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Continuous-Flow Chemo and Enzymatic Synthesis of Monoterpenic Esters with Integrated Purification

Carlos A.A. Adarme[a], Raquel A.C. Leão[b,c], Stefania P. de Souza[b], Ivaldo Itabaiana Jr[d], Rodrigo O.M.A. de Souza[b], Claudia M. Rezende[a]

[a] Aroma Analysis Laboratory, Chemistry Institute, Federal University of Rio de Janeiro, Rio de Janeiro, CEP 21941909, Brazil
[b] Biocatalysis and Organic Synthesis Group, Chemistry Institute, Federal University of Rio de Janeiro, CEP 21941909, Brazil
[c] Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, CEP 21941170, Brazil
[d] Departamento de Engenharia Bioquímica, Escola de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, CEP 21941909, Brazil

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ABSTRACT

Monoterpenic esters are very important flavor and fragrance compounds due to their organoleptic properties. Despite their importance, many drawbacks are found for the production of monoterpenic esters. Here in we report two different approach's (chemo and enzymatic) for the continuous production of monoterpenic esters with integrated purification arriving on the desired molecules with high yields (> 95%) and short reaction times.

1. Introduction

Short-chain esters are commonly flavor and fragrance compounds used in the food, cosmetic and pharmaceutical industries [1,2]. In particular, natural monoterpenic esters receive special attention for their organoleptic properties like rose (*e.g.* citronellol and geraniol esters), fruit (*e.g.* linalyl acetate) or mint (menthyl acetate) notes [3], for being natural flavors [4] and generally have higher prices than those produced synthetically. 5 According to the European [6] and US [7] legislations, a "natural flavor" substance should be obtained only by physical procedures (*e.g.* distillation) or by enzymatic or microbiological processes from natural sources (animal or vegetable). Nevertheless, some drawbacks are found for the production of monoterpenic esters such as low concentration of the desired compounds, variability in the composition and yield (seasonality) and others [8]. These factors make it difficult to transpose to an industrial scale, making the process more costly and expensive.

A cheaper and alternative method to obtain monoterpenic esters is by chemical synthesis that traditionally employs strong acid or basic catalysis [9] in a process environmentally unfriendly. In this way, there is an increasing interest in the development of new clean strategies for their production for industrial scale approaches.

Nowadays, the flavor and fragrance industries are interested in the biocatalysis for the production of flavor esters, because is a greener way to obtain this natural products derivatives due to the selectivity, reusability and softer reaction conditions required for biocatalysts [10]. From all enzymes that can be used for such transformations, lipases (triacylglycerol acylhydrolases E.C. 3.1.1.3.) have gained greater prominence over the years [11]. They have been used for the production of flavor compounds by direct esterification or transesterification in organic solvents or under solvent free reactions, being able to accept a wide range of substrates and do not need cofactors [12–19]. However, the cost of the biocatalyst make sometimes unviable their use for industrial proposes [20]. Continuous flow systems are an alternative to the traditionally batch process, with the advantage to improve the reuse of the biocatalyst and decrease the damage of the immobilized enzyme compared to batch reactions, due to the use of packed-bed reactors [21,22]. With the advent of continuous-flow technology researchers are able to improve important strategic parameters for industrial manufacturing such as energy efficiency, purification steps and reaction time [23–27].

Following our interest on the development of new methodologies for practical continuous-flow protocols [28–31], in this work we reported the continuous flow synthesis of monoterpenic esters comparing chemical and enzymatic catalysis for the acylation of five commercial monoterpenic alcohols (geraniol, citronellol, myrtenol, menthol and linalool) and two essential oils (*Cymbopogon martinii* and *Mentha arvensis*). We started with the biocatalized transesterification of the monoterpenic alcohols using ethyl acetate as acylating agent and for the chemical approach we have used acetic anhydride in a batch reactor were various parameters were studied such as temperature, concentration of the alcohol, concentration of enzyme and reaction time. The best reaction condition obtained was translated to a continuous flow system. Finally, we have integrated synthesis and purification in

E-mail addresses: souzarod21@gmail.com, souzarod21@yahoo.com.br (R.O.M.A. de Souza).

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order to arrive on the desired product in a single step.

2. Materials and Methods

2.1. Enzyme and chemicals

The kit of hydrolases was purchased from Selectazyme®. The immobilized enzymes Novozym 435®,Lipozyme RM IM® and the reagents *p*-nitrophenyl palmitate (pNPP), *p*-nitrophenol (pNP), ethyl acetate, acetic anhydride, sodium carbonate, geraniol, citronelol, mirtenol, mentol and linalol were purchased from Sigma-Aldrich Co. (St. Louis, USA). Lipase B from *Candida antarctica* (Lipozyme CaLB, soluble form) was purchased from Novozymes (Brazil). Purolite® ECR8205F and Purolite® ECR8214F (crosslinked copolymers of metacrylate containing oxirane groups (ECR8205F: 35-34% of oxirane; (ECR8214F 55-65% of oxirane)) were purchased from Purolite International Limited (Wales, UK). The essential oil of *Cymbopogon martinii* (Poaceae) was purchased from Ferquima® and the essential oil of *Mentha arvensis*, (Lamiaceae) was purchased from Mentol de Campinas S.A.

