ANNEX

A. MATERIALS AND METHODS

A.1. Materials

Table A.1. Reagents used during the project

Reagents	
Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (Pluronic® P-123) M_n ~5800 from Aldrich	Tetraethyl orthosilicate (TEOS) 99% from Aldrich
Dulbecco's Phosphate Buffered Saline (PBS) from biowest	Slide-A-Lyzer® MINI dialysis devices from Thermo Scientific
Hydrochloric acid from from Sigma-Aldrich	Ammonium Fluoride from Fluka
Heptane from from Sigma-Aldrich	Tween® 80 from Sigma-Aldrich
Ethanol Absolute from Panreac	Trypticasein soy agar (TSA) from Pronadisa
Ethyl acetate from Sigma-Aldrich	*S. aureus* strain ATCC 25923 from Ielab
4-Hydroxyphenethyl alcohol (Tyrosol) from Acros Organics	Trypticasein Soy Broth (TSB) from Pronadisa
Cinnamaldehyde from Sigma-Aldrich	Squalene from Sigma -Aldrich
Thymol from Sigma	Carvacrol from Sigma-Aldrich
Eugenol from Acros Organics	β-Caryophyllene from Sigma
Rosmarinic acid from Aldrich	Chlorhexidine Sigma -Aldrich
N-N-Dimethylformamide from Fisher	Methanol from Sigma-Aldrich
Dichloromethane from Sigma-Aldrich	Acetone from Sigma-Aldrich

A.2. Experimental methods

- **Synthesis of rod-shaped mesoporous silica support (SBA-15)**

SBA-15 material with a rod-shaped structure was prepared according to the hydrothermal synthesis method reported by Johanson *et al.* [28]. Firstly, the gel formation was achieved by mixing 1.2 g of Pluronic® P123 with 14 mg of ammonium fluoride, and dissolved in 40 mL of HCl 1.75 M. The mixture was stirred at 20˚C until the polymer dissolution. In another container 2.75 mL of TEOS and 8.5 mL of heptane were mixed. This solution was added to the

micellar solution, once the polymer complete dissolution. The blend was kept under vigorous stirring for 4 min for the reaction, immediately the solution was poured into a hydrothermal reactor (autoclave). After 10 min, the autoclave was closed and the hydrothermal treatment was performed at 100 ˚C for 24 h. After that, the material was washed and filtered with distilled water three times. Finally, the surfactant removal was performed by calcination at 550 ˚C for 5h with temperature ramp of 0.5 ˚C/min.

The resultant material was characterized by Nitrogen adsorption-desorption, ATR-FTIR, TGA, TEM, SEM and XRD.

- **Essential oil compounds loading**

Two EOC loading methods were evaluated. Incipient wetness impregnation and vacuum impregnation. Table A.2.1 summarizes the parameters evaluated to obtain the higher %LE. Carvacrol was used as model compound.

Table A.2.1. Experimental parameters evaluated to obtain a higher %LE.

Parameter	Variation
Concentration	Pure or diluted
Impregnation time (h)	1, 3.5, 15, 24
EOC quantity	1x, 5x
Final wash	Distilled water / ethanol
SBA-15 pre-drying	With / without
Solvent	Acetyl acetate / ethanol 70%

The theoretical free volume of SBA-15 was 1.5 µL EOC/mg_{SBA-15} and was denoted as 1x.

Vacuum impregnation method (VI)

The loading was carried out according to the procedure reported by Carmona [27]. 150 mg of SBA-15 was placed into a pear-shaped flask, heated at 120 ˚C for 6 h, under reduced pressure. Once the system was cooled at 25 ˚C the EOC was added, continued with stirring for 15 h under ambient pressure. After that, the sample was filtered and washed with 8 mL of water.

Incipient wetness impregnation (IW)

Impregnation process accomplished was described by Lillie, J. [3] and Qing-Zhou *et al.* [16] with some modifications. Briefly, 50 mg of calcined SBA-15 was placed into a 10 mL vial and

dried at 35 ˚C for 24 h. In order to evaluate the effect of the amount of EOC used, samples were impregnated with 1x and 5x times the SBA-15 theoretical capacity. Compound (pure or diluted) was added and mixed with a spatula. Afterward, when the pure compound was used the mixture was kept for impregnation at least for 1 h. Experiments with the diluted compound were kept under stirring at 250 rpm for 1 h.

After impregnation time the sample was washed with 8 mL of water or ethanol and then filtered. Finally, the sample was dried at 35 ˚C for 24 h.

Once the method was optimized, the loading was performed for carvacrol, thymol, and cinnamaldehyde. Experimental parameters used were: previous drying, ethyl acetate diluted EOC composition, 1h of impregnation, a final wash with water and finally dried at 35 ˚C for 24 h.
Loaded nanocomposites CRV/SBA, THY/SBA and CIN/SBA were characterized by TGA, SEM, FTIR, N_2 adsorption and Zeta potential.
Loading efficiency was determined from TGA results, following the next equation:

$$\%\ \mathbf{LE} = \frac{\text{loaded } \mu\text{L EOC per mgSBA}}{\text{pore volume } (\mu\text{L}) \text{ per mgSBA}} \times 100$$

– **Essential oil compound release profile**

Phosphate Buffered Saline (PBS) 0.1 M with Tween 2% v/v was employed as release media. Slide-A-Lyzer® MINI dialysis devices were used to perform the release experiment. 10 mg of nanocomposite was dispersed in 700 µL of PBS solution and placed into the dialyzer. A conical falcon was filled with 13.9 mL of buffer and then the dialysis device was introduced into the falcon. The system was stirred at 200 rpm and kept at 37 ºC. The EOC released was quantified by regularly sampling the buffer, up to 400 h. Each time the dialysis media were replaced with fresh solution. EOC released was quantified by UPLC analysis.

– **EOC organic extraction**

Organic extraction was employed to confirm the loaded amount of EOC into the SBA-15. 10 mg of sample was placed into a vial, then 4 mL organic solvent was added, stirred during 1 h, and filtered. Thymol, cinnamaldehyde and carvacrol extraction was fulfilled with methanol, ethanol

70%, and dichloromethane: acetone mixture (3:1), correspondingly**.** EOC concentration was quantified by GC-MS.

