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# Stability studies and determination of carnosic acid and its oxidative degradation products by gas chromatography–mass spectrometry

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ABSTRACT

The stability of carnosic acid (CA, an important abietane ferruginol-type diterpene, with antioxidant, anti-inflammatory, anti-bacterial, and anti-mutagenic properties) exposed to different stress conditions (daylight, high temperatures, different solvents, and humidity) was investigated. Gas chromatography–mass spectrometry (GC–MS) was used to analyse the degraded samples. Structural identification of degradation products was assigned based upon MS fragmentation patterns. The stability experiments were performed on pure CA (96% purity) as well as on commercially-available rosemary extract (containing 50.27% of CA and 5.65% of a diterpene lactone carnosol-C). For the extraction of target compounds three different techniques were compared: ultrasonic extraction (UE), Soxhlet (SE) and microwave extraction (MWE). It was shown that CA always occurs in combination with C, which is produced by oxidation and ring closure from CA, but with UE transformation of CA into C was minimal and constant when compared to SE or MWE. CA dissolved in tetrahydrofuran (THF) remained stable for at least 5 h at room temperature. After a certain time, under all tested conditions, the concentration of CA lowered as a result of the formation of degradation products such as C, rosmanol, 12-*O*-methyl-CA, 6,7-de-hydro-CA, 7-keto-CA and other unidentified degradants. In comparison to the solid state, the degradation of CA dissolved in organic solvents (THF and ethanol) was significantly faster. In general, protic solvents lead to faster degradation of CA. Only extremely high temperatures (200–300 °C) and long-term exposure to light and humidity (6 months) caused the degradation of CA in powdered samples.

The GC–MS method described was also used for determination of CA and C in five different species of the *Lamiaceae* family (*Rosmarinus officinalis* L., *Salvia officinalis* L., *Satureja Montana* L., *Salvia sclarea* L., and *Salvia glutinosa* L.).



## 1. Introduction

Carnosic acid (CA) or salvin and carnosol (C) or picrosalvin belong to the group of abietane ferruginol-type phenolic diterpenes and are present in many different plants. In plants the diterpenes are not distributed evenly; they are usually present within the aerial parts such as leaves, where they play diverse functional roles [1,2]. It has been confirmed that CA and C are powerful antioxidants, as their antioxidant activities are comparable to those of synthetic antioxidant BHT (2,6-di-*tert*-butyl-*p*-hydroxytoluene), BHA (butylated hydroxyanisole), and TBHQ (2-*tert*-butyl-hydroquinone) [3,4]. CA and C possess anti-bacterial, anti-inflammatory, anti-viral, anti-mutagenic, anti-carcinogenic and many other health-beneficial activities [2]. CA often occurs in combination with C, and both are commonly found in species of the *Lamiaceae* family such as rosemary (*Rosmarinus officinalis* L.) or sage (*Salvia officinalis* L.) [1–8]. The diterpenic content in plants can differ significantly depending on many factors such as concentration of $CO_2$ in the air, strong seasonal variations (exposure to thermal, water and UV-light stress), and fertilisation and harvest time [2,5,8].

The chemical structure of C was first described by Brieskorn in 1964 [9]. Later it was established that C is a diterpene $\delta$-lactone produced by oxidation and cyclization from CA and is an intermediate in the formation of CA's highly oxygenated derivatives [3,10–12]. C can be converted back to CA by catalytic hydrogenation where the lactone-ring is opened. The oxidative degradation process of CA leads to the formation of several different, more or less stable, degradation products. Most of these, except *O*-methylated products, indicate antioxidant properties [1,3]. Wenkert has described oxidative degradation of CA as the "oxidation cascade reaction of

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CA" [11]. The mechanism of CA oxidative degradation is very similar to that of its antioxidant activity [12].

High-performance liquid chromatography (HPLC) with gradient elution and ultraviolet-visible (UV) detection has been widely used for the separation and determination of diterpenes [1,3,4–6,8,10,12–14,16,19]. There are a few cases where in analysis of diterpenes, the hyphenated technique of LC–MS has been used [10,14–19]. In the literature, there are no reliable procedures that allow simultaneous separation and determination of CA and its degradation products using gas chromatography coupled to mass spectrometry (GC–MS). The reason is in its low thermal, photo and oxidative stability. Despite their unfavourable physicochemical properties, we have been able to develop a reliable analytical method that allows good separation, rapid, simultaneous unambiguous identification and determination of CA, C and other degradation products in different samples using GC–MS. In comparison with HPLC, a minor disadvantage is the necessity of a derivatisation step being required to ensure the thermal stability and volatility of the compounds. The method described includes derivatisation with trimethylsilylation (TMS). The spectra of the silylated investigated compounds are not available in common mass spectral libraries. This is the first report in which the identifying ions of TMS-derivatives of CA, C, rosmanol, 12-*O*-methyl-CA, 6,7-de-hydro-CA, and 7-keto-CA are presented and some of the fragmentation patterns are proposed and discused.

In this experiment, the stabilities of CA and C have been systematically tested by exposure to different factors including temperature, daylight, darkness, and moisture. Accelerated and long-term stability tests were performed and, with respect to the recorded mass spectra, some of the degradation products were identified. Additionally, three different extraction techniques (ultrasonic extraction, Soxhlet extraction, and microwave assisted extraction) were compared and the extracts were characterised not only in terms of extraction yields, as described in most articles [5–7,14,16,20]. but also regarding maximum stability of the CA and its minimal conversion to C and other degradation products. CA and C were successfully quantified by GC–MS in five different *Lamiaceae* species.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All reagents and solvents used were of analytical grade. Methanol (MeOH) and ethanol (EtOH) were purchased from Riedel-de Haën, tetrahydrofuran (THF), and pyridine (PYR) from Merck (Germany). Anhydrous sodium sulphate ($Na_2SO_4$) was obtained from J.T. Baker (Netherlands), *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) from Fluka Chemie (Switzerland), carnosic acid (CA, 96.4%) and carnosol (C, 98.2%) were from the Alexis corporation (Switzerland), cholesterol (99%) and cholesteryl acetate (95%) were supplied by Sigma-Aldrich (Germany).

