Direct Lipase Catalyzed Lipophilization of Chlorogenic Acid in Supercritical Carbon Dioxide

Carlos E. Hernandez 1, Ho-Hsien Chen 2, Chi-I Chang 3, Tzou-Chi Huang 2*

1. Department of Tropical Agriculture and International Cooperation, 2. Department of Food Science and 3. Graduate Institute of Biotechnology, National Pingtung University of Science and Technology

ABSTRACT

The enzymatic lipophilization of natural antioxidants is a process of enormous pharmaceutical interest; the direct esterification of such polar compounds, however, is a major challenge due to the heterogeneity of these reactions. In the present study, the Taguchi approach was employed to optimize the esterification of 5-CGA by immobilized Candida antarctica lipase B in SC CO2/t-butanol. The effects of temperature (35-55 °C), pressure (150-250 bar), t-butanol (2-10 % v/v), and the enzyme amount (10–30 mg/ml), were investigated. The maximum conversion reached 63 % in 25 hours at 150 bar, 55 °C, 10 % t-butanol (v/v), and 20 mg/ml of lipase. The SC CO2 selectivity towards the esterified product was the working principle of this study, by which minimized interphase transport limitations and enhanced mass-transfer phenomena substantially improved the reaction kinetics. This investigation offers an alternative towards the functionalization of natural antioxidants which harmonizes with the use of green technologies.

Key words: Candida antarctica lipase; Chlorogenic acid; Lipophilization; Supercritical CO₂
Introduction

Chlorogenic acid (5-cafeoyl-quinic acid, 5-CGA) has been associated to a broad range of bioactivities (Bonita et al., 2007; Clifford, 2000; Zheng et al., 2008), while its consumption from coffee water extracts determined to be safe (Watanabe et al., 2006). Biological properties of 5-CGA are primarily attributed to its capacity to donate hydrogen atoms of the phenolic ring to free radicals, therefore inhibiting oxidation processes. It has been proposed that such bioactivities as well as the bioavailability of natural phenolics can be enhanced by increasing their lipophilicity. The resulting amphiphilic structured phenolic would incorporate both emulsifying and antioxidant properties (Figueroa-Espinoza, and Villeneuve, 2005; Jayaprakasam et al., 2006; Sabally et al., 2006; Vosmann, Weitkamp, and Weber, 2006; Weitkamp, Vosmann, and Weber, 2006).

Towards the functionalization of 5-CGA in food and pharmaceuticals, enzymatic lipophilization has been considered by esterifying the carboxylic acid moiety of the quinic acid with a fatty alcohol (Figueroa-Espinoza, and Villeneuve, 2005). Guyot et al. (2000) reported the esterification of 5-CGA with fatty alcohols catalyzed by lipase (Novozyme 435) in free and added-solvent systems, with conversion rates of 40-75 % after 30 days. Even though relatively high yields were obtained, the characteristic heterogeneity of this reaction resulted in extremely slow conversion rates.

Most recently, an alternative chemo-enzymatic strategy was proposed, where 5-CGA was first chemically esterified with methanol and then transesterified with fatty alcohols. Although the overall yield of this two-step reaction was 61-93 % after 96 hours, a 9-hour methanol mediated esterification and the subsequent removal of this toxic reagent represent a major drawback.
The use of supercritical CO$_2$ (SC CO$_2$) as an alternative solvent for lipase catalyzed esterification has been a subject of much research due to its adjustable solvation and favorable transport properties (Knez, and Habulin, 2002; Laudani et al., 2007). Hence, opportunities arise to integrate 5-CGA biotransformation and the extraction/fractionation of less polar derivates by a simultaneous SC CO$_2$ process. Furthermore, the use of SC CO$_2$ is regarded as an alternative that harmonizes the functionalization of natural products with the uses of green technological processes.

Considering the multidimensional nature of a biotransformation process in SC CO$_2$ (multiple parameters, responses and interrelationships), the Taguchi method provides a convenient systematic approach. The Taguchi experimental design, based on the orthogonal arrays, allows a study of major process parameters in order to calculate their effect and determine the optimum condition having the least variability (expressed as signal-to-noise (S/N) ratio), (Kim et al., 2007; Teng, and Xu, 2007).

