Talanta 117 (2013) 102–111

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Optimization of the isolation and quantitation of kahweol and cafestol in green coffee oil

Agnes Chartier [a], Mathieu Beaumesnil [a], Alessandra Lopes de Oliveira [b], Claire Elfakir [a], Stephane Bostyn [c,*]

[a] Institut de Chimie Organique et Analytique (ICOA), Université d'Orléans-CNRS, Rue de Chartres BP 6759, 45067 Orléans cedex 2, France
[b] Departemento de Engenharia de Alimentos, Universitado Sao Paulo, Av Duaue de Caxias Norte 225, Caixa Postal 23, CEP 13635-900 Pirassununga SP, Brazil
[c] Institut Universitaire de Technologie – Université d'Orléans, 16 rue d'Issoudun, BP16724, 45067 Orléans Cedex 2, France

ARTICLE INFO

Article history:
Received 6 March 2013
Received in revised form
8 July 2013
Accepted 23 July 2013
Available online 29 August 2013

Keywords:
Kahweol
Cafestol
Quantification
Transesterification
Doehlert design
Flow chemistry

ABSTRACT

Kahweol and cafestol are two diterpenes that exist mainly as esters of fatty acids in green coffee oil. To recover them under their free form they have to be either saponified or trans-esterified. These two compounds are well known to be sensitive to heat, and reagents, therefore experimental conditions used in the transesterification reaction are critical. In this paper, a Doehlert experimental design plan is used to optimize the transesterification conditions using some key variables such as the temperature of the reaction, the reagent base concentration and the duration of the reaction. Therefore, the optimal parameters determined from the Doehlert design are equal to 70 °C, temperature of the reaction; 1.25 mol L^{-1} concentration of the reagent base; and 60 min reaction time. The contour plots show that the extracted quantity of kahweol and cafestol can depend greatly from the experimental conditions. After transesterification, the free form of the diterpernes is extracted from the lipid fraction using liquid–liquid extraction and analyzed using GC-FID without prior derivatization. The amount of kahweol and cafestol obtained from green coffee oil obtained by cold mechanical press of Catuai coffee bean is equal to 33.2 ± 2.2 and 24.3 ± 2.4 g kg_{oil}^{-1} respectively.

In an attempt to streamline the process, the transesterification reaction is performed in an in-flow chemistry reactor using the optimal conditions obtained with the Doehlert experimental design. The amount of kahweol and cafestol obtained from the same green coffee oil is equal to 43.5 and 30.072 g kg_{oil}^{-1}, respectively. Results are slightly higher compared to the ones obtained with the batch procedure. This can be explained by a better mixing of the coffee oil with the reagents and a faster transesterification reaction.

1. Introduction

Green coffee oil is extensively used in cosmetics [1] and pharmaceutical applications [2], due to its high content in triacylglycerol and fatty acids. After fractionation, it is also a source of other valuable products such as sterols, tocopherols and diterpenes. The latter have received a wider attention due to their anticarcinogenic properties [3–9] and protection against aflatoxin B1-induced genotoxicity [10,11]. These two pentacyclic diterpenes, kahweol and cafestol, (Fig. 1) exist in green and roasted coffee beans, and green coffee oil as esters of fatty acids [12,13] mostly as palmitate, and linoleate esters although other esters of fatty acids are also described. Thus 14 different fatty acids esters of cafestol [5,14] and 12 different fatty acids esters of kahweol have been identified [15]. Activity studies have shown that palmitate esters of kahweol and cafestol [16] and the free form of kahweol and cafestol [17,18] act as antioxidants and show anticarcinogenic property. Because the isolation of kahweol and cafestol as esters of fatty acids, from various matrices is a long and tedious process [19,20], the common method is to saponify or transesterify these esters in situ before recovering free forms of kahweol and cafestol. The saponification or the transesterification reaction is a critical step due to the sensitivity of the furan moiety of these compounds to acids, bases, and oxidants. This problem is associated to the heating procedure commonly used to obtain the free diterpenes. Then, the saponification or the transesterification step is followed with a liquid–liquid extraction using organic solvent such as hexane [21,22] diethyl ether [23], and methyl tert-butyl ether [24] followed with successive washes of the organic phase with water. Free kahweol and cafestol are then analyzed using gas chromatography (GC) after derivatization with silylating reagents [12,21,24,25]. Due to the lack of chromophore groups on the kahweol

* Corresponding author. Tel.: +33 238 494 323.
E-mail addresses: Agnes.chartier@univ-orleans.fr (A. Chartier), alelopes@usp.br (A.L. de Oliveira), stephane.bostyn@univ-orleans.fr (S. Bostyn).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.07.053

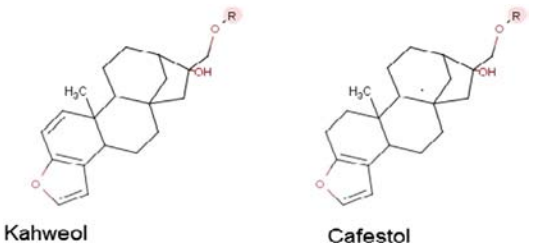

Fig. 1. Structures of diterpenes and diterpene esters and schematic of the transesterification reaction.

and cafestol molecules, the analysis of these compounds using high performance liquid chromatography (HPLC) followed by UV detection remains limited [19,22]. In addition, the fatty acid esters have to be saponified or transesterified prior the HPLC analysis [23]. An attempt was made by Oigman [26] to analyze kahweol and cafestol using LC-HRMS after microwave methanolysis.

In this paper our goal is to simplify the isolation and the determination of free diterpenes using GC-FID analysis. Our first approach is to optimize the transesterification reaction and the extraction of kahweol and cafestol from green coffee oil (Fig. 1). According to the authors' knowledge, the optimization of the transesterification parameters such as the reagent concentration, temperature and time of reaction using an experimental design has never been performed. Then, these optimal conditions will be applied to develop an in-line process in order to shorten this lengthy protocol. In certain applications such as the kinetic studies of diterpene extraction from coffee bean, and isolation of diterpenes for activity tests, a lot of samples can be generated. So in this paper, a flow chemistry system is used to streamline the transesterification reaction in order to reduce the experimental time. The reaction will be carried out into a continuous flow reactor. The reaction time and the yield of recovery will be compared to the batch procedure. In addition, in this paper, the author will also demonstrate that free diterpenes can be analyzed without derivatization using GC-FID or GC-MS. This will shorten the turnaround of samples and decrease the risk of degradation of the free diterpenes.

2. Experimental

2.1. Reagent and standards

The green coffee oil used as natural source of kahweol and cafestol and stored in a 1 L LDPE container at 4 °C in the refrigerator, is obtained by cold mechanical press of green Arabica coffee beans (Coffea Arabica l) variety Catuai Amarelo produced in the state of Sao Paulo in Brazil. The Arabica coffee cultivars are grown in Torrinha, Sao Paulo state, Brazil (22°25′34″S, 48°10′09′W, 802 m above sea level, and average annual temperature of 22 °C). Cherry fruits are selected from the harvest in June 2010, washed and sun-dried in patio.