– **Thymol loaded SBA-15 assembled to PCL fibers**

Preparation of PCL 10% m/v: 1.26 mg of PCL was dissolved in 5 mL of dimethylformamide (DMF) and 5 mL of dichloromethane (DCM), in all cases the polymer suspension was added at the flux rate of 1 mL/h for fiber formation. The hybrid fibers were produced by the electrospinner 2.2D500 de Yflow® by the double nozzle technique.

Briefly, system configuration utilized is shown in Figure A.2.1, two independent channels were used. Syringe one contained the polymer solution (PCL 10%), the second syringe was filled with a suspension of dispersed SBA-15 rods. The flux rate and solution concentration were 1 or 2 ml/h or 5 or 15 mg/mL respectively. The electrospinning voltages were -4.0 kV for the collector and +10.06 kV or +8.65 kV for the emitter.

Regarding the attachment of thymol loaded SBA-15 nanoparticles, a nanocomposite solution of 10 mg/mL (thymol) was prepared by mixing water: DMF (50:50), the suspension was sonicated for 30 min. Nanocomposite suspension was electrospun at a flux rate of 2 mL/h. The electrospinning voltages were -4.07 kV for the collector and +10.92 kV for the emitter.

All samples were characterized by SEM to confirm the presence of SBA-15 on fiber surface.

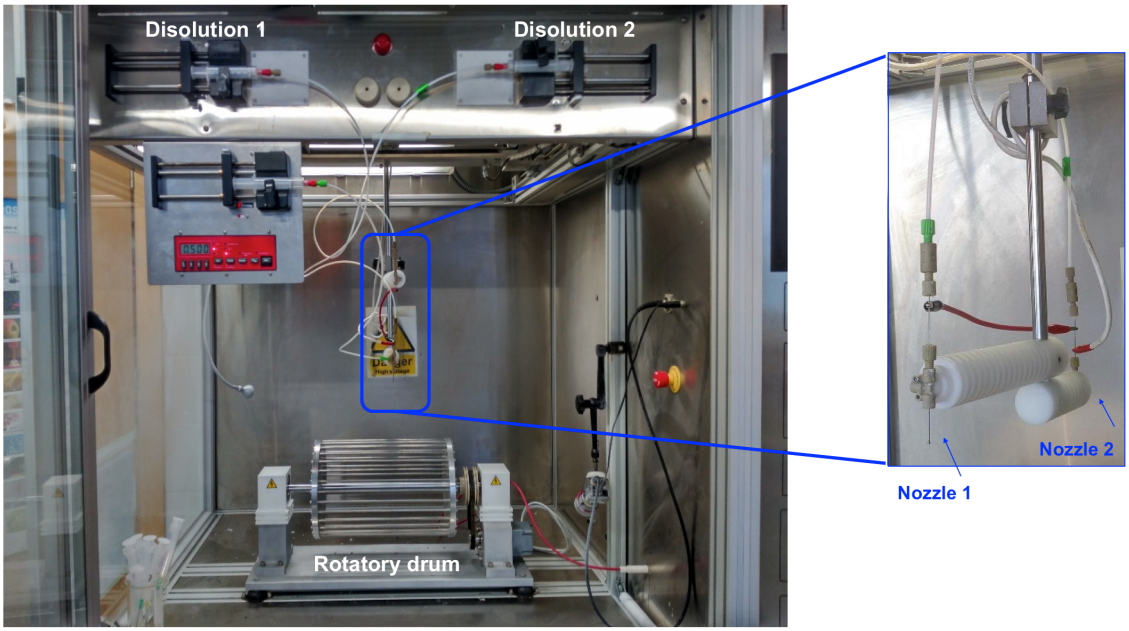


Figure A.2.1. Electrospinner equipment with double nozzle arrange and rotatory drum collector.

- **Bactericidal activity of EOCs and loaded nanoparticles**

MIC and MBC concentration of the EOC (carvacrol, cinnamaldehyde, thymol, eugenol, squalene, tyrosol, rosmarinic acid, and β-Caryophyllene**,** were evaluated in *S. aureus* strain, by using the broth dilution assay method. Three bacteria controls were prepared, a positive control was used to confirm the bacteria growth, the negative control with chlorhexidine (0.1 mg/mL) to verify the bacteria inhibition, and with tween (2%v/v) to evaluate its influence on the bacteria growth.

Briefly, bacteria strain was pre-cultured under stirring (150 rpm) at 37 ºC overnight in TSB growth broth to reach stationary phase (10^9 CFU/mL). After that, bacteria concentration was adjusted to exponential phase (10^5 CFU/mL), and inoculated with the EOC at concentrations from 0.1 mg/mL to 4.0 mg/mL. The mixtures were incubated under stirring (150 rpm) at 37 ºC overnight. After the incubation time, by using an MW96 plate sample serial dilution was performed, and 25 µL was plated onto nutrient agar plates by triplicate. Plates were incubated overnight at 37 ºC. Bacteria growth concentration (CFU/mL) was calculated according to this equation:

$$\textbf{Bacteria concentration } (\textbf{CFU/mL}) = \text{n colonies} \times \frac{1}{\text{drop volume (mL)}} \times \text{dilution}$$

MIC value is obtained when bacteria concentration decreases 2 $\log_{10}$, in our research from 10^9 to 10^7 CFU/mL.

The bactericidal activity of loaded nanoparticles was tested by the same method, with a different EOC concentration based on TGA analysis, with an empty SBA-15 as a growth control.

A.3. Characterization Techniques

- **X-Ray Diffraction**

XRD patterns were obtained using an Empyrean X-ray Diffractometer (PANalytical) with Cu-K_α incident radiation at 45 kV voltage and 40 mA current. Patterns were recorded in a sign range from 0.5˚ to 8˚ with step size of 0.01. Patterns were recorded from 0.5˚ 2θ for small angle.

- **Transmission Electron Microscopy**

TEM images were recorded with an FEI Tecnai T20 microscope operated at 200 kV. Samples were prepared by dispersing the product in distilled water and depositing it on a carbon grid with cupper mesh until water evaporation.