2.2. GC-MS

Analyses were performed on an Agilent 7820A gas chromatograph (GC) (Palo Alto, CA, USA) equipped with a G2613A auto-sampler coupled to a quadrupole mass spectrometer (MS) Agilent (MS 5977E). The identities of the terpenic esters were characterized by comparison with NIST 14 and ADAMS (2007) libraries. Helium carrier gas was at 1 mL min^{-1} with constant flow mode in a HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm). The oven was set at 60 °C to 150 °C in 3 °C min^{-1}, injector at 250 °C set in split mode (1:20) and injection the volume of 1.0 µ L. Mass spectrometer includes ion source at 230 °C, quadrupole at 150 °C, ionization voltage of 70 eV and mass spectra were obtained in full scan mode (40–350 *m/z*).

2.3. GC-FID

Analyses were performed on an Agilent 7890N gas chromatograph (GC) (Palo Alto, CA, USA) equipped with a G2613A auto-sampler coupled to flame ionization detector (FID). Hydrogen carrier gas was at 1,3 mL min^{-1} with constant flow mode in a DB-WAX capillary column (30 m × 0.25 mm × 0.25 µm). Oven was set at 90 °C (1 min) to 220 °C in 12 °C min^{-1}. Detector and injector were set at 250 °C. Injection was in split mode 1:20 and injection the volume 1.0 µ L.

2.4. Lipase selection

All experiments were carried out in batch conditions according to the section 2.6. One milliliter of a solution of 150 mg mL^{-1} of geraniol in ethyl acetate and 50 mg of each enzyme for the selection test were used. Reactions were performed at 50 °C for 48 h with magnetic stirring. Samples at 24 and 48 h were analyzed by GC-MS.

2.5. Lipase immobilization

In all experiments, immobilization efficiency and yields were determined by measuring the hydrolytic activities and the protein concentrations in the supernatant solution. Immobilization yields (Eq. (1)) were calculated after determining the amount of protein and enzyme units that disappeared from the supernatant and comparing with the initial protein and enzyme concentrations offered to the reaction (units per gram of support). Efficiency (Eq. (2)) was calculated after determining the activity of the immobilized enzyme and comparing with the number of enzyme units that disappeared from the supernatant (theoretically immobilized). Soluble protein was determined by the Bradford method using bovine serum albumin (BSA) as protein standard. 32

Table 1
Immobilization efficiency of new biocatalysts compared with N435.

Biocatalyst	Immobilization efficiency (%)[a]	Amount of protein (mg g^{-1} of support)	Initial rate (mM min^{-1})	Specific Activity (U/g)[c]
N435	–	30[b]	6.4	620
CALBEPO_A	55.1	4.3	3.6	800
CALBEPO_B	57.1	4.5	2.9	2500

[a] measured by desorption with Triton X-100 and Lowry assay.
[b] obtained after desorption assay with Triton X-100.
[c] obtained by hydrolytic activity assay. One unit of lipase activity was expressed as the release of 1 µmol pNP per minute under the assay conditions.

$$Immobilization\ \ yield = \frac{(total\ \ starting\ \ activity - total\ \ residual\ \ activity)}{Total\ \ starting\ \ activity} \times 100 \quad (1)$$

$$Immobilization\ \ Eficiency = \frac{Observed\ \ activity}{(total\ \ starting\ \ activity - total\ \ residual\ \ activity)} \times 100 \quad (2)$$

Purolite® ECR8205F and Purolite® ECR8214F polymers were applied as supports for lipase B from *Candida antarctica* immobilization, named respectively CALBEPO_A and CALBEPO_B. The difference between the two supports used is only the number of epoxy groups available for immobilization. For this purpose, 1 mL of the enzyme solution (0.62 U. mL^{-1} of specific activity) was diluted in 3 mL of phosphate buffer (25 mM, pH 7.0) and added to the appropriate support (1 g). The mixture was stirred during 4 h at 40 °C using a flask shaker, followed by vacuum filtering. The final biocatalysts were dried over night at ambient temperature. To check the covalent binding, supports were submitted to desorption with Triton X-100 according to Cabrera [33]. Soluble protein was determined by the Lowry method [34] using bovine serum albumin (BSA) as protein standard (Table 1).

2.5. Hydrolytic activity assay

The hydrolysis reaction was carried out using *p*-nitrophenyl palmitate (pNPP). Substrate solution was prepared by mixing one volume of 10 mM solution of pNPP in 2-propanol with nine volumes of 10 mM phosphate buffer solution pH 8.0 containing 0.44% (mass fraction) of Triton X-100 and 0.11% (mass fraction) of arabic gum. The lipase activity was measured using 100 µL of lipase solution or suspension and 900 µL of the substrate solution (10 mM pNPP) at 55 °C for 2 min. The absorbance of pNP released was spectrophotometrically monitored at 410 nm. One unit of lipase activity was expressed as the release of 1 µmol pNP per minute under assay conditions. The calibration curve was prepared using pNP as standard. Values are given as mean ± standard deviation in triplicate for each point.

2.6. Experimental design

For batch reactions, we first performed a full factorial design 2^3 with three central points to obtain the experimental error. For these experiments, we used the enzyme N435 that were carried out according to Table 1. In the case of chemical catalyzed reactions, we made a full factorial design 2^4 with three central points. The matrixes of experiment are shown in Tables 2 and 3.

