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Simultaneous determination of oleuropein and its metabolites in plasma by high-performance liquid chromatography

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Abstract

A method based on high-performance liquid chromatography (HPLC) with a diode array detection system was developed and validated aiming at the simultaneous determination of oleuropein (OE) and its metabolites, hydroxytyrosol (HT) and tyrosol (T), in human plasma. These phenolic components are believed to play a vital role in the prevention of coronary artery disease and atherosclerosis. The proposed method includes a clean-up solid-phase extraction procedure (using a C_{18} column) with high recovery efficiency (85–100%). The statistical evaluation of the method reveals good linearity, accuracy and reproducibility for all the compounds analyzed with RSD values less than 6.5%, while the detection limit is 50 ng/ml for both OE and T and 75 ng/ml for HT. This assay can be employed in bioavailability studies of olive oil phenolic compounds, thus assisting the evaluation of their pharmacological role.

Keywords: Oleuropein; Hydroxytyrosol; Tyrosol

1. Introduction

The Mediterranean diet, and even more so the Cretan Mediterranean diet, has been associated with longevity, and low rates of coronary artery disease (CAD) despite its high dietary fat content [1,2]. This was first shown in the Seven Countries Study [3] and later on in the Lyon Diet Heart Study [4,5]. The latter study has demonstrated that recurrent myocardial infarction, cardiovascular events, and all-cause mortality were reduced by 70% when a group of patients who had suffered a heart attack, was put on a Cretan diet. These studies concluded that the high life expectancy of the Cretans is credited to their dietary habits, and mainly in the high olive oil consumption. Olive oil serves as the principal source of dietary fat, and it has been shown that the olive oil antioxidant phenolic components prevent lipoprotein oxidation [6,7] which is considered to be a key factor in the pathogenesis of atherosclerosis [8,9]. Even though in vitro studies of the olive oil phenolic components, i.e. oleuropein (OE), tyrosol (T) and

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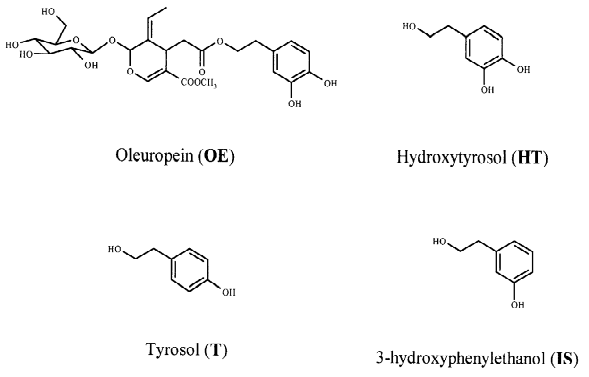

Fig. 1. Chemical structures of oleuropein (OE), hydroxytyrosol (HT), tyrosol (T) and μ-hydroxyphenylethanol used as internal standard (I.S.).

hydroxytyrosol (HT) (Fig. 1), have provided evidence for their beneficial effects, there is little information on the absorption and the bioavailability of these compounds. This is attributed to the lack of a sensitive and rapid assay for the quantitation of the aforementioned phenols in plasma, and the difficulty of obtaining pure standards, especially that of HT. Nevertheless, a number of analytical methods have been proposed for the determination of the above phenols from various sources, such as olive oil, olive drupes, and olive tree leaves. The level of the phenol content is determined by GC–MS [10], and by reversed-phase liquid chromatography using ultraviolet [11–13], fluorescence [11], electrospray mass spectrometric (MS) [11,14], tandem MS (MS–MS) [15], as well as atmospheric pressure chemical ionization (APCI) MS and MS–MS [16] detection. The determination of HT and T in biological fluids has also been investigated mainly in urine by GC–MS [17–19] and radioanalysis [20]. The assay of these phenolic substances in plasma has been reported only for HT by HPLC [21] and GC–MS [22].

This report describes the development of a sensitive method for measuring the olive oil phenolic compounds in plasma. It comprises of a sample preparation step, using solid-phase extraction (SPE) followed by chromatographic analysis with photodiode array detection. The OE and HT standards used in this study were isolated from olive leaf extracts [23] and produced from the purified OE, respectively. To date, this is the first method for the determination of OE in plasma, which can be used for the simultaneous determination of its metabolites.

