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Analysis of biologically active oxyprenylated henylpropanoids in Teatree oil using selective solid-phase extraction with UHPLC-PDA detection

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A B S T R A C T

An efficient analytical strategy based on different extraction methods of biologically active natu- rally occurring oxyprenylated umbelliferone and ferulic acid derivatives 7-isopentenyloxycoumarin, auraptene, umbelliprenin, boropinic acid, and 4'-geranyloxyferulic acid and quantification by UHPLC with spectrophotometric (UV/Vis) detection from Tea tree oil is reported. Absorption of the pure oil on Al_2O_3 (Brockmann activity II) prior washing the resulting solid with MeOH and treatment of this latter with CH_2Cl_2 resulted the best extraction methodology in terms of yields of oxyprenylated secondary metabo-lites. Among the five *O*-prenylphenylpropanoids herein under investigation auraptene and umbelliprenin were never detected while 4'-geranyloxyferulic acid was the most abundant compound resulting from all the three extraction methods employed. The UHPLC analytical methodology set up in the present study resulted to be an effective and versatile technique for the simultaneous characterization and quantifi- cation of prenyloxyphenylpropanoids in Tea tree oil and applicable to other complex matrices from theplant kingdom.

1. Introduction

Tea tree oil (TTO) is the volatile essential oil obtained by dis- tillation from leaves and branches of the native Australian plant *Melaleuca alternifolia* (Maiden & Betche) Cheel (Fam. Myrtaceae). This essential oil is nowadays well recognized among the most fash-ion herbal remedies used worldwide for the cure of a plethora of human disorders. TTO has in fact marked antiseptic properties andit was shown to be useful for the treatment of several skin dis- eases of viral, bacterial, and fungal origin. These are representedby acne [1], scabias [2], impetigo [3], seborrheic dermatitis [4],and several others [5]. Other reported biological activities include anti-inflammatory [6], anti-cancer [7], analgesic [8], insecticidal [9],and acaricidal [10] effects. In one of its monograph, the European Medicine Agency [11], TTO was considered as an optimal remedy for the treatment of small superficial wounds, insect bites, small boils, itching, and irritation in cases of athlete's foot. TTO has also recently found application in dentistry for the treatment of dental plaque, periodontitis, and other inflammatory-based oral diseases [12]. TTO is mainly composed by terpenes, among which monoter-pene and sesquiterpene hydrocarbons, and their alcohol deriva-tives [13]. However TTO phytochemical composition may greatlyvary, depending on key parameters like the amount of biomassused for the distillation of the essential oil, plant chemotypes,and the laboratory and/or industrial process employed for itsproduction (e.g. stem distillation, pressing, solvent extraction,etc.). To this concern, much of the attention has been dedicatedto the characterization of the pharmacological activity of themost volatile phytochemicals from TTO, like terpinen-4-ol and α-terpineol [14–16], while by far has been reported about compoundsfeatured by a higher molecular mass and/or relatively lower volatil-ity (e.g. phenolic ethers). Thus the exact chemical composition andpharmacological effects of TTO as a whole and those of its majorand/or minor components is yet to be exactly defined and this isstill a research field of current interest and with great potentialities.During the last decade we have been deeply involved in the characterization of the phytochemical profile of spontaneous edible herbs and vegetables, with a particular reference to the qualitative and quantitative of biologically active oxyprenylated secondary metabolites [17–19]. These latter are nowadays well recognized asan emerging class of novel natural products with a great therapeu- tic potential, mostly in terms of dietary feeding chemopreventionof severe acute and chronic syndromes affecting humans, like cancer, neurodegenerative disorders, metabolic diseases (e.g. dia- betes), cardiovascular diseases, stroke, and others [20–23]. As a continuation of our studies, we wish to report in the present manuscript an original and validated UHPLC-based method for the quantification of prenylated umbelliferone and ferulic acid biosyn- thetic derivatives, namely 7-isopentenyloxycoumarin **1**, auraptene **2**, umbelliprenin **3,** boropinic acid **4**, and 4'-geranyloxyferulic acid **5** (Fig. 1) in the essential

oil of *M. alternifolia* extracted with three different methodologies.

Such an investigation was also encouraged by acquisitions of literature data indicating that several plant species belonging to the Myrtaceae family exhibited a wide array of prenylated secondary metabolites [24].