The aim of this work was: 1) to preliminary optimize the lipase-catalyzed esterification of 5-CGA with 1-heptanol in SC CO$_2$, by estimating effects of major reaction parameter (temperature, pressure, co-solvent percentage, and enzyme concentration) on the overall reaction kinetics, as well as on the resulting ester concentration; and 2) to examine the effect of the alcohol chain length on the reaction course as well as on the overall conversion.
Materials and Methods

Materials

Immobilized lipase from *Candida antarctica* (lipase B, Novozym 435) was provided by Novo Nordisk. Chlorogenic acid (5-CGA), 2-methyl-2-propanol (t-butanol), 1-heptanol, 1-pentanol, and geraniol were obtained from Sigma-Aldrich. All solvents used were HPLC grade and from Merck.

Lipase Catalyzed Esterification in SC CO₂

A batch-operated stirred-system was specially designed and built in our laboratory to carry out the enzymatic reaction in SC CO₂. The system with a cell volume of 50 mL is described in Figure 1.

The reaction was run at 35-55 °C and 150-250 bar, for 25 hours. The reaction mixture composed of 5-CGA (100 mg), 1-heptanol (10 % v/v), t-butanol (2, 5, and 10 % v/v), and Novozym 435 (10, 20 and 30 mg/ml), was loaded into the reactor (Tables 1-2). After sealing, pressurization was achieved by pumping liquid CO₂ to the desired conditions. The reaction was then allowed to proceed and samples (2 mL each) were collected every 5 hour into 2 mL methanol for subsequent HPLC analysis. Samples were withdrawn by a sampling loop from the top of the reactor, and SC CO₂ was pumped to adjust pressure to the operational conditions. Further experiments were run with 1-pentanol and geraniol using the optimized parameters (as found for 1-heptanol), in order to examine the carbon chain effect. All experiments were run by duplication.

High Performance Liquid Chromatography (HPLC)

Samples obtained from the SC CO₂ reaction mixture were dissolved in methanol and filtered through a 0.45 µm syringe filter (Millipore). The analyses were performed
using a HPLC system consisting of a Hitachi L-6200A intelligent pump, a Hitachi L-7200 autosampler, and a Hitachi L-4500 diode array detector.

The chromatographic separations of ester and chlorogenic acid were done by a reverse-phase C\text{18} column (250 mm × 4.6 mm, 5 µm particle size, Hyperclone, Phenomenex). The temperature of the column was set at 35 °C and the injection volume was 20 µL. Gradient elution was employed with a mobile phase consisting of acetonitrile (A) and 1% glacial acetic acid (B). The flow rate was kept constant at 1.0 mL/min for a total run of 55 min. The system was run with a gradient program as follow: 0-10 % B in 15 min, 10-20 % B in 10 min, 20-40 % B in 5 min, 40-100 % B in 10 min, maintaining 100 % B for 5 min, and returning to 0 % B isocratic for 5 min as post run for equilibration of the column. Quantification was performed by the external standard method and the detection was achieved at 325 nm.

**Extraction and Isolation**

The formed heptyl chlorogenate was extracted from the SCO\textsubscript{2} soluble fraction by a 20 minutes dynamic mode at the reaction conditions, and collected in 50 mL of methanol. The solvent was then evaporated under vacuum at 45 °C to dryness, the remaining solids were re-dissolved in 2 mL of methanol-dichloromethane (1:20), and chromatographed on silica gel 60 (230-400 mesh, Merck) using mixtures of methanol-dichloromethane (1:20, 1:4, 1:2, 2:1). Fractions containing the reaction product were identified by TLC using silica gel 60 F\textsubscript{254} plates (Merck), and purified by crystallization.
**NMR Identification**

NMR spectrum was recorded in CDCl$_3$ at room temperature on a Varian Mercury plus 400 NMR spectrometer, and the solvent resonance was used as internal shift reference (TMS as standard). EIMS was recorded on a Finnigan TSQ-700 spectrometer.

**Taguchi Experimental Design**

For designing the experiment based on the Taguchi Method (Kim *et al.*, 2007; Teng, and Xu, 2007), and considering the number of factors and levels of this experiment (Table 1), an L$_9$ (3$^4$) orthogonal array has been used (arrangement of factors and levels in Table 2). For analyzing the effect of the control factors, the concentration of heptyl chlorogenate, the overall esterification yield, and corresponding reaction rates were used as the dependent variables.

In order to calculate the deviation of the experimental values from the highest desirable, the signal-to-noise ratio ($\eta_{ij}$) has been calculated:

$$\eta_{ij} = -10 \log \left( \frac{1}{n} \sum_{k=1}^{n} \left( \frac{1}{(y_{ijk})^2} \right) \right)$$

Where $y_{ijk}$ is the experimental value of the $i$th quality parameter in the $j$th experiment at the $k$th trial, and $n$ the number of trials.

