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Stability studies on *trans*-rosmarinic acid and GC–MS analysis of its degradation product

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ABSTRACT

The stability of *trans*-rosmarinic acid (*trans*-RA, an important phenolic compound with anti-oxidant, anti-inflammatory, anti-bacterial, and anti-viral properties) exposed to different stress conditions (daylight, higher temperatures, different solvents, and humidity) was investigated. Gas chromatography–mass spectrometry (GC–MS) was used to analyse the degraded samples, and structural identification of degradation products was assigned based upon MS fragmentation pattern. The GC–MS method was validated in terms of linearity, precision as repeatability, accuracy, limit of detection (LOD), limit of quantitation (LOQ) and recovery. The stability experiments were performed on pure *trans*-RA and on *trans*-RA present in commercially available rosemary extract. The *cis*-isomer of RA was the only degradation product. The results showed that *trans*-RA was readily isomerized into its *cis*-form within a few hours when dissolved in ethanol, methanol or tetrahydrofuran, and exposed to darkness or daylight at different temperatures. Isomerization took place to a greater extent in protic than in aprotic solvents. *Trans*-RA in the solid state was found to be stable for up to three months under all tested conditions. The described GC–MS method was also applied to the determination of *trans*-RA in eight different species of *Lamiaceae* family.

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1. Introduction

Trans-rosmarinic acid ((*R*)-2-(3,4-dihydroxytrans-cinnamoyloxy)-3-(3,4-dihydroxyphenyl) propionic acid) (*trans*-RA) is an ester of caffeic and 3,4-dihydroxyphenyllactic acid, the structure of which was elucidated by Scarpati and Oriente in 1958 as described elsewhere [1]. It belongs to a group of phenolic acids and is widely distributed throughout the nature. It was originally isolated from *Rosmarinus officinalis*, but can also be found in other species of the *Lamiaceae*, *Boraginaceae*, *Rubiaceae*, *Apiaceae* and *Araliaceae* families [1–4]. A multitude of biological activities have been described for RA, the main ones being anti-inflammatory, anti-oxidant, anti-bacterial, anti-viral, and anti-depression [1,3,5–8]. It has also therapeutic potential for the treatment or prevention of bronchial asthma, spasmogenic disorders, peptic ulcer, hepatotoxicity, atherosclerosis, ischaemic heart disease, cataract, and poor sperm motility [5]. More recently, RA has been reported to have anti-HIV [9], anti-allergenic [6,10], and anti-carcinogenic effects [11,12]. Over the last 10 years, several studies have focused on its quantification and determination in different plant materials and other matrices. Reversed-phase high-performance liquid chromatography (RP-HPLC) with gradient elution and ultraviolet-visible (UV) detection is especially widely used [3,13–22]. Due to the difficulties caused by the similar or almost identical UV spectra of similar compounds such as isomers [23–25] mass spectrometric detection providing also structural information is preferred for overcoming the drawbacks of UV detection [23,26,27].

Phenolic acids are unstable and sensitive to many different factors such as high temperatures, exposure to UV radiation or air oxygen, and metal ions [2,13]. Stability studies of *trans*-RA have been restricted so far to analyses performed under the daylight [4,13]. The aim of this work was the stability study of pure *trans*-RA and *trans*-RA in commercially available rosemary extract under stress conditions, i.e. the simultaneous determination of *trans*-RA and its main degradation product, *cis*-RA based on our previously published GC–MS method for the determination of *trans*-RA and other phenolic compounds in plant extracts [23]. The majority of phenolic acids occur in nature as *trans*-isomers, but when exposed to the mentioned factors they are transformed into *cis*-isomers [2,13,28].

GC–MS technique is mostly employed for the analysis of simple, low-molecular weight phenolic acids such as hydroxybenzoic and hydroxycinnamic acids [24,26,27,29,30]. This technique is not frequently used in the analysis of high molecular weight phenolics, such as RA, due to their thermolability and limited volatility [31]. In comparison with HPLC a minor disadvantage is the necessity of a derivatization step required to ensure the volatility of the compounds [24,27,32,33]. However, it offers also some advantages: good removal of matrix interferences, complete, high-resolution

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separation, sensitive detection, unambiguous identification and quantitation of a wide range of phenolics and terpenes (including *cis*- and *trans*-RA) in one chromatographic run.

2. Experimental

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 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), *trans*-rosmarinic acid (97%), caffeic acid (99%), cholesterol (99%) and cholesteryl acetate (95%) were supplied by Sigma–Aldrich (Germany).