Methanol, tert-butyl methyl ether (MTBE), hexane are HPLC grade, and are purchased from Carlo Erba (Rueil-Malmaison, France). Potassium hydroxide pellets with 87.5% purity is purchased from, Fisher Scientific (Illkirch, France). Cholesterol, purchased from Sigma-Aldrich (St. Quentin Fallavier, France) with 98% purity is used as internal standard. Cafestol and kahweol standards with 98% purity are purchased from Enzo Life Sciences (Villeurbanne, France). Filtered and deionized water with a Elix Advantage system (Millipore) and a Elga UHQ system (Millipore) is used. The conductivity obtained is lower than 1 μS and the particle size smaller than 100 μm.

2.2. Sample preparation

2.2.1. Transesterification method

Coffee oil is brought back to room temperature and stirred in order to obtain a homogeneous solution. Then, about 200 mg of coffee oil is weighted into a 10 mL borosilicate micro reactor vial (Interchim, Montluçon, France) to which 2 mL of a solution of potassium hydroxide in methanol is added. The temperature of the reaction is controlled with a Kret hot-plate (IKa Werke, Fisher Scientific, Illkirch, France) equipped with a thermo probe. The temperature, duration of the reaction and concentration of the potassium hydroxide solution are optimized according to a Doehlert experimental design. After transesterification, the whole solution is subjected to liquid–liquid extraction.

2.2.2. Liquid–liquid extraction

The previous solution is cooled down and brought to dryness under nitrogen to evaporate methanol. Then, 2 mL of water and 2 mL of organic solvent are added to the dry residue. The solution is mixed on a vortex, and centrifuged at 7000 RPM with a Jouan 4i multifunction centrifuge (Thermo Fisher Scientific, Ilkirch, France) during centrifugation, the temperature is maintained at 20 °C. Then, the organic phase is collected and the aqueous phase is again washed with the organic solvent and centrifuged. The total organic phase is collected and placed into a vial, and washed with 2 mL of a 1 mg mL^{-1} citric acid solution. The same experiment is repeated twice or until the pH of the aqueous solution is acidic.

Then the organic solution is brought to dryness under nitrogen and 3 mL of MTBE is added to the vial followed by 0.3 mL of a 200 mg mL^{-1} of cholesterol solution in MTBE, used as internal standard. Fig. 2 represents the flow chart with the optimal conditions obtained with the Doehlert experimental design.

2.3. Apparatus

A Trace GC Ultra, Thermo Fisher Scientific, equipped with a split/splitless injector and a flame ionization detector (FID) is used to determine the kahweol and cafestol content of each sample. A 5 μL aliquot is injected in split mode under constant flow on a Agilent J&W 5-MS 60 m length, 250 μm diameter and 50 μm film thickness column. Hydrogen is used as carrier gas and produced by a Parker Balston, H2PHEM-165 generator with 99.995% purity. The column flow rate is set at 1.2 mL min^{-1}. The injector temperature is set at 270 °C with a constant septum purge flow. The split flow is set at 30 mL min^{-1}. In the initial conditions, the oven temperature is maintained at 50 °C for 1 min and then ramped up to 250 °C at a 20 °C min^{-1} rate, followed by a second ramp at 5 °C min^{-1} until 300 °C. The final temperature is held for 4 min. The detector temperature is held at 300 °C. Gas flow rates used for the FID are set at 350, 35, 30 mL min^{-1} for air, hydrogen and nitrogen, respectively. Nitrogen is used as make-up gas.

A GC/MS, Trace Ultra-ISQ, thermo fisher scientific, equipped with a Restek 5-MS, 30 m length, 250 μm diameter and 50 μm film thickness column. Helium, 99.9995% purity is used as a gas carrier. The GC conditions are identical to the ones described above. A 1 μl

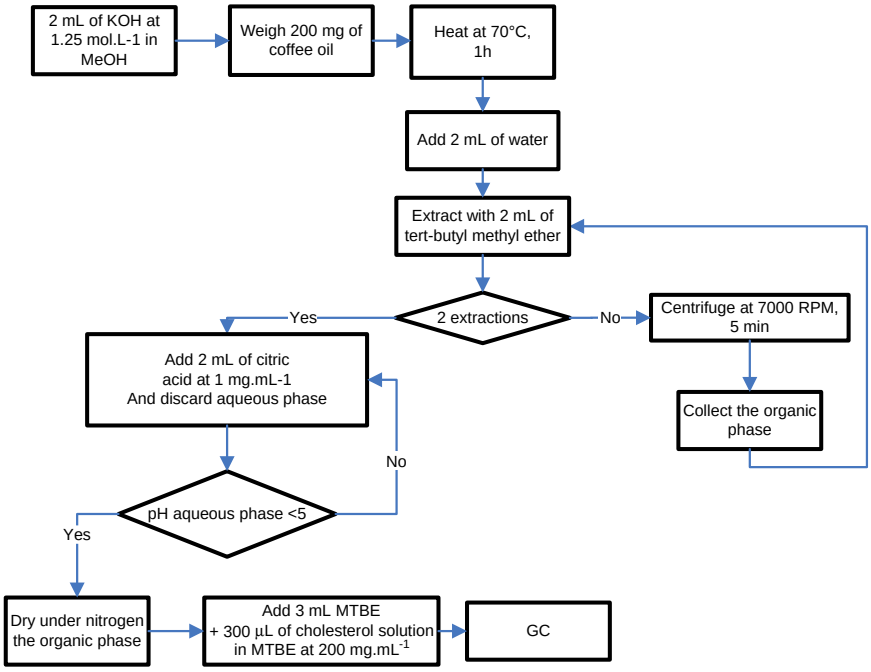

Fig. 2. Outline of the optimized procedure to obtain the analytical sample.

aliquot is injected with a split flow of 10 mL min^{-1}. The transfer line and the source temperatures are held to 300 °C and 200 °C, respectively. The solvent delay is 3 min. Data acquisition is obtained from m/z 40 to m/z 650. The scan time is set at 0.2 S.

2.3.1. *In-line transesterification reaction*

In order to streamline the transesterification reaction, the reaction is performed with a standard FlowSyn system from the Uniqsis Companies (Fig. 3). This system is equipped with two pumps, two injection loops and two reactors in series: a 2-input glass static mixer (chip), 1.6 mL total volume and a PTFE tubing reactor (coil) with a 1 mm internal diameter and a 20 mL total volume. Furthermore, two injection modes can be selected; with the mode B, the reagents are introduced via an injection loop before to be injected into the reactors. The advantage of this configuration is to avoid contact between the reagents and the pump mostly in the case of aggressive reagent. The injection loop size chosen is 2 ml. Therefore, reactor temperature and flow rate can be easily controlled.