- **Nitrogen adsorption-desorption**

Nitrogen sorption isotherms were obtained with a TriStar 3000 Micromeritics at -195.8˚C with samples degassed at 200 ˚C (40˚C for loaded samples) for 8 h. The BET surface area was measured from the relative pressure of 0.05-0.20. The pore size distribution was determined from adsorption isotherm using the Barret, Joyner y Halenda method (BJH). The total pore volume was calculated at $P_0/P=0.96$.

- **Scanning Electron Microscopy**

Images were acquired employing an SEM FEI Inspect™ F50, in an energy range between 10-15 keV.

Bacteria samples were prepared from growth phase bacteria (10^5 CFU/mL) previously treated individually with CRV, CIN and THY at MIC and MBC value, the control culture was kept untreated. All the samples were incubated at 37ºC overnight, spin-dried at 3000rpm and washed with PBS 0.1M. The bacteria fixation was carried out with glutaraldehyde 2.5% as a protein and lipid cross-linking reagent, several washes with PBS and centrifugations were performed. After that, the samples were filtered and dehydrated with graded ethanol series (30%, 50%, 70%, 80%, 90% and 100%). Finally, they were covered with a thin layer of metal (Gold/Palladium, 15 nm) and evaluated at different magnifications

- **Thermogravimetric Analysis**

TGAs were performed on a TGA/SDTA851^e (Mettler Toledo), using an oxidizing atmosphere with air flow rate of 50 mL/min, with a heating program with temperature ramp of 10˚C/min from 30˚C to 600˚C. All the samples were previously dried during 24 h at 35 ˚C to avoid the water decomposition interference in the thermograms.

- **ATR-FTIR**

Infrared analysis was carried out on a BRUKER Vertex-70 with Attenuated Total Reflectance Fourier infrared analyzer. Measurements was performed in absorbance mode and scans from 4000-600 cm^{-1}.

- **Zeta potential**

The zeta potential measurements of the loaded materials were performed using a Brookhaven Instrument, samples were dispersed in distilled water and the resulting sample pH was around 7.

- **Gas chromatography – Mass spectroscopy (GC-MS)**

EOC concentration from organic extraction were performed by GC-MS analysis using a Shimadzu GC-MS-QP2010 SE equipped with a Phenomenex® ZB-50 column. Helium was used as the carrier gas at 36.3 cm/s, injection temperature was 250 ˚C and injection volume of 1 µL. Temperature program settings were: 50˚C for 1 min, 10˚C/min until 160˚C and 20˚C/min until 200˚C. Retention time for carvacrol, cinnamaldehyde and thymol was 11.5 min, 12.6 min and 11.3 min correspondingly. Limonene was used as a standard reference with a retention time of 6.1 min.

- **Ultra-Performance Liquid Chromatography**

The released concentration of thymol, cinnamaldehyde, and carvacrol was measured by using a UPLC Acquity Class with a variable wavelength) photodiode array detector (PDA) at 275 nm. A C-18 BEH Acquity UPLC column was employed, with a mobile phase of acetonitrile/water, at 40˚C. The flow rate used was 0.3 mL/min with an injection volume of 2 µL, with a naproxen as a reference standard. Cinnamaldehyde quantification was in reference to *trans*-cinnamaldehyde isomer.

B. Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Table B.1.1 *S. aureus* concentration in control samples

Control		Concentration	Bacteria Concentration	
			CFU/mL	STD
Positive	TSB	-	$2.0x10^{09}$	$5.7x10^{08}$
	Tween	2.0 % v/v	$1.3x10^{09}$	$2.9x10^{08}$
Negative	Chlorhexidine	0.1 mg/mL	No growth	

Table B.1.2 MIC and MBC results obtained from dilution method against *S. aureus.*

EOC	MIC			MBC
	(mg/mL)	CFU/mL	STD	(mg/mL)
Carvacrol	**0.2**	$2.0x10^{04}$	$8.3x10^{03}$	**0.3**
Thymol	**0.2**	$4.0x10^{06}$	$9.6x10^{05}$	**0.3**
Cinnamaldehyde	**0.4**	$1.1x10^{05}$	$9.1x10^{04}$	**0.5**
Tyrosol	**1.0**	$7.7x10^{07}$	$6.7x10^{07}$	**4.0**
Eugenol	**1.3**	$7.9x10^{07}$	$9.7x10^{06}$	**1.5**
Rosmarinic Acid	**2.5**	$1.5x10^{05}$	$6.6x10^{04}$	**>4.0**
Squalene	**>4.0**	-	-	**>4.0**
β- Caryophillene	**>4.0**	-	-	**>4.0**

Figure B.1.1 Bacteria concentration at MIC values as an average from four independent experiments in triplicate.