### 2.2. Plant samples

Air-dried leaves of *Rosmarinus officinalis* L. (rosemary), *Salvia officinalis* L. (common sage), *Satureja Montana* L. (winter savory), *Salvia sclarea* L. (clary sage) and *Salvia glutinosa* L. (sticky sage), were used for the analyses. The dried plant material was ground, homogenised, and stored in darkness at room temperature. Commercially-available powdered rosemary extract containing carnosic acid (50.27%) and carnosol (5.65%) was also used in the analyses.

### 2.3. Preparation of plant samples

For the extraction of CA and C from plant samples, three different extraction techniques were compared: ultrasonic extraction (UE), Soxhlet extraction (SE) and microwave extraction (MWE). For UE, 1 g of an homogenised sample (dry powdered leaves of rosemary) was weighed into a centrifuge tube and spiked with an appropriate amount of ISTD-cholesterol. The sample was extracted three times by sonication with 20 mL of a mixture of THF, EtOH and PYR (v/v/v, 1:1:0.1). After each extraction the sample was centrifuged and the supernatants were combined, dried over $Na_2SO_4$, and concentrated into dryness by rotary evaporation. The residue was re-dissolved in 10 mL of the same organic mixture and an aliquot of 200 μL was firstly cleaned by solid phase extraction (SPE) and further by size exclusion chromatography (SEC).

SPE tubes (Superclean ENVI-Carb 6-mL) were preconditioned with EtOH (2 × 5 mL), and therafter the sample aliquot was loaded. The sorbent was never allowed to dry during the conditioning and sample loading step. The sample was rinsed with 2 mL of EtOH and the elution was carried out with an additional 30 mL of EtOH. The eluate was collected under gravity flow and evaporated to dryness. The solvent exchange to THF was performed by adding two 0.5 mL portions of THF and vortexing. For SEC, Bio-Beads S-X3 gel (200–400 mesh, Bio-Rad Laboratories, Richmond) was allowed to swell in THF for 24 h before use. The extract was quantitatively transferred into a glass column (15 mm I.D. and 30 cm long) filled with slurried beads. After the sample sank into the column bed it was rinsed with 2 mL of THF and additional 50 mL of THF was added as the mobile phase. Different fractions (0–10 mL, 10–20 mL, 20–30, 30–50 mL) were collected into glass-stoppered graduated test tubes. The third fraction (20–30 mL), which contained CA and C, was concentrated to dryness and re-dissolved in 1 mL of THF. An aliquot of 50 μL was evaporated by a gentle stream of dry nitrogen and the extract was derivatised by adding 100 μL of MSTFA, 50 μL of pyridine and by heating for 1.5 h at 70 °C. Prior to analysis, 200 μL of InjSTD was added and the solution was diluted with THF to the final volume of 1 mL. 1 μL was injected into the GC–MS.

For comparison, 1 g of homogenised sample was spiked with ISTD, 50 mL of an organic mixture was added (THF, EtOH and PYR, v/v/v, 1:1:0.1) and the samples were submitted to:

- MWE, using MW reactor (Pro Labo MICROWAVE 3.6, France), for 30 min at 30 °C or 80 °C,
- SE, refluxed for 3 h at 80 °C.

After each extraction the samples were further prepared as described above by UE.

### 2.4. Instrumentation and GC–MS conditions

The analyses were performed on two different GC–MS systems:

- HP GC 6890 coupled to an HP MS 5973 (Waldbronn, Germany). 1 μL of the sample was injected in the split mode (split ratio 10:1). Chromatographic separation was performed on a DB-5MS capillary column (J&W Scientific, Folsom, CA, USA; 30 m × 0.25 mm i.d., 0.25 μm thick) where the temperature program was as follows: initial 105 °C (0.8 min), 12 °C $min^{-1}$ to 200 °C (0.1 min), 7 °C $min^{-1}$ to 290 °C (6 min), 25 °C $min^{-1}$ to 320 °C (10 min). Helium was used as the carrier gas (flow 1.5 mL $min^{-1}$). MS was operated in the $EI^+$ mode, with electron energy at 70 eV, and MS data were collected in full scan mode (*m*/*z* 50–750 amu).
- A Polaris Q ion trap mass spectrometer coupled to a Trace GC ultra gas-chromatograph (Thermo electron corporation, USA) was used as well. Except for the oven temperature program, all other GC–MS conditions were the same as mentioned before;

**Table 1**
Storage conditions for photo or thermal stability studies of CA and C.

| Pure standards of CA (96.4%) and C (98.2%) and rosemary extract containing CA (50.27%) and C (5.65%) dissolved in EtOH or THF | | | | |
|---|---|---|---|---|
| Stability conditions | Storage conditions | | Temperature | Time of exposure |
| Short-term | Room temperature | daylight | 25 °C ± 2 °C | 5 h, 24 h, 4 days |
| | Refrigerator | darkness | 4 °C ± 2 °C | 5 h, 24 h, 4 days |
| Long-term | Room temperature | daylight | 25 °C ± 2 °C | 1 month |
| | Refrigerator | darkness | 4 °C ± 2 °C | 1 month |
| Accelerated | Thermostatically-controlled oven | darkness | 70 °C | 2 h |
| Rosemary extract containing CA (50.27%) and C (5.65%) in powdered/solid state | | | | |
| Stability conditions | Storage conditions | | Temperature | Time of exposure |
| Long-term | Room temperature | daylight | 25 °C ± 2 °C | 3 months |
| | Freezer | darkness | −16 °C ± 2 °C | 3 months |
| | Test chamber | relative humidity 75% | 40 °C ± 2 °C | 3 months |
| | Test chamber | relative humidity 75% | 40 °C ± 2 °C | 6 months |
| Accelerated | Thermostatically-controlled oven | darkness | 100 °C, 200 °C, 300 °C | 1 min |

the oven temperature program was: initial 105 °C (0.8 min), 12 °C min$^{-1}$ to 200 °C (0.1 min), 7 °C min$^{-1}$ to 290 °C (1 min), 15 °C min$^{-1}$ to 310 °C (4 min).