2.4. *Optimization of the transesterification reaction using a Doehlert experimental design*

To optimize the transesterification reaction, three variables are designed as important for the reaction: reaction time (t_r), potassium hydroxide concentration (C_{KOH}) and temperature (T). In our case, Doehlert experimental design is selected [28] because it needs fewer points to estimate the terms in a second-order (Eq. (1)) model for these three independent factors. The minimal number of experiment is 12 plus one in the domain center. The experimental points are circumscribed into a sphere, and each factor is analyzed at different numbers of level depending on its influence on the response. In our case the temperature factor is studied at 3 levels (62, 70 and 78 °C), the concentration one at 7 levels (between 0.6 and 1.9 mol L^{-1}) and the reaction time one at 5 levels (between 30 and 90 min) (Table 1). Therefore, the choice of the levels mainly depends on the easiness to control the factor and its impact on the response. In our cases, two responses are optimized: kahweol and cafestol concentrations.

The experimental results obtained when using a Doehlert matrix lead to the estimation of 10 coefficients for a second-order polynomial model, according to Eq. (1)

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (1)$$

The model consists of first-order terms (b_i), square terms (b_{ii}) and first-order interactions (b_{ij}). The estimation of the coefficients of the polynomial model (b_0, b_i, b_{ii} and b_{ij} in Eq. (1)) is calculated using the least-square method of Statgraphics Centurion XV Version Software (Sigma-Plus, Paris, France). The resulting model equation is used for drawing the response surface. The adequacy of the model is checked by analysis of variance (ANOVA). *F*-test is used to compare the variances at a probability level of 95%. The independent variables are represented by: X_1 for reaction time, X_2 for potassium hydroxide concentration and X_3 for temperature of the reaction. The experimental points according to this design are shown in Table 1. The experiment at the domain center (0, 0, 0) is carried out four times.

3. Results and discussion

Our first approach is to optimize the extraction, the washing procedure and the GC analysis conditions.

3.1. *Optimization of the extraction and choice of the organic solvent*

After transesterification the solution is brought to dryness under nitrogen and 2 mL of water and hexane are added to the residue. An emulsion is formed at the interface of the aqueous and organic phases and after centrifugation at 7000 RPM for 5 min, the interface remains cloudy. Nevertheless, the organic phase is collected and washed again, three times with 2 mL of water. The pH of the aqueous solution is checked and remains basic at pH 10. This experiment is repeated with different volumes of hexane and water in order to obtain a neat partition of the compounds between the two phases. After several attempts this procedure is

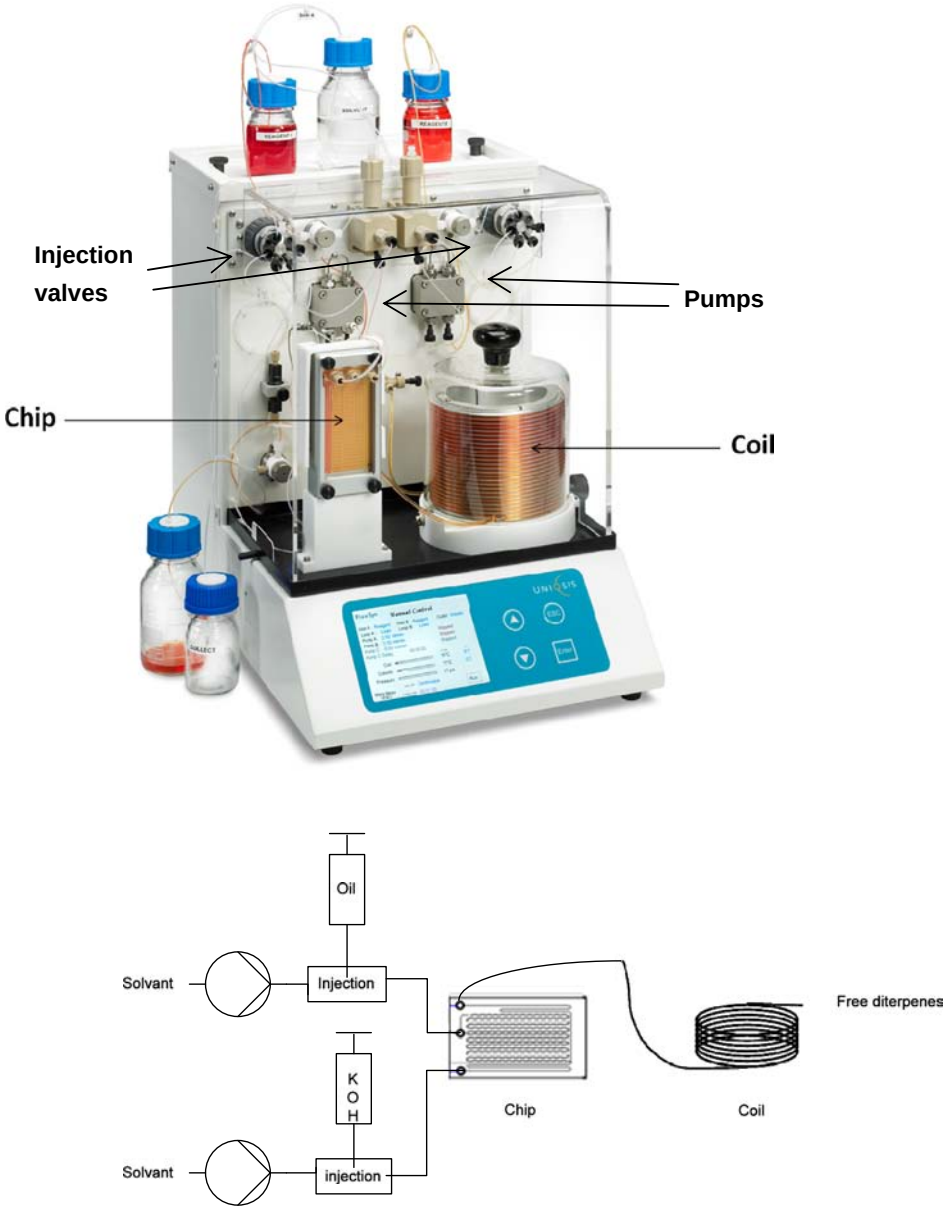

Fig. 3. Photo of the FlowSyn system from Uniqsis Companies and general flow reactor schematic for transesterification of oil.

Table 1
Experimental design and response values.

Run	X_1[a] (min)	X_2[b] (mol L^{-1})	X_3[c] (°C)	Kaweol[d] (mg kg^{-1})	Cafestol[d] (mg kg^{-1})	Ratio (Cafestol/Kahweol)
1	60 (0)	1.25 (0)	70 (0)	31,493	23,617	0.75
2	90 (1)	1.25 (0)	70 (0)	16,897	12,227	0.72
3	75 (0.5)	1.90 (0.866)	70 (0)	20,146	14,650	0.73
4	45 (−0.5)	1.90 (0.866)	70 (0)	19,421	13,665	0.70
5	30 (−1)	1.25 (0)	70 (0)	19,319	15,952	0.83
6	45 (−0.5)	0.60 (−0.866)	70 (0)	13,942	10,021	0.72
7	75 (0.5)	0.60 (−0.866)	70 (0)	25,967	20,123	0.77
8	60 (0)	1.25 (0)	70 (0)	33,690	24,896	0.74
9	45 (−0.5)	1.46 (0.289)	78 (0.816)	21,178	16,547	0.78
10	60 (0)	0.82 (−0.577)	78 (0.816)	26,898	19,892	0.74
11	75 (0.5)	1.46 (0.289)	78 (0.816)	20,142	14,850	0.74
12	45 (−0.5)	1.03 (−0.289)	62 (−0.816)	20,690	14,450	0.70
13	60 (0)	1.88 (0.577)	62 (−0.816)	22,669	16,221	0.72
14	75 (0.5)	1.03(−0.289)	62 (−0.816)	21,040	15,054	0.72
15	60 (0)	1.25 (0)	70 (0)	34,273	24,751	0.72
16	60 (0)	1.25 (0)	70 (0)	33,385	24,026	0.72

Value in brackets represents coded levels.

