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Validation of a fast gas chromatographic method for the study of semiochemical slow release formulations

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ABSTRACT

The validation of a fast GC-FID analytical method for the quantitative determination of semiochemical sesquiterpenes (E-β-farnesene and β-caryophyllene) to be used in an integrated pest management approach is described. Accuracy profiles using total error as decision criteria for validation were used to verify the overall accuracy of the method results within a well defined range of concentrations and to determine the lowest limit of quantification for each analyte. Furthermore it allowed to select a very simple and reliable regression model for calibration curve for the quantification of both analytes as well as to provide measurement uncertainty without any additional experiments.

Finally, this validated method was used for the quantification of semiochemicals in slow release formulations. The goal was to verify the protection efficiency of alginate gel beads formulations against oxidation and degradation of sesquiterpenes. The results showed that the alginate beads are adequate slow release devices which protect the bio-active molecules during at least twenty days.

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1. Introduction

Semiochemicals, which can be defined as chemical communication signals between living organisms, are more and more used in integrated pest management programs, acting as insect control or monitoring devices [1]. This increasing interest is linked to the need for reducing the pesticides treatments on the infested fields. However, the compounds used in such systems are generally obtained by chemical synthesis [2,3] instead of being extracted from natural sources, like plant matrixes.

Indeed, essential oils of many plant species contain a lot of molecules which are also reported in insect communication. E-β-Farnesene, the alarm pheromone of many aphid species [4], can be isolated, with a high purity degree, from *Matricaria chamomilla* L. (Asteraceae) essential oil [5] by means of a fast and simple process [6]. On a biological point of view, this sesquiterpene is also considered as attractant and oviposition inductor of predators (*Episyrphus balteatus* De Geer (Diptera: Syrphidae)) [7–9] and aphid parasitoids (*Aphidius ervi* Haliday (Hymenoptera: Braconidae)) [10]. β-Caryophyllene, identified recently as a potential component of the aggregation pheromone of the Asian ladybeetle *Harmonia axyridis* Pallas [11], is present as a major compound of *Nepeta cataria* L. (Lamiaceae) essential oil [6,12]. This molecule can also have a biological activity against aphid reproduction [13]. These two sesquiterpene compounds are therefore considered as allelochemicals (kairomones: receptor species benefits), being produced by members of one species and influencing the behaviour of individuals of another species.

An interesting way to promote the allelochemical properties of these molecules consists in the development of natural and biodegradable semiochemical slow release formulations for attracting and/or maintaining populations of predators and/or parasitoids on aphid infested fields in a biological control approach. Alginate gel beads were largely described as efficient releasers for aroma and flavour volatile compounds in the food industry [14] or for essential oils acting as antimicrobial agents [15,16]. The beads are rather simple to produce on a lab-scale, easy to manipulate and have low impact on the environment [17]. Furthermore, alginate, a polysaccharide derived from marine brown algae (*Phaeophyceae*), is a hydrophilic matrix with low oxygen permeability

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properties which can protect the volatile molecules from oxidation [18]. Indeed, the sesquiterpenes present double bonds which are preferential sites for oxidation reactions (hydroxylation, epoxidation, oxidative cleavage of double bonds like ozonolyse) [19]. Some papers relate the oxidation of cyclic sesquiterpenes like β-caryophyllene [20–22], but on our knowledge, only one experiment has been conducted on the oxidation of E-β-farnesene, a linear molecule [23].

The purpose of the present research consists in verifying this protection efficiency of alginate gel beads towards incorporated semiochemicals. The procedure developed involves the quantification of compounds in the formulations over time, when exposed to air and light, by means of fast gas chromatography (<5 min) coupled with a high frequency (300 Hz) flame ionisation detector (fast GC-FID).

The paper describes also the validation of a fast analytical GC method for the sesquiterpenes analysis with accurate results. The necessity for fast GC methods is growing for routine analyses. As a matter of fact, conventional GC methods are still time consuming, principally for the analysis of a great number of essential oil fractions, but also for validation steps. This validation was conducted by means of the accuracy profile concept based on the guidelines of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) [24–26]. The present procedure was largely described for pharmaceutical [27–32] and food [33,34] analytical methods. The method described herein is the first application of accuracy profile validation within the field of integrated pest management combined with fast GC – incorporating a direct resistively heated column (Ultra Fast Module) – analytical tool. Besides, most applications of this validation methodology used constant acceptance limits all over the concentration range investigated. At least since the work of Horwitz et al. [35], it is well known that the relative standard deviation of any assay increases with decreasing concentrations, thus leading to higher random error. Here, acknowledging that making more total error at small concentrations is acceptable, the acceptance limits were therefore settled larger at the expected lower limit of quantification to take into account this natural behaviour of analytical methods. Such larger acceptance limits for decreasing concentration is common place for the random errors as well as for the systematic errors in other fields of applications [36–38]. However, to our knowledge, it is the first time two levels acceptance limits are used with the accuracy profile validation approach.

Nonetheless in order to interpret and compare adequately results obtained by laboratories with their analytical methods, it is essential to estimate measurement uncertainty [39]. In this respect, measurement uncertainties of this fully validated method were also computed, without any additional experiments, thus increasing the reliability evaluation of the analytical results obtained and thus the adequacy of the developed method.

2. Experimental

2.1. Chemicals and reagents

Essential oil of *M. chamomilla* was purchased from Vossen & Co. (Brussels, Belgium) and was originated from Nepal (lot no. CHA06MI0406). Essential oil of *N. cataria* was purchased from Essential7.com (Roswell, NM, USA) and was originated from Canada (lot no. EO0020f).

E-β-Farnesene from chemical synthesis was kindly supplied by Dr. S. Bartram and Prof. W. Boland (Max Planck Institute for Chemical Ecology, Jena, Germany). β-Caryophyllene, used as reference compound for the method validation, was extracted by flash chromatography from *N. cataria* L. essential oil. (+)-Longifolene as internal standard was purchased from ABCR (Karlsruhe, Germany). The mean purities of the terpenes, shown in Table 1 with standard deviations (SDs) and relative standard deviations (RSDs), were determined by fast GC. A solution of each compound was prepared in *n*-hexane at a concentration of 1 μg μl^{-1}. Ten replicates were performed.

Table 1
Purity of compounds analysed by fast GC.

Compound	Retention time (min)	Mean purity (%)	SD	RSD (%)
E-β-Farnesene (from synthesis)	3.52	99.4	0.2	0.2
E-β-Farnesene (from F3 *Matricaria chamomilla*)	3.52	83.8	0.3	0.4
β-Caryophyllene (from F2 *Nepeta cataria*)	3.41	97.7	0.5	0.5
(+)-Longifolene (from synthesis)	3.39	100.0	0.0	0.0

n-Hexane of GC grade was purchased from VWR (Leuven, Belgium). *n*-Pentane extra pure was purchased from Acros Organics (Geel, Belgium).