2.2. Plant material

Air-dried leaves of *Borago officinalis* L. (borage), *Myrtus communis* (common myrtle), *Sideritis syriaca* (sideritis or greek's mountain tea), *R. 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 analyses. Samples were obtained from plants grown in their natural habitat in different regions of Slovenia (Maribor, Kočevje) and Croatia (Mljet, Lastovo, Umag) from April to October 2007. Dried plant material was ground, homogenized, and stored in darkness at room temperature. Commercially available powder of rosemary extract, containing 17% of *trans*-RA and some other active substances, was also used for analysis. Rosemary extract was supplied by Vitiva d.o.o., Slovenian nutraceuticals producer.

2.3. Preparation of plant samples

The samples were prepared according to the previously described method, with some modification [23]. 1 g of homogenized sample was weighed into a centrifuge tube and spiked with ISTD. The sample was extracted three times by sonication with 20 ml of mixture of THF, EtOH and pyridine (v/v/v, 1:1:0.1). After each extraction the sample was centrifuged and the supernatants combined, dried over Na_2SO_4, and evaporated to dryness using rotary evaporator. The residue was re-dissolved in 3 ml of the same solvent mixture and firstly cleaned by solid phase extraction (SPE) using Superclean ENVI-Carb 6 ml tubes filled with graphitized, nonporous carbon (surface area 100 $m^2\,g^{-1}$, 120–400 mesh, Supelco, Bellefonte, USA), and then further by size exclusion chromatography (SEC) using Bio-Beads S-X3 gel (200–400 mesh, Bio-Rad Laboratories, Richmond). After SEC, an aliquot of 50 μl from *trans*-RA fraction was derivatized and analysed. The schematic SPE and SEC procedures are shown in Table 1.

Table 1
Sample cleanup by SPE and SEC.

I. Superclean ENVI-Carb SPE 6 ml
1. Condition the SPE with 2 × 5 ml of EtOH
2. Add 200 μl of pre-treated sample dissolved in THF
3. Wash the sample with a small volume of EtOH
4. Elute the analytes with additional 30 ml of EtOH
5. Collect and concentrate the eluent to dryness and re-dissolve to 1 ml with THF
II. Bio-Beads S-X3 SEC
1. 24 h before use, swell the Bio-Beads S-X3 beads in THF
2. Fill 50 ml of the slurry into the glass column (30 cm × 15 mm i.d.) and wash with THF
3. Add the whole extract purified by SPE and wash with a small volume of THF
4. Elute the analytes with additional 50 ml of THF at flow rate 5 ml min^{-1}
5. Collect different fractions (0–10 ml, 10–20 ml, 20–30 ml, 30–50 ml)
6. Concentrate the *trans*-RA fraction (10–20 ml) to dryness and re-dissolve in 1 ml of THF

2.4. Instrumentation and GC–MS conditions

Analyses were performed on HP GC 6890 coupled to HP MS 5973 (Waldbronn, Germany). 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). Helium was used as the carrier gas with a constant linear velocity of 40 cm s^{-1}. The temperature program was as follows: initial 105 °C (0.8 min), 12 °C min^{-1}–200 °C (0.1 min), 7 °C min^{-1}–290 °C (6 min), 25 °C min^{-1}–320 °C (10 min). Injector temperature was set at 290 °C. Samples were injected in splitless mode. The injection volume was 1 μl. The transfer line temperature was held at 290 °C. MS was operated in the positive ionization mode (EI), with electron energy at 70 eV. Source temperature was 235 °C. The MS data were obtained in full scan mode (mass range 50–750 amu).

The identification of *trans*-RA was established by comparing its retention time and mass spectra with the derivatized standard compound, while the *cis*-RA was identified by comparing its spectral properties with those reported in the literature [4], and in the Wiley and Nist mass spectra libraries. At the time of the research the *cis* isomer was not commercially available.

2.5. Validation parameters

The method was validated for linearity, precision as repeatability, accuracy, limit of detection (LOD), limit of quantitation (LOQ) and recovery as per ICH guidelines [34,35].