[a] Coded value of reaction time.
[b] Coded value of hydroxide potassium concentration.
[c] Coded value of temperature.
[d] Concentration values do not included the recovery yield correction.

abandoned due to its lack of repeatability and the difficulty in obtaining a neat interface. The same experiment is then repeated with methyl tert-butyl ether (MTBE) in place of hexane. In this case, a neat interface is obtained between the organic and aqueous phases. An aliquot of the organic phase is injected on the GC. A typical chromatogram is reported in Fig. 4A showing the kahweol and cafestol peaks with some traces of fatty acids methyl esters. The extraction of kahweol and cafestol is optimized with two consecutive washings of the aqueous phase with 2 mL of MTBE.

To increase the extraction yield, the collected organic phase, 4 mL total is washed with different volumes of a 1 mg mL^{-1} of aqueous citric acid solution, until an acidic pH is obtained. Two successive washings of the organic phase with 2 mL of citric acid solutions are needed to remove more polar components, residue of potassium hydroxide, and obtain a pH below 7. Then, the organic phase is brought to dryness under nitrogen and 3 mL of MTBE is added with 300 μL of cholesterol solution as internal standard before GC analysis.

A known concentration of kahweol and cafestol in solution in methanol is extracted according to the procedure described above. After extraction, the solution is analyzed by GC-FID to determine the extraction yield of the two diterpenes. A yield of 80% and 73% is obtained for kahweol and cafestol, respectively.

3.2. Determination of the two diterpenes using GC-FID and GC/MS

An extract is injected on the GC/MS, Fig. 4A. The chromatogram shows two major peaks at 12.2 and 12.5 min and some minor peaks at 8.7, 9.5 and 16.7 min. The mass spectrum of the peak at 12.2 shows a base peak *m*/*z* 131.06 and two other fragments at m/*z* 314.19 (20%) and *m*/*z* 146.10 (70%). A good match is obtained with the mass spectrum of kahweol [13,19]. The molecular peak can be

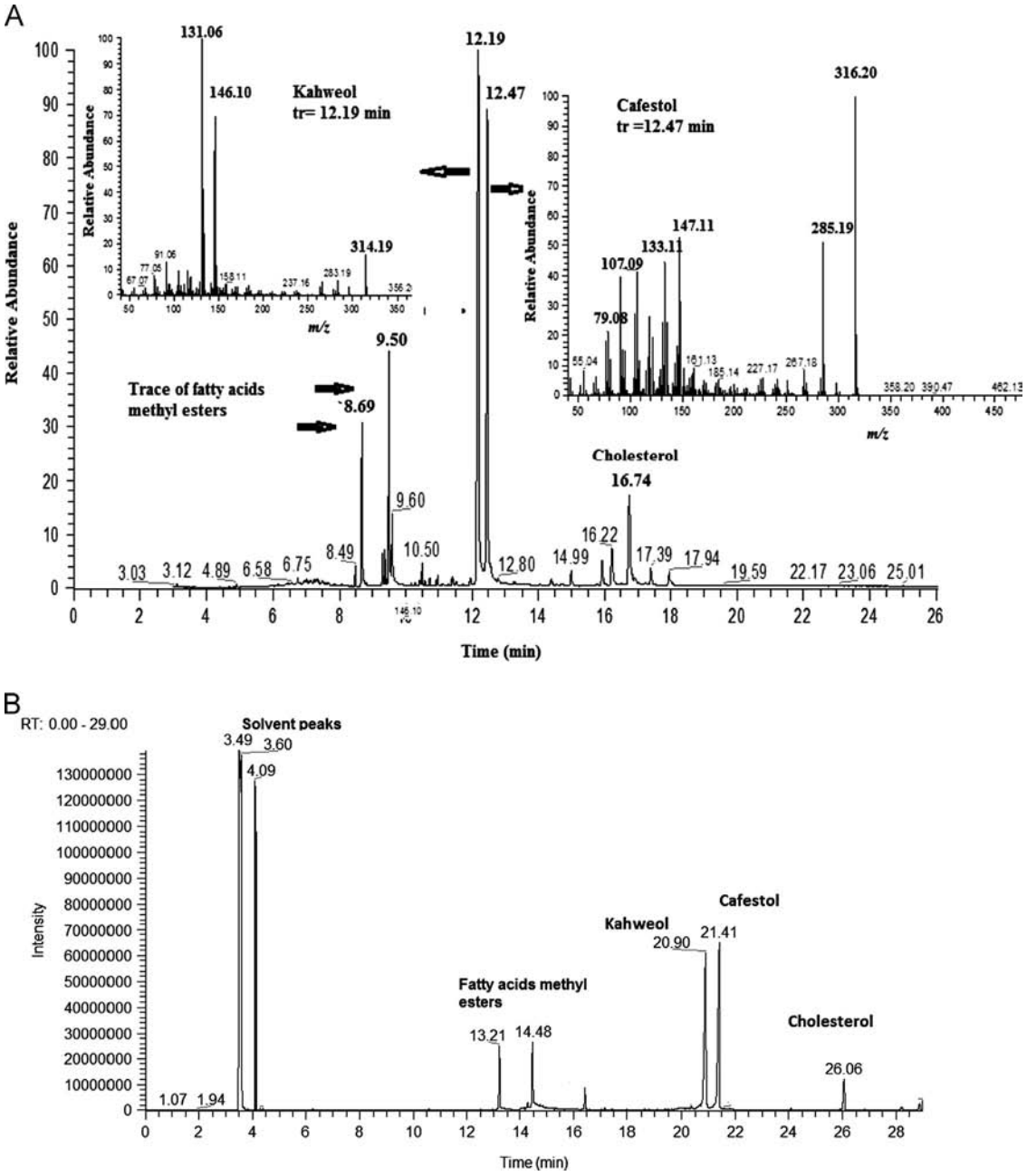

Fig. 4. Chromatograms of kahweol and cafestol obtained after extraction and transesterification by GC/MS analysis with mass spectra (A) and by GC-FID analysis (B).

attributed to m/z 314.2 and the two ions $[C_9H_8O]+$, $[C_{10}H_9O]+$. are *m/z* 131.06 and 146.70, respectively.