2.2. Fast GC analyses

Fast GC analyses were conducted on a Thermo Ultra Fast Trace GC gas chromatograph operated with a split/splitless injector and a Thermo AS 3000 autosampler (Thermo Electron Corp., Interscience, Louvain-la-Neuve, Belgium). The GC system was equipped with an Ultra fast module (UFM) incorporating a direct resistively heated column (Thermo Electron Corp.): UFC-5, 5% phenyl, 5 m × 0.1 mm I.D., 0.1 μm film thickness. The following chromatographic conditions were determined for good resolution of terpenes (mono- and sesquiterpenes) analyses in a previous paper [6]. Temperature programme for UFM was the following: initial temperature at 40 °C, held for 0.1 min, ramp 1 at 30 °C min^{-1} to 95 °C, ramp 2 at 35 °C min^{-1} to 155 °C, ramp 3 at 200 °C min^{-1} to 280 °C, final hold of 0.5 min at 280 °C. Injection temperature: 240 °C. Injection volume: 1 μl. Carrier gas: He, at constant flow rate of 0.5 ml min^{-1}. Split ratio = 1:100. The GC unit had a high-frequency fast flame ionisation detector (300 Hz FID), at 250 °C. H_2 flow: 35 ml min^{-1}; air flow: 350 ml min^{-1}; makeup gas flow (N_2): 30 ml min^{-1}. Data processing was realised by Chromcard software (Version 2.3.3). Fig. 1a and b shows the chromatograms obtained with this fast GC analytical method for the reference compounds (E-β-farnesene (81.6 ng μl^{-1}) and β-caryophyllene (80.5 ng μl^{-1}) with the internal standard longifolene (102.6 ng μl^{-1})) and for a blank sample (matrix without sesquiterpenes), respectively.

2.3. Flash chromatography

In a previous paper [6], the fractionation process was realised by a classic liquid column chromatographic separation of essential oils. The present research relates the use of flash chromatography (flash chromatography assembly with threaded joints, Sigma–Aldrich, Bornem, Belgium) to obtain purified extracts of relatively large scale quantities at higher speed (in less than 10 min). Ten millitres of essential oil (9.306 g for *M. chamomilla* and 9.525 g for *N. cataria*) were fractionated under pressure (N_2 at 0.5 bar) over 110 g of silica gel G60 (70–230 mesh: ref. no. 815330.1, from Macherey-Nagel) previously dried 16 h at 120 °C and packed in a glass column (35 mm I.D.) with glass wool plug at the bottom. The silicagel bed was 34 cm high. Essential oil of *M. chamomilla* was eluted with 1200 ml *n*-pentane to yield five fractions of 250 ml (F1), 200 ml (F2), 400 ml (F3), 200 ml (F4) and 150 ml (F5), respectively. Essential oil of *N. cataria* was eluted with 1050 ml *n*-pentane leading to three fractions of 250 ml (F1), 350 ml (F2) and 450 ml (F3), respectively. Fifty microlitres of each fraction were diluted 30

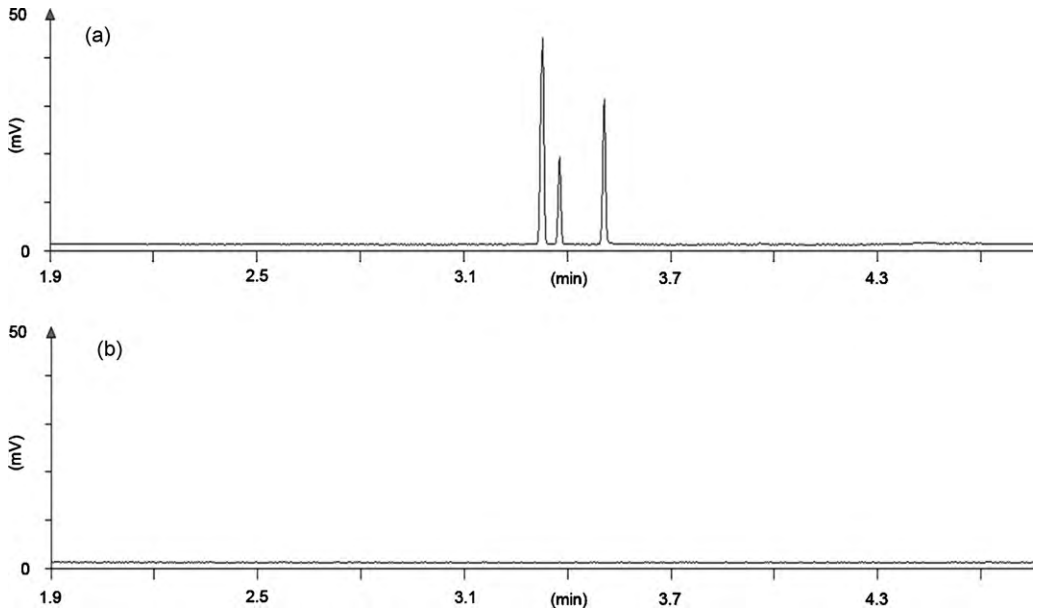

Fig. 1. Chromatograms of analytes (E-β-farnesene at 81.6 ng μl^{-1} and β-caryophyllene at 80.5 ng μl^{-1}) and internal standard (longifolene at 102.6 ng μl^{-1}) (a) and of a blank alginate beads matrix sample (b) analysed with optimised fast GC method. For analysis conditions, see text.

times in *n*-hexane prior to fast GC analyses. Solvent-free purified compounds were obtained after solvent evaporation from fractions at atmospheric pressure and at 40 °C with a Büchi rotatory evaporator (rotation: 1.6 tour s^{-1}). The recovery of this evaporation mode was measured in five replicates (96.3% ± 0.94%) and judged satisfactory according to the AOAC norm (2006) which requires recoveries comprised between 90% and 108%. Solvent-free fractions were diluted in *n*-hexane and analysed by fast GC. The large amounts (approximately 5 ml and 8 ml for E-β-farnesene and β-caryophyllene, respectively) of purified semiochemicals obtained by that way were stored at 4 °C until use.

2.4. Formulation of alginate gel beads

A solution of sodium alginate (Sigma Low viscosity, Sigma–Aldrich, Bornem, Belgium) was prepared in distilled water at 1.5% (w/v). In the same time, a 0.2 M calcium chloride (Acros Organics, Geel, Belgium) solution was prepared in distilled water. The ionic strength of this solution was fixed at 0.5 M with sodium chloride.