2.5.1. Preparation of calibration solutions and calibration curves

THF was used as the solvent for the preparation of standard solutions. Standard stock solutions of *trans*-RA, cholesterol and cholesteryl acetate were prepared by accurately weighing 10 mg of each of them into 100 ml volumetric flasks, and then dissolving in THF. Cholesterol was used as an internal standard (ISTD) for recovery evaluation, and cholesteryl acetate as an injection standard (InjSTD) for volume correction. Five calibration solutions were prepared by combining various volumes of *trans*-RA stock solution (10, 50, 100, 200 and 500 μl) with 100 μl of ISTD (100 mg l^{-1}). The solvent was evaporated by a gentle stream of dry nitrogen, and trimethylsilyl (TMS) derivatives were prepared by adding 100 μl of MSTFA, 50 μl of pyridine and heating for 2 h at 70 °C. Prior to analysis, 100 μl of InjSTD (100 mg l^{-1}) was added and the solutions were diluted with THF to the same final volume of 1 ml. 2 μl of calibration solutions, containing 1–50 mg l^{-1} of *trans*-RA were injected into the GC–MS system in triplicates. In addition, three analyses of the calibration solutions were performed within a day. Curves were constructed by linear regression of the peak-area ratio of *trans*-RA to the ISTD (y), vs. the concentration (x).

2.5.2. Precision

The injection repeatability (system precision) was established by seven successive injections of two calibration solutions (1 ng μl^{-1} and 50 ng μl^{-1}) and the relative standard deviation (RSD) of the peak area ratio of *trans*-RA and ISTD was calculated. The repeatability of the analyses was evaluated by %RSD of three replicate analyses of five calibration solutions within a day. The sample preparation repeatability (method precision) was established by carrying out the analysis of the analytes for three times.

2.5.3. Accuracy

The accuracy of the assay method was evaluated at three concentration levels (5, 10 and 50 μg/ml), and the recoveries were calculated for each individual level.

2.5.4. Limit of detection and limit of quantitation

The limit of detection (LOD) was calculated based on a signal-to-noise ratio (S/N: 3.3). The limit of quantitation (LOQ) was then calculated based on the signal-to-noise ratio (S/N: 10).

2.5.5. Recovery

Recovery experiments were performed to evaluate the extraction, clean-up and derivatization procedures of *trans*-RA from plants extracts and to assess the suitability of the developed GC–MS for the analysis of *trans*-RA. This was achieved by spiking known concentrations of ISTD to pre-analysed samples. InjSTD was used for volume correction. The recovery percentages were determined by comparing the concentrations found from spiked samples with the concentration added. In addition, recoveries were confirmed by the re-extractions of the solid residues.

2.6. Stability tests

2.6.1. Stability of pure trans-RA in organic solvents and in the solid state

Trans-RA was dissolved in MeOH, EtOH and THF, to obtain concentrations of 100 mg l^{-1}. An aliquot of 200 μl from each solution was taken, evaporated, derivatized, and measured by GC–MS immediately after preparation. The content of *trans*-RA was determined from the corresponding calibration curve.

Each solution was divided into several equal parts and transferred into graduated glass-stoppered test tubes. For photostability studies, four parts of each solution were exposed to daylight at room temperature for 5 h, 24 h, 4 days and 1 month; another four parts of each solution were stored in the refrigerator in darkness at −18 °C for 5 h, 24 h, 4 days and 1 month; and for thermal stability studies one part of each solution was placed in a hot air oven maintained at 70 °C, for 2 h. After exposure to the mentioned conditions, an aliquot of 200 μl from each solution was derivatized and analysed. The content of *trans*-RA was determined.

In order to test the stability of pure *trans*-RA in the solid state, the standard compound powder was stored in darkness at −18 °C or at daylight and room temperature for 1 week, 1, 3 and 6 months. The powder was also exposed to the temperature +40 °C and relative humidity of 75% for 3 and 6 months. Thereafter the powder was dissolved in THF in order to obtain a concentration 100 mg l^{-1}. An aliquot of 200 μl was taken for derivatization and analysis. The content of *trans*-RA was determined.

2.6.2. Determination of trans-RA from rosemary extract and its stability in the solid state

The stability of *trans*-RA present in commercially available rosemary extract was also examined. First, the amount of *trans*-RA in the extract was determined. 50 mg of rosemary extract was weighed into a 100-ml volumetric flask and dissolved in THF/pyridine (v/v, 1:0.1), 200 μl of this solution was evaporated, derivatized, and analysed. The amount of *trans*-RA was determined from the corresponding calibration curve.

