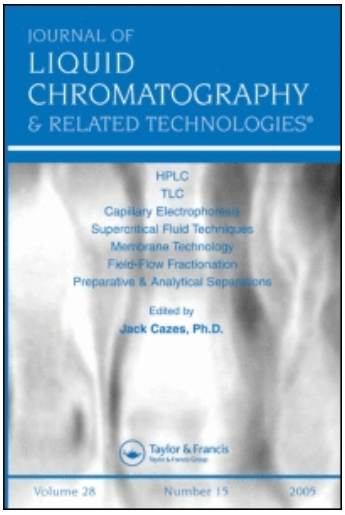

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Abstract: The hydrolytic stability of Oleuropein (OE), the major bioactive molecule of olive oil was investigated under acidic conditions. For this purpose a reversed-phase liquid chromatography (HPLC) method was developed and validated using a C_8 column and a diode array detection system. Statistical evaluation of the proposed HPLC method showed good linearity, precision and accuracy so that it can be used for analytical purposes. The relative standard deviation (RSD) of the method was less than 3.76% and the LOQ was established to be 0.5 μg/mL. OE was hydrolyzed at three different HCl concentrations (1, 0.1, and 0.01 N) under different temperatures. The results obtained were treated as logarithms of concentration vs. time because it was realized that the hydrolysis followed first order kinetics. The observed degradation rate constants k_{obs} were determined as well as the corresponding half-lifes $t_{1/2}$. Arrhenius plots were also constructed from the hydrolysis data. The results indicate that OE is stable under acidic conditions.

Keywords: Olive oil polyphenols, Antioxidants, HPLC DAD, Hydrolysis of oleuropein

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INTRODUCTION

Numerous reports exist on the beneficial effects that the so-called Mediterranean diet exerts on human health in terms of lowering the occurrence of chronic diseases[1,2] such as CAD (Coronary Artery Disease), atherosclerosis[3] and cancer.[4] Recently it has been shown that such a diet may also prove helpful in the case of AD (Alzheimer's Disease).[5] These human health effects are believed to be closely related to the consumption of olive oil, which represents the main source of fat for the Mediterranean population. Oleuropein (OE) (Figure 1), which is the ester of hydroxytyrosol (HT) and the glycoside of the secoiridoid elenolic acid (EL), is considered to posses the main biological activity of olive oil. It has been shown that olive oil antioxidant polyphenolic components prevent lipoprotein oxidation,[6,7] which is a key factor in the pathogenesis of atherosclerosis.[8] Nevertheless little is known about its metabolic fate after oral administration. In recent years, a significant research effort has been spent towards the hydrolysis of the sugar moiety of the molecule.[9–11] The hydrolysis of the two esteric functions of the molecule has been studied by ^{1}H, ^{13}C and COSY NMR spectroscopy.[12,13]

Given the fact that HT, a potential hydrolysis product of OE, is one of the most potent antioxidants and may be closely related with its bioactivity, the hydrolytic stability of the OE molecule is considered of major importance towards the elucidation of its biological role. Therefore, it is crucial to evaluate the hydrolytic stability of OE under acidic conditions, because the *per os* administration of the molecule involves its metabolic degradation in the stomach under pH 1–3. In our study, three different concentrations of HCl have been used, simulating the stomach conditions during digestion. The establishment of Arrhenius equations may be also of significant importance because they can be employed to predict the hydrolysis of the molecule at different conditions, especially during the industrial processing of olive oil.

EXPERIMENTAL

Reagents and Chemicals

All solvents used throughout the experiments were of HPLC grade and have been were obtained by Merck, (Darmstad, Germany). μ-Hydroxyphenylethanol (used as internal standard) was purchased from Sigma-Aldrich GmbH, (Steinheim, Germany). Ammonium acetate was obtained from Riedel de-Haen GmbH, Germany. The purity of all organic reagents was checked by HPLC prior to their use. Oleuropein (OE) was isolated from olive leaves according to the procedure previously described[14] and μ-hydroxyphenylethanol (used as internal standard) was purchased from Sigma-Aldrich GmbH (Steinheim, Germany).