2. Materials and methods

2.1. Chemicals

Tea tree oil (Mystic Moments, UK, batch: MM4220202) was purchased from a local market. Ferulic acid, umbelliferone, dichloromethane, 3,3-dimethylallyl bromide, geranyl bromide, *trans*, *trans*-farnesyl bromide, dry potassium carbonate, acetone, *n*-hexane, silica gel (high pure grade, pore size 60 Å, 70–230 Ámesh), aluminium oxide (activated neutral) were purchased from Merck Sigma-Aldrich (Milan, Italy) and all used without further purification. Boropinic acid, 7-isopenteniloxycoumarin, auraptene, umbelliprenine, boropinic acid, and 4'-geranyloxyferulic acid (GOFA), have been synthesized in our laboratories as reported previously [20], and their purity (>97.5%) assessed by HPLC and ^{1}H NMR. Methanol and acetonitrile (both UHPLC grade), acetic acid, and triethylamine were purchased from Carlo Erba Reagents (DasitGroup-Carlo Erba Reagenti, Milan, Italy). HPLC-grade water (> 18.2 M▲ cm resistivity) was obtained by a Milli-Q Ultrapure water purification system (Millipore, Bedford, MA, USA). All chem- icals and reagents used were of the highest purity commercially available.

2.2. Extraction procedures

The extraction of TTO was accomplished using three different methodologies: a) TTO (100 µL) was poured into an Eppendorf Tube (1.5 mL capacity), then a saturated $NaHCO_3$ aqueous solution with (50 µL) and *n*-hexane (50 µL) were added and the resulting mixture shaked in a vortex apparatus for 1 min and subsequently centrifu- gated at 13000 rpm for 5 min. The aqueous and organic phases were separated, each filtered on 0.22 µm PTFE filter and aliquots from both phases of 5 µL were injected into the UHPLC apparatus; b) TTO (100 µL) was poured into an Eppendorf Tube (1.5 mL capacity) then silica gel (15 mg, previously treated with 1.5% of Et_3N) was added and the resulting mixture shaked in a vortex apparatus for1 min and subsequently centrifugated at 13000 rpm for 5 min. The surnatant was eliminated and the resulting solid was washed with MeOH (2 × 100 µL), the obtained solutions discarded and finally CH_2Cl_2 (100 µL) was added to the remained solid. After havingbeen shaked and centrifuged under the same experimental condi- tions as above, the surnatant was filtered on 0.22 µm PTFE filter andan aliquote of 5 µL was injected into the UHPLC apparatus; c) TTO (100 µL) was poured into an Eppendorf Tube (1.5 mL capacity) thenaluminium oxide (15 mg, Brockmann activity II) was added andthe resulting mixture shaked in a vortex apparatus for 1 min and subsequently centrifugated at 13000 rpm for 5 min. The surnatant was eliminated and the resulting solid was washed with MeOH (2 100 µL), the obtained solutions discared and finally CH_2Cl_2(100 µL) was added to the remained solid. After having been shakedand centrifuged under the same experimental conditions as above, the surnatant was filtered on 0.22 µm PTFE filter and an aliquote of5 µL was injected into the UHPLC apparatus.

2.3. Preparation of standard solutions

Stock solutions of target analytes at the concentration of 1.0 mg/mL were individually prepared by dissolving 10 mg of each reference powder into 10 mL volumetric flask with MeOH. Thestock solutions were finally collected to provide one solution. The standard solutions were stored in amber vials and cool ambient (1–3 °C).

2.4. Analytical conditions

Analysis of the five analytes under investigation were performedon a Waters Ultra Performance Liquid Chromatography system (ACQUITY H-Class). Chromatographic separation was achieved using a Acquity UHPLC® BEH C_{18} (50 ×2.1 mm I.D. 1.7 µm par-ticle size) column protected by a disposable Security Guard C_{18} (4.6 × 2.1 mm I.D.) (Phenomenex, Torrance, CA, USA). The column was thermostated at 25 ± 1 °C. All injected solutions were stored in the auto-sampler at 5° C. The partial loop with needle overfill mode was set up to inject 5 µL. The "strong" wash was accom- plished with CH_3CN, while the "weak" one with a 1:1 MeOH/H_2O mixture. Mobile phase consisted of H_2O/CH_3CN (90/10) contain- ing 0.04% of formic acid (A) and CH_3CN containing 0.04% of formicacid (B) in gradient elution. To perform the best separation of the analytes a linear gradient elution program was used as reported in Table 1. The solvents were filtered before use through a 0.22 µm type GVmembrane (Millipore). For quantification purposes, the UV detec- tion was set at 322 nm for each analyte. Under these conditions,the total run time was 14 min and then the column was washed for7 min. Empower v.3 software (Waters) was used for setting-up the analysis and for data management. A Sartorius CPA225D analyti- cal balance was used to weigh the analytes for the preparation of stock solutions and calibration standards. Evaporations of all sol- vent were carried out in a Visiprep Vacuum Manifold equipped with a Visidry Drying Attachment System (Supelco, Bellefonte, USA).Samples were sonicated in an ultrasonic bath DU-32 (ArgoLAb,Carpi, Italy).

