Stability and solubility of *trans*-resveratrol are strongly influenced by ph and temperature

Špela Zupančič, Zoran Lavrič, Julijana Kristl

PII: S0939-6411(15)00168-X
DOI: http://dx.doi.org/10.1016/j.ejpb.2015.04.002
Reference: EJPB 11892

To appear in: *European Journal of Pharmaceutics and Biopharmaceutics*

Received Date: 30 December 2014
Revised Date: 7 March 2015
Accepted Date: 6 April 2015

Please cite this article as: Š. Zupančič, Z. Lavrič, J. Kristl, Stability and solubility of *trans*-resveratrol are strongly influenced by ph and temperature, *European Journal of Pharmaceutics and Biopharmaceutics* (2015), doi: http://dx.doi.org/10.1016/j.ejpb.2015.04.002

STABILITY AND SOLUBILITY OF *TRANS*-RESVERATROL ARE STRONGLY INFLUENCED BY pH AND TEMPERATURE

Špela Zupančič[1], Zoran Lavrič[1], Julijana Kristl[1*]

[1] University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia

E-mails: spela.zupancic@ffa.uni-lj.si, zoran.lavric@ffa.uni-lj.si, julijana.kristl@ffa.uni-lj.si

* Corresponding author.

University of Ljubljana, Faculty of Pharmacy, Aškerčeva cesta 7, 1000 Ljubljana, Slovenia.

Tel.: + 386 1 476 9521;

Fax: + 386 1 425 8031

E-mail: julijana.kristl@ffa.uni-lj.si

ABSTRACT

Recently *trans*-resveratrol (*trans*-RSV) has received great attention due to its prophylactic and therapeutic properties. Its limited bioavailability provides compelling evidence of the need for more suitable formulations in order to attain better clinical effectiveness. Some physicochemical properties of *trans*-RSV are still unknown or research findings are contradictory. Therefore, this paper presents newly determined *trans*-RSV solubility and stability at various pH and temperatures, and the importance of such data for the studies of novel *trans*-RSV-loaded nanofibers. In acidic pH *trans*-RSV was stable, whereas its degradation started to increase exponentially above pH 6.8. Consequently, it is worthwhile to note that special consideration has to be dedicated to long dissolution testing or biological assays on cell lines in order to obtain relevant data. Measurements were done by validated UV/VIS spectroscopy, HPLC, and newly developed UPLC methods. Specificity was confirmed for HPLC and UPLC method, whereas UV/VIS spectroscopy resulted in false higher *trans*-RSV concentrations in conditions under which it was not stable (alkaline pH, light, increased temperature). The study is of interest because it draws attention to the importance of careful selected experimental conditions, their influence on the *trans*-RSV stability and the implications this has for formulation development, storage, and maintenance of therapeutic doses.

Keywords: resveratrol, stability, solubility, analytical methods, validation, isomerization, degradation, nanofibers, release, drug delivery

Chemical compounds

Acetonitrile (PubChem CID: 6342); Chloroform (PubChem CID: 6212); Formic acid (PubChem CID: 284); Methanol (PubChem CID: 887); Phosphoric acid (PubChem CID: 1004); Potassium dihydrogen phosphate (PubChem CID: 516951); Sodium hydroxide (PubChem CID: 14798); Sodium iodide (PubChem CID: 5238); Sodium tetraborate decahydrate (PubChem CID: 22435386); *Trans*-resveratrol (PubChem CID: 445154)

1 INTRODUCTION

Trans-resveratrol (3,5,4′-trihydroxystilbene; *trans*-RSV), one of the most promising naturally occurring compounds, has been shown to exhibit a wide range of therapeutic and preventive properties. Several studies have confirmed its anti-inflammatory, cardioprotective, antitumor, neuroprotective, and antioxidant activities [1, 2]. Unfortunately, its *in vivo* applications are severely limited due to low bioavailability as a consequence of its poor aqueous solubility, limited stability, and extensive liver metabolism [2, 3]. The research community is working to solve these problems by formulating new drug delivery systems that stabilize and protect *trans*-RSV from degradation, increase its solubility in water, achieve sustained release, and target *trans*-RSV to specific locations via multiparticulate and nanodelivery systems [4-7]. In addition, *trans*-RSV has attracted attention not only from the pharmaceutical but also cosmetics [8] and food [9] industries.