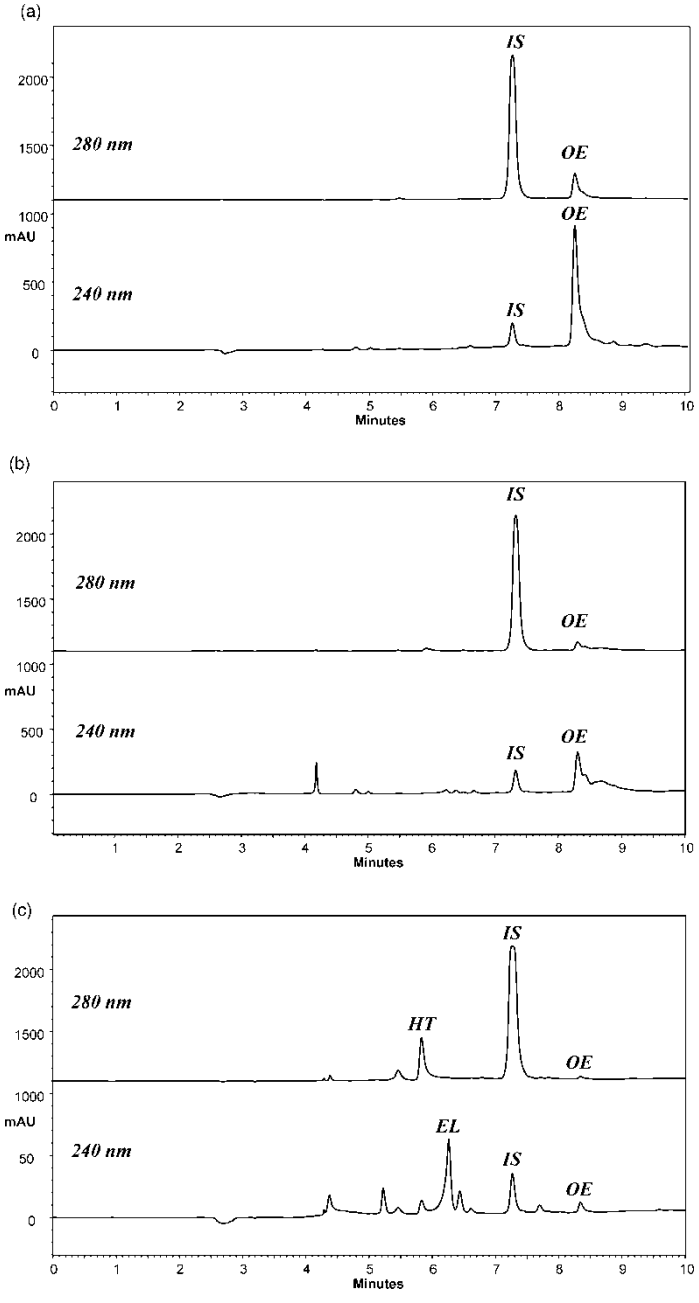

Figure 1. Panel A shows the chromatographic separation of OE and IS prior to hydrolysis (t = 0 min, 0.01 N HCl and 41°C). Panel B shows the hydrolysis pattern of OE after t = 315 min at 0.01 N HCl and 41°C, whereas panel C depicts the hydrolysis products after 20 days at 0.1 N HCl.

Instrumentation

The HPLC system comprising a Finnigan SPECTRA system P4000 quaternary pump equipped with a Rheodyne 7725i injector, an 100 μL loop, a Finnigan on line degasser and coupled to a Finnigan SPECTRA system UV6000LP diode array detector was utilized. HPLC controlling, data acquisition and processing was performed by the ChromQuest v.2.51 software connected to the HPLC by a Finnigan SN4000 controller. An Altech C_8 reversed-phase column (250 × 4.6 mm, I.D. 5 μm) equipped with a C_8 Altech precolumn was used throughout all chromatographic experiments. All solvents used were HPLC grade and they were vacuum filtered through a 0.2 μm Titan Membrane Filter and sonicated for 5 min prior to their use. The column was maintained at 40°C throughout all experiments with the aid of an electronically controlled oven.