The identification of CA and C from the samples was established by comparing their retention times and mass spectras with the derivatised standard compounds, whilst other compounds were identified by interpretations of their fragmentation patterns, by comparing their spectral properties with those reported in the literature [21] or in the Willey and NIST mass spectra libraries.

### 2.5. Preparation of calibration solutions and calibration curves

According to the results of the stability tests, THF was used as a solvent for the preparation of standard solutions. Standard stock solutions of CA, C, cholesterol (ISTD) and cholesteryl acetate (InjSTD), were prepared by weighing 10 mg of each into four 10-mL volumetric flasks, then dissolving in THF ($\gamma$ = 1000 mg L$^{-1}$). Calibration solutions were prepared in conical glass flasks by combining different volumes (50–500 μL) of CA and C stock solutions with 100 μL of ISTD stock solution.

The solvent was evaporated to dryness and trimethylsilyl (TMS) derivatives were created by adding 100 μL of MSTFA, 50 μL of PYR and by heating for 1.5 h at 70 °C. Prior to analysis, 100 μL of InjSTD ($\gamma$ = 1000 mg L$^{-1}$) was added and the solutions were diluted to 1 mL with THF. 1 μL of these solutions were injected into the GC–MS system in triplicate. In addition, three replicate analyses of the calibration solutions were performed within 2 weeks. Curves were constructed by linear regression of the peak-area ratio of CA or C to the ISTD (y), versus the concentrations (x) in mg L$^{-1}$.

### 2.6. Stability tests

The stability tests were performed on pure CA (96% purity), as well as on commercially-available rosemary extract (containing 50.27% of CA and 5.65% of C). Because of their more affordable prices, most of the stability tests were performed on commercially-available rosemary extracts, and a small number were carried out on pure standards. The stabilities of the compounds were studied in the solid state as well as after dissolving in THF or EtOH.

In order to investigate the stabilities of CA and C in organic solvents, pure compounds as well as commercially-available rosemary extract were dissolved in EtOH and THF, separately ($\gamma$ = 1000 mg L$^{-1}$). A 200 μL aliquot from each solution was combined with 100 μL of ISTD (1000 mg L$^{-1}$), evaporated, derivatised, spiked with InjSTD, diluted to 1 mL with THF and measured by GC–MS immediately after preparation. The initial contents of CA and C were determined from the corresponding calibration curves. The results were used to control the decreasing contents of both compounds and for monitoring the progress of the degradation process during exposure to the stress conditions. Then several equal parts from each solution were transferred into graduated glass-stoppered test tubes and the prepared samples were exposed to different storage conditions for different periods of time (Table 1). After exposure to these conditions, an aliquot of 200 μL from each solution was combined with 100 μL of ISTD (1000 mg L$^{-1}$), evaporated, derivatised, spiked with InjSTD, diluted to 1 mL with THF and measured by GC–MS.

The rosemary powdered extract (500 mg in a solid state) was also exposed to different storage conditions (Table 1). After exposure, the powder was dissolved in THF (10 mg/10 mL), and a 200 μL aliquote was taken for further analysis.

## 3. Results and discussion

### 3.1. Plant sample extraction

In this study, the plant material was extracted using three different extraction techniques (UE, SE and MWE) and the results were compared. The aim was to choose the optimal extraction procedure which would ensure maximum stability of the compounds and minimal formation of degradation products. CA appears to be a thermo-sensitive compound. The concentrations of CA and C differed significantly in the final extracts depending on the extraction method used. The concentration of CA was approximately six times higher (33 mg g$^{-1}$) in the samples extracted by UE than when extracted by SE (5.7 mg g$^{-1}$) or MWE (6.8 mg g$^{-1}$) (Fig. 1). Using UE the concentration of C was minimal (1.8 mg g$^{-1}$) and the area ratios between CA/C, CA/ISTD, and C/ISTD were constant. SE and MWE resulted in an increase in the C concentration because of CA degradation. The higher temperatures used in the SE (approx. 80 °C) and long-term extraction (3 h) promoted CA degradation and its conversion to C and some other degradants, which are explained in Section 3.2. Similar results were obtained in the extractions using microwaves. Based on the results, for further investigation the UE at room temperature was adopted as the optimal one.

The mixture of organic solvents THF-EtOH-PYR proved to be an effective solvent for the extraction of CA from different aromatic plants. Cleaning and separation methods using a combination of SPE and SEC were suitable for removing interferences and for isolating the investigated compounds. After SEC, different fractions were collected and derivatised; the major compounds of the third