Although there are currently more than 6,830 publications referenced on the US National Library of Medicine's PubMed service about *trans*-RSV, some basic physicochemical properties of *trans*-RSV are still unknown or the research findings frequently contradict each other. Comprehensive studies of *trans*-RSV stability and solubility in water are scarce, but these represent fundamental information required for developing a dosing regimen, preparing carrier systems and their loading ability, and choosing experimental conditions for *in vitro* and *in vivo* experiments. Some studies have claimed that *trans*-RSV has limited stability under the influence of light, certain pH levels, and temperature, which can cause *trans*-RSV isomerization or degradation [10]. *Trans*-RSV is highly photosensitive and rapidly isomerizes to *cis*-RSV when exposed to ultraviolet or visible light [2]. Unfortunately, the less stable *trans*-RSV is more biologically active [11]. Isomerization kinetics is affected by irradiation time, wavelength, the physical state of *trans*-RSV, and temperature [12, 13].

By contrast, the effect of pH on *trans*-RSV stability has been investigated in very few studies. Trela and Waterhouse in 1996 proved that *trans*-RSV is stable in buffers with pH 1, 3.5, and 7.0 for 28 days, whereas at pH 10 it rapidly degraded with a half-life of only 1.6 hours [12]. To the best of our knowledge, there are no published data on *trans*-RSV degradation kinetics at specific pH levels relevant to physiological fluids.

The impact of temperature on *trans*-RSV stability was studied at very different temperatures. For example, Liazid et al discovered that when *trans*-RSV was exposed for 20 minutes to increased temperatures the extent of degradation is 17% at 125 °C, 39% at 150 °C, and 70% at 175 °C [14]. On the other hand, Sessa et al. also evaluated *trans*-RSV stability and found a higher degradation rate at 4 °C than at 30 and 55 °C during 30 days [9]. Comparing different studies' findings shows that *trans*-RSV in solution is not stable at either low or high temperatures [9].

Concerns have also been raised regarding the correct values of *trans*-RSV water solubility due to different data observed in the literature. The data for *trans*-RSV solubility obtained by different authors varies from 50 ng/L (at pH 6 and 8 at 37 °C) [15] to 19.6 mg/L (in water at 25 °C) [16] or 21 mg/L (at pH 7.4 and 37 °C) [17], despite the relatively similar experimental conditions.

For identifying and quantifying *trans*-RSV in solutions and plasma various analytical methods have been used involving UV/VIS spectroscopy (UV/VIS) [13, 18, 19], high performance liquid chromatography (HPLC) coupled with fluorescence, UV/VIS [9, 20-22] or mass spectrometry [23] detection, and gas chromatography with mass selective detection (GC-MS) [24]. An overview of the literature about *trans*-RSV showed that validations of the HPLC method were usually performed for determining the *trans*-RSV content in wine [24] or in *in vivo* pharmacokinetic studies [23], covering the samples with several metabolic or degradation compounds. By contrast, in the reports that have dealt with formulation development, generally no validation of analytical methods is specified, with a rare exception in which the validation was provided and results reported [22].

Considering the low stability and solubility of *trans*-RSV, our hypothesis was that careful selection of particular experimental conditions and analytical methods would improve the consistency of findings. Therefore, a new analytical method, UPLC with photodiode array detection (UPLC) for analysis of *trans*-RSV separated from *cis*-isomer and degradation products, was developed and validated. The findings were compared with those of the UV/VIS and HPLC methods commonly used. The *trans*-RSV stability and solubility were determined at the conditions, which resemble as much as possible physiological or storage conditions. In addition, as a case study, the release of *trans*-RSV from newly developed nanofibers was determined and the influence of pH and the selection of analytical method on the release profiles was investigated. All the findings are compared with some published data in order to determine the

key points for careful analysis, formulation, and preparation of products that consistently maintain therapeutic dose of *trans*-RSV.