Stock Standard Solutions

Stock standard solutions of OE and IS were prepared at the 1 mg/mL level by dissolving appropriate amounts of these compounds in methanol and at the 100 μg/mL level diluting appropriate volumes of the aforementioned solutions in HPLC water. The corresponding mixed working solutions were prepared every day diluting appropriate volumes of the stock standard solution of OE whereas a standard volume of the IS at the 10 μg/mL level was added separately at each dilution level of OE.

HPLC

The chromatographic separation of the two substances and the monitoring of OE hydrolysis were performed using a gradient elution program described in Table 1. Solvent A was 0.05 M ammonium acetate buffer adjusted to pH 5.0 with glacial acetic acid and Solvent B was acetonitrile. The flow rate was kept constant at 1 mL/min flow. After each run (mobile phase composition 90% A–10% B) a delay time of 3 minutes before the next injection was necessary in order to equilibrate the column.

Detection

UV spectra of the two substances were recorded with the aid of the diode array detector and the maxima of absorbance determined to be 240 nm for OE and 280 nm for the IS. These two wavelengths were used for data acquisition in all the chromatographic experiments. Additional confirmation of the UV spectra was performed by recording the spectrum of each separate substance by means

Table 1. Gradient elution program for HPLC separation of OE and IS on a C8 column

Time (min)	Solvent A (%)	Solvent B (%)	Flow (mL/min)
0	90	10	1
8	40	60	1
11	30	70	1
15	90	10	1
17	90	10	1

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

Solvent B: Acetonitrile.

of a Unicam UV-300 UV-Vis spectrophotometer using 10 mm optical path length quartz cuvettes.

Peak assignment was primarily based on retention time R_t (peak window 10% of R_t) and secondly on spectral identification based on diode array data.

Hydrolysis Procedure

An 200 μL aliquot from OE stock standard solution (1 mg/mL) was mixed with 50 μL of the IS stock standard solution (1 mg/mL). The resulting mixture was further diluted in 4.75 mL of a preheated HCl solution of suitable concentration (1, 0.1, and 0.01 N) in a screw capped amber-coloured vial. The latter was placed in an electronically controlled heated aluminum block. At given time intervals a 100 μL aliquot was drawn and mixed with 100 μL of an appropriate NaOH solution (1, 0.1, or 0.01 N) in order to adjust the solution pH to 7. This was done in order to quench the hydrolysis reaction, and avoid injecting a very acidic mixture to the column. This mixture was kept in an ice-water bath (i.e., 0°C) prior to its injection (100 μL). It should be noted that the first sample was drawn from the hydrolysis solution immediately after the addition of OE.

Statistical Evaluation

Linearity

For the estimation of the linearity region and the evaluation of the linearity relationship between concentration and response, six samples were analyzed (i.e., 20, 15, 10, 5, 2, 1, 0.5 μg/mL containing a standard amount of 10 μg/mL IS) and the resulting data were evaluated following least

squares regression analysis. Neither forcing through 0,0 nor weighting was applied.

Precision

The precision of the proposed method was estimated by injecting five replicates of a two substances mixture at three different levels (20, 10, and 2 μg/mL OE containing a IS standard amount corresponding to 10 μg/mL). The results were evaluated by calculating the %RSD.

The inter day reproducibility was estimated by injecting a sample at the 20 μg/mL level at four separate weeks and calculating the %RSD of the method.

Accuracy

The accuracy was evaluated as the %Er at three different concentration levels, which are different from those used in the linearity estimation, i.e., 20, 10, and 2 μg/mL.