2. Experimental

2.1. Reagents and chemicals

Tyrosol (4-hydroxyphenylethanol) and μ-hydroxyphenylethanol (3-hydroxyphenylethanol) (the latter was used as internal standard, I.S.) were purchased from Sigma–Aldrich (Steinheim, Germany). Ammonium acetate was obtained from Riedel de-Haen (Germany). All solvents used throughout the experiments were obtained by Merck (Darmstadt, Germany). The purity of all organic reagents was checked by HPLC prior to their use.

2.2. Isolation of OE and HT from olive leaf extracts

Chemically pure HT (3,4-hydroxyphenylethanol) was isolated from fresh olive fruits as follows. The extract from drupes (100 g corresponding to 25 olive oil fruits from *Olea europaea* var. koroneiki) was prepared in distilled water (5 ml) after cutting into pieces, refluxed for 5 min and subjected to filtration under reduced pressure. The solution was then extracted with petroleum ether (5 ml), the water layer was separated and removed under reduced pressure and subjected to chromatography (MPLC Si gel R18 Merck 20–40 μm, H_2O) yielding 40 mg of chemically pure compound. Oleuropein (OE) was isolated from olive leaves according to a previously described procedure [23]. The purity of the isolated OE and HT reference standards was greater than 99% as it was assessed by NMR analysis.

2.3. Stock standard solutions

Stock standard solutions of all analytes were prepared in methanol at the 1 mg/ml level. Working solutions containing OE, HT and T were prepared every week by diluting appropriate volumes of the corresponding stock standard solutions in HPLC-grade water at the 100 μg/ml level, whereas a standard volume of the I.S. at the same level was added separately.

2.4. Plasma samples

Human plasma samples were obtained by venipuncture (University Clinic, School of Medicine, University of Crete) from eight healthy adult volunteers (four male and four female, nonsmokers with body mass index 20–30) who have been fasting from olive oil for 3 days. Plasma samples were collected in vacutainer tubes containing EDTA as anticoagulant, centrifuged for 10 min at 4000 rpm in a dark room and were kept at −80 °C until use. Spiked plasma samples containing OE, HT, T and the I.S. were prepared daily by adding appropriate volumes of the corresponding working solution (100 μg/ml) into 1 ml of human plasma to yield concentrations of 1.5, 1.0, 0.75, 0.5, 0.3 and 0.166 μg/ml of OE, T and HT and a fixed concentration of 500 ng/ml of the I.S.

2.5. Instrumentation

An HPLC system comprising of a Spectra system P4000 (Finnigan, Riviera Beach, FL, USA) quaternary pump equipped with 7725i injector (Rheodyne, Rohnert Park, CA, USA) with a 100-μl loop, a Finnigan on-line degasser and coupled to a Finnigan Spectra system UV6000LP diode array detector was utilized. The whole process was computer-controlled by the CHROMQUEST v.2.51 software through the Finnigan SN4000 controller. Chromatographic separation was performed on an C_8 reversed-phase column (250×4.0 mm, I.D. 5 μm) (Alltech, Deerfield, IL, USA) equipped with a C_8 Alltech precolumn. The solid-phase extraction (SPE) procedure was developed on an automated AspecXL (Gilson, Middleton, WI, USA) SPE system equipped with a Gilson 402-syringe pump and a 10-ml transfer tubing.

2.6. HPLC

A gradient elution program was used for the separation of the four substances as shown in Table 1. Solvent A was 0.05 *M* ammonium acetate buffer (adjusted to pH 5.0 with glacial acetic acid), solvent B was acetonitrile, and the flow-rate was 1 ml/min.

Table 1
Gradient elution program for reversed-phase high performance liquid chromatographic separation of OE, HT, T and I.S.

Time (min)	Solvent A[a] (%)	Solvent B[b] (%)	Flow-rate (ml/min)
0	90	10	1
4	80	20	1
8	40	60	1
11	30	70	1
15	90	10	1

[a] Solvent A: 0.05 *M* ammonium acetate buffer adjusted to pH 5 with glacial acetic acid.

[b] Solvent B: acetonitrile. Chromatography was performed on an C_8 reversed-phase column (250×4.0 mm I.D., 5 μm) equipped with a C_8 Alltech precolumn.

At the end of each run, i.e. 15 min, the column was left to equilibrate at the starting mobile phase composition (i.e. 90% A–10% B) for an additional 5 min, giving a total chromatographic analysis time of 20 min. All mobile phases were vacuum filtered through a 0.2-μm Titan membrane filter (Scientific Resources, USA) and degassed in an ultrasonic bath prior to HPLC analysis. The column was maintained at 40 °C throughout all experiments with the aid of an electronically controlled oven.