2 METHODS AND MATERIALS

2.1 Materials

Trans-RSV was purchased from LGC Standards GmbH, Germany with a declared purity of 99.6% according to the manufacturer. Orthophosphoric acid 85%, potassium dihydrogen phosphate, formic acid, acetic acid, sodium chloride, sodium iodide, hydrochloric acid fuming 37%, sodium tetraborate decahydrate, sodium hydroxide, chloroform, and acetone (for analysis) were obtained from Merck KGaA (Germany). Poly(ε-caprolactone) PCL (Mr 70.000–90.000 g/mol) was purchased from Sigma Aldrich (Germany). Acetonitrile and methanol of HPLC grade were purchased from J. T. Baker (the Netherlands). Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA) and used for preparing the mobile phases. Deionized water (type 2, analytical grade) was obtained from Millipore Elix 35 Water Purification System (Millipore Corp., Burlington, MA, USA) and used throughout the experiments. Buffers with pH 1.2, 6.8, and 7.4 were prepared according to Ph. Eur. Chapter 5.17.1. [25]. The pH of each freshly prepared buffer was adjusted to the prescribed pH using a pH meter (220 SevenCompact™ pH/Ion meter, Mettler Toledo, Switzerland). Filters with pore size 0.2 µm (Minisart RC 15, Sartorius Stedim Biotech GmbH, Germany) were used prior to each sample analysis. All RSV solutions were protected from light during the experiments unless otherwise stated.

2.2 Standard solution preparation

Initial standard stock solutions of *trans*-RSV with concentrations of 1 mg/mL were further diluted with water supplemented with 0.1% formic acid in order to obtain seven standard solutions in the range of 0.5–15 µg/mL.

2.3 Analytical methods

2.3.1 UV/VIS spectroscopy

UV–VIS absorption spectra of the samples were recorded in the range of 240–370 nm with 1 nm resolution using a UV/VIS spectrophotometer (Hewlett Packard 8453, Germany) at room temperature. Maximal *trans*-RSV absorption in aqueous solution at 306 nm was used to calculate the *trans*-RSV concentration.

2.3.2 HPLC method

Trans-RSV was analyzed using the HPLC method previously described by Bolko et al. with small modifications [20]. Briefly, *trans*-RSV was determined with HPLC (Agilent 1100 Infinity system, Agilent Technologies Inc., USA) using reverse phase column C18 (3.145 μm particle size, 250x4.6 mm, YMC Europe GmbH, Germany) at 30 °C. The mobile phase was a mixture of water and methanol 50:50 (v/v) supplemented with 0.25% (v/v) acetic acid. The auto-sampler temperature was set at 4 °C. The injection volume was 20 μL, flow rate 1 mL/min, and run time 15 min. A UV scan was obtained from 240 to 370 nm with resolution 2 nm and area under the curve of *trans*-RSV peak at 306 nm was chosen for detection and further calculation of the *trans*-RSV concentration.

2.3.3 UPLC method

The UPLC method was developed to separate *trans*-RSV from *cis*-RSV and degradation products with better resolution than with HPLC. *Trans*-RSV was determined by the chromatographic system Acquity UPLC (Waters Corp., USA). A UV-VIS photodiode array module (PDA) equipped with a high-sensitivity flow cell was used for detection. The column with pre-column was Acquity UPLC HSS C18 SB 1.8 μm 2.1 × 100 mm (Waters Corp., USA). A gradient elution was used to achieve chromatographic separation with a mobile phases A (water, containing 0.1% formic acid and 10% acetonitrile) and B (acetonitrile, containing 2% water). The mobile phase in the gradient elution progress was: 0–1 min 0% B, 1.5 min 1.7% B, 2.5 min 9.1% B, 7 min 17.6% B, 8 min 28.3% B, 9–11 min 52.4% B, and 11.1–13 min 0% B. The flow rate was set at 0.4 mL/min and the column temperature was maintained at 40 °C. The auto-sampler temperature was 4 °C and the injection volume was 5 μL. The analytical run time for each sample was 13 min. UV

scan was obtained from 240 to 370 nm with resolution 0.6 nm and the *trans*-RSV concentration was calculated as above..