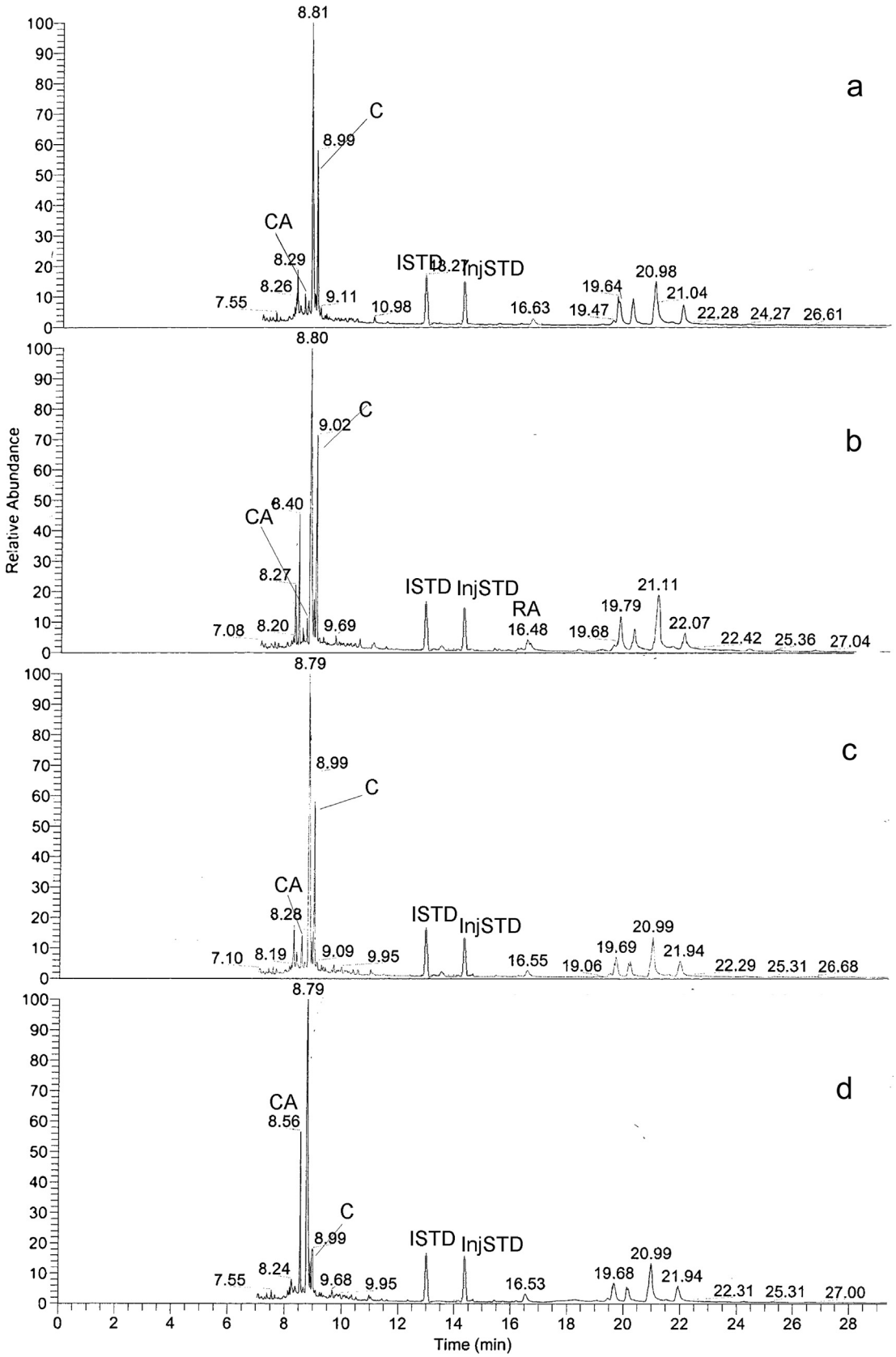



**Fig. 1.** Rosemary leaf extracts obtained after (a) SE, for 3 h at 80 °C, (b) MWE for 30 min at 80 °C, (c) MWE for 30 min at 30 °C, and (d) UE at room temperature.

fraction (20–30 mL) were CA and C. Pentacyclic triterpenoids, such as betuline, betulinic acid, oleanolic acid, and ursolic acid were also eluted in this fraction. These were identified by comparison with standard compounds [22], but they were not quantitatively evaluated (Fig. 2a). The CA and C contents from commercially-available rosemary extract were determined from the corresponding