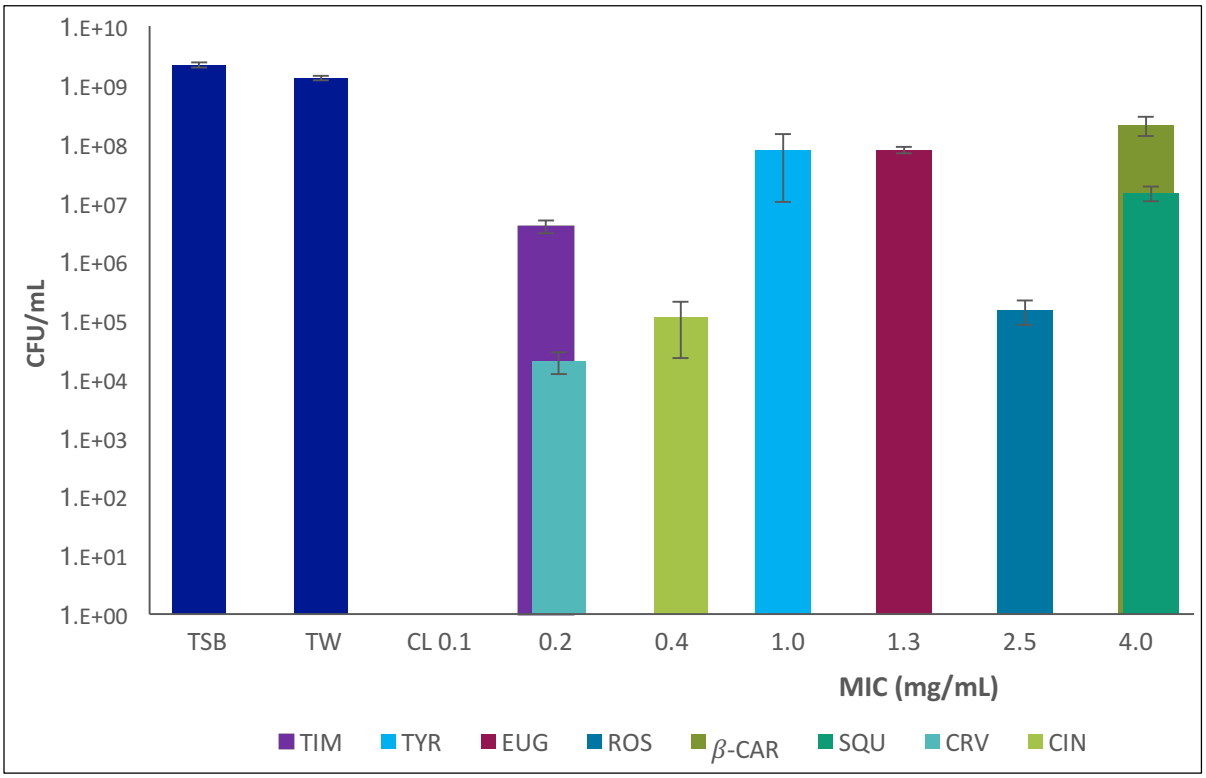
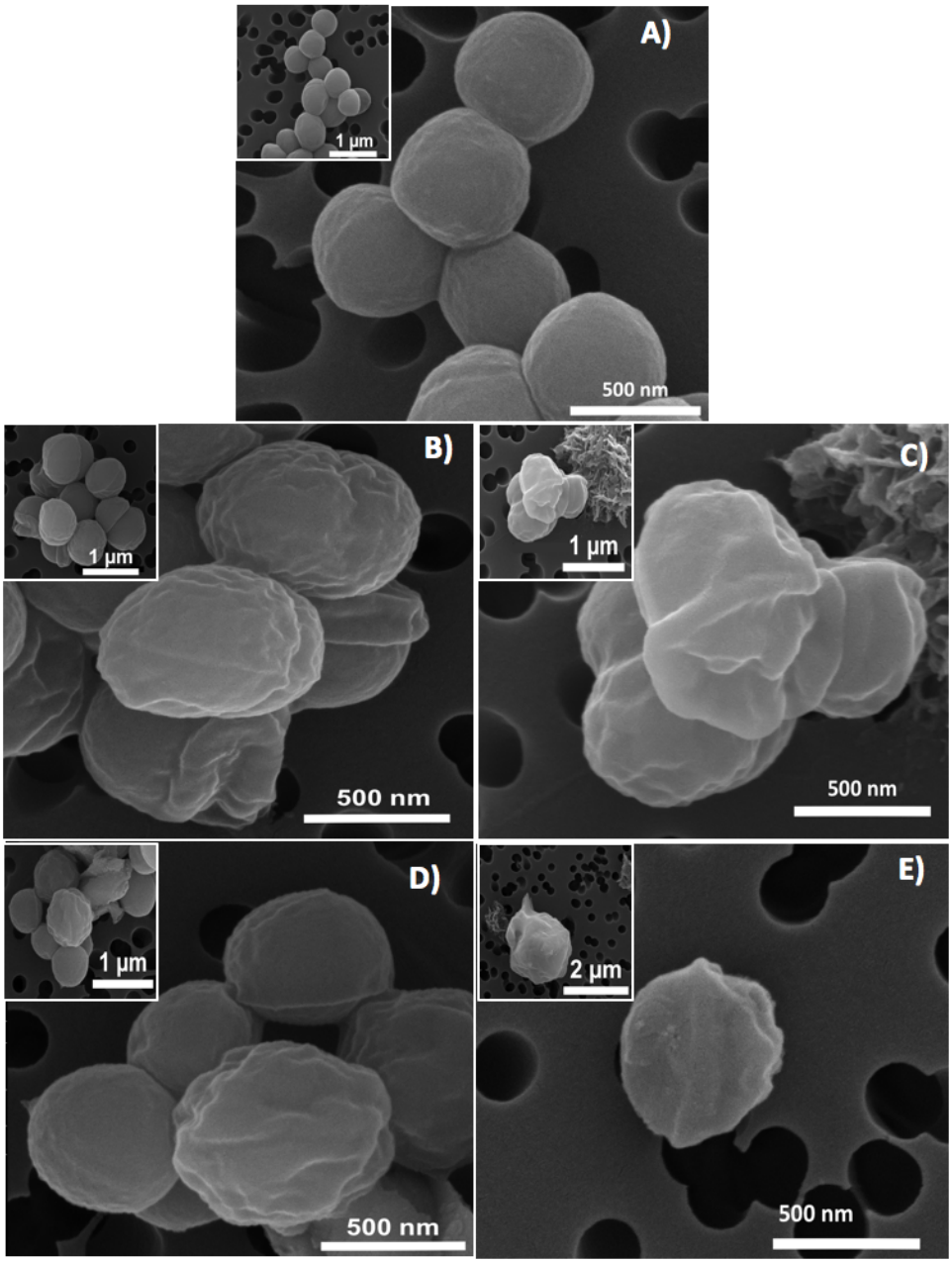


Figure B.1.2 SEM micrographs of *S. aureus.* A) Untreated bacteria. Treated bacteria: B) CRV at MIC value. C) CRV at MBC value. D) CIN at MIC value. E) CIN at MBC value.