The stability of *trans*-RA from rosemary extract was examined in the dry state. The dry powder was stored in darkness at −18 °C and at daylight at room temperature for 1 week, 1, 3 and 6 months. Powder extract was also exposed to the temperature +40 °C and relative humidity of 75% for 3 and 6 months. After exposure to the mentioned conditions, 50 mg was dissolved in 100 ml of THF/pyridine (v/v, 1:0.1). Aliquots of 200 μl were evaporated, derivatized, and analysed. The content of *trans*-RA was determined.

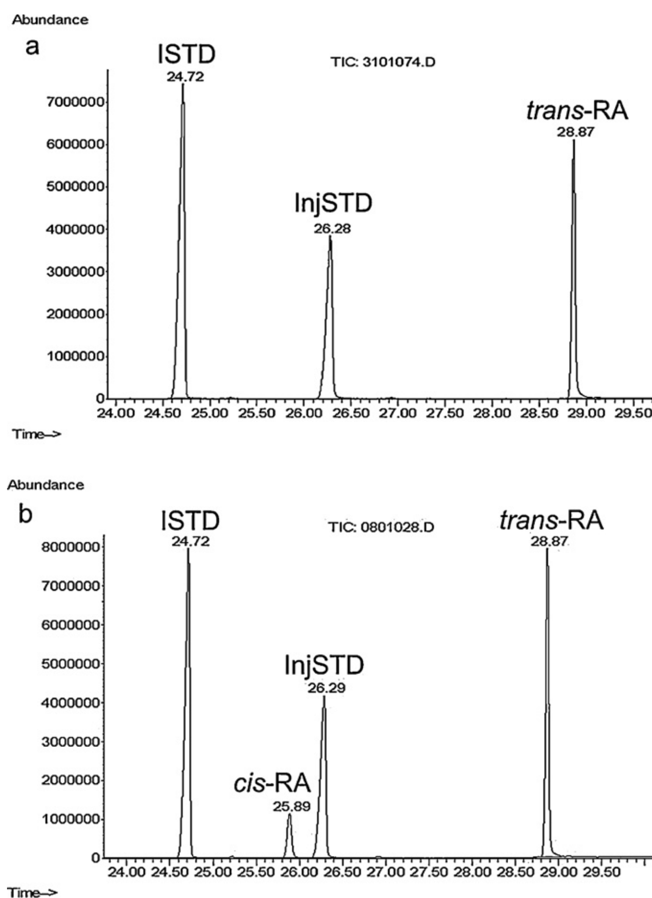

Fig. 1. TIC chromatograms of silylated compounds in freshly prepared solution of *trans*-RA in THF (a) and solution of *trans*-RA in THF after being stored in the refrigerator at −18 °C for 4 days (b).

3. Results and discussion

3.1. Method validation

Linear calibration range was found to be from 1 to 50 ng μl^{-1} for *trans*-RA. R^2 values were in the range from 0.984 to 0.999. The repeatability of chromatographic analyses was evaluated by the relative standard deviation (RSD) of three replicate analyses of five calibration solutions. RSD was between 4.1% and 8.4%. The injection repeatability was evaluated by seven successive injections at two concentration levels (LOQ and 50 ng μl^{-1}) and the peak-area ratio of *trans*-RA and ISTD was evaluated. RSD was between 3.1% and 3.6%. The values of the relative standard deviations for sample repeatability also lie well within the limits (RSD < 10%) indicating the sample repeatability of the method. The recovery of *trans*-RA ranged from 96.6% to 100.2% for all concentration levels (5, 10 and 50 ng μl^{-1}). The LOD and LOQ for *trans*-RA were 0.3 ng μl^{-1} and 1.0 ng μl^{-1}, respectively.

3.2. Stability of trans-RA

After being stored for 5 h in the refrigerator in darkness at −18 °C, the *trans*-RA dissolved in THF was found to be stable, and no isomer conversion occurred (Fig. 1a). However, as the storage time increased, a new peak appeared in the chromatogram (Fig. 1b) and its relative peak area gradually increased over time. The peak was