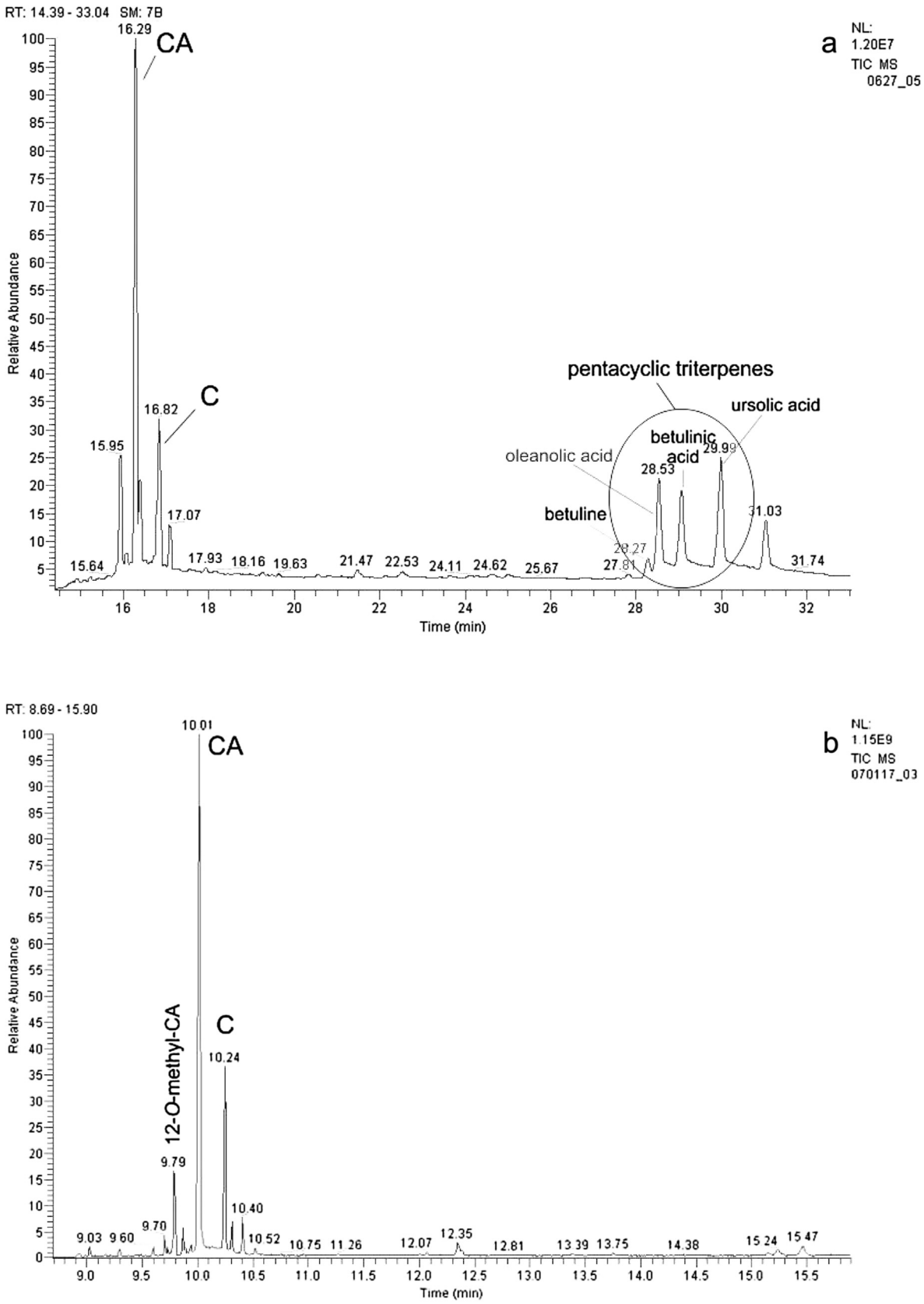



**Fig. 2.** Chromatograms of silylated compounds present in *Rosmarinus officinalis* L. leaf extract obtained after SPE and SEC by collecting different fractions, (a) fraction 20–30 mL; (b) a typical chromatogram of a commercially available rosemary extract with declared contents of CA and C (50.27% and 5.65%).

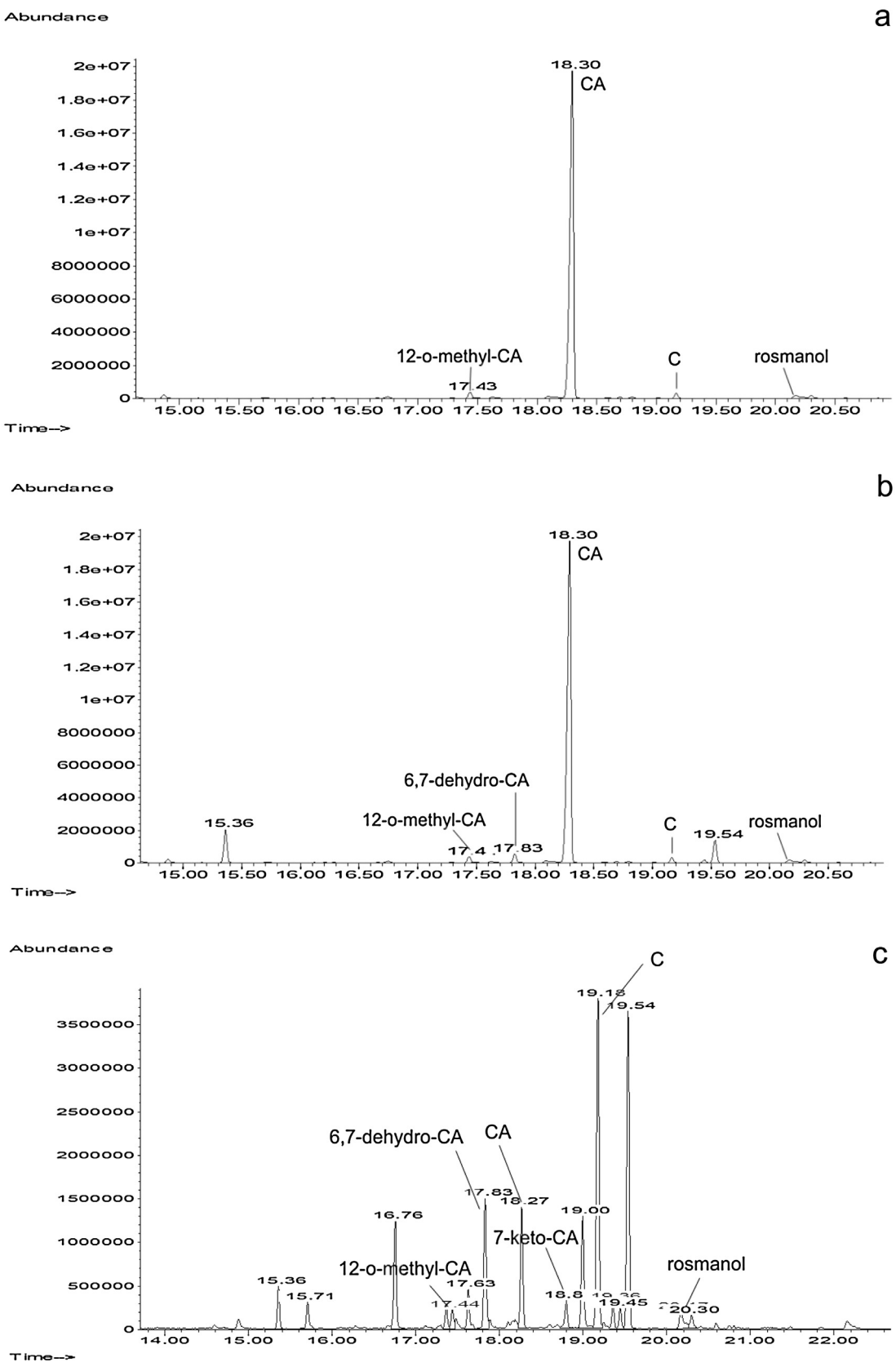



**Fig. 3.** Chromatograms of silylated compounds investigated present in a solution of CA in THF: (a) freshly prepared (b) after being stored in the refrigerator at 4 °C for 4 days (c) after being stored in the refrigerator at 4 °C for 1 month.