Eight ml of sodium alginate solution added to 1.8 ml of sunflower oil and 0.2 g of E-β-farnesene (solvent-free fraction (F3) from fractionation of *M. chamomilla* essential oil) or β-caryophyllene (solvent-free fraction (F2) from fractionation of *N. cataria* essential oil) were mixed with an ultraturax system (IKA T18 Basic, QLab, Vilvoorde, Belgium) at 24,000 rpm during 20 s to obtain a thin and homogeneous emulsion. For the α-tocopherol (Sigma–Aldrich, Bornem, Belgium) alginate beads type, 150 mg of this component were added in the mix before the ultraturax emulsion process.

The emulsion was extruded by needle (0.4 mm I.D.) and the drops fell into agitated (magnetic stir bar at 600 rpm) $CaCl_2$ solution to form the alginate gel beads containing semiochemical compounds. The distance between needle and $CaCl_2$ solution was fixed at 20 cm to obtain spherical beads. The beads stayed 48 h in the $CaCl_2$ solution to stabilise the syneresis phenomenon. The beads were dried before use to eliminate surface water. First, they were drained off on a filter paper during a few seconds. Then they were dried under air pressure at 2 bars during 30 min.

2.5. Determination of the sesquiterpenes protection efficiency of formulations

For each tested sesquiterpene (E-β-farnesene and β-caryophyllene) obtained by flash chromatography, four different formulations were compared in terms of protection efficiency during 20 days. For each day of analysis, three replicates of each formulation were prepared. The flasks were put under sunlight at room temperature until analysis, except for the day 0 where the analysis took place directly after the preparation of the samples. A Hobo data logger (Miravox, Hoevenen, Belgium) was installed near the flasks all along the 20 days of experiment to measure the lab temperature.

The first formulation consisted in compounds, isolated by flash chromatography, and not formulated in solvent or encapsulated in matrix. The purities of E-β-farnesene and β-caryophyllene from essential oils are presented in Table 1. Eighty milligrams of extract were introduced in a 10 ml flask and let in the previous described conditions until analysis. For the analysis, the flasks were filled up to the mark with *n*-hexane. One and a half ml of this solution was transferred into another 10 ml flask with 250 μl of longifolene (internal standard) at 10 μg μl^{-1}. The flask was filled up to the mark with *n*-hexane. The solutions were then analysed by fast GC for internal quantification.

For the second tested formulation, the compounds were mixed with sunflower oil in a 1:10 (w/w) ratio. Seven hundreds mg of the mix were introduced in a 10 ml flask. For the experiments, the flask was filled up with *n*-hexane. Before the fast GC analysis, sunflower oil containing triglycerides had to be discarded from the solution. For this purpose, 1 ml of the solution (sesquiterpene – sunflower oil – *n*-hexane) was fractionated over 1.5 g of silica gel G60 (70–230 mesh: ref. no. 815330.1, from Macherey-Nagel) previously dried 16 h at 120 °C and packed in a glass column (10 mm I.D.) with glass wool plug at the bottom. The silicagel bed was 3 cm high. The deposited sample was eluted with 50 ml *n*-pentane. Nine ml of this eluted extract were introduced with 250 μl of longifolene (internal standard) at 10 μg μl^{-1} in a 10 ml flask and filled up to the mark with *n*-hexane. The solution was then analysed by fast GC for internal quantification.

The elution volume (50 ml) necessary to elute the semiochemicals from the column was determined by measuring the elution recovery of a known quantity of E-β-farnesene deposited on the silica gel. The recovery of 91.2% ± 0.4% was judged satisfactory according to the AOAC norm (2006) which requires recoveries comprised between 85% and 110% considering the concentrations tested.

The two other formulations to test consisted in sesquiterpene alginate beads with or without added α-tocopherol, respectively. The α-tocopherol was added in the beads formulations as an antioxidant. The protocol of protector effect determination was the same for the two types of formulations. Ninety mg of alginate beads were introduced in a SOVIREL tube. Two ml of pentasodium tripolyphosphate ($Na_5P_3O_{10}$), at 25 μg μl^{-1} in water, were added to destabilize the alginate beads cohesion and liberate the semiochemicals and the sunflower oil contained in the beads. In the same time, 250 μl of internal standard (longifolene) at 10 μg μl^{-1} were added in the tube for further quantification. The tube was let at rest during 30 minutes. Three successive extractions of sesquiterpene compounds were conducted with *n*-pentane as extraction solvent. For each extraction, 5 ml *n*-pentane were added to the tube, the solution was homogenised for 10 min and centrifuged at 5000 rpm at 20 °C ± 2 °C. The pentane phases coming from the 3 extractions were transferred cautiously in a flask. The solvent was then evaporated until 1 ml at atmospheric pressure and 40 °C with a Büchi rotatory evaporator. The same fractionation process than the one previously described was elaborated to quantify semiochemical compounds without injecting sunflower oil on the GC column. For this purpose, the 1 ml extract was deposited on 1.5 g of silica gel G60 previously dried and packed in a glass column (10 mm I.D.) with glass wool plug at the bottom. *n*-Pentane was used as elution solvent. Fifty millilitres were collected. This extract was analysed by fast GC for internal quantification of E-β-farnesene or β-caryophyllene.

The protection efficiency of each formulation was expressed by the mean residual percentage of compounds at each day of analysis compared to the mean values of day 0 (day 0 = 100%).

2.6. Method validation

The validation step was performed using the accuracy profile concept [24–26]. The range of concentration levels for E-β-farnesene and β-caryophyllene was from 80 ng μl^{-1} to 1000 ng μl^{-1} in *n*-hexane for the calibration standards and in a reconstituted matrix for the validation standards. Longifolene was used as internal standard (I.S.) for these sesquiterpene components at 102.6 ng μl^{-1} in each level of concentration for the calibration and for the validation standards.

The choice of longifolene as internal standard was made for different reasons: this compound belongs to the same family than the analytes (sesquiterpenes), the retention time of longifolene was close to the retention time of the analytes without coelution (see Section 3.1) and longifolene was absent of the samples to analyse in routine.

2.6.1. Solutions used for calibration

For each component, three standard solutions were prepared in three replicates for three series (three separated days) of analyses. The concentration levels are shown in Table 2. Each solution was analysed by fast GC. The calibration curves were obtained for each series of analyses by plotting the ratio of analysed peak area/I.S. peak area, *versus* the concentration of analyte.

2.6.2. Solutions used for validation

Five independent standard solutions were prepared in three replicates for three series (days) of analyses. The solutions consisted in matrix of alginate gel beads without sesquiterpene treated as explained in Section 2.5, and spiked with fixed amounts of reference sesquiterpenes. The concentration levels of the validation standards are shown in Table 3. These standards were treated like real samples on fast GC.

Table 2
Levels of calibration standards for E-β-farnesene and β-caryophyllene.