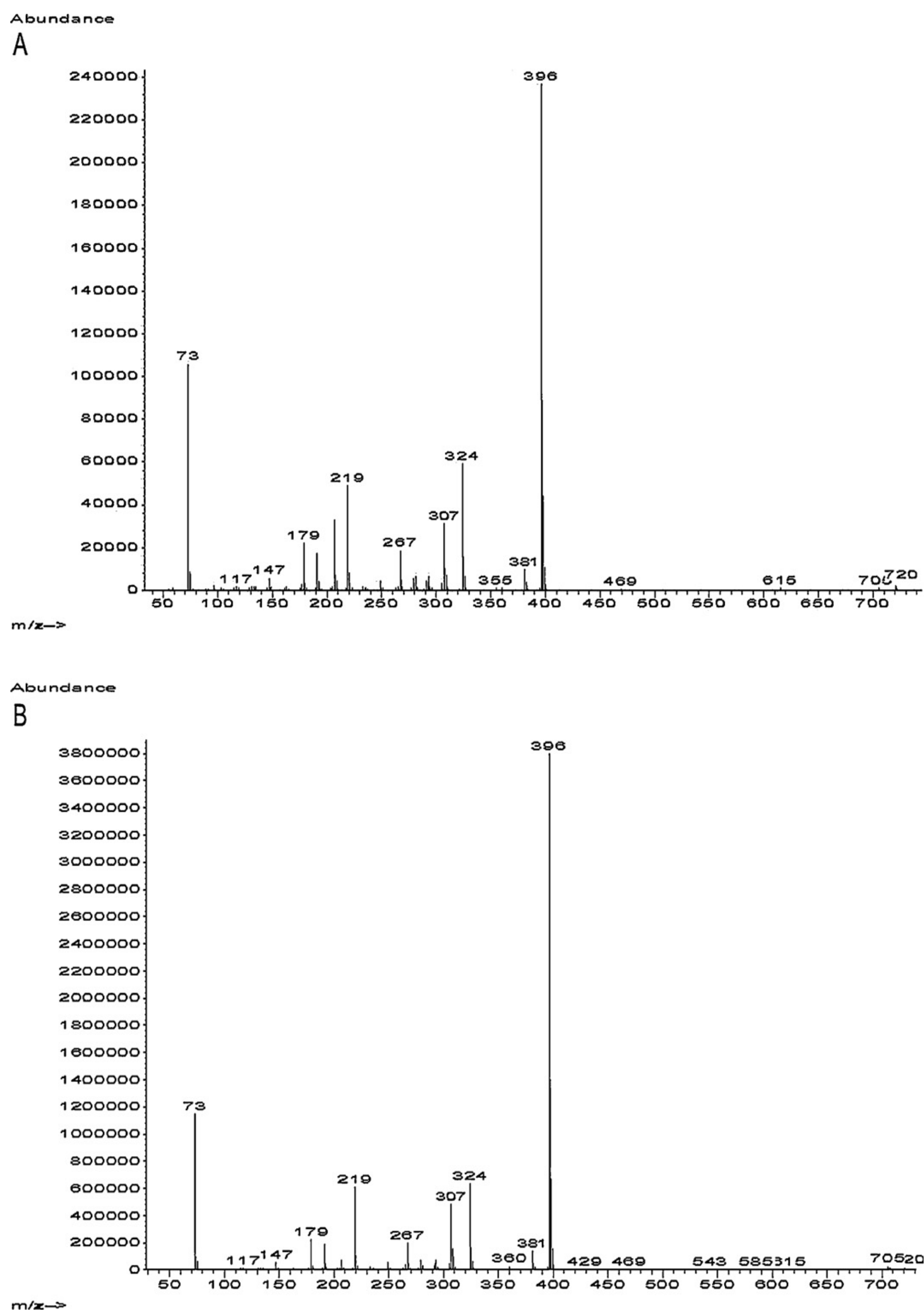

Fig. 2. Typical EI mass spectra of *cis*-TMS-rosmarinic acid (a) and *trans*-TMS-rosmarinic acid (b).

identified as *cis*-RA (see Section 3.3). As stated by other authors [4,13,28] and also according to our results, it can be assumed that *cis*-RA is not a naturally occurring compound, as it was not present in the analysed plants but was produced as a result of isomerization from *trans*-RA. The results showed that the reduction in *trans*-RA content dissolved in various organic solvents and exposed to different stress conditions was in accordance with the increase of the amount of *cis*-RA. After being dissolved in THF and stored in the refrigerator in darkness at −18 °C, the *trans*-RA content decreased after 24 h by 9.5%, after 4 days by 15.1%, and after 1 month by 18.3%. Photo degradation studies were carried at room temperature by exposing the solutions to daylight for varied lengths of time. The results showed that the content of *trans*-RA dissolved in THF lowered after 5 h by 2.5%, after 24 h by 14.2%, after 4 days by 15%, and after 1 month by 22.3%.

