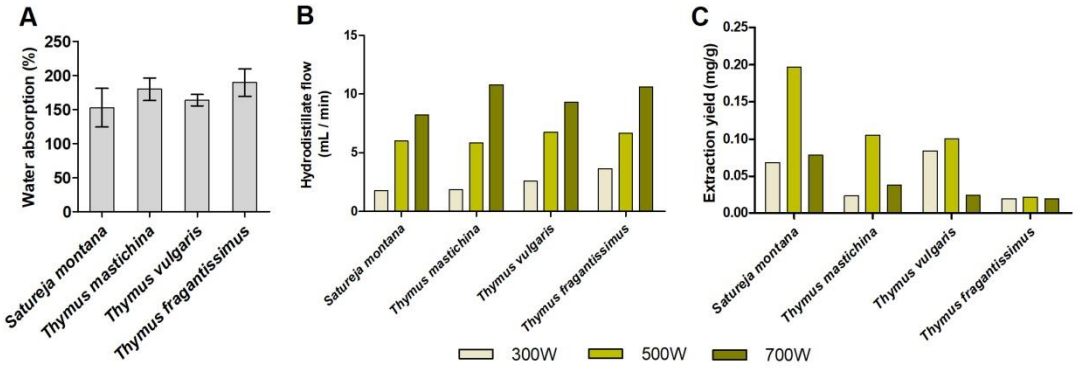

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774 **Figure 1** – Behavior of aromatic plant byproducts harvested in 2016, using
775 solvent-free microwave extraction of EOs: (A) water absorption percentage, (B)
776 microwave hydrodistillate flow (mL/min), and (C) yield of extraction (mg/g).
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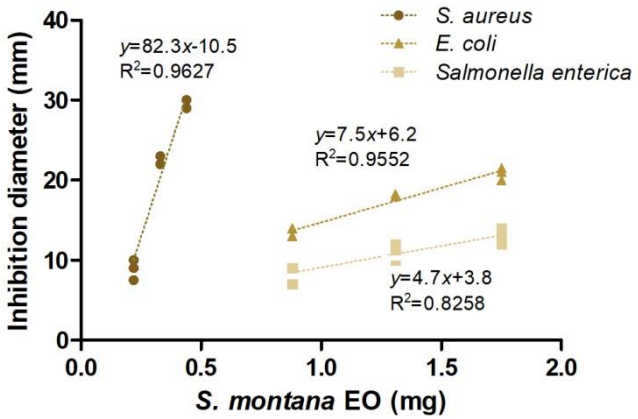

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780 **Figure 2** - Correlation between the amount of *S. montana* essential oil and total
781 inhibition zones (mm) against *E. coli* ATCC 25922, *S. enterica* sv Anatum SF2,
782 and S. *aureus* ATCC 6538 using the agar disc diffusion method. Each *S. montana*
783 EO volume was tested in triplicate.
784