2.5. Method validation

The method was validated according to the ICH guidelines usingstandard mixtures (linearity, limit of detection [LOD] and limit of quantification [LOQ]) and TTO extract samples (accuracy and pre- cision). Linearity was calculated in the range 1–100 µg/mL for each analyte. Method precision was tested at three concentration lev-els (QC_L, QC_M and QC_H) in three replicates for each kind of TTO extract sample in order to calculate the percentage of RSD of the determination. TTO extract samples spiked with standard solution at three concentration levels (QC_L, QC_M and QC_H) were used for the determination of the accuracy of the method. Samples were

spiked in triplicates. Runs were accomplished in triplicates. Selectivity of the method was evaluated comparing standard calibration curves in methanol with standard calibration curves using the TTO extractcontaining the lowest concentration of the selected analyte.

3. Results and discussion

The aim of the present investigation was to further character- ize and define the phytochemical composition of TTO in termsof oxyprenylated phenylpropanoids. Such a class of secondary metabolites is nowadays known to occur in plants and other nat- ural sources in very low amount [25]. Thus it is conceivable to hypothesize that their presence in essential oils is virtually unde- tectable using the most common analytical techniques used forqualitative and quantitative analysis of such phytopreparations (e.g. gas chromatography). To this concern we decided to further process commercially available TTO to obtain extracts enriched in the five analytes herein under investigation. Three methodologies have been employed to accomplish this experimental step: a) par- tition of the oil between a saturated $NaHCO_3$ aqueous solution and *n*-hexane, and absorption of TTO on solid matrices like silica gel (pre-treated with Et_3N) and alumina (Brockmann activity II). Inthe first case the aim would be to obtain sodium salts of ferulicacid derivatives and the alkaline hydrolysis of the coumarin ring to yield the corresponding disodium salt of the o-hydroxycoumarate and separate the most abundant terpene fraction in the organic phase. On the other hand the use of silica gel and alumina have been proved to be a powerful mean to promote the deterpenation of essential oils [26,27].

3.1. UHPLC method validation

The UHPLC method was validated using analytes mixtures in order to evaluate linearity, LOD, and LOQ, and TTO extract samples to evaluate accuracy and precision.

3.1.1. System suitability

The system suitability was assessed by ten replicate analyses ofthe analytes at a concentration of 5 μg/mL. As reported in Table 2, all retention times, capacity factors, resolutions, theoretical plate numbers, and peak asymmetries were within acceptable values.

3.1.2. Linearity

The linearity of the five compounds under investigation was estimated using TTO samples fortified at different concentration levels (QC_L, QC_M and QC_H). The slopes obtained from oil samples were different from that of the standard solution. Standard addi- tion methodology was used during the quantification step in order to obtain reliable results. The calibration curves were drawn inthe range of 1–100 μg/mL for all analytes. Data were obtained by plotting concentration (μg/mL) to the peak area of pure chemi-cal standards. The calibration curves were linear over the range tested for each analyte, and the determination coefficients (r^2) werehigher than 0.9990. In order to describe the relationships between concentration of the analytes and the detector response, a linear regression analysis with weighting factors consisted in $1/x^2$ values was used. Data are summarized in Table 3.

3.1.3. LOD – LOQ

The LOQ of the method was defined according to International Guidelines ICH Q2 (R1) 2005 as the concentration of the lowest standard on the calibration curve for which (a) the analyte peakis identifiable and discrete, (b) the analyte response is at least ten times the response of the blank sample, and (c) the analyte responseis reproducible with a precision less than 20% and true-ness better of 80–120%. LOD and LOQ were estimated on the basis of the results for three replicates of real samples spiked at different concentration levels, considering a signal-to-noise ratio of 3 and 10, respectively. The LOQ values for each analytes were assessed to be 0.5 μg/mL. On the basis of the signal-to-noise ratio of the chromatograms, theLOD of the method was also set at 0.3 μg/mL.