Level	Concentration of E-β-farnesene (ng μl^{-1})	Concentration of β-caryophyllene (ng μl^{-1})	Concentration of I.S. (ng μl^{-1})
1	25.5	24.9	102.6
2	509.8	499.6	102.6
3	1019.7	999.2	102.6
Total	9 samples/series (day)/compound		

3. Results and discussion

3.1. Resolution and selectivity of the method

The selectivity of the chromatographic method depends on the resolution of the targeted compounds and on the absence of interference. In the present paper, the total resolution was defined between the two nearest compounds, longifolene and β-caryophyllene as:

$$R_s = \frac{2(t_{R\,\beta\text{-caryophyllene}} - t_{R\,longifolene})}{W_{\text{longifolene}} + W_{\beta\text{-caryophyllene}}},$$

where t_R is the retention times and W is the peak widths of the two nearest compounds.

The resolution was good with R_s (1.65) higher than 1.5.

Moreover, by comparing 4 replicates of a blank injection (alginate beads with α-tocopherol and sunflower oil, but without sesquiterpene, treated as explained in Section 2.5) to a diluted mixture (6 replicates) of E-β-farnesene (81.6 ng μl^{-1}), β-caryophyllene (80.5 ng μl^{-1}) and longifolene (102.6 ng μl^{-1}), no peak or interference was observed at the retention times corresponding of the analytes and the internal standard. Fig. 1b shows the chromatogram of a blank injection compared to the chromatogram of analytes (Fig. 1a). The retention times of the reference compounds are presented in Table 1.

3.2. Validation by use of accuracy profile approach

This concept of validation largely described in practice and theoretically explained by Hubert et al. [24–26] and by Rozet et al. [40–42], can be summarized as follows:

- Step 1: analyse the three series of calibration standards and draw calibration curves as explained in Section 2.6.1. Test different regression models for the calibration curves.
- Step 2: analyse the validation standards of each series. Back-calculate the predicted concentrations by means of the peak area

Table 3
Levels of validation standards for E-β-farnesene and β-caryophyllene.

Level	Concentration of E-β-farnesene (ng μl^{-1})	Concentration of β-caryophyllene (ng μl^{-1})	Concentration of I.S. (ng μl^{-1})
1	81.6	80.5	102.6
2	163.2	160.9	102.6
3	367.1	362.1	102.6
4	734.2	724.2	102.6
5	1019.7	1005.8	102.6
Total	15 samples/series (day)/compound		

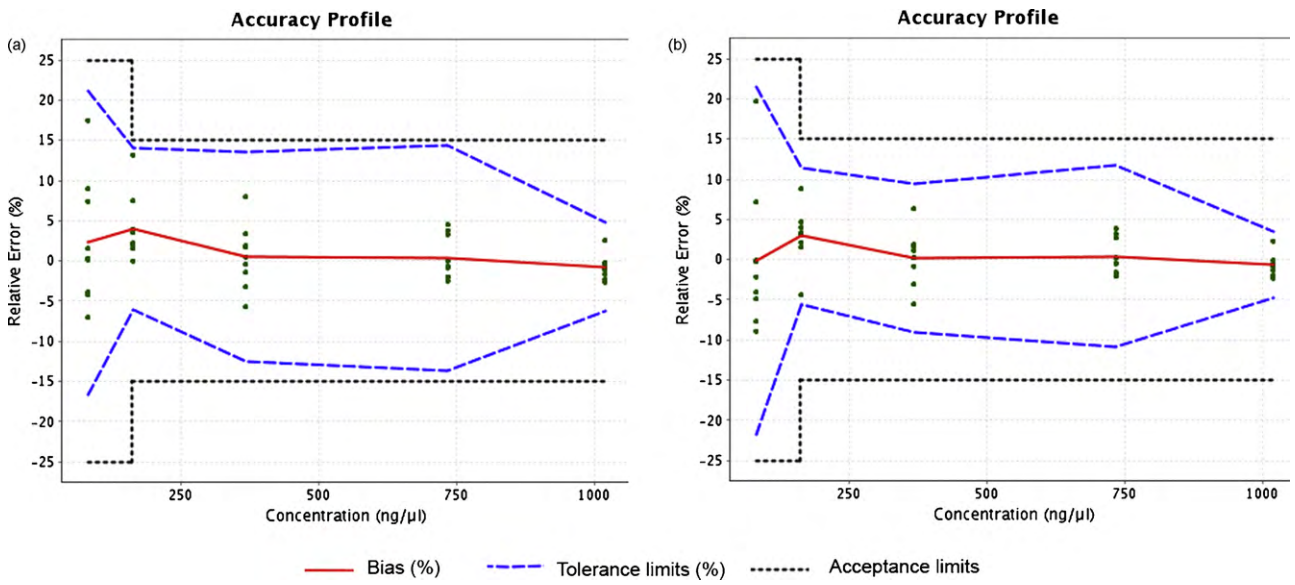

Fig. 2. Accuracy profiles of E-β-farnesene obtained by considering a simple linear regression model (a) and by considering a linear regression model through zero fitted with the maximum level of concentration (b); plain line: relative bias, dashed lines: β-expectation tolerance limits (β = 95%), dotted curves: acceptance limit (±25% and ±15%) and dots: relative back-calculated concentrations of the validation standards.

ratio obtained and introduced in the corresponding regression equation.

- Step 3: determine the mean bias (estimating the trueness) for each concentration level, which corresponds to the systematic error.
- Step 4: calculate the precision parameters: repeatability and intermediate precision for each concentration level, which correspond to the random error.
- Step 5: determine the relative tolerance limits (β-expectation tolerance interval) for each validation standard concentration level with a prespecified probability level β.
- Step 6: plot the accuracy profile as the mean bias, the relative tolerance limits and the acceptance limits in function of the concentrations, in relative values.
- Step 7: determine the linearity of the method by plotting the back-calculated concentrations of all the series (N = 45) in function of the introduced concentrations (concentration levels of the validation standards). This step is necessary to verify that the analytical method gives results (in terms of predicted concentrations) strictly proportional to the tested concentrations.

3.2.1. Analysis of the response functions and determination of the best regression models

For each analyte and for each series of calibration standards, using the three calibration levels ranging from 80 ng µl^{-1} to 1000 ng µl^{-1}, different regression models were tested for E-β-farnesene and β-caryophyllene in order to define the most adequate one. The regression models tested were the simple linear regression and the linear regression model through zero fitted with the maximum concentration level of the calibration standards.