C. EOC loading into synthesized SBA-15 rod-shaped particles and compounds release study

C.1 SBA-15 synthesized characterization

Figure C.1.1 Average rods size (external diameter and length) from SEM micrographs and distribution histograms

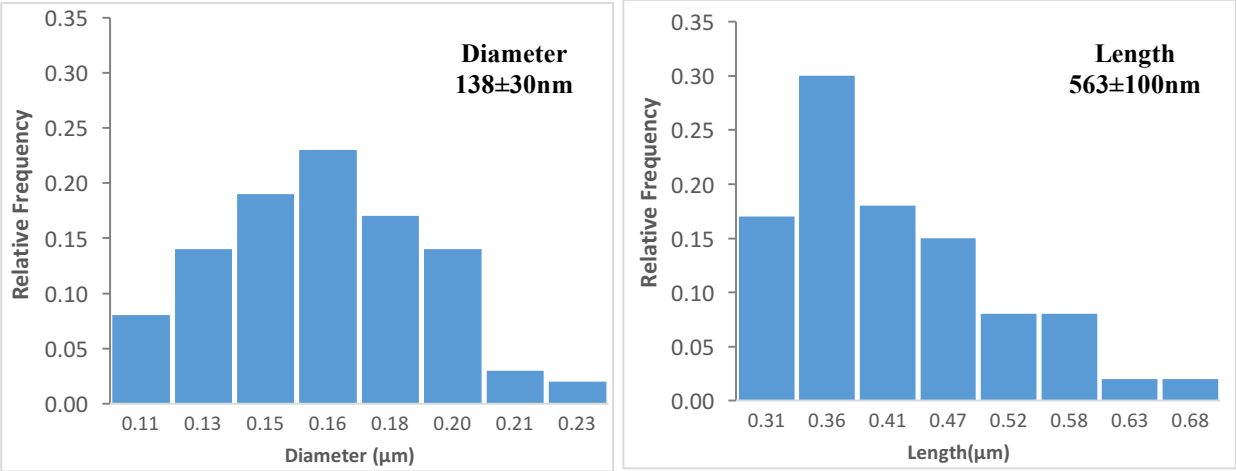


Figure C.1.2 SBA-15 pore size distribution from N_2 analysis

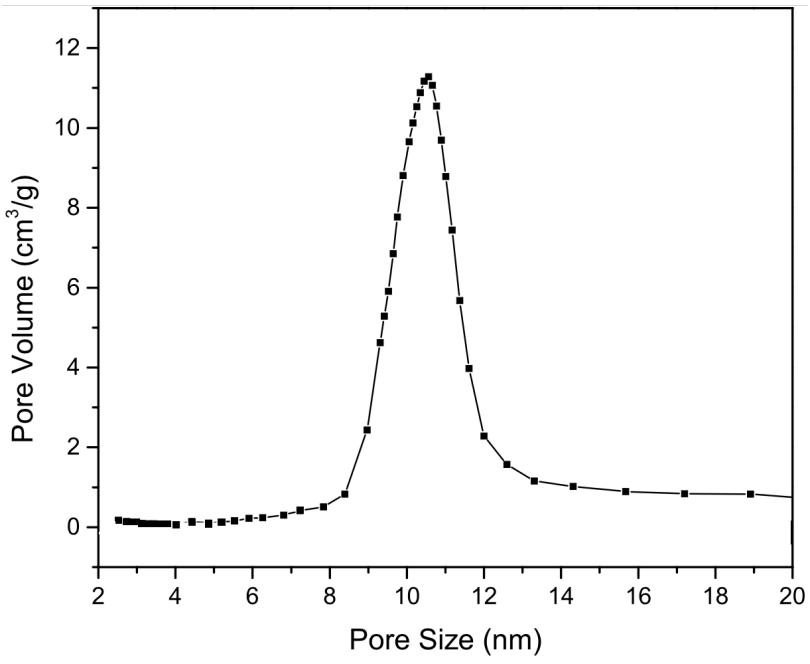
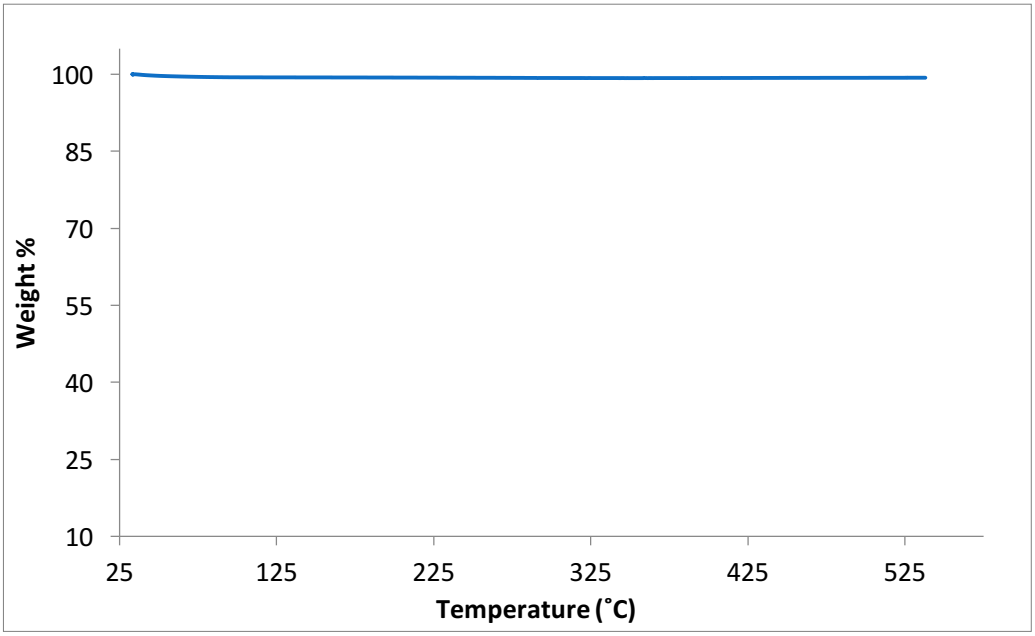


Figure C.1.3 TGA analysis for SBA-15 sample (after calcination)



C.2. EOC loading into SBA-15

Figure C.2.1 Thermogravimetric curves for SBA-15 samples loaded with carvacrol (pure and diluted) by incipient wetness impregnation