2.7. Detection

UV spectra of all substances were recorded with the aid of the diode array detection system and the maxima of absorbance were determined to be 240 nm for OE, and 280 nm for the other three substances (T, HT and I.S.). The recording of the chromatogram was, therefore, performed at the two aforementioned wavelengths (Fig. 2A and B). Additional confirmation of the UV maxima was obtained by recording the spectrum of each separate substance using a Unicam UV-300 UV–Vis spectrophotometer with a pair of 10-mm optical path length quartz cuvettes. Identification of the eluting peaks was performed by comparing their retention time values (t_R) and the corresponding UV spectra (obtained from the diode array data) with those of the standards. The time window for peak identification was set to 10% of t_R.

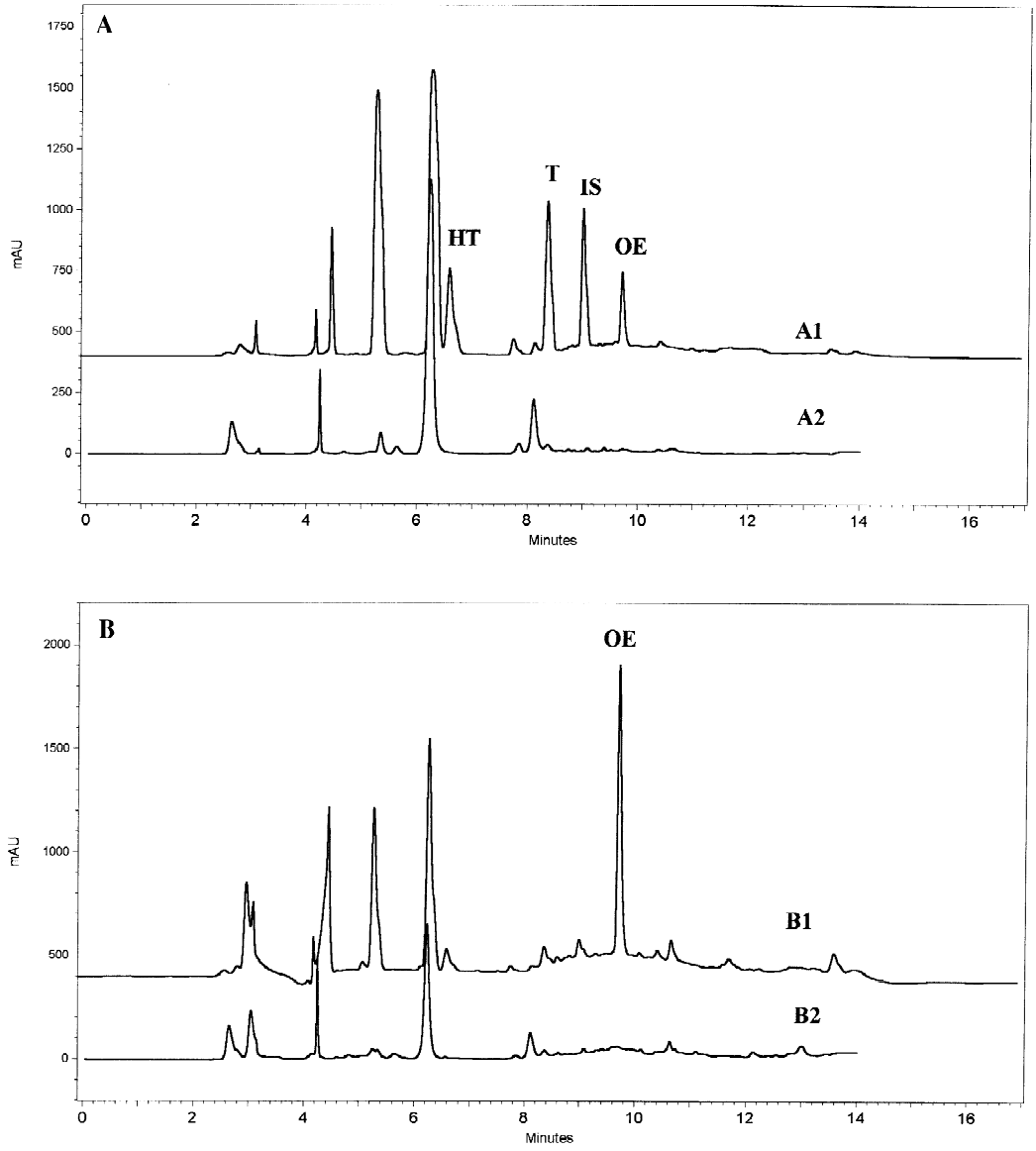

Fig. 2. Chromatographic separation of the three phenolic components from *Olea europea*, recovered from spiked plasma by solid-phase extraction, at 280 nm (A1 and A2 traces) and at 240 nm (B1 and B2 traces). A2 and B2 correspond to blank plasma samples subjected to the same clean-up procedure as the phenol-spiked plasma samples. Peaks labeled OE, HT, T and the I.S. correspond to oleuropein, hydroxytyrosol, tyrosol and the internal standard, respectively.