2.7. Batch reactions

Reagents were added to 4 mL vials on silicon carbide plates under controlled temperature (40–60 °C for the biocatalyzed reaction and 70–110 °C for the chemical reaction) and magnetic stirring. The amount

Table 2
Real and code values (-1: lower level; 0: intermediate level; +1: high level) for the full factorial design 2^3 of the biocatalyzed reaction.

Variable	-1	0	+1
Temperature (°C)	40	50	60
Time (h)	1	2	3
Substrate concentration (mg mL^{-1})	50	150	250

Table 3
Real and Code values (-1: lower level; 0: intermediate level; +1: high level) for the full factorial design 2^4 of the chemical reaction.

Variable	-1	0	+1
Temperature (°C)	70	90	110
Time (h)	0,5	1	1,5
Sodium acetate concentration (%)	2	6	10
Acetic acid/acetic anhydride (v/v)	0.0250	0.2625	0.5000

of enzyme and reagents was added according to the experimental design. At the end, the reaction mixture was analyzed by GC-FID.

2.8. Continuous flow reaction

The reaction mixtures were pumped using the Asia flow system equipped with a stainless steel column filled with the enzyme (packed bed reactor). For each experiment, ethyl acetate was initially pumped through the system during 3 min. The same procedure was made after the experiment in order to clean the system from any remaining reactant or starting material. For the biocatalyzed continuous flow reaction, we used a column with an inner volume of 2.4 mL (i.d. = 8.0 mm, h = 4.9 cm) and for the chemical esterification reaction, a coil with inner volume of 1,0 mL (i.d. = 1.5 mm, h = 57.0 cm) was employed.

2.9. Continuous flow reaction and purification

This system consisted in 3 steps: reaction, continuous work-up and liquid-liquid separation. Different flow rates between the organic and the aqueous phase have been studied to determinate the best rate (1:1, 1:3, 1:4). Different concentrations of sodium carbonate solutions were tested (3, 5 and 10%), as well as several approaches towards a better mixing efficiency between the two phases (Scheme 1).

3. Results and Discussion

3.1. Lipase selection

We began our studies evaluating different free (Table S1) and immobilized enzymes (Table S2) on the transesterification of geraniol with ethyl acetate at 50 °C for 48 h. The main results are shown in Tables 4 and 5 (for further details with all results obtained see Supporting Information).

Table 4
Selected results for the evaluation of different free enzyme from the esterification reaction between geraniol and ethyl acetate.

Entry	Lipase Source	Conversion (%)	
		24 h	48 h
1	Lipase B – *Candida antarctica*	88	96
2	Lipase E – *Alcaligenes* sp	87	90
3	Lipase F- *Alcaligenes* sp	85	90
4	*Pseudomonas stutzeri*	70	81
5	Lipase C- *Alcaligenes* sp	72	85
6	Lipase D- *Alcaligenes* sp	68	84

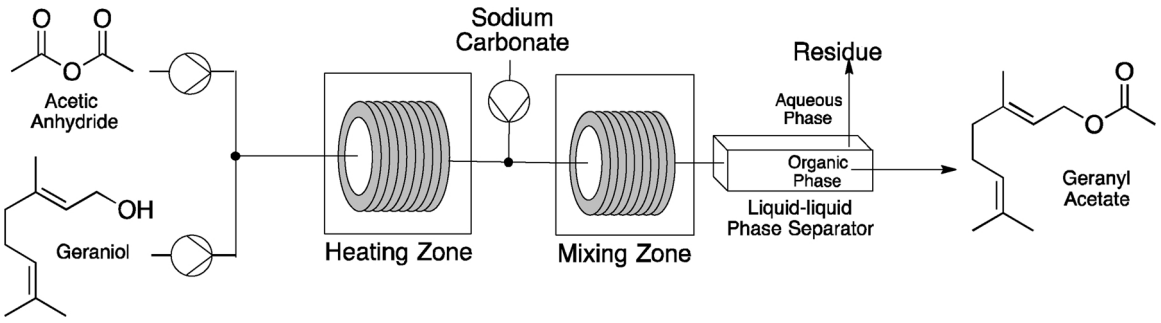
Reaction conditions: 1 mL solution containing 150 mg of geraniol in ethyl acetate and 50 mg of each enzyme. at 50 °C for 48 h under magnetic stirring. Conversions were determined by GC-MS.

As shown on Tables 4 and 5, both free and immobilized enzymes could lead to desired products with good conversions after 24 and 48 h. The best conversion after 48 h was obtained for the free enzymes *Candida antarctica* B (96%), *Alcaligenes* sp E (90%) and F (90%), and for the immobilized enzyme N435 (90%). Another interesting performance was observed with the immobilized *Candida antarctica* B on epoxy resins (Entries 4 and 5, Table 5) where a similar conversion was obtained when compared to N435. But a closer look into the amount of protein present in CALBEPO_A and CALBEPO_B (Table 1) revels that the reaction catalyzed by these enzymes was much more efficient then N435, since we have only 4.5 mg of protein loaded in each gram of support against 30 mg. g of support^{-1} presented by commercial preparation. This difference in the amount of protein between the home-made enzymes and the commercial biocatalyst is crucial for increasing the productivity of the reaction. In these biocatalysts, lipase B from *Candida antarctica* was immobilized on two epoxy resins through covalent bonds. This type of interaction restricts lipase to a more active and stable conformation, turning the final biocatalyst more robust, since the conformational restriction turns the enzyme more resistant to deleterious effects of solvent, as well high temperatures. Previous work by our research group [35] has demonstrated that these biocatalysts have excellent performance in reactions of kinetic dynamic resolution of amines and in esterifications when compared to the commercial enzyme Novozyme 435. Since there was not a big difference on conversion between free and immobilized enzymes, we decided to move forward using the immobilized enzyme N435. It is important to note that industrial application of free enzymes can be limited by a poor long-term operational stability, difficulty of recovering and re-use of the enzyme.