The signal-to-noise ratios ($\eta_{ij}$) were further transformed into grades, expressed as:

$$x_{ij} = \frac{\eta_{ij} - \min_{j} \eta_{ij}}{\max_{j} \eta_{ij} - \min_{j} \eta_{ij}}$$

Where $x_{ij}$ represents the normalized $\eta_{ij}$ (transformed into a value between 1 and 0) for the $i$th performance characteristic in the $j$th experiment.
Analysis of variance (ANOVA) yielded the percent contribution of each factor. The average effect of a factor at a level was calculated by examining the orthogonal array, the factor assignment, and the experimental results.

The graph of factor influence was obtained by plotting the average factor-level effect (numerical value of results along the y-axis) against the corresponding factor level (x-axis).
Results and Discussion

In this work, the possibility of performing a lipase-catalyzed esterification of 5-CGA and 1-heptanol in SC CO₂, while simultaneously extracting the formed compound, was demonstrated.

The biotransformation of 5-CGA via esterification with 1-heptanol was used to incorporate the corresponding seven-carbon aliphatic chain, and as a result modify its polarity. As shown by HPLC analysis of samples withdrawn at specific intervals from the reaction mixture (Figure 2), lipophilization resulted in a significant increase of the acid original retention time (from 24 to 38 min) and Log P values (from -0.75 to 2). The resulting modified lipophilicity consequently enhanced the selectivity of the reaction medium towards the formed ester.

The Taguchi method allowed a systematic estimation of the process parameter effects towards the identification of the optimum condition. The design of this experiment based on a L₉ (3⁴) orthogonal array and the corresponding response values are shown in Table 2. The values of S/N ratios were transformed into a normalized grade (x), in order to calculate the deviation of the experimental values from the highest possible (desired value, x =1).

Chlorogenic Acid/ Heptyl Ester Concentration in SC CO₂/t-butanol

As stated by several researchers, the solubility of phenolic acids in SC CO₂ is essentially minimized by the degree of OH-ring substitutions (Chafer et al., 2007; Murga et al., 2003). Accordingly, it was shown by preliminary experiments run in pure SC CO₂ at 200 bar and 50 °C, a considerable low solubility of 5-CGA at 2 hours (0.03 ± 0.01 mM/ 100 mg), and a resulting low production of heptyl chlorogenate, reaching 0.10 ± 0.01 mM after 14 hours.
Noteworthy is the low dielectric constant of SC CO\textsubscript{2}, which makes essential the addition of a co-solvent capable of hydrogen-bonding and dipole-dipole interactions (Diaz-Reinoso \textit{et al.}, 2006; Villeneuve \textit{et al.}, 2000). Thus, \textit{t}-butanol was chosen as the solubility enhancer, while on the other hand it would not interfere on the reaction as a lipase substrate since it is sterically hindered.

An improved 5-CGA solubility was obtained at the highest \textit{t}-butanol percentage (10 % v/v), resulting in concentrations of 0.14 ± 0.03 mM (trial 3), 0.22 ± 0.02 mM (trial 5), and 0.11 ± 0.05 mM (trial 7).

Addition of \textit{t}-butanol was found to be the most influential factor determining solubility of heptyl chlorogenate, as it was for its acid precursor. However, due to an increased lipophilicity, the solubility of the heptyl chlorogenate in the SC CO\textsubscript{2} phase was found to be considerably higher in comparison with 5-CGA (as shown in Figure 2).

The experimental parameter effects on the ester partition in SC CO\textsubscript{2} were estimated based on its concentration and total yield at 25 hours (Figure 3). Heptyl chlorogenate was distributed between the polar phase (mixture of immobilized lipase and 5-CGA), and the SC CO\textsubscript{2} phase, with equilibrium concentration in the latter increasing with co-solvent addition and temperature. An increased concentration of the ester was attributed to high levels of \textit{t}-butanol, which was observed as an increased partitioning in SC CO\textsubscript{2}, reaching 46, 44, and 40 % in trials 5, 7 and 9, respectively. In contrast, a lower concentration was associated to high amounts of enzyme, and evidenced as poor ester partitions in SCO\textsubscript{2}, attaining 21 (trial 3), 20 (trial 4), and 14 % (trial 8).

While the effect of \textit{t}-butanol alone on heptyl chlorogenate partition was 47 %, that of lipase concentration was 35 %, due to an increased area of absorption in the reaction
mixture. Given the high effect of \( t \)-butanol on the solubility enhancement, adjusting temperature and pressure was less significant (12 and 1.6 \%, respectively).