2.8. Solid-phase extraction

Sample clean-up was performed using C_{18} SPE cartridges obtained from Varian Associates (500 mg, 3 ml). The whole procedure was automated using an AspecXL SPE system. The cartridges were equilibrated initially with 1 ml methanol and subsequently rinsed with 2 ml of HPLC-grade water. The sample was applied to the cartridge with a flow of 1 ml/min and passed through it by applying a stream of air at a flow-rate of 1 ml/min. The cartridge pressure was maintained stable after the end of the procedure for

30 s. The cartridge was then washed twice with 1 ml of water in order to remove plasma compounds and dried under vacuum for 15 min. Consequently, it was placed back to the AspecXL SPE instrument, and eluted with 1 ml methanol–acetic acid (99:1, v/v) mixture.

2.9. Stability of the analytes

In order to minimize possible degradation of the analytes, they were stored in dark colored vials and kept refrigerated at −35 °C. All phenolic substances were found to be stable for at least 2 months. Nevertheless a slow degradation of standard solutions was observed after a 6-month storage, as evidenced by the appearance of other peaks in the chromatogram. In the case of plasma analysis, immediate analysis of the samples was performed due to the probability of HT degradation (possibly through oxidation).

2.10. Validation of the assay method

At the beginning of every laboratory day two standard mixtures of the four substances at the 1000 and 300 ng/ml levels, serving as system-suitability working standard solutions, were injected for assessing the performance of the chromatographic procedure in terms of retention time stability and signal sensitivity.

2.10.1. Linearity

For the linearity study six spiked plasma samples (containing 1500, 1000, 750, 500, 300 and 166 ng/ml of OE, T and HT and a fixed concentration 500 ng/ml of I.S.) were analyzed, and the linearity of the data was checked by performing linear least-squares regression analysis.

2.10.2. Precision

The assay precision was evaluated by performing the overall assay (SPE and chromatographic method) at two levels (1000 and 500 ng/ml) in five replicates ($n=5$) and calculating the RSD values. The reproducibility study (system precision) was performed by injecting all the calibration curve standards in five replicates ($n=5$).

2.10.3. Accuracy

The accuracy of the method was assessed by three quality control standards at three concentration levels, which were different from the calibration curve standards, i.e. 1.25, 0.9 and 0.4 μg/ml, and was evaluated as the relative percentage error.

2.10.4. Recovery

Plasma samples were spiked at all the concentration levels of the calibration curve, and then analyzed employing the described method. Peak areas were employed for the calculation of concentration, and recovery was evaluated as the ratio of the peak area for every substance in the spiked sample against that of the standard.

2.10.5. Limits of detection and quantitation

The LOD and LOQ were determined by measuring the background response, and running six blank samples of plasma at maximum sensitivity. The signal-to-noise (S/N) ratio of 3:1 (peak area ratio of the analyte vs. baseline noise) and 10:1 were used for the calculation of the LOD and LOQ, respectively.

3. Results and discussion

3.1. Internal standard

The choice of the I.S. was based on its chemical structure similarity to the substances under analysis. The two preferential candidates were 2-hydroxyphenylethanol and 3-hydroxyphenylethanol, which are isomers of the oleuropein metabolite tyrosol. From these two substances the first one co-eluted with tyrosol, whereas the second one was clearly separated and furthermore presented no interference with other plasma substances.