3.1.4. Accuracy and precision

Data for intra- and inter-day precision and accuracy, obtained from the analysis of three batches of LLOQ and the QC samples at three different concentration levels of all analytes (LLOQ, LQC, MQCand HQC) in duplicate on the same day and for five consecutive days,are shown in Table 4. The intra- and inter-day precision (RSD values) did not exceed 6.05%, while the intra- and inter-day accuracy (BIAS%) variedbetween 3.57% and + 3.24%. According to ICH guidelines, these results suggest that the method assessed in this study has satis-factory accuracy, precision, and reproducibility.

3.1.5. Carry over

Carry over was investigated by injecting into the UHPLC appa- ratus two blank samples of the essential oil followed by two blank samples spiked with the analytes at the LOQ concentration, fol- lowed by at least three blank samples of the processed essential oil. Non-significant carry over effect (< 0.25%) was evident.

3.1.6. Recovery and stability

Recoveries of all the five analytes herein under investigation were >97.9% with a good precision (RSD <3.1%). Their stability was quantified under different storage conditions in the studied matrix using the QC samples, that have been analysed immediately after preparation and after the applied storage conditions (temperature,freeze-thaw cycles, short-term, and long-term storage). QC sampleshave been analysed against a calibration curve and the obtained concentrations have been compared with the nominal concentra- tions. Stock solutions were stable at room temperature for 48 hand at 4 °C for two weeks. The analytes were also stable up to 24 h at room temperature in oil samples with no chemical degradation having been observed at 20 °C up to five weeks. No degradation was observed after three cycles of freezing and thawing. The sta- bility of the investigated analytes in extracts was confirmed after 24 h when stored at 4 °C.

3.1.7. Selectivity and robustness

The selectivity of the analytical method allowed to quantify selected analytes (prenyloxy compounds in the present case) in the presence of components that may be impurities, degradation products, and/or components of the matrix. The selectivity of the method was evaluated by analyzing blank TTO extracts. In the evaluation of the selectivity, all peaks related to prenyloxy phytochemicals were well resolved from baseline and separated from each other and there was no matrix effect in the TTO extracts. The peak purity index belonging to their analysis in TTO extracts were found to be higher than 0.9936.

Robustness test of an analytical method aims to explore how sensitive the responses are upon little modification in the factor settings. The robustness of the method was tested using exper- imental design in order to study the simultaneous variation of the factors. An eleven-run fractional factorial design, three exper- iments within the optimized conditions, were chosen to evaluate whether a change in factor value produced a statistically significantvariation in the observed responses. In the study the ranges examined were small deviations from the method settings with regardto acetonitrile% (0–10%), mobile phase pH, flow rate, and column temperature. To evaluate the robustness of the method, ANOVA testwas applied and *p* values of regression coefficient and regression equation were calculated. The *p* values were 0.03 thus indicating that small changes did not effectively affect the peak area ratio.

3.1.8. Quantification of oxyprenylated phenylpropanoids in TTOprocessed extracts

The validated UHPLC method was applied to the measurement of concentration of the five oxyprenylated phenylpropanoids 1–5in the three TTO extracts obtained as depicted above. Calibration curves were used for quantification. Results of the quantification are reported in Table 5.

Processed TTO samples were prepared as described above and at least three independent extractions and analyses were performed to obtain the final reported concentrations. An example of a UHPLCchromatogram (samples obtained by absorption on Al_2O_3) is shownin Fig. 2.

Adopting the above described experimental conditions the retention times of detected analytes (compounds **1**, **4**, and **5)** in TTO processed samples matched those obtained from the analyses carried out with pure chemical standards. Data reported in Table 5 shows that considering total yields extraction with silica gel pre- treated with Et3N was the less effective methodology. Attempts to get a selective extraction by partition between a 1:1 $NaHCO_3$

saturated solution *n*-hexane biphasic mixture did not provide the expected results. All the three oxyprenylated phenylpropanoids detected, namely 7-isopentenyloxycoumarin **1**, boropinic acid **4**, and 4'-geranyloxyferulic acid **5**, were virtually soluble in both solvents and no better results have been obtained increasing the percentage and/or concentrations of the water solution and/or of the organic solvent. Thus the most effective methodology was the TTO absorption on alumina (Brockmann grade II) followed by desorption with CH_2Cl_2. It is noteworthy to underline that increasing up to IV or decreasing the grade of alumina did not results in any improvements in selectivity of extraction and yields of the investi- gated oxyprenylated secondary metabolites.