Table 2 evidenced the dynamic behavior of product concentration determined by the variable combination of factors. Thought, as stated, a solubility enhancement effect was firstly determined by the \( t \)-butanol percentage, higher concentrations of heptyl chlorogenate (faster reaction rates) were rather determined by the effect of temperature (51 \%) and secondary by the co-solvent (25 \%). Minor contributions were associated with lipase concentration (14 \%) and pressure (9.2 \%) at the studied ranges.

As shown in Figure 4, the optimum condition for higher concentrations of heptyl chlorogenate in the reaction mixture was 55 °C, 150 bar, \( t \)-butanol at 10 \%(v/v), and 20 mg/ml of Novozym 325. As high temperature (55 °C) and co-solvent levels (10 \% v/v) were associated with faster reaction rates and better partitioning, an intermediate lipase level (20 mg/ml) favored optimum ester concentrations, since higher amounts of enzyme would interfere by an agglomeration effect.

The effect of pressure on the heptyl chlorogenate concentration in SC CO\(_2\) was minimized by the high contributions of co-solvent and temperature. However, under the conditions of this experiment, concentrations of heptyl chlorogenate decreased with pressure in the range of 150 to 200 bar (Figure 4). Therefore, experiments at low pressure and high temperature (lower SC CO\(_2\) densities) were rather associated with higher heptyl chlorogenate selectivity.

**Esterification Efficiency and Reaction Rate**

Various studies have been published for investigating Novozym 435-catalyzed esterification of phenolic acids with alcohol substrates in conventional organic solvents (Figueroa-Espinoza, and Villeneuve, 2005; Guyot \textit{et al.}, 2000; Lopez Giraldo...
et al., 2007; Stevenson et al., 2007; Villeneuve, 2007; Weitkamp, Vosmann, and Weber, 2006). Alternatively, numerous experiments have been done to improve the stability and efficiency of this biocatalyzer in SC CO₂, for esterification of a diversity of substrates under a multitude of conditions (Habulin et al., 2007; Knez, and Habulin, 2002; Laudani et al., 2007). A few works, however, have explored the possibility of performing a lipase catalyzed reaction of phenolic acids in this non-conventional medium.

In this work, RP-HPLC with UV detection at 325 nm was used to monitor the lipase-catalyzed synthesis of heptyl chlorogenate in SC CO₂/t-butanol. The esterification yields and conversion rates of this reaction are shown in Table 2.

As it is noted, the conversion of the esterification reaction took place at relatively high rates in trials 9, 7 and 5. The revealed rapidly increasing conversions were found to be highly influenced by the effect of temperature (51 %), while it was inversely correlated to large amounts of lipase (38 %). As observed (Lopez Giraldo et al., 2007), addition of lipase up to a certain amount could induce a non-desirable biocatalyst agglomeration. In a less extend, reaction rates were increased with pressure and t-butanol due to substrate aggregation and an enhanced solubility, respectively (Habulin et al., 2007). It is worthy to mention that since pressure and co-solvent effects were less significant (3.8 and 5 %, respectively) at the studied levels, selecting the overall optimum condition must be rather systematic, considering other responses.

In general, trial 7 (55 °C, 150 bar, 10 % (v/v) t-butanol, and 20 mg/ml of lipase) showed optimum conditions towards the synthesis of heptyl chlorogenate at relatively high rates, reaching 63 % of product formation after 25 h of incubation.

As shown in Figure 5, a steady maximum velocity was attained at 20 hours, while prolongation of experiments up to 35 hours did not yield higher results. Water
formation was assumed to be the control factor of both the kinetics and thermodynamic equilibrium of the reaction. As it has been recognized elsewhere (Colombié et al., 1998), water formation at the beginning of the reaction provides the lipase with the required conditions to be active, and as the reaction proceeds water would reverse the reaction and slow down the conversion rates.