3.2. Biocatalyzed acetylation of geraniol with ethyl acetate

3.2.1. Experimental design

In order to find the best reaction conditions, we performed a 2^3 full factorial design in batch conditions, using as model the enzyme N435



Scheme 1. Representation of the continuous-flow reaction followed by in-line purification with liquid-liquid phase separator.

Table 5
Selected results for the evaluation of different immobilized enzyme for the esterification reaction between geraniol and ethyl acetate.

Entry	Lipase Source	Conversion (%)	
		24 h	48 h
1	Amano IM, *Burkholderia cepacia*	69	83
2	Lipozyme RM IM® (*Rhizomucor miehei)*	67	67
3	N435® *(Candida antarctica* B)	89	90
4	*Candida antarctica* B immobilized on epoxy resin (CALBEPO_A)	89	91
5	*Candida antarctica* B immobilized on epoxy resin (CALBEPO_B)	90	91

Reaction conditions: 1 mL solution containing 150 mg of geraniol in ethyl acetate and 50 mg of each enzyme at 50 °C for 48 h under magnetic stirring. Conversions were determined by GC-MS.

for the biocatalyzed acetylation of geraniol with ethyl acetate. The independent variables tested were temperature, time and substrate concentration. The respective levels of the variables for the 2^3 full factorial design are present on Table 2. The results obtained based on the experimental design can be found on Table 6.

As shown in Table 6, the best conversions were obtained when running the reaction at the lower substrate concentration at 40 °C or 60 °C for at least 1 h (Entries 1, 2, 8 and 9, Table 6). Nevertheless, for the substrate concentration of 150 mg mL^{-1} at 50 °C (Entries 5, 6 and 7, Table 6), the results were also high (87%), which seems that the use of a higher concentration of starting material can maximize product formation leading to a better productivity of the process developed. According to the estimate effects for the full factorial design 2^3, the increase in substrate concentration let to a negative effect, but the interaction of temperature and substrate concentration have a positive one (See Supporting information for further details, Table S3). In this way, we have chosen 50 °C and 150 mg mL^{-1} of substrate as the best condition for our biocatalyzed reaction.

3.2.2. Biocatalyzed transesterification reaction under continuous flow conditions

With these results, we started to study the lipase-catalyzed reaction under continuous flow conditions. The temperature was set at 50 °C and the concentration of substrate was fixed at 150 mg mL^{-1}. Besides the fact that we have optimized these conditions to N435, we decided to evaluate also the performance of other immobilized enzymes under continuous flow conditions. The column was filled with 1 g of immobilized enzyme (N453, Lipozyme RM IM, CALBEPO_A or CALBEPO_B) and experiments were performed at different residence times (from 1 min to 60 min) in order to investigate the best conversion towards the desired product. The maximum residence time was established at 1 h based in our batch optimization. The results are shown in Table 7.

Table 6
Results for the N435 biocatalyzed acetylation of geraniol with ethyl acetate (full factorial design 2^3).

Entry	Temperature (°C)	Time (h)	Substrate concentration (mg/mL)	Conversion** (%)
1	40	1	50	87
2	40	3	50	89
3	40	1	250	59
4	40	3	250	73
5(C)*	50	2	150	87
6(C)*	50	2	150	87
7(C)*	50	2	150	87
8	60	1	50	89
9	60	3	50	89
10	60	1	250	71
11	60	3	250	82

Reaction conditions: Temperature, time and concentration of geraniol were used according to the experimental design using a 1 mL solution of geraniol and ethyl acetate at appropriate concentration and 50 mg of N435. *Central point. **Conversions were determined by GC-FID.

Table 7
Continuous flow biocatalyzed transesterification reaction between geraniol and ethyl acetate catalyzed by different immobilized enzymes.

Entry	Res. Time (min)	Conversion (%)[a]			
		N435	LipoRMIM	CALBEPO_A	CALBEPO_B
1	1	66	39	64	42
2	2	73	46	65	51
3	4	84	54	78	70
4	6	88	56	82	77
5	8	90	57	85	81
6	10	89	60	87	83
7	30	90	66	90	89
8	60	90	74	90	89

Reaction Conditions: A solution containing 150 mg of geraniol in ethyl acetate was pumped through a packed-bed reactor containing 1 g of immobilized enzyme. The column dimension were: i.d = 8.0 mm, h = 4.9 cm. Reactions were carried out at 50 °C.
[a] Conversions were determined by GC-FID.