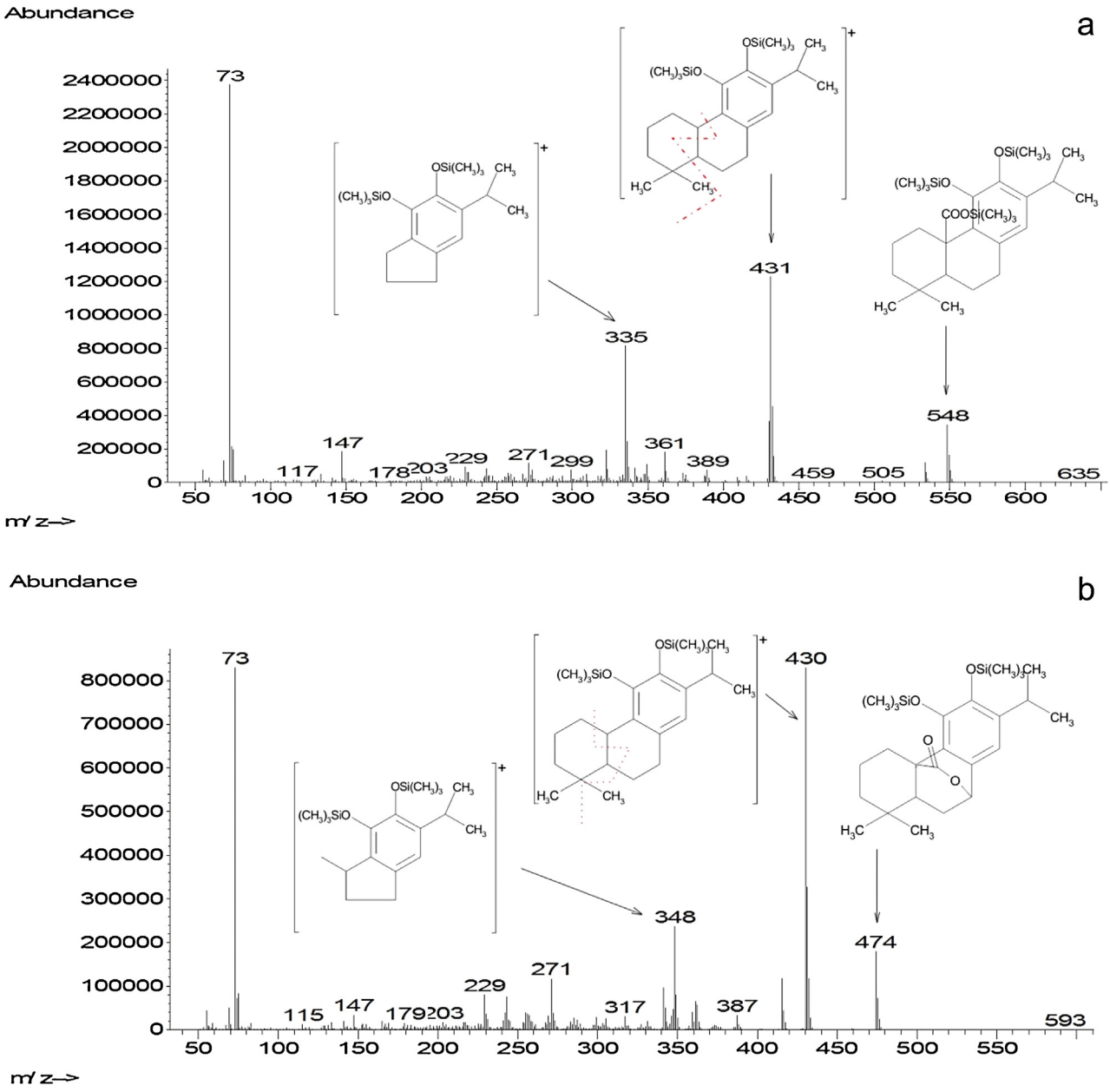



**Fig. 4.** Typical EI mass spectra of (a) CA-3TMS derivative (b) C-2TMS derivative.

calibration curves, and compared with the declared values specified by the manufacturer. After ten independent analyses it was confirmed that the extract contained 50.3 ± 1.27% of CA and 5.5 ± 0.42% of C (Fig. 2b).

### 3.2. Stabilities of CA and C in organic solvents

It is well known that diterpenic compounds are very unstable and sensitive to many different factors and that they can be rapidly decomposed or transformed into other compounds [2,3,5,8–14,18–20]. The stabilities of pure CA and C dissolved in organic solvents have been described previously, and it has been confirmed that CA is more stable in non-polar organic solvents such as DMSO and less so in polar ones such as MeOH [12,13]. Thorsen and Hildebrandt reported that pure CA dissolved in DMSO is stable for several days, whilst C showed significant degradation within a few hours in all solvents tested (MeOH, DMSO, DMSO–acetonitrile, or ethyl acetate–acetonitrile). Interestingly, both compounds present in rosmemary extract were stable up to 21 h when the extract was dissolved in DMSO [13]. Cuvelier showed that pure CA dissolved in MeOH and exposed to daylight and air-oxygen completely decomposed in 15 days and the more stable compound C was formed [10]. Our results confirmed that pure CA (96.4%) was stable for at least 5 h (at room temperature) when it was dissolved in the aprotic solvent THF. During this time the concentrations of CA and C were constant and the peak areas of CA, C, and ISTD were constant. In the freshly prepared CA (96.4%) in THF, besides CA, the minor contents of 12-*O*-methyl-CA and rosmanol were also identified (Fig. 3a). Prolonged storage time resulted in CA degradation. After being dissolved in THF and stored in the refrigerator for 4 days the CA partially degraded (by app. 10%) and at least three new compounds were formed, of which only 6,7-dehydro-CA was identified (Fig. 3b). Longer storage time (1 month in the refrigerator) resulted in further degradation of CA (by 90%) which

produced at least ten new compounds where 6,7-dehydro-CA and 7-keto-CA could be identified (Fig. 3c). Degradation was faster at room temperature and daylight than in a refrigerator. Degradation took place to a greater extent in a protic solvent (EtOH) than in an aprotic one (THF). Degradation products were the same. Identification of degradation products is very difficult as the pure standards are not commercially available, mass spectra could not be found in the literature, and only few of them are present in the spectral libraries. Therefore, in the future, additional efforts will be needed in order to identify all of the degradation products of CA.

Furthermore, the stability of pure C (98.2%) was studied. In a freshly prepared solution of C in THF, the presence of 6,7-de-hydro CA was observed. After 4 days in a refrigerator, C slightly degraded (by 8%) into two new compounds, which were not identified. After one month in a refrigerator, large-scale degradation of C occured (by 87%) and led to the formation of at least five new compounds, which could not be identified. Degradation was faster at room temperature and daylight than in a refrigerator. Degradation was faster and greater in EtOH than in THF. From the results we concluded that under the same conditions, C degraded slightly more slowly than CA and formed a significantly lower number of new compounds.