The mass spectrum of the peak at 12.5 min shows a base peak at *m/z* 316.2 and some major fragments at *m/z* 147.1 (55%), *m/z* 285.2 (50%), and *m/z* 133.1(45%). This spectrum is a good match with the one of the cafestol [13–19]. The fragment *m/z* 285.2 corresponds to the loss of *m/z* 31 [CH_2OH]. The base peak is the molecular ion of the cafestol compound. The elution order can be justify by the presence of a supplementary double bond on the kahweol structure and a slightly higher volatility for kahweol than cafestol.

On the chromatogram, the minor peaks are identified against the 2011 NIST electronic library. The peaks at 8.7 and 9.5 min are identified as palmitic and linoleic acid methyl esters. The peak at 16.7 min is corresponding to cholesterol. With GC-FID analysis (Fig. 4B), the identification is confirmed using the retention time of kahweol and cafestol standards.

Therefore, kahweol and cafestol can be analyzed using GC–MS or GC-FID without previous derivatization on two different 5-MS type columns.

3.3. Evaluation of the method

To quantify kahweol and cafestol obtained by extraction from coffee oil, multiple point internal calibration curves using cholesterol as internal standard are constructed. An internal standardization is preferred due to the precision of the method for quantitative analysis. Cholesterol is chosen as internal standard for its similarity in functional group types to the diterpenes and for its stability under the required analytical conditions.

The linearity is observed for a concentration range of 94–1500 mg L^{-1} and 80–1200 mg L^{-1} for kahweol and cafestol, respectively. Five standard solutions are analyzed in triplicate. The equation of the calibration curves is established using regression analysis; the correlation coefficient obtained is equal to 0.9984 and 0.9887 for kahweol and cafestol, respectively (Table 2). Then, the concentration of kahweol and cafestol in the unknown samples is obtained from the signal response (y) with the confidence interval for the true value of the concentration. This interval depends on two factors, the uncertainty of the slope and the intercept, and the uncertainty of the response reading that is the mean of three replicates. A common way to take these two sources of error is the application of the error propagation to the estimated concentration. Thus the experimental error is equal to 2.2 mg kg^{-1} and 2.4 mg kg^{-1} for kahweol and cafestol, respectively. Statistical analysis is given in Table 2.

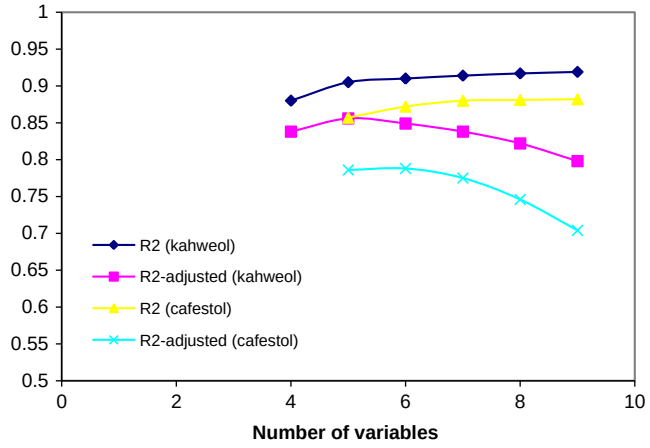

Fig. 5. Evolution of the determination coefficients (R^2 and adjusted R^2) versus the number of coefficients of the quadratic polynomial model.

Table 2
Regression analysis of the calibration curves, $y=mx+b$ and determination of the experimental error on the concentration of kahweol and cafestol.

	Kahweol	Cafestol
Linearity range mg L^{-1}	94–1500	80–1200
R^2	0.9983	0.9887
Number of calibration standards (M)	5	5
Number of experimental points (N)	15	15
Slope (m)	0.5037	0.5233
Intercept (b)	−0.3781	1.8884
Degree of freedom	13	13
Residual variance of the regression ($s^2_{y/x}$)	0.6209	1.4518
Standard deviation of the slope $\sigma_{(m)}$	0.0056	0.1555
Standard deviation of the intercept $\sigma_{(b)}$	0.0243	0.5674
Standard deviation of the signal $\sigma_{(y)}$	1.946	4.299
Standard deviation of the unknown read from the calibration curve $\sigma_{(x)}$	4.614	4.607

Table 3
Analysis of variance (ANOVA) of variables of the quadratic polynomial model of kahweol concentration.

Source	Sum of squares	*df*	Mean square	*F*-ratio	*p*-value
X_1	3.25803E6	1	3.25803E6	0.40	0.5522
X_2	2.0394E6	1	2.0394E6	0.25	0.6361
X_3	2.43261E6	1	2.43261E6	0.30	0.6060
X_1^2	3.04104E8	1	3.04104E8	37.00	**0.0009**
$X_{1\times2}$	3.19225E7	1	3.19225E7	3.88	0.0963
$X_{1\times3}$	1.27957E6	1	1.27957E6	0.16	0.7068
X_2^2	2.16895E8	1	2.16895E8	26.39	**0.0021**
$X_{2\times3}$	1.40935E7	1	1.40935E7	1.71	0.2383
X_3^2	1.4099E8	1	1.4099E8	17.15	**0.0061**
Total error	4.93157E7	6	8.21928E6	–	–
Corrected total	6.09099E8	15	–	–	–

$R^2=91.9\%$.
Adjusted $R^2_{adjusted}=79.8\%$.

3.4. Experimental design

One of the specifications of the Doehlert design is the different number of experimental level (3, 5, and 7) for the studied variables. Therefore from these three parameters reaction time, t_r, potassium hydroxide concentration, C_{KOH}, and temperature of the reaction, T, a choice is made. The choice must be done according to the ease of control of the variable and its impact on the response variation. From that, temperature is chosen to have three experimental levels, reaction time, five, and base reagent concentration, seven. Finally from the literature, the initial values of the chosen parameters are: for t_r 60 min ($X_1=0$) with a step size value of 30 min, for C_{KOH} 1.25 mol L^{-1} ($X_2=0$) with a step size value of 0.75 mol.L^{-1}, for T 70 °C ($X_3=0$) with a step size value of 10 °C.

A minimal number of 12 experiments is required to obtain the coefficients of the model but generally to have more degree of freedom and to be able to estimate the model and experimental errors, the point at the center is carried out several times; four times in this paper. Therefore, 16 experiments are carried out. Each of the 16 solutions is analyzed in triplicate using GC-FID. The concentrations of kahweol and cafestol expressed in mg kg^{-1} of coffee oil are given in Table 1.