In terms of biological activity, the most part of citations in the literature about essential oils refer to their terpenoid fraction, despite it is well established that some contain also an appreciable amounts of phenols and similar phytochemicals. This is the case of TTO, for which, however very few reports about pharmacological effects attributable to the phenylpropanoids and/or polyphenols portions have been described [28]. Quite recently we have also demonstrated by means of HPLC analysis that, despite their rela- tively high molecular weights, also secondary metabolites of mixed biosynthetic origin like oxyprenylated phenylpropanoids, can be effectively considered as components, although minor, of essen-tial oils, like those extracted from *Citrus* fruits peels and skins [29]. Consequently it can be hypothesized that such natural productscan represent additional determinants of the observed biological activity featuring essential oils. For example TTO has been recentlyshown to efficiently have an *in vivo* topic anti-cancer effect in subcutaneous tumours [30]. This effect could be partly explained by
the presence of 7-isopentenyloxycoumarin **1**, boropinic acid **4**, and
4'-geranyloxyferulic acid **5**, all able to act as powerful anti-cancer agent on several cancer cell lines both *in vitro* and *in vivo* [23]. Thus it is of extreme use to investigate the minor high molec-ular weight components of essential oils (e.g. phenylpropanoids, polyketides, etc.) and this can be accomplished only by setting up more and more sensitive analytical methodologies, like the UHPLC-based one developed and validated in the present manuscript. Furthermore our experimental protocol is among the first exam- ples of application of UHPLC for the qualitative and quantitative analysis of oxyprenylated phenylpropanoids in vegetable matri-ces, and in absolute the first example for their determination in essential oils. The in so far reported methodologies for the analysisof this class of secondary metabolites were in fact all HPLC-based ones [18,19]. Although we recently applied UHPLC for the deter- mination of the same compounds in vegetable oils [17], the overall experimental protocol we employed in that case was different. Itis finally worthy to point out how the key step of the overall pro- cedure described herein was the adsorption of the oil on alumina allowing us to easily detect and quantify the analytes under inves- tigation practically without any interferences of the matrix, due forexample to a massive presence of monoterpenic and sesquiterpenichydrocarbons and oxygenated related products (e.g. alcohols). Themethod reported is also among the few examples in the literature about the usefulness of alumina for the total or partial deterpe- nation of essential oils. The findings reported herein enforce also the concept of the effectiveness of selective extraction of plantsecondary metabolites using solid matrices over “classic” phyto- chemical procedures (e.g. maceration) [31] and represent the first application of this technique to oxyprenylated phenylpropanoids. This pre-treatment coupled to the UHPLC protocol we described can be easily and effectively applied in the next future to the analysis of oxyprenylated secondary metabolites to a wide arrayof phytopreparations and/or food or nutraceutical products not only represented by essential oils, but also extracts obtained using apolar solvents like petroleum ether, chlorinated solvents, diethyl ether, acetone, etc.

4. Conclusions

In this paper an innovative UHPLC/PDA method for the quantifi-cation of selected phenylpropanoid compounds in three different kind of TTO extracts was developed. The method combined a selec-tive extraction method and high separation efficiency of UHPLC.A complete validation of method was achieved and its applicabil- ity of the method was verified on real samples of TTO extracts.The further insight into the phytochemical item provided in this manuscript may be of help in the assessment of the authentic-ity and genuineness in contexts like quality control of production processes, determination of commercial value, and chemical finger-printing in general. Results outlined in the present study give new insights into the chemical composition of TTO and suggest that 7- isopentenyloxycoumarin, boropinic acid, and 4'-geranyloxyferulic acid have to be considered as additional components of the in sofar phytochemical pool reported for TTO.

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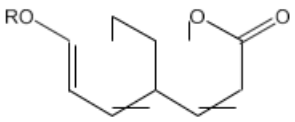
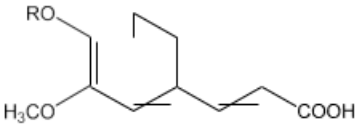


1 R = 3,3-dimethylallyl

2 R = geranyl

3 R = farnesyl



4 R = 3,3-dimethylallyl

5 R = geranyl

Fig. 1. Structure of prenyloxyphenylopropanoids under investigation as compo- nents of TTO.