The calculation of the different validation parameters (trueness, precision, accuracy, linearity, limits of detection (LOD) and quantification (LOQ) and range) was realized from each regression model. Moreover, in each case, an accuracy profile, with a maximum risk limit of 5%, was constructed as it can be seen in Figs. 2 and 3 for E-β-farnesene and β-caryophyllene, respectively. Furthermore, in order to take decision about the validity of the method, acceptance limits in terms of maximum total error have to be set. For this application, two levels of acceptance limits were defined. First, for the concentration levels ranging from 160 ng µl to 1000 ng µl^{-1}, the acceptance limits was settled at ±15%, meaning that a symmetric maximum total error of 15% around the true concentration of analyte present in the sample could be accepted. Second, for the targeted lower limit of quantification, a slightly larger acceptance limits was defined: ±25%. Indeed, it is reasonable to authorize increasing error for small concentrations as on one hand the absolute value of this error (in concentration for e.g.) will still be acceptable and on the other hand this concentration dependant behaviour of error has been reported since long time ago [43] and is integrated in various validation guidelines [36,44].

For the E-β-farnesene, two models were tested: a simple linear regression model (Fig. 2a) and a linear regression model through zero fitted with the maximum level of concentration (Fig. 2b). In both cases, the relative β-expectation tolerance limits were inside the acceptance limits fixed at ±15% and ±25%. Nevertheless, considering on one hand the risk profiles illustrated in Fig. 4, and on the second hand its practical advantage the second model seemed to be the most appropriate with a maximum risk of 2.8% (Fig. 4a) instead of 4.4% in the first model. Indeed, these risk profiles express that the probability to obtain future results outside the specified acceptance limits for the concentration range tested are of maximum 4.4% for the simple linear regression model and at most 2.8% for the highly simple and economic one level calibration scheme.

In the case of β-caryophyllene, the study of accuracy profiles was more complex due to the presence of a systematic bias at each concentration level for the two tested regression models. Fig. 3a and b shows the accuracy profiles obtained for, respectively, the simple linear regression model and the linear regression through zero fitted with the maximum concentration level. In both profiles, the β-expectation tolerance limits were completely outside the acceptance limits fixed at ±15%. Indeed, a strong proportional systematic error was observed as given by the following equations of the linearity between true concentration (X) and back-calculated concentration (Y) for the simple linear and the forced through zero regressions, respectively:

$$Y = 2.218 + 0.7361X$$
$$Y = 4.629 + 0.7348X$$

In order to correct this systematic error a correction factor was determined as the inverse of the slopes of the linearity equations

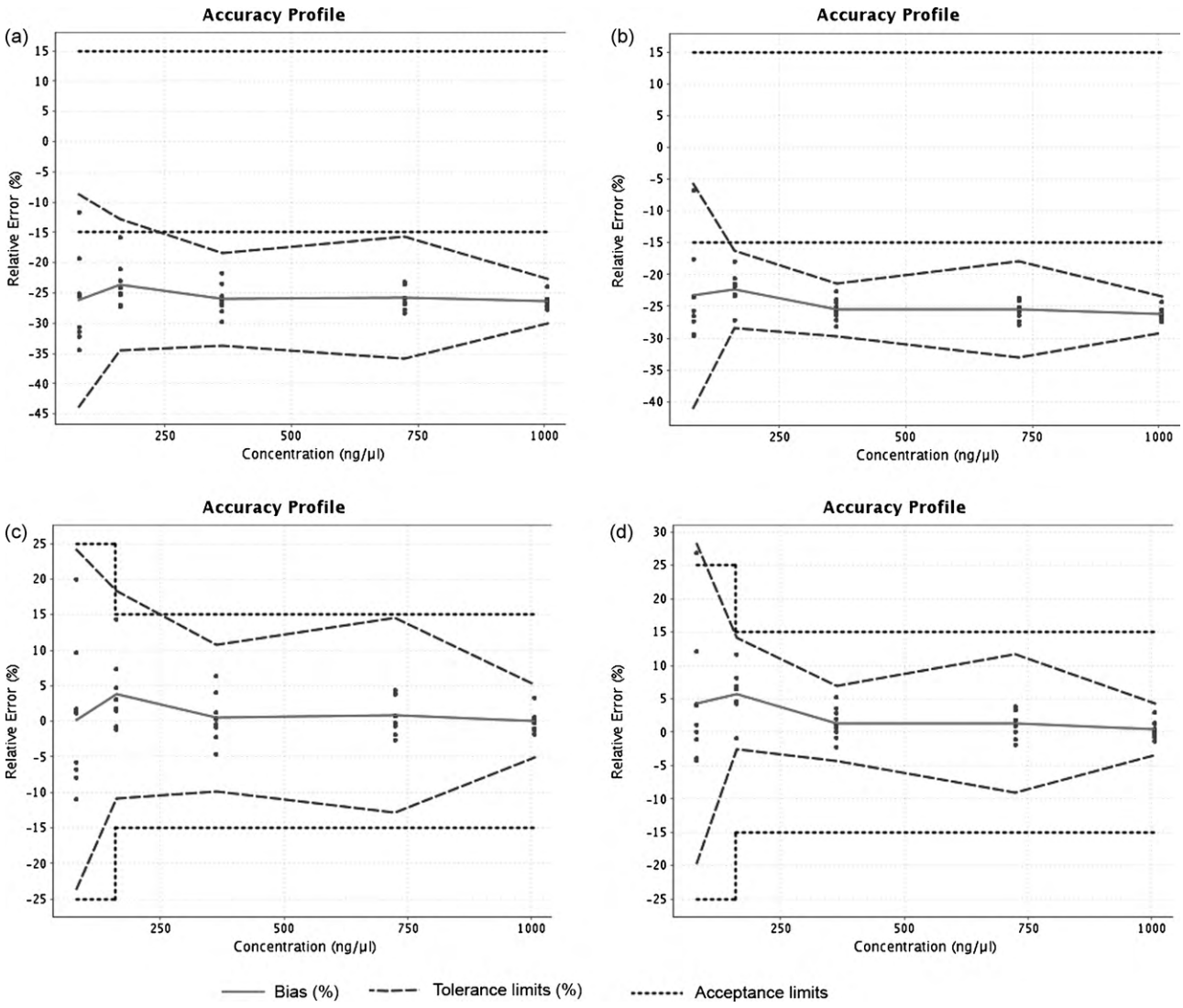

Fig. 3. Accuracy profiles of β-caryophyllene obtained by considering a simple linear regression model (a) and considering a linear regression model through zero fitted with the maximum level of concentration (b) without correction of the bias. Accuracy profiles of β-caryophyllene obtained by considering a simple linear regression model (c) and considering a linear regression model through zero fitted with the maximum level of concentration (d) with a correcting factor of the bias. Plain line: relative bias, dashed lines: β-expectation tolerance limits (β = 95%), dotted curves: acceptance limit (±25% and ±15%) and dots: relative back-calculated concentrations of the validation standards.