3.2. Clean-up procedure

Our first attempt was to use liquid–liquid extraction for the sample clean-up. Four different solvents were used, namely *n*-hexane, chloroform, ethylacetate and ethylacetate–acetone (2:1, v/v). The corresponding recoveries of oleuropein ranged from almost 0% (*n*-hexane and chloroform) to nearly 50% for ethyl acetate. On the other hand lowering or

raising the sample pH significantly (which converts the phenolic hydroxyl groups in all the molecules to their corresponding salts for backextraction) may result in hydrolysis of oleuropein [24]. Therefore, a SPE procedure was employed. A standard amount of all three substances and the I.S. in 1 ml water was loaded onto a C_{18} SPE column and the elution procedure was monitored by HPLC. The use of methanol (an excellent solvent for obtaining oleuropein from olive oil and olive fruit) for the equilibration and elution step gave a nearly quantitative recovery for OE, T and the I.S., but only 50% for HT. At the next step, sample acidification was attempted for disrupting the hydrogen bond formed between the plasma proteins and HT. The use of phosphoric acid gave very good results in terms of recovery for all the phenolic substances, but also increased the degree of interference as significantly larger amounts of plasma substances were eluted. Similar results were obtained with either TFA or acetic acid. The incorporation of additional clean-up steps to this procedure—washing with *n*-hexane or with a 2.5 or 5% (v/v) water–methanol mixture—did not result in any improvement in the chromatogram. On the other hand, elution with ethyl acetate or acetonitrile instead of methanol gave less interfering peaks but low recovery. Therefore, the SPE procedure, which found to be advantageous both in terms of maximum recovery and minimum chromatographic interference involved no sample acidification and elution of the phenols with a solution of 1% (v/v) acetic acid in methanol. Briefly, the SPE cartridge was equilibrated with 1 ml methanol and washed with 2 ml water. It was then loaded with the plasma sample, washed twice with 1 ml of water, dried and eluted with 1 ml of 1% (v/v) acetic acid in methanol. Finally, the SPE eluate was dried under a nitrogen stream and light heating (45 °C) and reconstituted with 150 μl of 5% (v/v) methanol in water. It was found that the peak shape was greatly improved, as the reconstitution solution was weaker in terms of eluting power (i.e. less organic modifier) than the initial gradient chromatographic conditions (90% A–10% B). The last step prior to the HPLC injection was a centrifugation step (10 000 rpm for 2 min) for discarding any particulate matter.

All samples were analyzed immediately after their pretreatment because the potentially most powerful antioxidant HT could be oxidized. In case of analyzing batch samples all together an appropriate antioxidant [10], such as ascorbic acid or tocopherol, should be added if the samples are not to be processed immediately.

3.3. In process stability

A plasma sample was spiked with HT at the 5 μg/ml level and processed by SPE as described in Experimental. The reconstitution volume was 1000 μl with no antioxidant added. Five 100-μl portions from this solution, each one corresponding to 500 ng of HT on column, were injected at the following time intervals: 0, 30, 90, 120 and 1200 min. The reconstituted sample remained at ambient temperature. No signal reduction was observed for HT even after 20 h. The RSD of the HT peak area was 4.15%. These results indicate that the samples could remain in an autosampler for at least 20 h without any signal degradation. Therefore, the addition of an antioxidant was not considered necessary (given the fact that the samples were processed immediately after plasma collection).

3.4. Method validation

The whole SPE–chromatographic procedure was validated for its linearity, precision, accuracy, specificity, sensitivity, and recovery.

3.4.1. Linearity

The linearity of the method was evaluated by linear regression analysis using six concentrations of T, HT and OE versus a standard concentration of I.S. employing the [analyte peak area]/[I.S. peak area] ratio for every substance as the analytical response. The results reported apply to the overall procedure, i.e. both the SPE and the chromatographic separation. Good linearity was achieved for all the analytes, as indicated by the equations listed in Table 2.

3.4.2. Precision

The intra-day precision of the overall procedure was determined by analyzing five replicates of spiked plasma at two concentration levels (0.5 and 1 μg/ml). The intra-day precision, expressed as the relative standard deviation (RSD), ranged from 3.9

Table 2
Calibration curve for OE, HT AND T

Ratio[a]	Regression equation	Correlation coefficient, r^2
OE/I.S.	$y = 0.003x + 0.0386$	0.990
HT/I.S.	$y = 0.0007x + 0.028$	0.990
T/I.S.	$y = 0.0014x + 0.280$	0.999

[a] Expressed as the ratio of the peak area of the analyte against that of the internal standard (I.S.).

to 6.5% for the two concentration levels, as shown in Table 3. The inter-day precision was determined by analyzing three replicates of a spiked plasma sample at the 0.5 μg/ml level, prepared on 3 different days. The inter-day precision was found to be 6.84% for OE/I.S., 5.21% for HT/I.S. and 3.5% for T/I.S. (Table 3). These low RSD values indicate the suitability of the method for bioavailability purposes. The reproducibility (system precision) was evaluated for all the calibration curve concentrations, and found to be 1.98–6.1% for OE, 0.75–6.5% for HT, and 0.6–6.5% for T.