Table 7 shows that similar results were obtained for 8 min − 60 min residence time for N435 and CALEPO_A. In the case of RM IM, the best result was obtained for 60 min of residence time, being far away from the excellent results achieved by the other immobilized enzymes. For the immobilized enzyme CALEPO_B, similar results were obtained for 30 and 60 min of residence time. A brief look to the space-time-yield of N435 and CALEPO_A can show us that both enzymes reach high values, 2.4 g h^{-1} of product and 2.26 g h^{-1} respectively. Taking into account the amount of protein present in each support, CALEPO_A is 6 times more productive then N435, delivering 680 mg of product h^{-1}.mg ptn^{-1}. Unfortunately, the long term stability of CALEPO_A is still low which compromises its industrial application. In this way, we monitored the continuous-flow esterification reaction of geraniol at the best conditions found on Table 7 (Entry 5) a period of 8 h, when aliquots were taken every 30 min in order to determine the stability of N435. Fig. 1 shows the long-term stability results for N435.

As we can see in Fig. 1, the immobilized enzyme N435 retained its activity after a period of 8 h and the conversion remained constant at approximately 90%. These results show that the immobilized enzyme was stable for the experimental condition tested, allowing continuous operation for prolonged times an important feature for a biocatalyst aiming to be applied on production scale.

With these results in hands, we decided to extend the scope of the protocol developed and decided to use the immobilized enzyme N435 as a catalyst for the esterification of other monoterpenic alcohols (myrtenol, citronellol, menthol, linalool and the essential oil of *Cymbopogon martini*), at the best condition obtained previously. Table 8 shows the results for these experiments at 50 °C and 8 min of residence time.

As expected, citronellol and myrtenol show high conversions, similar to those obtained with geraniol, since they are all primary alcohols. Unfortunately, the residence time applied for menthol and linalool was not enough in order to achieve a significant conversion and this is probably due to the fact that this monoterpenic alcohols are more sterically hindered.

3.3. Chemical esterification reaction

Additionally to the biocatalyzed esterification reaction, we studied the esterification reaction between geraniol and acetic anhydride without the addition of a catalyst, which would need laborious