LOD and LOQ

The limit of quantification and limit of detection for the proposed method were assessed as 3 and 10 times the S/N ratio. The last has been evaluated by injecting three blank samples (tree samples containing no OE and estimating the noise at the USP window of OE).

Robustness

The robustness of the HPLC method was evaluated for two major contributing parameters, the pH of the mobile phase and the AMA concentration. First, in the analysis of a 20 μg/mL sample the AMA concentration of the mobile phase was kept constant while its pH was adjusted to the values of 5, 6, and 6.8. On the other hand, the pH was kept constant (pH = 5) and the AMA concentration was altered from 30 mM to 40 and then, 50 mM.

Kinetic Investigation

From the results obtained from the hydrolysis of OE the corresponding log's of concentration were calculated and have been plotted vs time. The data were treated according to classical least squares regression analysis as it has been realized from the plot that the OE hydrolysis follows a first order mechanism. The hydrolysis experiments were conducted at three different temperatures and three pH's so the corresponding Arhenius plots can be extracted from these data.

RESULTS AND DISCUSSION

The accelerated kinetic investigation of substances like OE is of significant importance in order to determine two major goals namely its hydrolytic stability versus the low pH of the stomach (OE is an olive oil ingredient, hence its main pathway of administration to the body is *per os*) and to investigate its hydrolytic stability during the processing of olive oil. The chromatographic separation of OE and the IS is shown in Figure 1A. Figure 1B depicts a representative chromatogram of OE degradation after 315 min in 0.01 N HCl at 41°C. This chromatogram shows the formation of an intermediate product at $r_t \sim 4.2$ min, which was greatly reduced under prolonged hydrolysis conditions (at 0.1 N HCl) producing HT and EL (Fig. 1C). That illustrates that OE is not hydrolyzed at the esteric bond between the HT and EL moieties under the tested conditions.

Linearity, Reproducibility, and Accuracy

Following the experimental condition described previously a linear relation was established between response (calculated as the ratio of OE peak area vs that of the IS) and concentration. The correlation can be described by the following equation:

$$S_{OE/IS} = 0.0296\ (\pm 0.00093) * C_{OE} + 0.028\ (\pm 0.102)$$

where $S_{OE/IS}$ is the signal (peak area) ratio between OE and IS and C_{OE} is the added concentration of OE. The correlation coefficient r of the determination is $r = 0.998$ showing good linearity whereas the standard error of estimation was determined to be $Er = 0.017$.

The intra-day precision was evaluated by injecting multiple samples ($n = 5$) at three different concentrations corresponding at the high, middle and low area of the calibration curve (i.e. 20, 10, and 2 μg/mL respectively). The precision of the method was evaluated calculating the % relative standard deviation (%RSD). The values were found to be 3.40, 3,76, and 2.24 for the 20, 10, and 2 μg/mL concentration respectively.

The inter-day precision was calculated by injecting a 10 μg/mL sample at four different laboratory weeks. The %RSD value calculated was found to be 5.8% which together with the intra-day precision proves the validity of the proposed method in terms of reproducibility.

The accuracy of the method was determined by calculating the % Er (% relative percentage error) for the three concentration levels (2, 10, and 20 μg/mL). The mean OE/IS of the five assayed replicates was calculated against the nominal concentration found from the calibration curve and the corresponding values were found to be −2.84, 3.53, and −4.58% for the 20, 10, and 2 μg/mL concentration levels respectively.

Robustness

The robustness of the method was evaluated for three different values of pH (5, 6, and 6,8) and three different AMA concentrations (0.03M, 0.04 M, and 0.05 M). The robustness of the method was evaluated calculating the %RSD values of the retention time and peak area of the OE peak for the aforementioned AMA concentrations. It was found that upon changing the AMA concentration (under a stable pH value, i.e., 5) the retention time (%RSD = 0.3) as well as the peak area (%RSD = 2.8) of the OE peak remained practically unaffected. Similarly, change of the mobile phase pH (i.e., 5, 6, and 6.8) resulted in no change of the retention time (%RSD = 0.8) and peak area of (%RSD = 1.082) the OE peak.