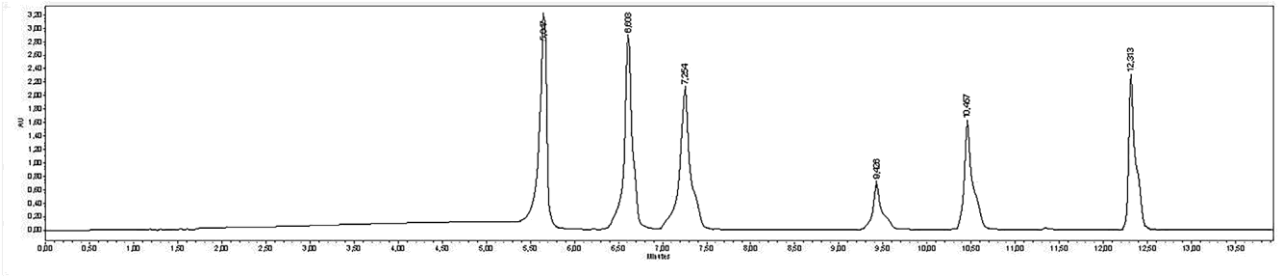


Fig. 2. Chromatogram of a five analytes mix at the concentration of 45 µg/mL, under investigation at 322 nm.

Table 1
Chromatographic gradient program.

Time	Flow (mL/min)	%A	%B	Curve
Initial	0.1	100	–	6
3.0	0.1	100	–	6
4.0	0.2	100	–	6
4.1	0.2	50	50	6
4.2	0.3	50	50	8
7.0	0.3	50	50	8
12.0	0.3	–	100	6
18.0	0.3	–	100	6
18.1	0.3	100	–	6
19.0	0.1	100	–	6
21.0	0.1	100	–	6

Table 2
System suitability parameters.

Compound	**4**	**1**	**5**	**2**	**3**
Retention time (min.)[a]	6.6 (0.4%)	7.2 (0.5%)	9.4 (0.2%)	10.4 (0.3%)	12.3 (0.5%)
Capacity factor[b]	3.5	4.0	5.5	6.1	7.5
Resolution[c]	2.20	1.77	5.25	2.85	5.42
Theoretical plate numbers	4356	3504	16044	11025	27335
Peak symmetry (10%)	1.0	0.6	1.2	1.0	1.0

[a] Values in brackets represent RSD% of retention time (10).
[b] T_0 was calculated uracil eluted using the same conditions (t_0 = 1.46).
[c] Values represent resolution of adjacent peaks.

Table 3
Calibration data of the five analytes.

Compound	Slope	Intercept	r^2
1	167358(154836–179880)	−26600(−186000 to 132776)	0.9881
2	100376(92799–107954)	3310(−93130 to 99752)	0.9863
3	121888(112150–131626)	−6425(−130400 to 117516)	0.9854
4	181346(167960–194733)	25069(−145300 to 195443)	0.9869
5	46568(42599–50536)	10810(−39690 to 61313)	0.9779

Table 4
Precision and accuracy data of the five analytes.

Compound	**1**	**2**	**3**	**4**	**5**
Precision					
Intra-day ($n = 6$)	2.8–5.3	2.7–4.6	2.4–6.8	3.5–6.8	1.5–4.3
Inter-day ($n = 6$)	3.0–6.2	3.1–7.2	3.5–7.3	4.2–7.9	0.9–5.6
Accuracy					
Intra-day ($n = 6$)	2.1–5.4	0.5–2.6	1.2–3.4	1.4–3.6	0.9–3.0
Inter-day ($n = 6$)	0.8–2.5	1.4–3.6	1.8–4.3	1.5–3.8	1.2–2.9

Table 5
Quantification of compounds **1-5** in TTO processed extracts (values expressed as μg/g TTO).

Compound/Extract	Hexane	$NaHCO_3$ (sat.)	SiO_2/Et_3N	Al_2O_3 (II)
1	19.88	27.06	33.24	44.03
2	ND	ND	ND	ND
3	ND	ND	ND	ND
4	0.63	0.47	0.77	1.01
5	42.95	66.60	84.25	130.51

ND = not detected or below LOQ.