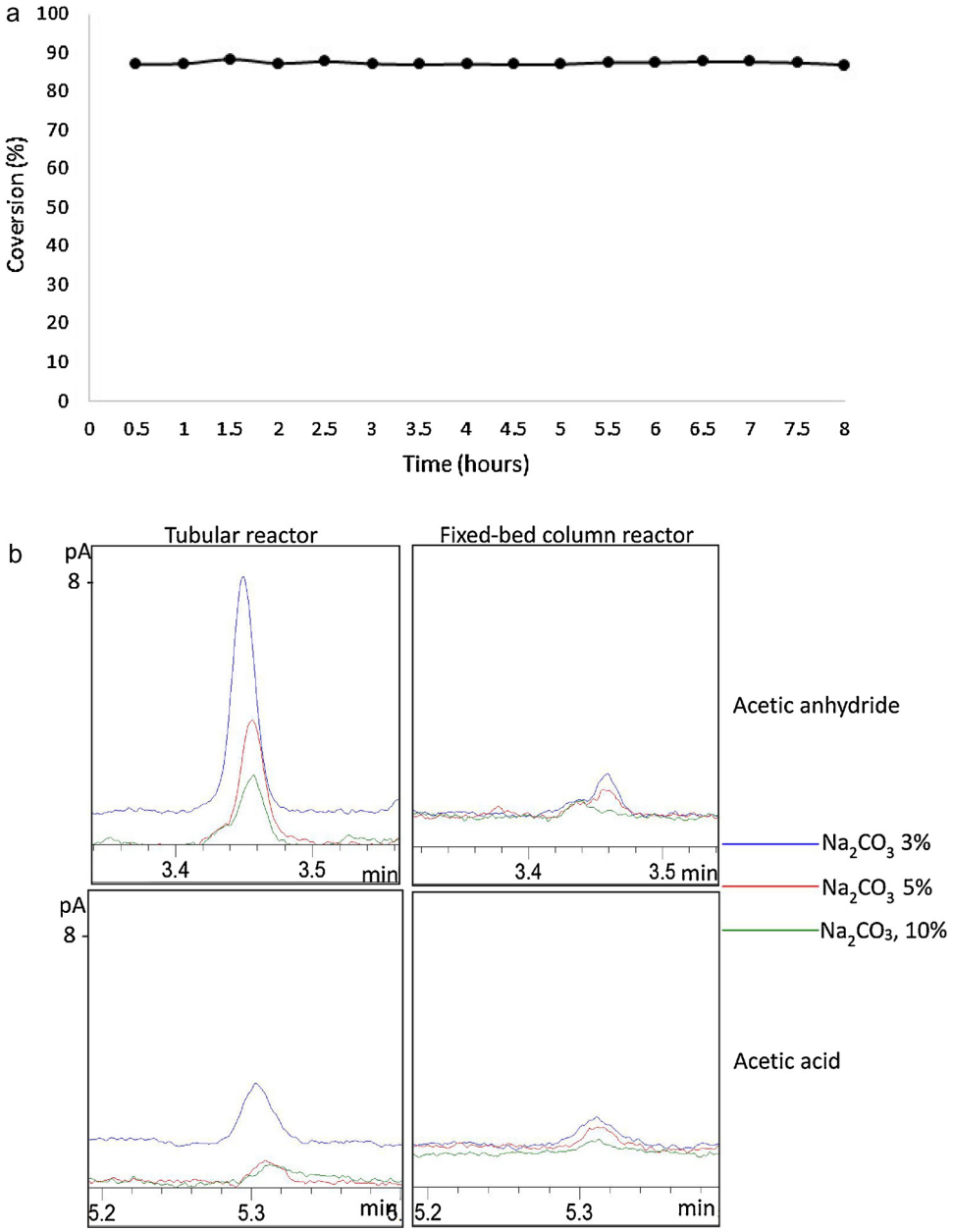


Fig. 1. (a)Long-term stability test for N435 immobilized enzyme. (b) Chromatographic region of GC-FID analysis, for each type of reactor, at different sodium carbonate concentration under continuous flow purification.

separation by the end of the reaction time. Traditionally, esters are prepared from an alcohol and a carboxylic acid in a reaction catalyzed by a base or an acid. To avoid the use of the catalyst, we choose acetic anhydride that is more reactive than carboxylic acids. To control the pH of the reaction media, we use sodium acetate that acts as an acid scavenger. The addition of sodium acetate makes the reaction media heterogeneous, hindering the translation of batch conditions to continuous-flow conditions. In a previously solubility test, we found that the addition of acetic acid make the reaction media homogenous at 60 °C. Since acetic acid can act as acyl donor and also as a catalyst, we evaluated its addition through the variable acetic acid/acetic anhydride ratio in the experimental design.

3.3.1. Experimental design

As usual, we started our optimization performing a full factorial design 2^4 with three central points, and studied the influence of the variables temperature, time, concentration of salt and the acetic acid/acetic anhydride ratio under batch conditions. It was observed that salt concentration is statistically insignificant to the reaction conversion and the addition of acetic acid, evaluated through the variable acetic acid/acetic anhydride ratio, has a negative effect on the reaction outcome (for further details see Supporting Information, Tables S4 and S5).

3.3.2. Chemical esterification reaction under continuous flow process

Aiming at improving the esterification process to obtain geranyl acetate, we performed the reaction under continuous flow. We evaluated temperature (90 °C − 150 °C) and the residence time (2 min − 10 min). Reagents were pumped separately at a molar ratio of 1:1.35 (geraniol: acetic anhydride) and results are summarized on Table 9.

Table 9 shows that conversions around 90% could be obtained at 110 °C with only 2 minutes of residence time. Nevertheless, the best conversions (> 99%) were obtained in the range of 4 to 10 min of residence time at 150 °C. For 2 min at 150 °C, the conversion was about 95%, with an isolated yield of 93%. Definitely, temperature plays an

Table 8
Continuous-flow esterification reaction catalyzed by N435 for different monoterpenic alcohols.

Monoterpenic alcohol	Conversion[a] (%)
Citronellol	91
Myrtenol	86
Menthol	0.2
Linalool	0.1
Essential oil of *Cymbopogon martinii*	87[b]

Reaction Condition: A solution containing 150 mg of alcohol or essential oil in ethyl acetate was pumped through a packed-bed reactor containing 1 g of immobilized enzyme. The column dimension were: i.d = 8.0 mm, h = 4.9 cm. Reactions were carried out at 50 °C and 8 min of residence time.

[a] Conversions were determined by GC-FID.

[b] Conversion is based on total geraniol content (75%) of the essential oil.

Table 9
Continuous-flow esterification reaction between geraniol and acetic anhydride at different reaction conditions.

Entry	Res. Time (min)	Conversion (%)[a]			
		90 °C	110 °C	130 °C	150 °C
1	2	61	90	91	95
2	4	79	93	95	99
3	6	83	95	97	99
4	8	86	96	98	99
5	10	88	96	99	99

Reaction conditions: reagents were pumped separately at different flow rates in order to achieve a molar ratio of 1:1.35 (geraniol: acetic anhydride). Coil dimensions: i.d. = 1.5 mm, h = 57.0 cm.

[a] Measured by GC-FID.

Table 10
Continuous flow chemical esterification of different monoterpenic alcohols with acetic anhydride.

Monoterpenic alcohol	Conversion (%)[a]
Citronellol	99
Myrtenol	99
Menthol	94
Linalool	–
Essential oil of *Cymbopogon martinii*	99[b]
Essential oil of *Mentha arvensis*	89[b]

Reaction conditions: Reagents were pumped separately at different flow rates in order to achieve a molar ratio of 1:1.35 (geraniol: acetic anhydride) Coil dimensions: i.d. = 1.5 mm, h = 57.0 cm. Reaction were carried out at 150 °C.

[a] Measured by GC-FID.

[b] Conversion is based on total geraniol content (75%) of the essential oil from *Cymbopogon martini* and total menthol content (45%) of the essential oil from *Mentha arvensis*.

important role and continuous-flow process can lead this chemical esterification to outstanding results with very short residence times. Again, these results prompted us to perform the chemical esterification of other monoterpenic alcohols (citronellol, myrtenol, menthol, linalool, and the essential oils of *Cymbopogon martini* and *Mentha arvensis*). Temperature of 150 °C and a residence time of 4 min were used in order to evaluated the related reactions and results are shown on Table 10.