The isomerization into *cis*-isomer took place to a greater extent in protic solvents such as MeOH, EtOH, than in THF, an aprotic solvent. After being stored for 24 h in the refrigerator in darkness at −18 °C, the content of the *trans*-RA dissolved in EtOH decreased by 17.9%. After being stored for 24 h in daylight at room temperature, the content of the *trans*-RA dissolved in EtOH decreased by approximately 25%.

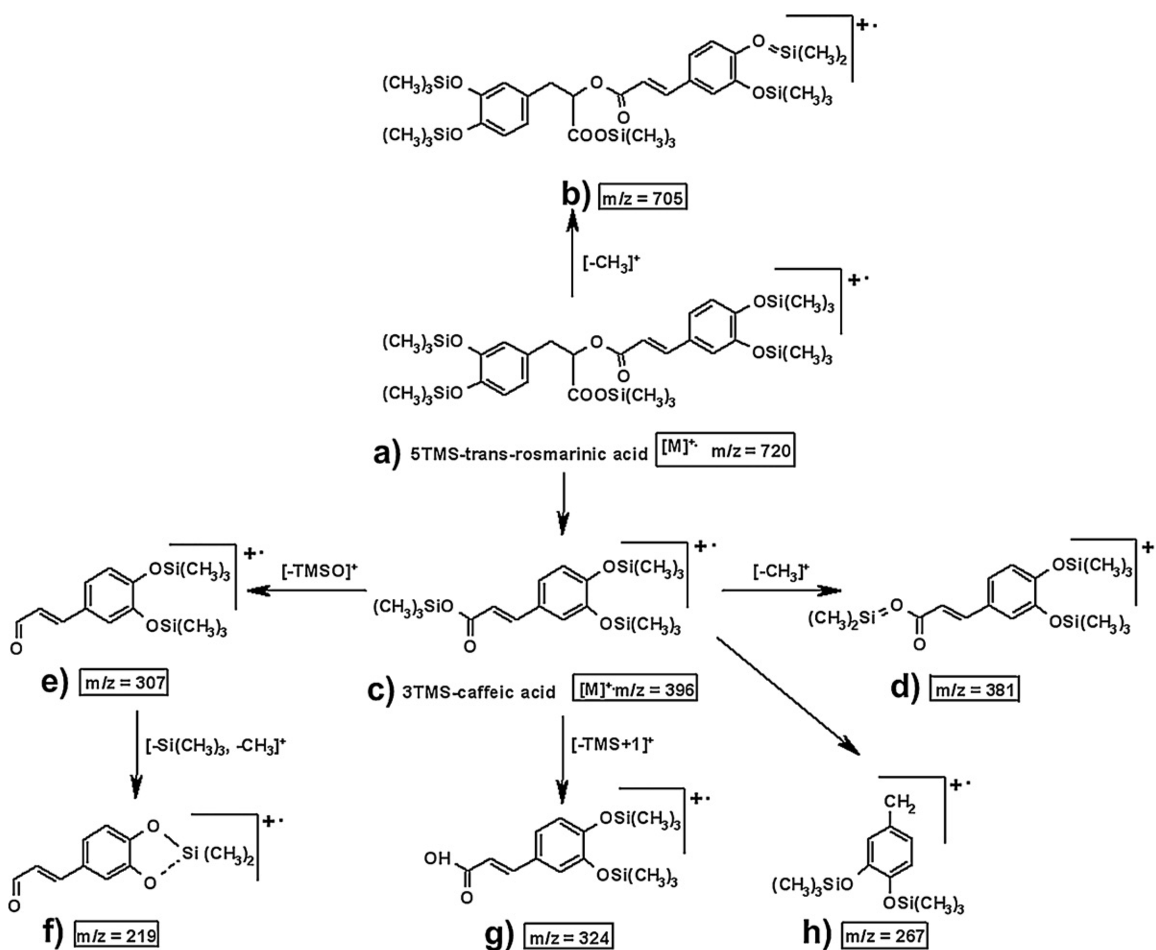

Fig. 3. Fragmentation pathway of TMS derivative of *trans*-RA obtained under EI conditions.

Finally, when *trans*-RA was dissolved in solvents, accelerated heating did not markedly promote isomerization. After being heated at 70 °C for 2 h, the content of *trans*-RA in EtOH or MeOH decreased by approximately 6%, while in THF isomerization it was insignificant (less than 3%).