(1.3585 and 1.3609 for the simple linear and the forced through zero regressions, respectively) [45]. The correction factors were then applied to the results obtained using their respective regression model, as explained in Hubert et al. [46]. Fig. 3c and d show the corrected accuracy profiles with acceptance limits fixed at ±15%, except at the lowest concentration level (±25%). In the simple linear regression model (Fig. 3c), the upper tolerance limit stepped outside the 15% acceptance limit at the second concentration level (160 ng µl^{-1}). For the second model (Fig. 3d), the tolerance limits were over the whole concentration range tested inside the acceptance limits except for the first concentration level.

Nonetheless, after examination of the risk profile obtained using the forced through zero calibration curve (Fig. 4b), the true risk associated to the first concentration level was very close (5.15%) to the maximum risk limit fixed initially at 5%. Indeed, this slight increased risk to obtained future results outside the ±25% is perfectly acceptable with respect to the final use of the method. This regression model was thus finally chosen as the most appropriate for the quantification of β-caryophyllene.

3.2.2. Trueness of the method

The trueness expresses the closeness of agreement between the mean value obtained from a series of measurements and the value which is accepted as the true value [25]. Trueness is expressed in terms of bias (in relative (%) and absolute (ng µl^{-1}) values) which corresponds to the systematic error. As presented in Table 4, the relative biases are not too high ranging from −0.67% and from 0.38% for E-β-farnesene and β-caryophyllene, respectively, illustrating the good trueness of the method developed for each analyte when using a linear regression curved forced through zero and using only the maximum concentration level of the calibration standard.

3.2.3. Precision of the method

The precision expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same sample [25]. The precision is evaluated in terms of repeatability (same analytical procedure, same operator, and same day) and intermediate precision (same analytical procedure but different operators and different days) expressed by relative standard deviations (RSDs %).

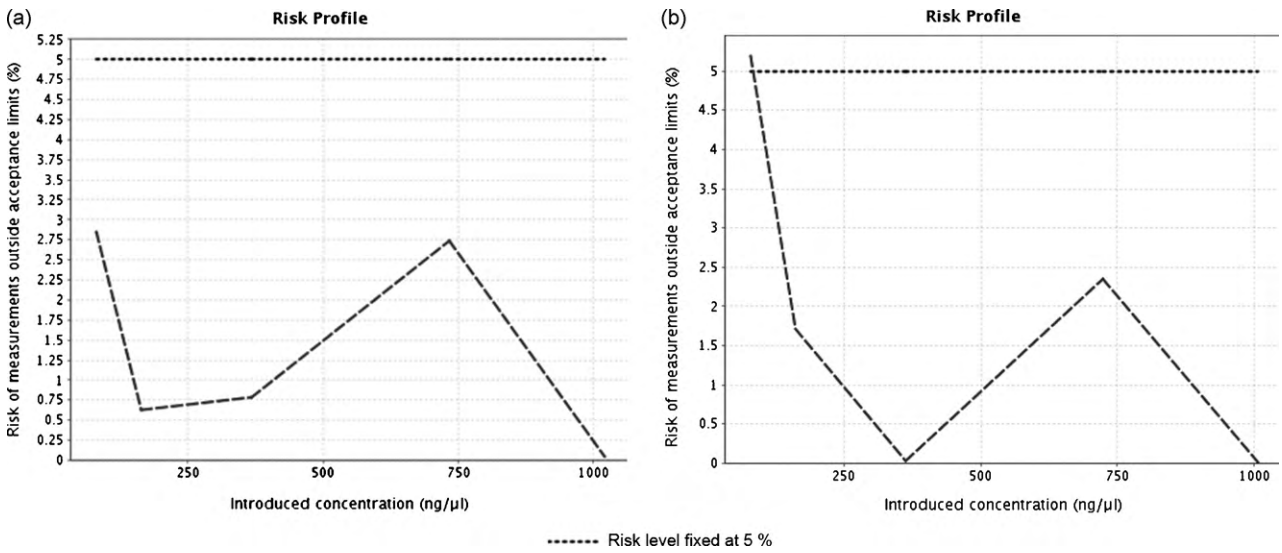

Fig. 4. Risk profiles of E-β-farnesene (a) and β-caryophyllene (b) for the chosen regression models. Dotted line: maximum risk of 5%; dashed line: effective risk of having results falling outside the specified acceptance limits.

As shown in Table 4, the relative standard deviations of repeatability and intermediate precision are lower than 5% for both analytes, except at the lowest concentration levels where, nevertheless, they never exceeded 10%.

3.2.4. Accuracy of the method

The accuracy of an analytical procedure expresses the closeness of agreement between the value found and the value accepted as the conventional true value. The closeness of agreement observed is the resultant (total error) of the sum of the systematic and random errors, also the sum of the trueness and the precision [25].

Figs. 2b and 3d show the accuracy profiles of E-β-farnesene and β-caryophyllene for the chosen regression models. The plain central lines represent the relative biases. The dashed lines are the 95% β-expectation tolerance limits (the probability that each future results will fall inside these β-expectation tolerance lim-

Table 4
Validation results for E-β-farnesene and β-caryophyllene for the chosen regression models.

	E-β-Farnesene			β-Caryophyllene		
Range (ng µl^{-1})	81.6–1019.7			80.5–1005.8		
Response function ($m = 3$, $n = 3$)						
	Series 1	Series 2	Series 3	Series 1	Series 2	Series 3
Slope	0.0089	0.0091	0.0089	0.0071	0.0072	0.0070
Trueness ($n = 3$, $p = 3$)						
Concentration levels	Absolute bias (ng µl^{-1})	Relative bias (%)		Absolute bias (ng µl^{-1})	Relative bias (%)	
1	−0.1	−0.1		3.5	4.3	
2	4.8	2.9		9.3	5.7	
3	0.8	0.2		4.9	1.3	
4	2.9	0.4		9.7	1.3	
5	−6.8	−0.7		3.8	0.4	
Precision ($n = 3$, $p = 3$)						
Concentration levels	Repeatability (RSD, %)	Intermediate precision (RSD, %)		Repeatability (RSD, %)	Intermediate precision (RSD, %)	
1	8.8	8.8		9.8	9.8	
2	3.4	3.4		3.4	3.4	
3	2.9	3.4		2.3	2.3	
4	0.8	2.6		0.7	2.3	
5	1.0	1.4		1.1	1.4	
Accuracy ($n = 3$, $p = 3$, $\beta = 0.95$)						
Concentration levels	β-Expectation tolerance limits (ng µl^{-1})	β-Expectation tolerance limits (%)		β-Expectation tolerance limits (ng µl^{-1})	β-Expectation tolerance limits (%)	
1	[63.9–99.1]	[−21.7 to 21.4]		[64.7–103.2]	[−19.6 to 28.3]	
2	[154.2–181.7]	[−5.5 to 11.4]		[156.8–183.6]	[−2.5 to 14.1]	
3	[333.9–401.9]	[−9.0 to 9.5]		[346.6–387.4]	[−4.3 to 6.7]	
4	[654.3–820.0]	[−10.9 to 11.7]		[659.1–808.6]	[−9.0 to 11.6]	
5	[970.5–1055.0]	[−4.8 to 3.5]		[970.7–1049.0]	[−3.5 to 4.3]	
Linearity ($n = 3$, $m = 5$, $p = 3$), $N = 45$						
Range (ng µl^{-1})	81.6–1019.7			80.5–1005.8		
Slope	0.9928			0.9998		
Intercept	3.7050			6.3450		
r^2	0.9989			0.9991		
Lower LOQ (ng µl^{-1})	81.6			80.5		
Lower LOD (ng µl^{-1})	40.8			49.5		