The results presented on Table 10 shows that primary alcohols (citronellol and myrtenol) have a high conversion, similar to the results obtained for geraniol in the previous test. In the case of menthol, very good conversions were obtained (94%), something not observed for the biocatalyzed reaction. For linalool, we observed degradation probably associated to the high temperature (150 °C) used during the continuous flow reaction.

3.4. Continuous flow purification

Taking these results into account, we decided to integrate synthesis and purification in order to arrive at the end of our continuous flow process with a product which would not need any further step of purification. The best reaction condition found for the continuous flow chemical esterification reaction was used to investigate the continuous flow purification of geranyl acetate. A continuous flow liquid-liquid extraction was envisioned in order to extract remaining impurities from the chemical synthesis (acetic acid and unreacted acetic anhydride), by a sodium carbonate work-up at different concentrations (3, 5 and 10% p/v).

First, we decided to determine the best proportion between organic and aqueous phase (1:1; 1:3; 1:4) at a fixed sodium carbonate concentration (3%). In order to explore these changes, we needed to keep the total flow rate at 0.5 mL min^{-1} to maximize the efficiency of our continuous flow liquid-liquid separator (see further details on Supporting Information). The criterion chosen to define the relationship between the organic and aqueous phase used in the purification process was the reduction of the percentage area of the acetic acid.

After the heating zone, where the acetylation reaction was taking place, we added a second T-junction in order to deliver the sodium carbonate solution for the work-up process. The extraction process went through a mixing zone (2.2 mL coil, i.d = 1.0 mm, h = 280 cm) and then through the liquid-liquid separator in order to arrive on the desired purified product. The best proportion between organic and aqueous phase to remove impurities was 1:4, as shown in Scheme 2. For this proportion, was observed the less intense chromatographic peaks, indicating that a major quantity of impurities were separated from the geranyl acetate.

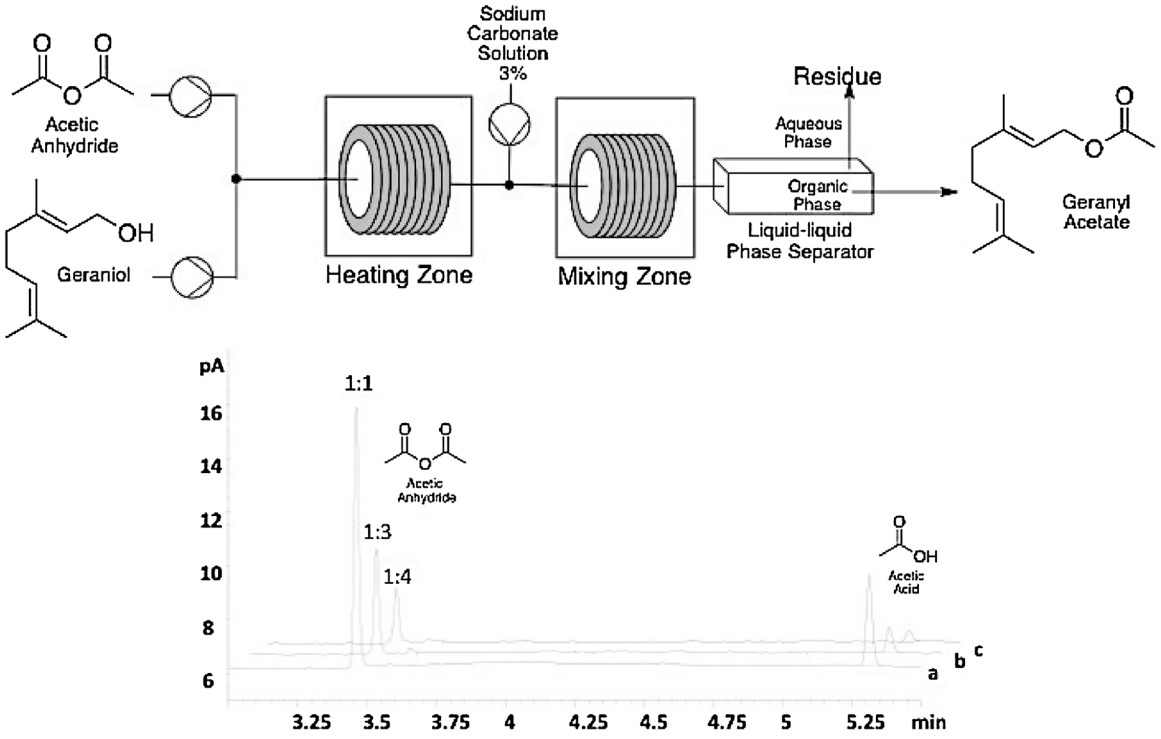
Based on the best proportion found between organic and aqueous phases, we decided to investigate increasing sodium carbonate concentrations in order to evaluate the effect on the purification step of our process. Another variable also investigated by us was the type of reactor used as mixing zone. A packed-bed reactor filled with glass pieces, in order to enhance mass transfer and mixing, was used instead of the coil reactor and results are presented on Fig. 1. The efficiency of the purification process at different sodium carbonate concentrations were evaluated based on the peaks observed in GC-FID for acetic anhydride and acetic acid, the two major impurities found in this process.