The stability of CA (50.27%) was studied in the rosemary extract dissolved in THF and EtOH exposed to different conditions (described in Section 2.6). It was found that the CA quickly (in less

**Table 2**
Structural formulas and names of identified TMS-diterpenes, and the molecular weights of their TMS derivatives with characteristic fragmentation ions and characteristic fragmentation ions of all unidentified TMS-degradation compounds.

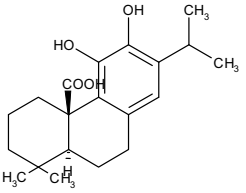
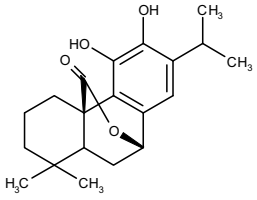
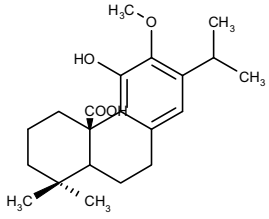
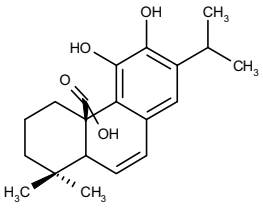
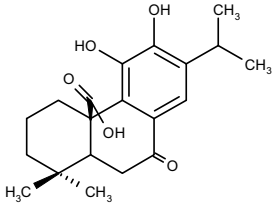
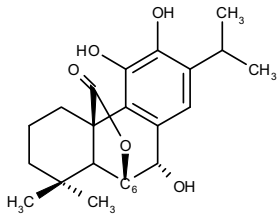
| Identified diterpenic compounds | Molecular weight of TMS derivative (g/mol) | Characteristic fragmentation ions *m*/*z* (relative intensity%) |
|---|---|---|
|  <br>Carnosic acid (CA)<br>Carnosic acid (CA) | 548 | 335 (100), 431 (90), 548 (15) |
|  <br>Carnosol (C)<br>Carnosol (C) | 474 | 348 (80), 430 (100), 474 (20) |
|  <br>12-*O*-methyl-carnosic acid<br>12-*O*-methyl-carnosic acid | 490 | 277 (60), 373 (100), 490 (30) |
|  <br>6,7-dehydro-carnosic acid<br>6,7-dehydro-carnosic acid | 546 | 387 (60), 429 (100), 546 (30) |
|  <br>7-keto-carnosic acid | 562 | 359 (99), 444 (100), 562 (30) |

**Table 2**
(*Continued*)

| Identified diterpenic compounds | Molecular weight of TMS derivative (g/mol) | Characteristic fragmentation ions *m/z* (relative intensity%) |
|---|---|---|
| 7-keto-carnosic acid  | | |
| Rosmanol (epi-, iso-) Rosmanol (*epi-*, *iso-*) | 562 | 433 (90), 518 (100), 562 (40) |

| Unidentified compounds | Characteristic fragmentation ions m/z |
|---|---|
| »unknown 1« | 341, 387, 415, 430 |
| »unknown 2« | 284, 372, 428 |
| »unknown 3« | 341, 357, 369, 474 |
| »unknown 4« | 284, 372, 428, 460, 555 |
| »unknown 5« | 301, 371, 489, 504 |
| »unknown 6« | 301, 371, 489, 504 |
| »unknown 7« | 474, 517, 634 |
| »unknown 8« | 429, 447, 555, 673 |

than 5 h) transformed into C and other degradants under all conditions tested. Again, degradation took place to a greater extent in EtOH than in THF. Degradation products were the same as those formed after the one-month aging of pure CA (96.4%) in THF. 12-*O*-Methyl-CA, 6,7-dehydro-C, 7-keto-CA, C, and rosmanol could be identified. After accelerated heating at 70 °C for 2 h, the content of CA in EtOH decreased by approximately 50%, whilst in THF degradation was even more rigorous, as CA was completely degraded and quantitatively converted to C.

From the results we can conclude that temperature is the major factor affecting the degradation of CA and C in solutions, and light exposure further accelerates the degradation of both compounds. In general, more protic solvents lead to faster degradation.

### 3.3. Stabilities of CA and C in the solid state

In the next step, the stability of rosemary extract (containing 50.27% of CA) in the solid state was tested. It was confirmed that powder extract was stable for up to 3 months under all conditions tested and during this time its composition did not change. After 6 months of exposure to +40 °C and relative humidity of 75%, significant changes occurred: the content of CA decreased by 28%, whilst the C content increased by 97%. A change in the colour and odour of the extract was also observed. Powder was also heated for 1 min at 100, 200 and 300 °C separately, and analysed. The purpose of this study was to examine the possibility of controlled thermal transformation of CA to C and other compound(s). No changes appeared after heating to 100 °C; as usual, besides CA, minor traces of 12-*O*-methyl-CA were also present in the extract. When heated to 200 °C, degradation of CA appeared and at least three new unidentified degradation products were formed. At 300 °C, CA was completely degraded and at least four new unidentified degradation products were formed.

### 3.4. Confirmation of CA and C

$EI^+$ mass spectra of a CA-3TMS derivative is shown in Fig. 4a. In all chromatograms the diterpenic compounds investigated were well distinguished by order of elution during gas chromatography (CA is eluted before C) and by intensities of the fragment ion signals in their mass spectra (Table 2). The molecular ion of CA-3TMS derivative is easily visible at *m/z* 548 (Fig. 4a). The most important and intense fragment ion can be observed at *m/z* 431 (elimination of TMSCOOH group) and is characteristic of diterpene compounds of ferruginol type [21]. The characteristic ion at *m/z* 335 is formed by the cleavage of 3 bonds (between C4-C5, C5-C10, and C9-C10 atoms) within the CA molecule.