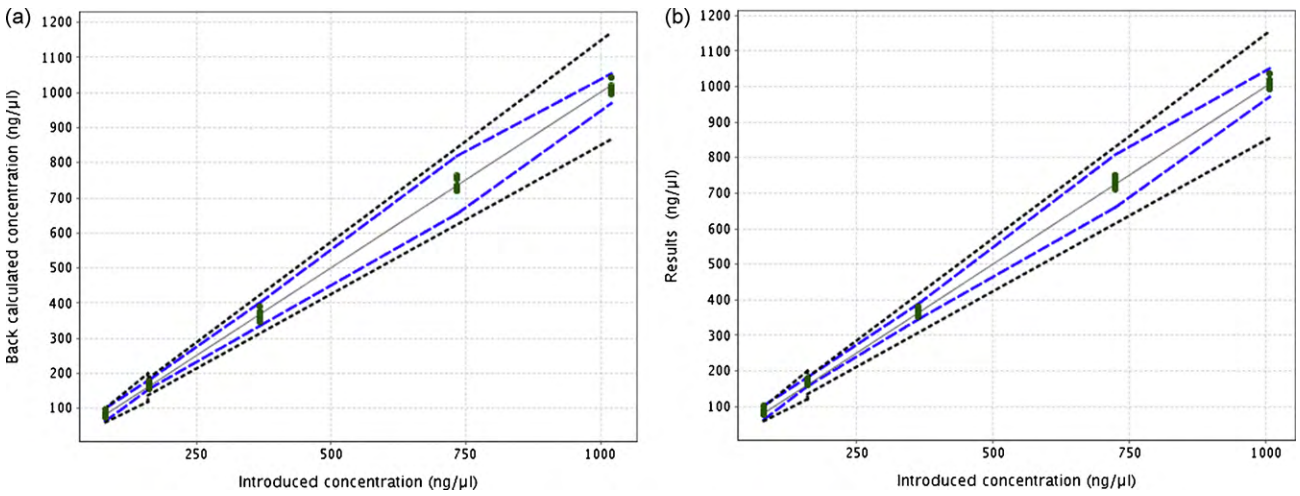

Fig. 5. Linearity profiles of (a) E-β-farnesene and (b) β-caryophyllene (after correction of the results). Plain line: identity line ($Y = X$), dashed lines: β-expectation tolerance limits ($\beta = 95\%$), dotted curves: acceptance limit expressed in ng μl^{-1} and dots: back-calculated concentrations of the validation standards.

its is of 95%) and the dotted lines represent the acceptance limits. The method is considered giving accurate results as long as the β-expectation limits do not cross the acceptance limits. Table 4 gives the β-expectation intervals for each analyte at all concentration level tested of the validation standard.

3.2.5. Limits of detection, quantification and range

The lower LOQ (LLOQ) is the lowest amount of the targeted analyte in the sample which can be quantitatively determined under the experimental conditions prescribed with a well defined accuracy [25]. Therefore, the LLOQ of both analytes are the smallest concentrations tested for which the β-expectation tolerance intervals are included inside the acceptance limits previously settled. As discussed earlier, based on a risk analysis the LLOQ was defined as 80 ng μl^{-1} for β-caryophyllene. The LOD (limit of detection) was arbitrarily defined as 1/2 LOQ. The limit of detection is generally defined as the lowest quantity of a substance that can be distinguished from the blank but which cannot be quantified. LODs and LOQs (in ng μl^{-1}) are presented in Table 4.

3.2.6. Linearity

The linearity of the results generated by an analytical procedure is the ability within a given range to obtain test results that are directly proportional to the concentrations (amounts) of an analyte in the sample [25,47].

In practice, the linearity was determined, for the two compounds, by drawing a regression line of the back-calculated concentrations (for all the series, $N = 45$) in function of the introduced concentrations (validation standards, 5 levels ranging from 80 ng μl^{-1} to 1000 ng μl^{-1}).

For each compound, the linearity of the results obtained by the analytical method was demonstrated using β-expectation tolerance limits ($\beta = 95\%$) fully included with the acceptance limits expressed in concentration units as shown in Fig. 5a and b for E-β-farnesene and β-caryophyllene (after correction of the results), respectively. The determination coefficients (r^2) as well as the linearity equations are given in Table 4.

3.3. Measurement uncertainty

To allow a correct interpretation of results obtained by an analytical procedure, their reliability must be demonstrated. Validation ensures that the method is fit for its future purpose, however it is not sufficient if one aims at interpreting and comparing results correctly. Uncertainty of measurements should therefore be evaluated to ensure this. One major advantage of the applied validation methodology is that it can, without any additional experiments, give estimation of uncertainty of measurements. Indeed Feinberg et al. [48] demonstrated the mathematical link between the variance used to compute the β-expectation tolerance interval and the uncertainty of the measurements as defined in the ISO/DTS 21748 [49]. Therefore, as long as the experimental design used for the validation is representative of the sources of variability that will be encountered during routine analysis, this uncertainty estimate is relevant for the results obtained in the laboratory having validated the analytical procedure. Several estimations of uncertainty were

Table 5
Estimates of the measurement uncertainties related to E-β-farnesene and β-caryophyllene, at each concentration level investigated in validation using the selected regression models.