The best result was obtained using a fixed-bed column reactor and an aqueous solution of 10% sodium carbonate. With these conditions, were did not observe the peaks corresponding to acetic acid and acetic anhydride, indicating the removal of the impurities during the continuous purification. Nevertheless, we observed that for these experimental condition there is a slight hydrolysis (≈ 4%, based on relative areas) of the geranyl acetate. This was concluded based on the presence of a chromatographic peak corresponding to geraniol on the final product.

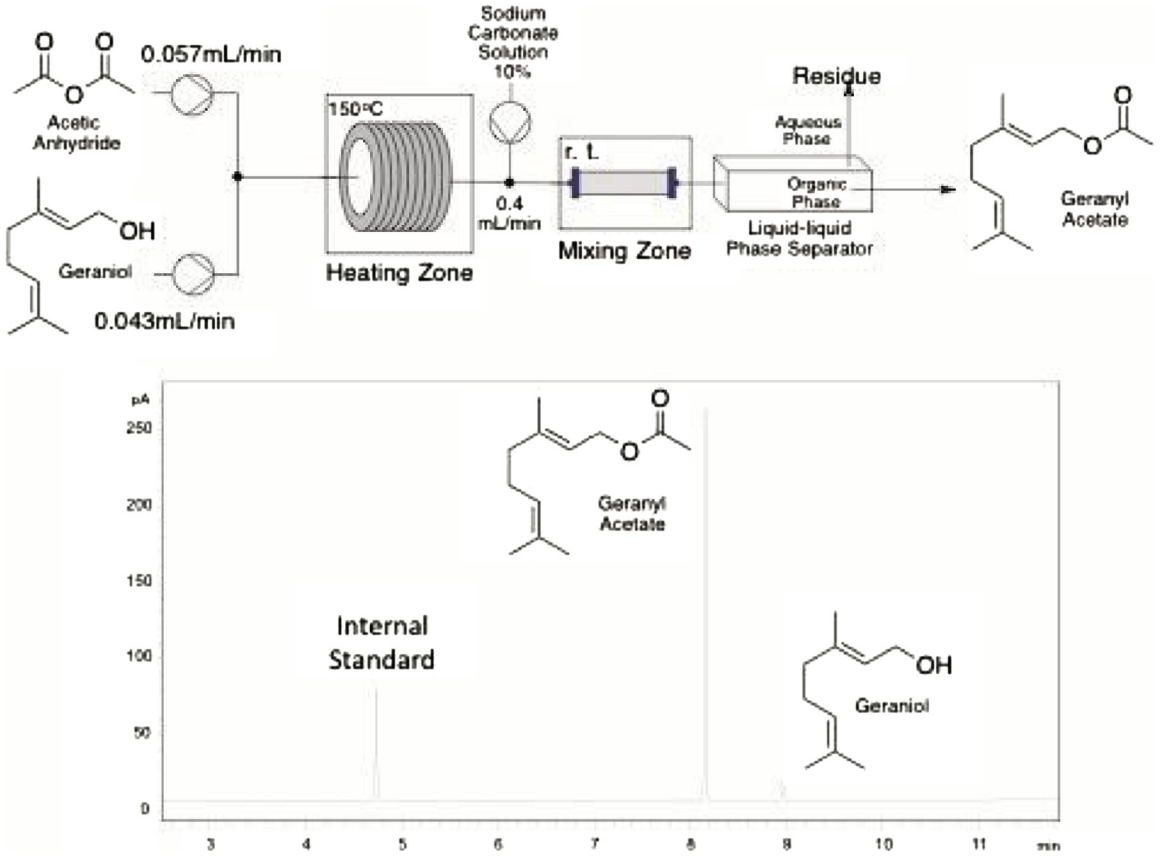
Finally, we performed an experiment with the continuous synthesis and purification based on the best conditions established for the production of geranyl acetate. The optimized process could lead us to the production of geranyl acetate with a final chromatographic purity of 94.1% (Scheme 3).

4. Conclusion

In conclusion, we have developed a continuous flow approach for monoterpenic esters production through biocatalysis or chemical catalysis under continuous-flow conditions. For the biocatalyzed approach, we found that the Novozyme 435 was the best choice for the continuous flow environment due to the long-term stability, leading to high conversion in short residence time and low temperatures. For the chemical



Scheme 2. GC-FID analysis for different proportions tested under continuous flow purification protocol. Proportions of organic to aqueous phase: a: 1:1; b: 1:3 and c 1:4.



Scheme 3. Schematic representation of the continuous-flow synthesis and characterization of geranyl acetate and GC-FID chromatogram of the final product.

approach, we found that acetic anhydride is a good acyl donor, that lead to the desired geranyl acetate with high conversion (> 99%) in only 4 min at 150 °C. The best experimental conditions for the chemical esterification reaction was applied with success for the esterification of citronellol, myrtenol, menthol and the essential oils of *Cymbopogon martinii* and *Mentha arvensis*, which also presented high conversions. Finally, we were able to establish a continuous flow synthesis and purification of geranyl acetate with a chromatographic purity of 94.1% of the final product, being possible to apply the process developed to biocatalyzed or chemical catalyzed reactions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mcat.2018.04.007.

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