LOQ and LOD

The LOD and LOQ were determined as three and ten times the S/N ratio for OE and found to be 0.15 μg/mL and 0.5 μg/mL respectively.

Specificity

The specificity of the method was demonstrated by its ability to give baseline resolution for the two injected substances (OE and IS) whereas in the same retention time the baseline drift presented no interfering peaks. Additionally the full UV spectrum of the substances recorded with the aid of the diode array detector showed no change in terms of peak shape and λ_{max} indicating that no other substance was co-eluted at the given retention time. Furthermore, the specificity of the chromatographic method is also shown by the baseline separation of OE and its two major constituents/metabolites, namely HT and EL.

Kinetic Data

The data from the chemical kinetic experiments of OE hydrolysis (peak area ratio of OE vs IS) were transformed to the corresponding logarithms (log) in order to facilitate their statistical analysis. This was feasible on the grounds of the realization that the hydrolysis followed first-order kinetics at least for the time interval selected for its monitoring. The last was determined as twice the $t_{1/2}$ of OE for each experiment. The $t_{1/2}$ was estimated as the time at which the area of the OE peak was reduced by 50% of its initial concentration.

As mentioned before the kinetic measurements were performed at three different HCl concentrations namely 1, 0.1, and 0.01 N and three different

temperatures at each HCl concentration (33°C, 37°C, and 41°C for 0.1 N HCl and 41°C, 51°C, and 61°C for 0.01 N HCl), except for the case of 1 N HCl where only two temperatures (27 and 33°C) were used. Figure 2 shows the decay of OE under different temperatures and 0.1 N HCl concentrations. In all steps of the hydrolysis experiments effort was made to avoid organic solutions (e.g., methanol for the preparation of the standard solutions of the calibration curve) in order to prevent other reactions to occur (e.g., transesterification of the free carboxylic acid moieties).

The fitting of the data to the proposed logarithmic linear model (which corresponds to first-order kinetics) with acceptable correlation coefficients not less than 0.99, indicates the close agreement of experimental and theoretically calculated data. The statistical evaluation of the hydrolysis is summarized in Table 2. From these data, it can be clearly seen that the hydrolysis is definitively acid catalyzed, and it can be readily anticipated that increasing the acid concentration the rate of hydrolysis increases. This