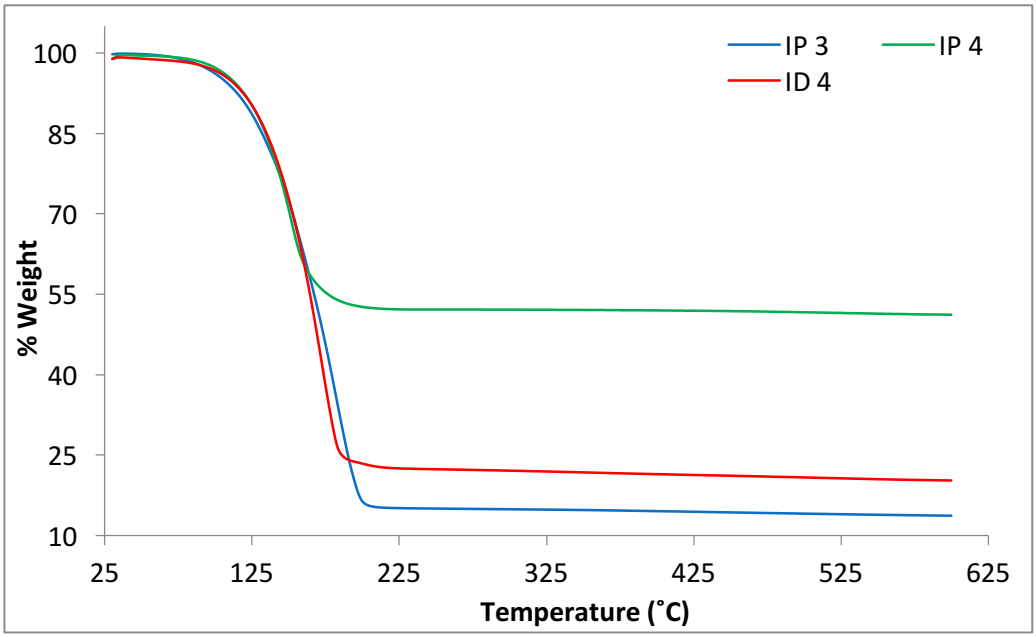


Table C.2.1 Supplementary results of EOC loading experiments obtained from different loading methods and conditions.

Sample	Method	Quantity *	Concentration	Impregnation time (h)	Final wash	Pre-drying	%LE
ID1	Incipient wetness	5x	Diluted/ ethanol	3.5	ethanol	no	82.1
IP2	Incipient wetness	5x	pure	1.0	water	yes	41.8
VI (P)	Vacuum impregnation	5x	Diluted/ ethyl acetate	15	water	yes	190.7

*EOC amount added referred to the theoretical capacity

Figure C.2.2 TGA thermograms of supplementary results of EOC loading experiments obtained from different loading methods and conditions

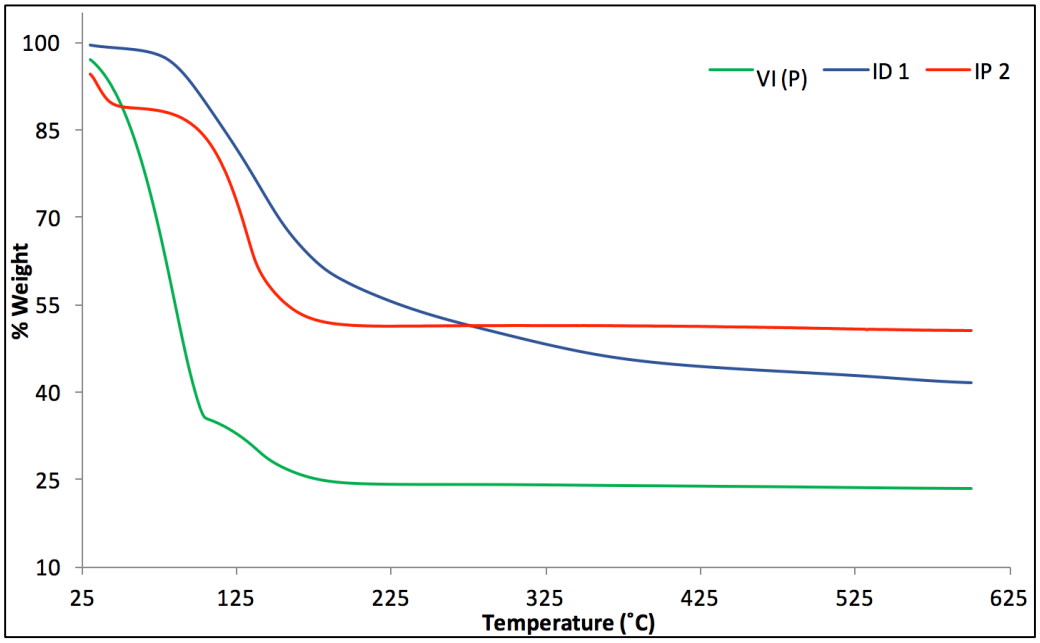


Figure C.2.3. CRV/SBA particles characterization. **A)** Thermogravimetric analysis**. B)** SEM image of loaded rods. **C)** N_2 Adsorption-desorption Isotherm. **D)** FTIR comparative spectrum in absorbance mode