**Effect of the Alcohol Chain Length**

In addition to 1-heptanol, 5-CGA was subject to esterification with 1-pentanol and geraniol at: 55 °C, 150 bar, 20 mg/ml of lipase, and 10 % v/v t-butanol, for 25 hours. As it has been demonstrated (Guyot et al., 2000; Lopez Giraldo et al., 2007), the length of the carbon chain plays an important role on the reaction rate. Figure 5 shows variable rates for different fatty alcohols, which points out an effect from the differences in the reagent Log P values. 1-heptanol (Log P = 2.22) and 1-pentanol (Log P = 1.39) yielded comparatively high esterifications (63-65 %, respectively), while a lower percentage was observed when geraniol (Log P = 2.49) was used (42 %). Likewise, the variable concentration of esters on the supercritical medium was influenced by their respective solubilities, which was a function of the modified polarity (depending on the carbon chain length). In this way, while higher Log P values (longer carbon chains) contributed to a better solubility in SC CO₂, lower Log P values (shorter carbon chains) allowed higher conversions due to a better substrate aggregation. Thus, although heptyl chlorogenate showed higher concentrations than the pentyl derivative, their final yield was not significantly different.

Overall, this supercritical medium provided an enhanced selectivity for a less hydrophilic product, as well as a reaction thermodynamically shifted towards synthesis rather than hydrolysis. SC CO₂ lipase-catalyzed reaction greatest advantage
was the simple downstream processing and fractionation of the ester and unreacted acids, by simple adjusting operating conditions.

NMR Characterization of Heptyl Chlorogenate

\(^1 H\) and \(^{13} C\) NMR data are shown in Table 3. The EIMS spectrum for the purified heptyl chlorogenate shows the molecular ion at m/z 452 (M\(^+\), 12), and other major ions at: 392 (4), 351 (5), 336 ([M-heptoxyl]\(^+\), 5), 336 (20), 272 ([M-caffeic acid]\(^+\), 3), 180 (caffeic acid, 43), 163 (cafferoyl, 100), 147 (20), 134 (25), 111 (38), 70 (68), 55 (67). The corresponding structure of heptyl chlorogenate is illustrated in Figure 2.
Conclusions

The Taguchi experimental design provided valuable insights into the lipase-catalyzed esterification of 5-CGA in SC CO₂.

Immobilized lipase B from Candida antarctica (20 mg/ml) was used to successfully lipophilize 5-CGA by esterification with 1-heptanol in SCO₂/1-butanol (10 % v/v) at 150 bar, and 55 °C. Under these conditions, reaction rates approached 2.32 µM ester/g lipase per minute with conversions of 63 %.

The SC CO₂ selectivity towards the esterified product was the working principle of this study, by which minimized interphase transport limitations and enhanced mass-transfer phenomena substantially improved the reaction kinetics.

Coupling lipase-catalyzed esterification of 5-CGA with SC CO₂ combined the biotransformation of an originally polar compound with the extraction of a rather lipophilic derivate. SC CO₂ offers great advantages for controlling the conversion rate of this reaction, as well as facilitates fractionation and extraction of products by pressure/temperature manipulations.

Further experiments are needed regarding studies on solubility of reagents and products, in addition to phase behavior of the mixtures with SC CO₂ in order to understand in detail the occurring phenomena. It is also necessary the study of water control alternatives in this reaction system.
References


Habulin, M., S. Sabeder, M. Paljevac, M. Primozic, and Z. Knez. 2007. "Lipase-Catalyzed Esterification of Citronellol with Lauric Acid in Supercritical
Carbon Dioxide/Co-Solvent Media." *Journal of Supercritical Fluids* 43 (2): 199-203.


<table>
<thead>
<tr>
<th>Parameter</th>
<th>Level 1</th>
<th>Level 2</th>
<th>Level 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Temperature (°C)</td>
<td>35</td>
<td>45</td>
<td>55</td>
</tr>
<tr>
<td>B Pressure (bar)</td>
<td>150</td>
<td>200</td>
<td>250</td>
</tr>
<tr>
<td>C t-BuOH (%)</td>
<td>2</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>D Lipase (mg/ml)</td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
</tbody>
</table>
Table 2. Heptyl Chlorogenate Concentration, Overall Esterification Yield, and Reaction rate in \( \text{SCO}_2 \) at 25 Hours (Orthogonal Array L9 \( (3^4) \))