$EI^+$ mass spectra of a C-2TMS derivative is shown in Fig. 4b. From the mass spectra in Fig. 4b the molecular ion of C-2TMS derivative at *m/z* 474 can be clearly seen. The main and most intense ion occurs at *m/z* 430, as the result of COO-group elimination and is specific for diterpenic lactones [21]. The secondary fragment at *m/z* 348 is formed from the ion at *m/z* 430 as a result of the cleavage of three C—C bonds between C1 and C10, C10 and C5 and C5 and C6 atoms, and a transfer of the $CH_3$ group within the molecule.

Structural formulas and the names of all identified TMS-diterpenes, including the molecular weights of their TMS derivatives, with characteristic fragmentation ions, are presented in Table 2. MS data of all unidentified compounds are also reported in Table 2.

### 3.5. Plant extracts analysis

CA and C were successfully quantified by GC–MS in five different *Lamiaceae* species. Fig. 5 presents the GC chromatogram of the silylated extract of *Salvia officinalis* L. From CA and C, different biologically-active components in plants, such as rosmarinic acid, caffeic acid, betuline, betulinic acid, oleanolic acid, ursolic acid, and different monosaccharides were separated and identified, but they were not quantified. The average contents of CA and C were determined in mg $g^{-1}$ per dry weight (Table 3). The content of CA ranged from traces (<0.1 mg $g^{-1}$) up to 33 mg $g^{-1}$. The lowest concentration of CA was determined in *Salvia glutinosa* and the highest in *Rosmarinus officinalis*, whilst in other plants it was undetected. C was detected only in rosemary and sage in concentrations from 0.80 to 1.78 mg $g^{-1}$.

## 4. Conclusions

The GC–MS method described is sensitive and suitable for simultaneous quantification of structurally-related phenolic diterpenes such as carnosic acid (CA) and carnosol (C) and their degradation products in different plant samples. Before GC–MS analysis, the method requires derivatisation of the compounds. In this way the

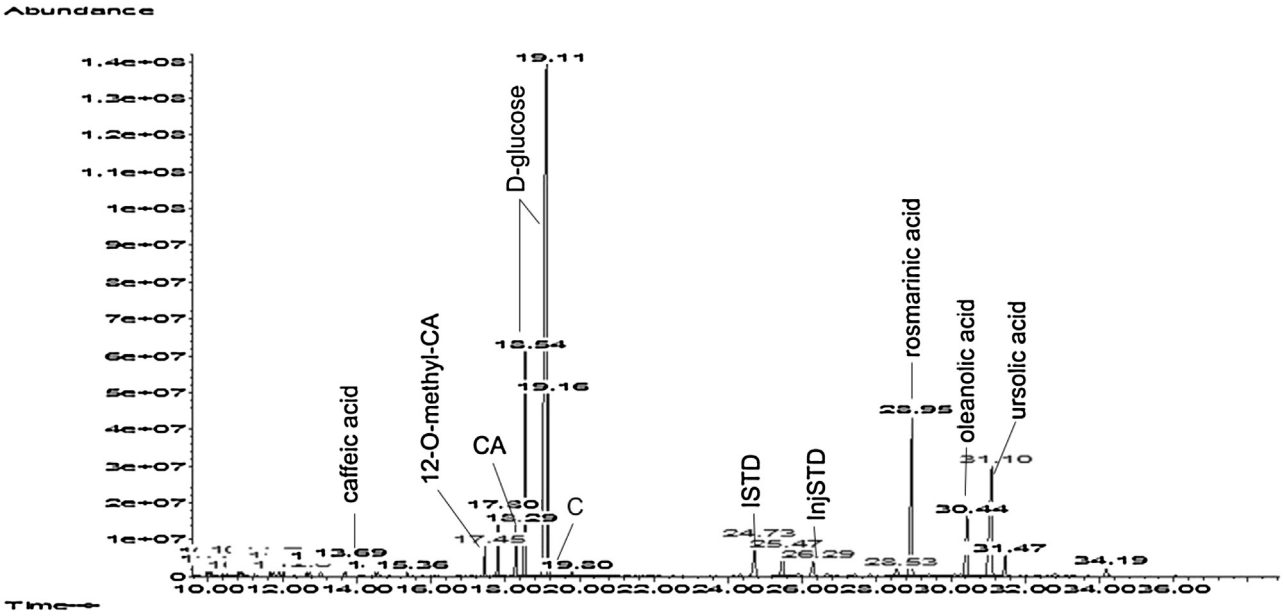



**Fig. 5.** Chromatogram of silylated compounds present in *Salvia officinalis* L. extract.

**Table 3**
Contents of carnosic acid (CA) and carnosol (C) in different plant extracts (mg $g^{-1}$ per dry weight); each content value is the mean of two replicated analyses.

| | Plant species | | | | |
|---|---|---|---|---|---|
| Compound | *Rosmarinus officinalis* | *Salvia officinalis* | *Salvia sclarea* | *Salvia glutinosa* | *Satureja montana* |
| *Carnosic acid (CA)* | 33.02 | 3.51 | * | <0.1 | * |
| *Carnosol (C)* | 1.78 | 0.80 | * | * | * |

* Not identified.

compounds are converted to more stable and more volatile TMS derivatives. Although UV–VIS provides useful information for the identification of antioxidant phytochemicals such as diterpenes, the use of conventional approaches based on spectra is often limited when samples contain similar compounds whose absorption spectra are almost identical. Unambiguous identification can be done using other detection techniques like mass spectrometry (MS) which provides additional structural information for easier identification and quantification of the compounds.

Extraction of diterpenic compounds from plant samples using ultrasound at room temperature is more suitable than Soxhlet extraction or extraction using microwaves. With ultrasonic extraction, conversion of CA into C is minimal and constant. The stability of CA and C were studied in the extracts as well as in the pure standards. From the results it is clear that the degradation process of CA is solvent, temperature, light and time-dependent. At all tested conditions, the concentration of CA was lowered as a result of the formation of C, rosmanol, 12-*O*-methyl-carnosic acid, 6,7-dehydro-CA, 7-keto-CA and other unidentified degradation products.

Finally, it has to be emphasised that the maximum stability of CA can be achieved only by careful laboratory work which includes the selection of a suitable extraction method, solvent, using an inert atmosphere, and avoiding humidity and higher temperatures.

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