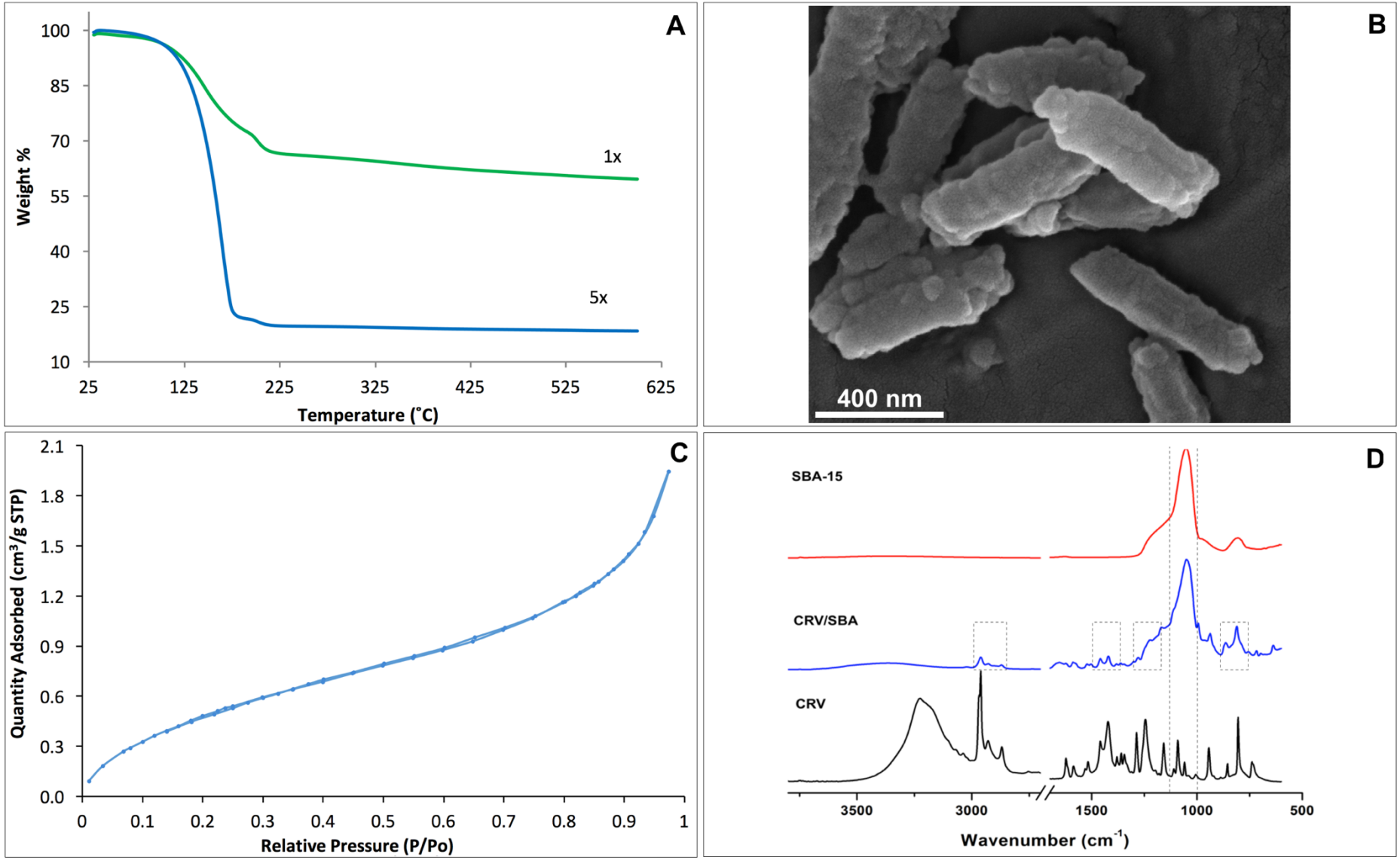


Figure C.2.4 CIN/SBA particles characterization. **A)** Thermogravimetric analysis. **B)** SEM image of loaded rods. **C)** N_2 Adsorption-desorption Isotherm. **D)** FTIR comparative spectrum in absorbance mode

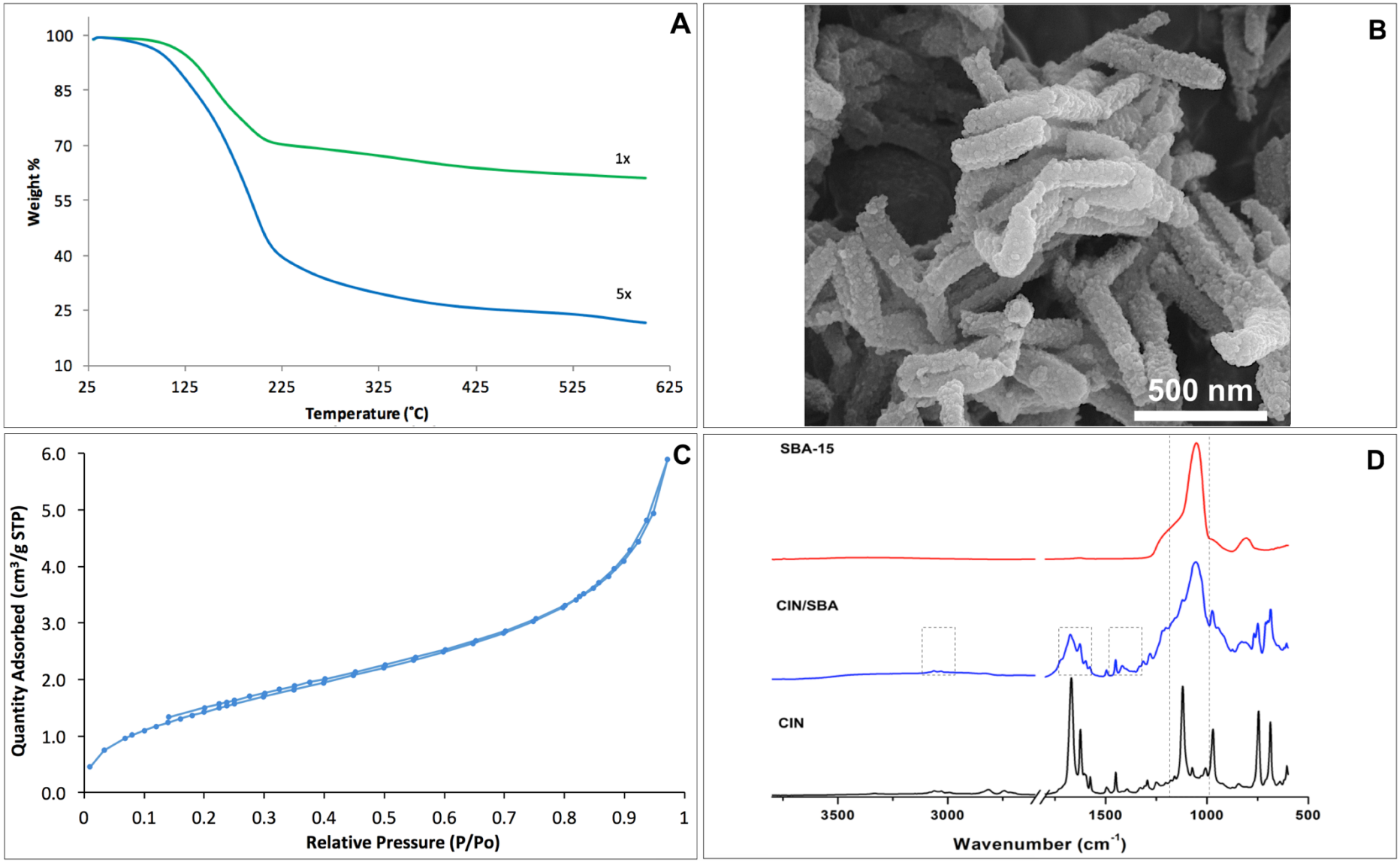


Figure C.2.5. Thermal degradation and differential thermogravimetric curves of **A)** Thymol **B)** Carvacrol, **C)** Cinnamaldehyde