<table>
<thead>
<tr>
<th>Trial</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Ester Concentration</th>
<th>Esterification Yield</th>
<th>Reaction Rate</th>
</tr>
</thead>
</table>
|       |   |   |   |   | mM | %  | µM/g | min | %
| 1     | 1 | 1 | 1 | 1 | 0.18 | 0.04 | 13.76 | 0.01 | 1.03 | 0.53 |
| 2     | 1 | 2 | 2 | 2 | 0.20 | 0.09 | 14.66 | 0.05 | 0.55 | 0.23 |
| 3     | 1 | 3 | 3 | 3 | 0.16 | 0.00 | 13.62 | 0.00 | 0.34 | 0.00 |
| 4     | 2 | 1 | 2 | 3 | 0.34 | 0.33 | 29.45 | 0.48 | 0.74 | 0.37 |
| 5     | 2 | 2 | 3 | 1 | 0.65 | 0.62 | 26.51 | 0.45 | 2.01 | 0.84 |
| 6     | 2 | 3 | 1 | 2 | 0.28 | 0.23 | 31.48 | 0.49 | 1.18 | 0.59 |
| 7     | 3 | 1 | 3 | 2 | 1.54 | 1.00 | 62.57 | 1.00 | 2.32 | 0.91 |
| 8     | 3 | 2 | 1 | 3 | 0.37 | 0.36 | 45.42 | 0.80 | 1.14 | 0.57 |
| 9     | 3 | 3 | 2 | 1 | 0.82 | 0.72 | 46.95 | 0.82 | 3.10 | 1.00 |
Table 3. $^{13}$C and $^1$H-NMR Data for Heptyl Chlorogenate (400 MHz in CDCl$_3$)

<table>
<thead>
<tr>
<th>No.</th>
<th>$\delta_C$</th>
<th>$\delta_H$</th>
<th>No.</th>
<th>$\delta_C$</th>
<th>$\delta_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75.7</td>
<td>1.89 br t (12.0)</td>
<td>13</td>
<td>22.5</td>
<td>0.83 t (6.4)</td>
</tr>
<tr>
<td>2</td>
<td>38.6</td>
<td>2.31 br d (12.0)</td>
<td>14</td>
<td>14.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>70.3</td>
<td>5.43 t (9.6)</td>
<td>1’</td>
<td>126.6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>73.8</td>
<td>3.77 d (9.6)</td>
<td>2’</td>
<td>115.1</td>
<td>6.85 br s</td>
</tr>
<tr>
<td>5</td>
<td>70.7</td>
<td>4.28 br s</td>
<td>3’</td>
<td>144.1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>36.9</td>
<td>2.05 br d (12.4)</td>
<td>4’</td>
<td>147.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.19 d (12.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>174.1</td>
<td>5’</td>
<td>5’</td>
<td>115.6</td>
<td>6.68 d (8.0)</td>
</tr>
<tr>
<td>8</td>
<td>66.6</td>
<td>4.12 t (6.4)</td>
<td>6’</td>
<td>122.3</td>
<td>6.62 d (8.0)</td>
</tr>
<tr>
<td>9</td>
<td>25.7</td>
<td>1.24 m</td>
<td>7’</td>
<td>144.1</td>
<td>7.30 d (15.6)</td>
</tr>
<tr>
<td>10</td>
<td>28.4</td>
<td>1.59 m</td>
<td>8’</td>
<td>113.8</td>
<td>5.95 d (15.6)</td>
</tr>
<tr>
<td>11</td>
<td>28.8</td>
<td>1.24 m</td>
<td>9’</td>
<td>168.1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>31.8</td>
<td>1.22 m</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Scheme of the Experimental Batch-Stirred Apparatus for Synthesis Under High Pressure: (1) CO₂ Tank, (2) High Pressure Pump, (3) Reactor, (4) Stirrer, (5) SC CO₂ Sampling Loop (From Bottom of the Reactor), (6) SC CO₂ Sampling Loop (From Top of the Reactor), (7) collector, (8) Temperature Controller, (9) Pressure Gauge.
Figure 2. UV Chromatogram (325 nm) Corresponding to Samples Withdrawn from the Reaction Mixture every 5 Hours (A-E) in Trial 7 (150 bar, 55 °C, 10 % (v/v) of t-butanol, and 20 mg/ml of lipase). Chemical Structure of Heptyl Chlorogenate (peak y) is Shown with Numeration Corresponding to Table 3, while 5-CGA is Indicated as Peak x.
Figure 3. Operational Parameter Effects on the Average Heptyl Chlorogenate Partition in SC CO$_2$. (□) Temperature, (△) Lipase Concentration, (▲) t-butanol, (■) Pressure.
Figure 4. Operational parameter effects on the average heptyl chlorogenate concentration. (□) temperature, (△) lipase concentration, (▲) $t$-butanol, (■) pressure.
Figure 5. Concentrations of heptyl chlorogenate (▲), pentyl chlorogenate (○), and geranyl chlorogenate (x) in SC CO₂ at: 55 °C, 150 bar, 20 mg/ml of lipase, and 10 % v/v t-butanol.