In the solid state, whether exposed to darkness or daylight, humidity and different temperatures, pure *trans*-RA was stable for up to six months. The *trans*-RA present in commercially available rosemary extract was stable for up to three months in the dry state, under the same conditions. After six months of exposure to 40 °C and relative humidity of 75%, the content of *trans*-RA in the extract decreased by 65%, when considering the initial value. The colour and the odour of the extract were also changed.

3.3. Confirmation of degradation product cis-RA

Since the MS fragmentation patterns of *trans*-RA and of the degradant were almost identical, we suspected that these compounds were isomers. Unambiguous identification of *cis*-RA was established by comparing its spectral properties with those in the literature [4] and in the standard mass spectra libraries (Wiley, NIST). In the literature mass spectra of *cis*-TMS-RA was presented. It contained characteristic molecular peak at *m*/*z* 720, and fragmentation products at *m*/*z* 705, 396, 324, 307, 267, 219, 179, 149, 73. Exactly the same fragment profile was obtained in our results as shown in the fragmentation pathway in Fig. 3.

The geometric isomers *cis*-RA and *trans*-RA can be well distinguished by the order of elution during gas chromatography (*trans*-RA is retained longer than *cis*-RA) (Fig. 1b), and by the relative intensities of some fragment ion signals in their very similar mass spectra (Fig. 2a and b). Differences are the intensities of the fragment ion signals at *m*/*z* 307 and *m*/*z* 324, where both are more intense for *cis*-RA than for *trans*-RA (Fig. 2a and b). For both compounds molecular ion can be observed at *m*/*z* 720 (Figs. 2a, b and 3a), but its intensity is very low (about 0.8% for *cis*-RA and <0.8% for *trans*-RA). The loss of a methyl group from the molecular ion resulted in fragment ion *m*/*z* 705 (Fig. 3b), which is more intense for *trans*-RA than for *cis*-RA (Fig. 2a and b). The most important and intense fragment ion shows up as *m*/*z* 396 for both compounds (Figs. 2a, b and 3c). It is formed upon the cleavage of an ester bond within the RA molecule. This ion indicates clearly the presence of caffeic acid in the RA molecule, since the same ion was observed in the EI mass spectra of the caffeic acid-TMS derivative. All other fragments are also characteristic for TMS caffeic acid, as published elsewhere [26,27,29,30]. The loss of a methyl unit produces the *m*/*z* 381 (Figs. 2a, b and 3d) and loss of the TMSO group provides the *m*/*z* 307 ion (Figs. 2a, b and 3e). The relatively intense fragment at *m*/*z* 219 (Figs. 2a, b and 3f) also appears to be from the caffeic acid portion of the RA molecule, but its structure is difficult to propose [24,30]. However, the difference in mass 88 u, between *m*/*z* 307 and *m*/*z* 219 ions suggests further elimination of the TMS group and loss of a single methyl group from the other TMSO group on the caffeic acid moiety (Figs. 2a, b, 3e and f) [26]. Other fragments at *m*/*z* 324, 267 and 179, resulting from different rear-

Table 2
Contents of *trans*-rosmarinic acid in different plant extracts (mg g^{-1} per dry weight); each content value is the mean of three replicate analyses for two different samples of the same plant.

Compound	Plant species							
	Borago officinalis	*Myrtus communis*	*Sideritis syriaca*	*Rosmarinus officinalis*	*Salvia officinalis*	*Salvia sclarea*	*Salvia glutinosa*	*Satureja montana*
Trans-rosmarinic acid	0.85	<0.1	<0.1	4.56	10.14	10.66	8.80	1.53