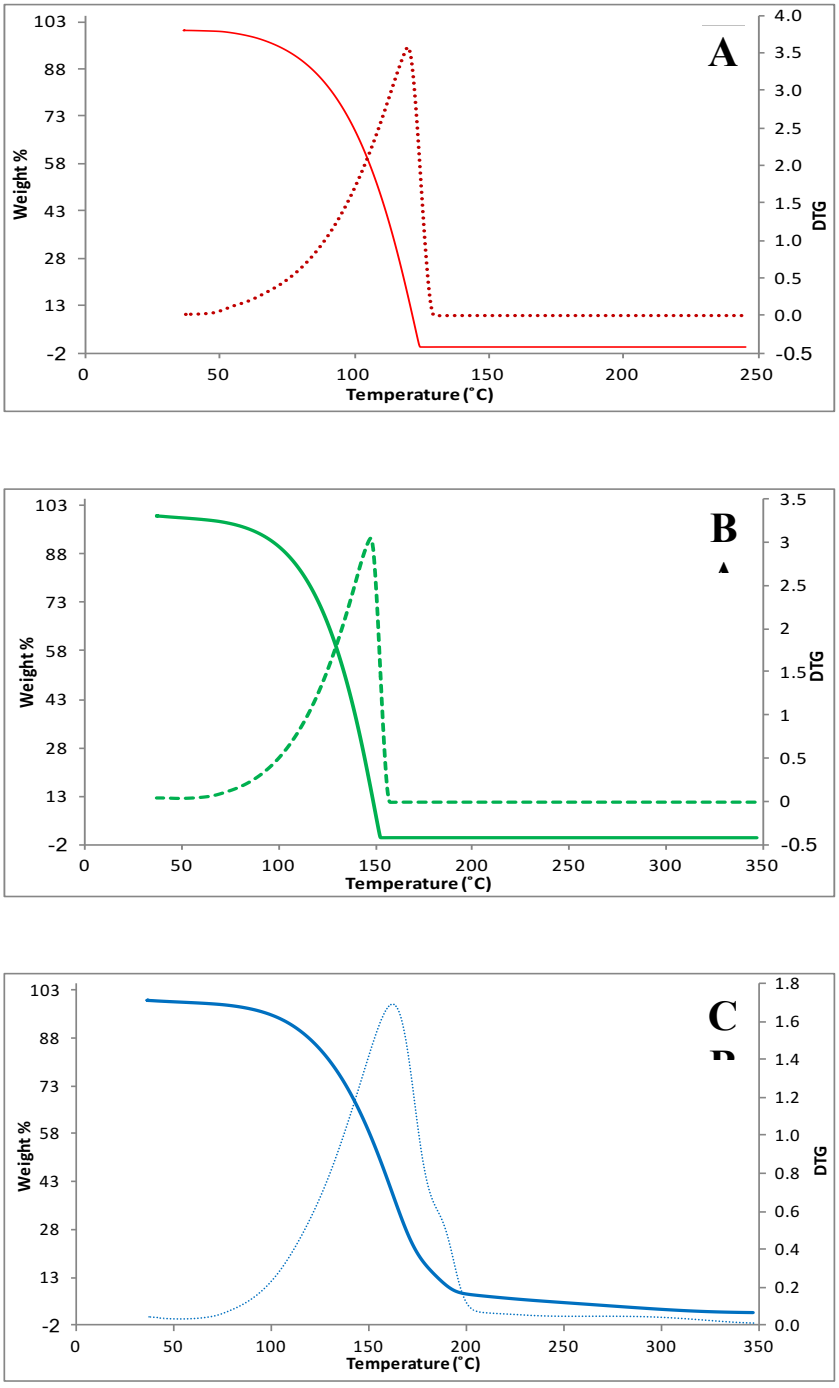


Figure C.2.6 MS chromatogram of extracted cinnamaldehyde from SBA-15 particles

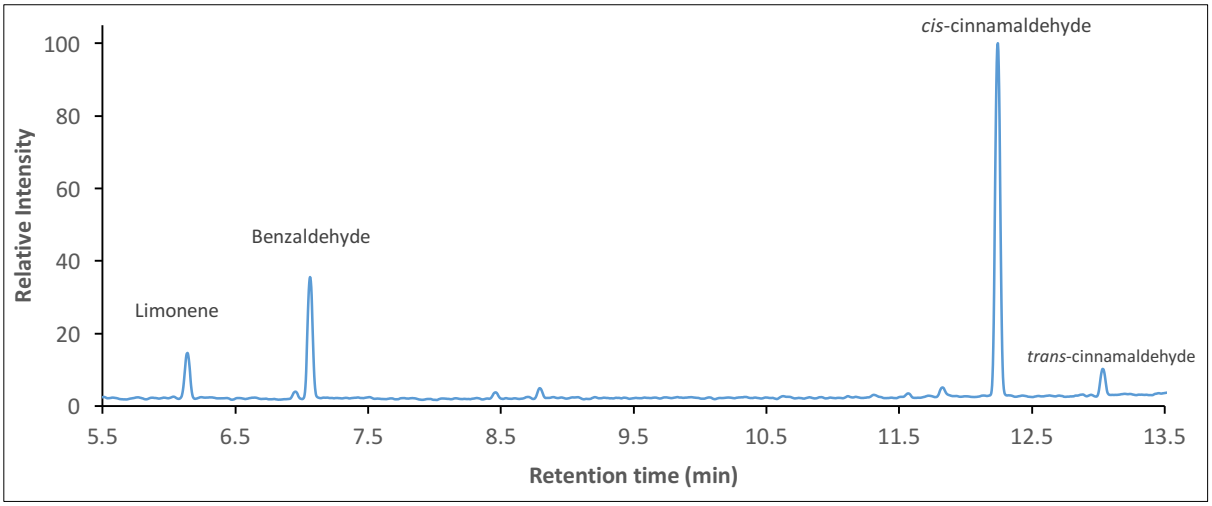


Figure C.2.7 Overlay diagrams of pore volume distribution for EOC loaded SBA-15, from N_2 adsorption-desorption analysis. Inset scaled up diagram