3.4.1. Optimization of the kahweol transesterification reaction using the Doehlert experimental design

Each of the 16 solutions is analyzed in triplicate using GC-FID. The results of ANOVA are reported in Table 3. A coefficient is defined to be significant if the *p*-value is less than 0.05. Table 3

shows that only 3 coefficients are significant: X_1^2; the duration of the reaction, X_2^2; the concentration of the reagent base and X_3^2; the temperature of the reaction. In these conditions, the model will be established with only 3 parameters and its robustness can be seriously diminished. Therefore the variation of the coefficients of determination R^2 and adjusted R^2 are plot in function of the number of coefficient used to defined the model (Fig. 5). R^2 gives the goodness of fit of the data, and adjusted R^2 adjusts for the number of explanatory terms needed in the model. For kahweol, the adjusted R^2 reaches a maximum with 5 coefficients and decreases afterwards, therefore, the ideal combination of having the best fit without excess parameters requires five parameters corresponding to the following equation:

$$Y = 33210.5 - 15102.5X_1^2 - 6111.0X_1X_2 - 12755.0X_2^2 - 4849.0X_2X_3 - 9707.0X_3^2 \quad (2)$$

The influential parameters are determined by successive iteration. In this case, the value of the coefficient of determination (R^2) is 92% and adjusted R^2 is 85.6%. This value indicates that 8% of the variation is not explained by the model. The value of the standard error of estimate is 2.4 g kg^{-1} and is slightly greater than the calculated experimental error that is equal to 2.2 g kg^{-1}. Fig. 6A shows that the maximum concentration of free kahweol is obtained at the center of the domain and its average value obtained from Table 1 is equal to 33.2 g kg^{-1}. Fig. 7A represents the contour plot according to the reaction time and concentration for a temperature equal to level 0 (70 °C). The surfaces are ellipsoidal and their axes are oriented in the direction of the points $(+1,-1: X_1, X_2)$ and $(-1,+1: X_1, X_2)$. So the decrease of one of these parameters must be counterbalanced by the other one. The worst case is where both parameters decrease or increase in the same direction. The concentration ratio between the worst conditions (6.2 g kg^{-1}) and the best ones (33.7 g kg^{-1}) as defined by the contour plot is 5.6 showing the real influence of the experimental conditions on the transesterification reaction of kahweol.

3.4.2. Optimization of cafestol transesterification reaction using Doehlert experimental design

The results of ANOVA are reported in Table 4. Table 4 shows that only 3 coefficients are significant. In these conditions, the model will be established with only 3 parameters and its robustness (accuracy) can be seriously diminished. As explain above for kahweol, the variations of the determination coefficient, R^2 and adjusted R^2 are plot in function of the number of parameters used to defined the model (Fig. 5). For cafestol, the adjusted R^2 reaches a maximum with 6 parameters and decreases afterwards, therefore, the ideal combination of having the best fit is defined with 6 parameters corresponding to the following equation:

$$Y = 24322.5 + 1136.5X_3 - 10233X_1^2 - 5135.5X_1X_2 - 9533.0X_2^2 - 3751.0\times_2X_3 - 7296.0X_3^2 \quad (3)$$

In this case, the value of R^2 is equal to 87.3% and R^2-adjusted 78.7%. The value of the standard error of estimate is 2.2 g kg^{-1}. This result is in good agreement with the calculated experimental error that is equal to 2.4 g kg^{-1}. The contour plots for different values of temperature are reported on Fig. 6B. The maximum concentration of 24.0 g kg^{-1} is obtained for a X_3 (temperature of the reaction) value equal to 0.0 (70 °C). With these conditions, in Table 1, the average maximal concentration of cafestol is equal to 24.3 g kg^{-1}. Fig. 7B represents the 2D contour plots according reaction time and concentration for a temperature equal to level 70 °C or level 0. The surface contours are ellipsoidal too and their directions are similar to the kahweol response but more pronounced. A variation in the extreme conditions such as high reaction time, and high base reagent concentration superior at 80 min and 1.7 mol L^{-1}, respectively or short reaction time and low base reagent concentration inferior at 40 min and 0.7 mol L^{-1}, respectively leads to the lowest sensitivity. In the case of cafestol, the concentration ratio

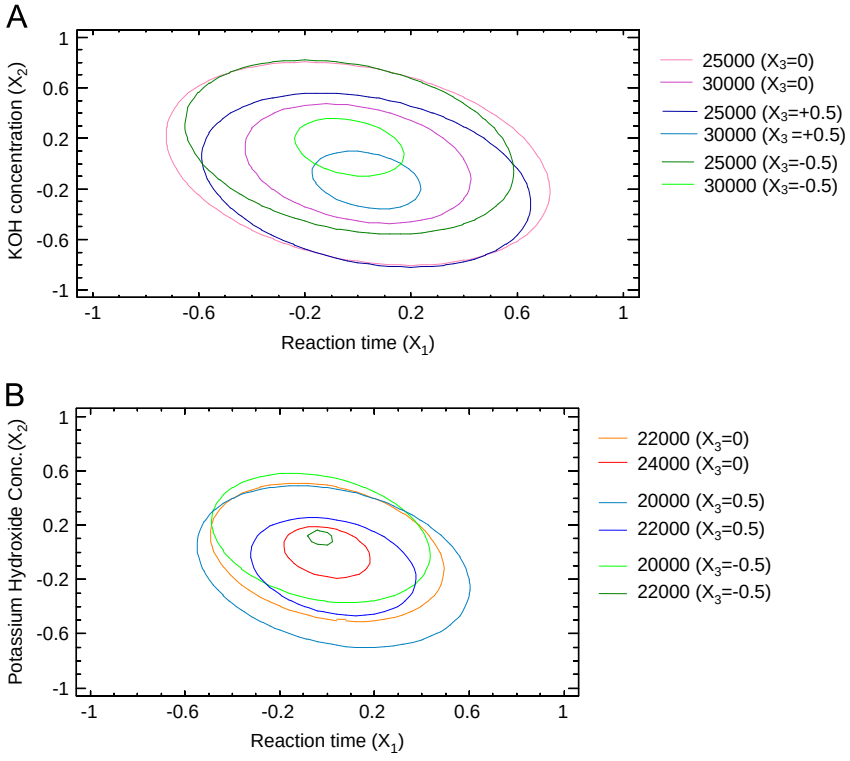

Fig. 6. 2D contour plot of reaction time (X_1) vs. Potassium hydroxide concentration (X_2) for different values of temperature (X_3) on the response: kahweol concentration (A) (g $kg^{-1}$of oil), cafestol concentration (B) (g $kg^{-1}$of oil).