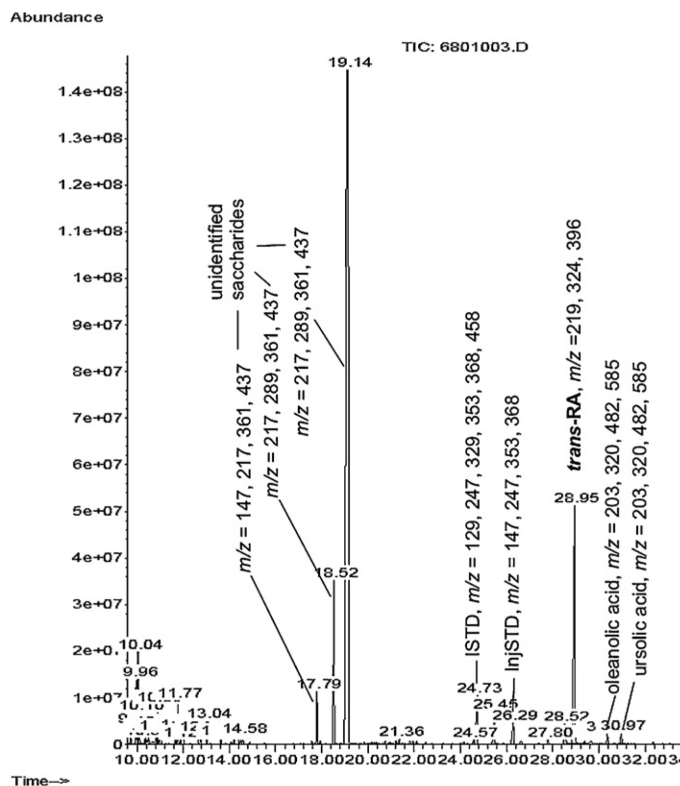

Fig. 4. TIC chromatogram of silylated compounds, present in clary sage (*Salvia sclarea* L.) extract; for identification and quantification, fragment ions with greater intensities were selected.

rangements are also observed in the EI spectra of both compounds (Figs. 2a, b, 3g and h).

3.4. Plant sample analysis

The mixture of organic solvents THF–EtOH–pyridine has proved to be an effective solvent for the extraction of *trans*-RA and other phenolic compounds, usually present in plant extracts. The best recoveries of RA (above 94%) were obtained after triple extraction, while the double extraction gave lower recoveries (about 77%). Recovery was evaluated using ISTD and was confirmed by the re-extraction of the solid residue.

Cleaning methods using a combination of SPE and SEC were suitable for removing interferences and for isolating the phenolic acid fraction. After trimethylsilylation, *trans*-RA was successfully separated, identified, and quantified by GC–MS in eight different plant species. Fig. 4 presents the TIC chromatogram of TMS derivatives of the investigated compounds, present in clary sage (*S. sclarea* L.) extract. The average contents of *trans*-RA were determined and expressed in mg g^{-1} per dry weight (Table 2). The contents of *trans*-RA ranged from traces (<0.01 mg g^{-1}) up to 10.7 mg g^{-1}. The lowest concentrations of *trans*-RA were determined in *M. communis* and *S. syriaca*. It was present in higher amounts in *S. sclarea* and *S. officinalis*.

3.5. Rosemary-extract analysis

It was established that the commercially available rosemary extract contains approximately 18.2 ± 0.72% of *trans*-RA and that the results are comparable with the declared content. In addition to *trans*-RA, certain other compounds such as phenolic acids (caffeic, cinnamic, syringic, p-coumaric, ferulic and sinapinic acid) and sugars, were also detected in the extract, but the results were not quantitatively evaluated.

4. Conclusions

In nature, some phenolic acids are present as *trans*-isomers, but the gradual formation of *cis* isomers can occur upon exposure to different stress conditions. An example is rosmarinic acid (RA). Although its instability has already been mentioned, this is the first report where the stability of *trans*-RA has been systematically tested. It degraded (isomerized) rapidly in different organic solutions when stored under different lighting conditions and different temperatures for varied lengths of time. *Cis*-RA was confirmed as the only conversion product in this isomerization process. Solid *trans*-RA remained stable for up to six months under all tested conditions. It was found that the isomerization of *trans*-RA to *cis*-RA was solvent, temperature, and time-dependent. In general, more protic solvents, higher temperatures, and longer times led to greater isomerization. Therefore, *trans*-RA should be handled with caution when considering lighting, usage of protic organic solvents, and higher temperatures. Longer analysis time should also be avoided, in order to minimize this isomerization.

Both geometric isomers were successfully separated and determined by GC–MS. The developed method was found to be linear, accurate and precise for the quantitation of *trans*-RA. Although HPLC-UV seems to be the preferred choice, our results indicated that GC–MS can be a challenge for the analysis of RA and related compounds. The proposed GC–MS method is more time consuming than HPLC, but it has advantages: it offers complete separation, unambiguous identification, and quantitative determination of many different compounds in one chromatographic run, even if the compounds are present at trace levels.

The applicability of the described GC–MS method is also demonstrated by the determination of *trans*-RA in eight different species of the *Lamiaceae* family (see Table 2).

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