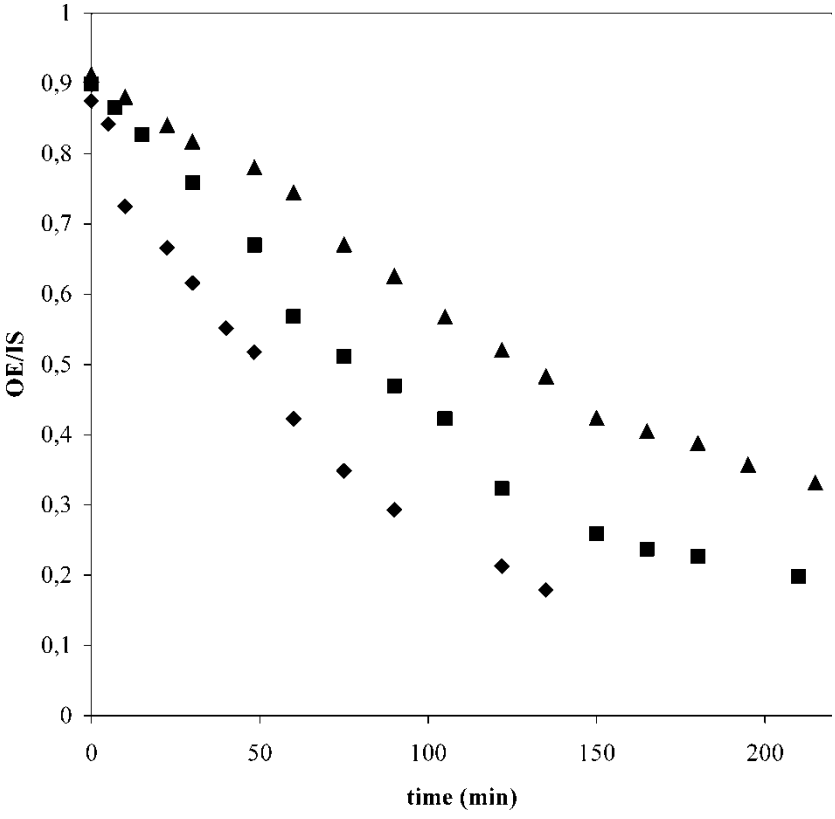

Figure 2. Hydrolysis profile of OE at 0.1 N HCl, at 41°C (◆), 37°C (■) and 33°C (▲).

Table 2. Statistical evaluation of the hydrolysis data of OE

HCl concentration (N)	Temperature (°C)	k_{obs} (min^{-1})	Correlation coefficient (r)	$t_{1/2}$ (min)
1	27	0.009	0.99	77
	33	0.0265	0.99	26
0.1	33	0.0022	0.99	315
	37	0.0034	0.99	204
	41	0.0052	0.99	133
0.01	41	0.005	0.99	1386
	51	0.013	0.99	533
	61	0.0047	0.99	154

can give a general idea for the metabolic fate of OE in the stomach, as its pH fluctuates generally between 1 and 3.

From the resulting data Arrhenius plots can be constructed for different temperature and pH values, giving the estimated reaction rate constants k_{obs}, expressed in min^{-1} according to the equation

$$K_{obs} = Ae^{E_a/RT}$$

In the preceding equation A is a experimentally determined nonthermal constant termed the frequency factor, E_a is the activation energy of the reaction, R is the gas constant ($R = 8.315\,J/mol\,K$) and T the absolute temperature (expressed in °K). The results are summarized in Table 3. The Arrhenius plots were constructed only for two HCl concentrations (0.1 N and 0.01 N) as at least three temperature levels are required. The data are summarized in Table 3. Extrapolating the equation for the 0.01 N HCl concentration at 37°C, the $t_{1/2}$ value calculated to be 2357.14 which combined with the corresponding value at 37°C and 0.1 N HCl namely 203.8 min indicate that the hydrolytic stability of the analyte is relatively high at the conditions of the stomach (pH ranging from 1–3).

Table 3. Statistical evaluation of the hydrolysis data of OE

HCl concentration (N)	Arrhenius equation	Correlation coefficient (r)
0.1	$\ln k_{obs} = -10331.41\ (\pm 4.6)\ 1/T + 27.643\ (\pm 0.0148)$	1
0.01	$\ln k_{obs} = -11502.71\ (\pm 1071.45)\ 1/T + 28.97\ (\pm 3.31)$	0.99

CONCLUSIONS

The proposed method has been demonstrated to be reliable in terms of linearity, reproducibility, accuracy, precision, specificity and robustness. Thus it can be used for the monitoring of OE content in various samples. In addition, it has been proved to adequately separate the major metabolites of OE, such as HT and EL. The large values of $t_{1/2}$ indicate that OE is relatively stable in the acidic conditions of the stomach (37°C and pH 1–3), therefore indicating that its main metabolic pathway is governed by enzymatic hydrolysis. Nevertheless, OE is readily hydrolyzed in higher HCl concentrations (e.g., 1 N) even at lower temperatures.

The chromatographic data also point out that OE, under the tested conditions, is not hydrolyzed at the esteric bond between the HT moiety and the EL, even at 1 N HCl at $t_{1/2}$. This is shown by the formation of an intermediate product detected at $r_t \sim 4.2$ min, which is greatly reduced under prolonged hydrolysis (at 1 N HCl at 27°C) producing HT and EL. Nonetheless, the exact mechanism of OE hydrolysis is currently under investigation in our laboratory using liquid chromatography-mass spectrometry methodology.

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