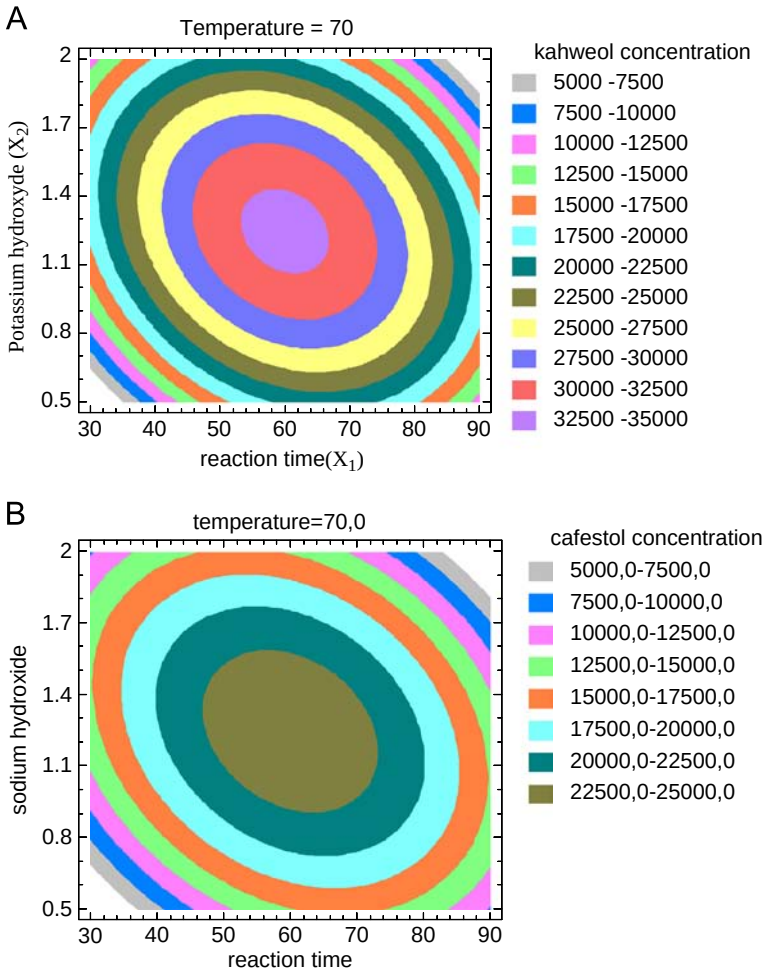

Fig. 7. 2D contour plot showing the effects of reaction time (X_1) and potassium hydroxide concentration (X_2) for a temperature value (X_3) equal to 70 °C on kahweol concentration (g $kg^{-1}$of oil) (A) and on cafestol concentration (g $kg^{-1}$of oil) (B)

between the worst conditions, 6.2 g kg^{-1} and the best ones, 23.8 g kg^{-1} as defined by the Doehlert experimental design is 3.8. So cafestol is less sensitive to the conditions of transesterification reaction in comparison to kahweol in this experimental domain. Although, according to Table 1, the concentration ratio of kahweol to cafestol one is fairly constant (0.7) under the experimental conditions used to define the Doehlert plan. Consequently, under mild experimental conditions used to define the Doehlert experimental design, Kahweol and cafestol are not as much sensitive to the transesterification reaction conditions. But under extreme experimental conditions such as a potassium hydroxide concentration above to 1.7 mol L^{-1} and duration of reaction above 85 min, both compounds are impacted and the impact on kahweol is more pronounced than the one on cafestol; these results confirmed the ones described by Oigman et al. [26].

Therefore, the optimal conditions for both molecules are at the domain center where $X_1=60$ min, time of reaction, $X_2=1.25$ mol L^{-1}, potassium hydroxide temperature and $X_3=70$ °C, temperature of the reaction.

3.5. Flow chemistry application and optimization

To optimize the time of analysis and make easy the automation, the transesterification is carried out in flow chemistry. The flow chemistry is based on the use of a continuous reactor with a diameter size comprised between the micrometer to millimeter. Residence time in the reactor is controlled by the length of the reactor. In our case, the Uniqsis system is used with the two reactors in series. The first one is the glass static mixer followed by the tubing reactor. A loop is used to add a solution of KOH at 1.25 mol L^{-1} in methanol. This concentration has been chosen from the previous results and corresponds to the domain center. The other loop is used to introduce a solution of coffee oil. Tests of solubility have permitted to choose a mixture of acetone and methanol (1:2 v/v) as solvent for diluting the coffee oil. Therefore for the experiments, a coffee oil solution of 100 mg mL^{-1} is prepared in acetone/methanol (1:2, v/v) in order to inject 200 mg of coffee oil via a 2 mL injection loop. After adding the two solutions each pump delivers methanol into the two reactors.

The experimental matrix is reported on Table 5. The retained parameters are temperature and residence time. To obtain the desired residence time, the pumps are programmed with a specific flow rate. The total flow rates used in these experiments are given in Table 5. Selected residence times from 10 to 40 min are inferior to the reaction time of the batch process (Table 1). Furthermore, temperatures superior to the methanol boiling point (65 °C) are selected to test the capability of the system to work under pressure. Fig. 8 shows the result of free kahweol and cafestol concentrations in function of residence time and temperature. In the case of kahweol at 100 °C (Fig. 8A), the curve has an asymptote at 20 min. This kind of profile can be explained by the fact that before 20 min, the reaction is incomplete and after 20 min the concentration decreases due to degradation of the compound. The same kind of profile is observed for 80 °C but the asymptote is

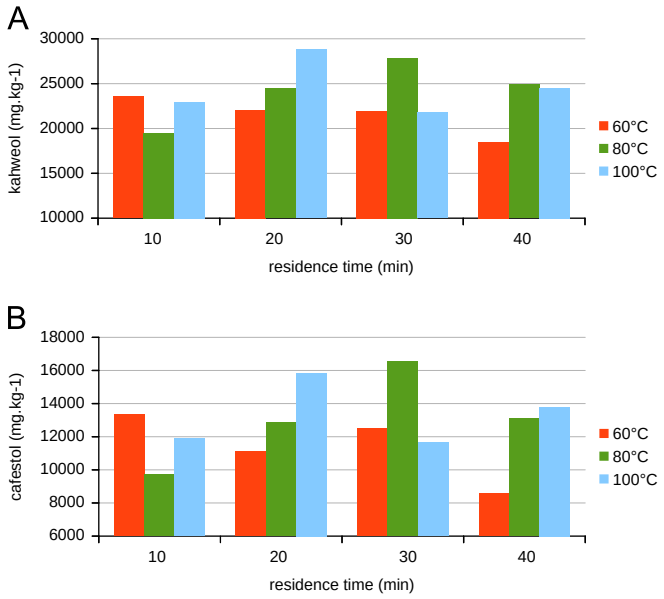

Fig. 8. Flow chemistry application: concentration of diterpenes concentration: Kahweol (A), cafestol (B) versus residence time in the reactor and temperature of the reactor.

Table 4
Analysis of variance (ANOVA) of the variables of the quadratic polynomial model of cafestol concentration.

Source	Sum of squares	*df*	Mean square	*F*-Ratio	*P*-Value
X_1	404,496	1	404,496	0.06	0.8124
X_2	2.49159E6	1	2.49159E6	0.38	0.5609
X_3	5.16261E6	1	5.16261E6	0.78	0.4098
X_1^2	1.39619E8	1	1.39619E8	21.22	**0.0037**
$X_{1\times2}$	2.07799E7	1	2.07799E7	3.16	0.1259
$X_{1\times3}$	123,685	1	123,685	0.02	0.8954
X_2^2	1.21162E8	1	1.21162E8	18.41	**0.00**51
$X_{2\times3}$	8.43316E6	1	8.43316E6	1.28	0.3008
X_3^2	7.96571E7	1	7.96571E7	12.11	**0.0132**
Total error	3.94796E7	6	6.57993E6	–	–
Corrected total	3.33648E8	15	–	–	–

$R^2=88.2\%$.
Adjusted $R^2=70.4\%$. The values are in bold to mention that the *p-value* is <0.05.