3.5. Accuracy

Three quality control standards at three concentration levels, which were different from the calibration curve standards, namely 1.25, 0.9 and 0.4 μg/ml were analyzed in order to determine the accuracy of the method. The results are listed in Table 4 and are expressed as the relative percentage error, defined as [assayed concentration − nominal concentration]/[nominal concentration] × 100. The estimated accuracy values with the proposed method are within acceptable levels for all the three phenolic components. The obtained data indicate that the method could be considered as accurate for bioanalytical purposes.

Table 3
Precision data of the OE, HT and T determination in human plasma

Ratio[a]	Intra-day precision RSD (%)		Inter-day precision RSD (%)[b]
	1 μg/ml	0.5 μg/ml	0.5 μg/ml
OE/I.S.	5.8	6.5	6.8
HT/I.S.	4.9	5.0	5.2
T/I.S.	3.9	3.9	3.5

[a] Peak areas were employed for the calculation of concentration of the analyte and I.S.
[b] Three replicates at 3 different laboratory days.

Table 4
Accuracy data of the OE, HT and T determination in human plasma

Ratio[a]	Accuracy (Er. %)[b]		
	1.25 μg/ml	0.9 μg/ml	0.4 μg/ml
OE/I.S.	5.9	2.9	9.6
HT/I.S.	4.2	5.2	7.5
T/I.S.	0.2	1.7	8.2

[a] Peak areas were employed for the calculation of concentration of the analyte and I.S.
[b] Er %, Relative percentage error.

3.6. Recovery

Plasma samples were spiked with standard amounts of OE, T, HT and the I.S. at the calibration curve concentrations ($n = 5$). The recovery was calculated by comparing the ratio of [peak area of the analyte]/[peak area of the I.S.] after the SPE to that of the unextracted standard injected the same day. The recoveries were found to be 84–86% for OE, 99–100% for HT and 93–97% for T, thus indicating a nearly quantitative recovery of all the three substances.

3.7. Selectivity

The selectivity of the method was assessed by acquiring the full UV spectrum (220–450 nm) of every substance analyzed. That was used as an additional confirmation to the chromatographic retention time data. Quantitation of the phenolic substances was performed at their λ_{max} values, i.e. 280 nm for HT, T and I.S., and 240 nm for OE. The specificity of the method was checked for any possible interference from endogenous plasma components. Several blank plasma samples were analyzed with the proposed method and indicated no interfering peaks at the expected retention times of the analytes or the I.S. (Fig. 2, traces A2 and B2). Therefore, the method's ability to efficiently separate the analytes from any possible interference, combined with the diode array UV spectral data, indicates the specificity of the developed assay.

3.8. Sensitivity

The sensitivity of the method as presented by its limit of detection (LOD) was found to be 75 ng/ml for HT and 50 ng/ml for both OE and T. The higher detection limit for HT was attributed to the fact that it exhibits significantly lower molar absorptivity than the other substances. The LOQ was 166 ng/ml (the lower concentration of the calibration curve) for all the three substances, even though it could be somewhat lower for T and OE.

4. Conclusions

An HPLC method has been developed and validated for the determination of OE and its major metabolites T and HT in plasma. The method includes a SPE step with high recovery efficiency (85–100%) for the phenolic compounds. The developed assay has been shown to be accurate, specific, sensitive, precise, and reliable and it can be easily automated. Preliminary experiments with humans (with average mass of 70 kg) using a daily dose of 28 mg oleuropein (OE) (in a total leaf extract form) showed no detectable level of OE and its metabolites in the volunteers' plasma. On the other hand, the detected levels of HT in rat plasma (1.2 μg/ml after administering a dose of 20 mg/kg) [21], proves that the developed methodology has the required sensitivity for measuring the phenol levels in plasma. In light of that, we are currently modifying the administration scheme in order to increase the intake dose of OE.

Nevertheless, there is still a need for more sensitive determination of OE, T and HT, and improvement of their already low LOQ values. Incorporating MS detection into the developed assay will satisfy this requirement. Moreover, we are currently working on improving the sensitivity of the analysis by using GC–MS/MS detection for HT and T, and LC–MS/MS for OE and other glycosides. Our preliminary GC–MS/MS data showed that we could detect HT and T in the sub-ng/ml level; this would greatly assist the bioavailability studies of olive oil phenolic components, which are currently carried out in our laboratory.

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