Analyte	Concentration (ng μl^{-1})	Uncertainty of the bias (ng μl^{-1})	Uncertainty of the correction factor (ng μl^{-1})	Combined uncertainty (ng μl^{-1})	Expanded uncertainty (ng μl^{-1})	Relative expanded uncertainty (%)
E-β-farnesene	81.6	2.400	–	7.58	15.16	18.6
	163.2	1.880	–	5.94	11.88	7.3
	367.1	5.290	–	13.71	27.41	7.5
	734.2	10.610	–	21.80	43.59	5.9
	1019.7	6.630	–	15.78	31.56	3.1
β-Caryophyllene	80.5	2.623	0.003286	8.30	16.60	20.8
	160.9	1.822	0.003286	5.81	11.61	7.3
	362.1	2.784	0.003286	8.95	17.90	5.0
	724.2	9.410	0.003286	19.51	39.02	5.4
	1005.8	6.147	0.003286	15.76	31.52	3.2

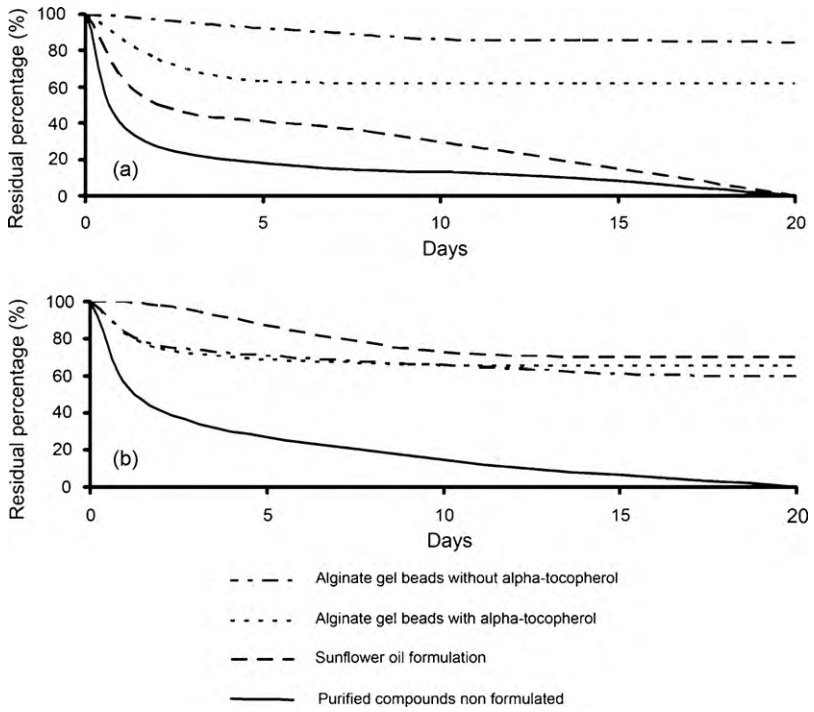

Fig. 6. Residual percentage of E-β-farnesene (a) and β-caryophyllene (b) in formulations during 20 days.

thus computed without any additional experiments and are presented in Table 5. The expanded uncertainty was computed using a coverage factor of $k = 2$ [39,50,51], representing an interval around the results where the unknown true value can be observed with a confidence level of 95%. For the particular case of β-caryophyllene, a correction factor was introduced in order to alleviate the strong systematic error observed. The introduction of this factor has two effects. First, it expands the values of the uncertainty of the uncorrected results by a factor corresponding to its value, as expected in theory. Second, the uncertainty of this factor should be taken into account. Since this correction factor is the inverse of the slope, its uncertainty is the standard error of the slope obtained from the least square linear regression. Its value is given in Table 5. As can be seen in this table, the uncertainty of this correction factor is far from being the most important source of uncertainty. Nonetheless, the combined standard uncertainty, expanded uncertainty and relative expanded uncertainty were computed by incorporating this supplementary uncertainty and are given in Table 5. It is also for this reason that the minor additional uncertainty conveyed by the correction factor was not introduced in the accuracy profiles of Fig. 3c and d.

As shown in Table 5, the relative expanded uncertainty of each semiochemical irrespective of the concentration levels did not exceed 10%, except for the smallest concentration levels for which it is around 20%. In other words, this means that with a confidence level of 95% the unknown true value is situated at maximum ±10% around the measured result for samples ranging from 160 ng μl^{-1} to 1000 ng μl^{-1} and at maximum ±20% around the measured result for samples at 80 ng μl^{-1}.

3.4. Protection efficiency of the formulations

During the twenty days of analysis, the temperature was measured in the lab where the experiments were conducted. The mean observed temperature was of 23.06 °C ± 1.90 °C.

The evolution of the protection capacity of the different formulations, expressed in terms of residual percentage of compound, is presented in Fig. 6a and b for E-β-farnesene and β-caryophyllene, respectively.

For E-β-farnesene, the most stable formulation is the alginate beads without α-tocopherol. The residual percentage of semiochemical in this formulation decreases slowly until day 10, and then stays relatively stable until the end of the experiment at a value close to 85%. The residual percentage in the beads with α-tocopherol becomes stable after 5 days at a lower value close to 60%. The quantity of E-β-farnesene formulated in sunflower oil and in the non-formulated pure E-β-farnesene decreases rapidly with a half-life period of 2 and 1.2 days, respectively.

The case of β-caryophyllene is slightly different from that of E-β-farnesene in terms of protection efficiency of formulations. There is no important difference in the residuals percentage evolution for the three formulations (sunflower oil, beads with α-tocopherol and beads without α-tocopherol), the stability being comprised between 60% and 70%. The non-formulated compound is rapidly degraded with a half-life period of 1.6 days.

The alginate beads are more protective for the components formulated and are easier to manipulate as slow release devices to put on fields in integrated pest management programs. Slow release studies of semiochemicals are presently conducted on these alginate beads to determine a mathematical kinetic model of release considering the impact of physico-chemical parameters like temperature, relative humidity, light intensity and wind. The results of this experiment will be presented in a following paper.

4. Conclusion

The method for the quantification of semiochemical sesquiterpenes by fast GC-FID was completely validated by applying the concept of total error using the accuracy profile as decision tool. The accuracy profiles were constructed for the two analytes (E-β-farnesene and β-caryophyllene) by considering a probability of 95% and a linear regression through zero fitted with the maximum level of concentration as calibration curve. The different validation criteria were evaluated and the lowest limits of quantification were

determined. The method developed is providing accurate results along the concentration range evaluated for the two analytes, i.e. from 80 ng μl^{-1} to 1000 ng μl^{-1}. In addition the measurements uncertainties were estimated without any additional experiments thanks to the validation methodology, allowing correct interpretation and comparison of the results in a cost effective manner.

Moreover, the alginate gel beads formulations were estimated in terms of protection efficiency of sesquiterpenes. The results showed that the beads protect the compounds at relatively high levels (between 60% and 85% of residual percentages with a stabilisation of the degradation) during minimum twenty days. Some experiments presently in study (lab controlled conditions) show that the release of such formulations can be conducted during at least 80 days. The biological effects of the slow release devices (tested on *E. balteatus* De Geer and on aphid parasitoids) have also been demonstrated in lab experiments and in naturally conditions (field experiments) (unpublished results).

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