Table 5
Experimental matrix for the trans-esterification reaction in flow chemistry.

Run	Temperature (°C)	Residence time (min)	Total flowrate (mL min^{-1})	Kaweol (mg kg^{-1})	Cafestol (mg kg^{-1})
1	60	10	2.14	4608	9983
2	60	20	1.07	4229	9369
3	60	30	0.71	4200	9755
4	60	40	0.53	3323	8667
5	80	10	2.14	3577	8979
6	80	20	1.07	4839	9848
7	80	30	0.71	5702	10,869
8	80	40	0.53	4960	9911
9	100	10	2.14	4455	9581
10	100	20	1.07	5952	10,676
11	100	30	0.71	4165	9518
12	100	40	0.53	4855	10,109

delayed. This delay could be explained by a slower kinetic. At 60 °C, the curve is constant. The plot of cafestol concentration versus the residence time in the reactor (Fig. 8B) exhibits the same profile.

The maximum concentration average for kahweol, is 43.5 g kg_{oil}^{-1} against 32.2 g kg_{oil}^{-1} average value at the center domain to the previous results. For cafestol, this average is 30.1 g kg_{oil}^{-1} against 24.3 g kg_{oil}^{-1}. With faster transesterification reaction, a higher yield of extraction is obtained with the flow chemistry reactor.

4. Conclusions

Each step from the extraction of the diterpenes from the unsaponifiable fraction to the transesterification reaction is optimized. The optimization of the parameters of the transesterification using a Doehlert experimental design allows the development of a robust method to extract kahweol and cafestol from green coffee oil. It shows that the optimal conditions for the three selected parameters (reaction time, potassium hydroxide concentration and temperature) are at the center of the defined domain. The optimal values are 60 min, 1.25 mol L^{-1}, 70 °C. Moreover due to Doehlert design analysis, the two model equations obtained give a coefficient of determination upper than 87%. The contour plots obtained show the importance to work with the optimal conditions. Indeed, a variation of the parameters can lead to a decrease in the concentration by a factor 5.47 and 3.8 for kahweol and cafestol, respectively. The optimal concentrations obtained are 33.2 ± 2.2 g kg_{oil}^{-1} and 24.3 ± 2.4 g kg_{oil}^{-1} for kahweol and cafestol, respectively.

In flow chemistry, the results show that the reaction time is equal to the residence time and can be shortened to 30 min. Concentrations of kahweol and cafestol obtained with the in-flow system are equal to 43.2 and 30.1 g kg_{oil}^{-1}, respectively. Both concentrations are slightly higher than the ones obtained with the batch procedure and this result can be explained by a better mixing of the coffee oil with the reagents, and a faster transesterification reaction. In addition, a shorter residence time of the compounds in the reactor may avoid any possible degradation of the free diterpenes. This method allows the transesterification of higher amount of kahweol and cafestol in a shorter time.

This optimized method can be used to compare the content of diterpenes from one variety of coffee beans to another one. In addition, for nutraceutical and pharmaceutical industries, interested in extraction of free diterpenes, this method gives the opportunity to control the recovery yield of free diterpenes and also to stream-line the process.

Acknowledgments

The authors thank Cofecub to have supported part of this work and to have made possible the meeting of the different research teams.

References

[1] A.M.R. Alvarez, M.L.G. Rodriguez, Grassas aceites 51 (2000) 74–79.
[2] J.F. Follier, S. Plessis, US Patent 4793990 (1988).
[3] K.J. Lee, H.G. Jeong, Toxicol. Lett. 173 (2007) 80–87.
[4] C. Cavin, D. Holzhauser, A. Constable, G. Scharf, Food Chem. Toxicol. 40 (2002) 1155–1163.
[5] L.K.T. Lam, V.L. Sparnins, L.W. Wattenberg, Cancer Res. 42 (1982) 1193–1198.
[6] R. Ugert, M.B. Katan, Annu. Rev. Nutr. 17 (1997) 305–324.
[7] L.L. Gershbein, Anticancer Res. 14 (1994) 1113–1116.
[8] E.G. Miller, W.A. Formby, F. Rivera-Hidalgo, J.M. Wright, Oral Surg. 65 (1988) 745–749.
[9] L.W. Wattenberg, Cancer Res. 43 (1983) 2448–2453.
[10] C. Cavin, D. Holzhauser, A. Constable, A.C. Huggett, B. Schilter, Carcinogenesis 19 (1998) 1369–1375.
[11] W.W. Huber, G. Scharf, W. Rossmanith, S. Prustomersky, B. Grasl-Kraupp, B. Peter, R.J. Turesky, R. Schulte-Hermann, Arch. Toxicol. 75 (2002) 685–694.
[12] G. Gross, E. Jaccaud, A.C. Huggett, Food Chem. Toxicol. 35 (1997) 547–554.

[13] B.C. Petitt, J. Agric. Food. Chem. 35 (1987) 549–551.
[14] I. Kölling-Speer, S. Strohschneider, K. Speer, J. High Resol. Chromatogr. 22 (1999) 43–46.
[15] T. Kurzrock, K. Speer, J. Sep. Sci. 24 (2001) 843–848.
[16] J.Y. Kim, K.S. Jung, K.J. Lee, H.K. Na, H.-K. Chun, Y.-H. Kho, H.G. Jeong, Cancer Lett. 213 (2004) 14–154.
[17] K.J. Lee, H.G. Jeong, Toxicol. Let. 173 (2007) 80–87.
[18] R.C.E. Dias, F. Gonçalves Campanha, L.G. Esteves Vieiras, L. Pires Ferreira, D. Pot, P. Marracini, M.De Toledo Benassi, J. Agric. Food Chem. 58 (2010) 88–93.
[19] T. Kurzrock, K. Speer, Food Rev. Int. 17 (2001) 433–450.
[20] K. Speer, I. Kölling-Speer, Braz. J. Plant Physiol. 18 (2006) 201–216.
[21] G. Lercker, N. Frega, F. Bocci, M.T. Rodriguez-Estrada, Chromatographia 41 (1995) 29–33.
[22] J.M. Araújo, D. Sandi, Food Chem. 101 (2006) 1087–1094.
[23] B. de Roos, G. van der Weg, R. Urgert, P. van de Bovenkamp, A. Charrier, M. Katan, J. Agric. Food Chem. 45 (1997) 3065–3069.
[24] D. Pacetti, E. Bocelli, M. Balzano, N.G. Frega, Food Chem. 135 (2012) 1569–1574.
[25] J.A. Silva, N. Borges, A. santos, A., Alves, Food Anal. Methods, http://dx.doi.org/10.1007/s12161-012-9387-5.
[26] S.S. Oigman, R.O.M.A. de Souza, H.M. dos Santos Júnior, A.M.C. Hovell, L. Hamerski, C.M. Rezende, Food Chem. 134 (2012) 999–1004.
[28] P. Araujo, S. Janagap, J. Chromatogr. B 910 (2012) 14–21.