2.4 Validation of analytical methods

UV/VIS spectroscopy, HPLC, and UPLC methods were validated in terms of specificity, linearity, accuracy, precision, detection limit, and quantitation limit. The various validation parameters and values for accepting the range of validation parameters were in accordance with both ICH and FDA guidelines [26, 27].

Specificity was evaluated by measuring the blank solution, the *trans*-RSV standard (5 µg/mL), a mixture of *trans*- and *cis*-RSV, and 85% or 7.5% of *trans*-RSV with degradation products in the sample. The resulting mixtures were then analyzed for peak purity by scrutinizing UV/VIS spectra from diode array detector. The mixture of *trans*- and *cis*-RSV was prepared from the 50% methanol *trans*-RSV solution by exposure to UV light (366 nm) for 2 min at room temperature, as was similarly described by Camont et al. [13]. The mixture of *trans*-RSV and its degradation products were formed by *trans*-RSV degradation at alkaline pH. Resolution from the nearest eluting peaks was determined according to ICH guidelines in the sample with a mixture of 85% *trans*-RSV and degradation products [26].

Linearity was determined by calculating a regression line from the plot of the peak area versus concentration for the seven standard solutions (0.5, 1, 2, 5, 7, 10, and 15 µg/mL) using the least squares method.

Detection limit and *quantitation limit* were determined from their respective calibration curves with equations described in ICH guidelines [26].

Accuracy was assessed using three concentration levels (1, 5, and 15 µg/mL) with three replicates which were prepared by three different stock solutions and reported as average percent recovery by the difference between the mean and the accepted true value.

Precision was assessed at two levels: repeatability and intermediate precision. *Repeatability* was evaluated with concentrations 1, 5, and 15 µg/mL, which were injected three times into UPLC system over short interval of time. *Intermediate precision* was evaluated by analyzing three

different standard samples (1, 5, and 15 µg/mL) on three different days. The results were reported as the relative standard deviation (RSD).

2.5 Determination of *trans*-RSV chemical stability

Solutions with approximate 10 µg/mL of *trans*-RSV were prepared in buffers with pH 1.2, 6.8, and 7.4 and stored at −22, 4, 25, and 37 °C for 90 days. At different time points the samples were taken, filtered, and analyzed. Due to rapid degradation of *trans*-RSV at higher pH, stability studies were performed a bit differently. Solutions with approximate 20 µg/mL of *trans*-RSV were prepared in buffers with pH 8.0 (50 mM phosphate buffer), 9.0 (25 mM borate buffer), and 10.0 (50 mM carbonate buffer) stored at 37 °C. Samples (4 mL) were withdrawn and immediately added to 4 mL of 50 mM HCl to stop the degradation process (final pH was below 4). The samples were analyzed by UV/VIS, HPLC, and UPLC method after filtration. The order of the degradation reactions was determined with the graphic method [28]. Additionally, the effect of temperature on the degradation rate of *trans*-RSV was characterized by the Arrhenius equation:

$$ln\, k = ln\, A - \frac{E_a}{RT}$$

where A is pre-exponential factor typical for *trans*-RSV degradation, E_a is activation energy (J/mol), R is universal gas constant (8.314 J/Kmol), and T is the absolute temperature.

2.6 Determination of *trans*-RSV solubility at different pH

The solubility of *trans*-RSV was determined by adding an excess of *trans*-RSV into 100 mL buffers with pH 1.2, 6.8, and 7.4. The suspensions were continuously stirred with a magnetic stirrer at 200 rpm and 37 °C for 72 h. At three different time points (24, 48, and 72 h) samples were withdrawn, immediately filtered, properly diluted and analyzed.

2.7 Preparation of nanofiber mats with *trans*-RSV and release study

Trans-RSV-loaded PCL nanofibers were prepared by electrospinning method. First, a 10% (w/w) PCL solution was prepared by dissolving polymer and 0.03% (w/w) NaI in mixture of chloroform

and acetone in ratio 3:1 (w/w). *Trans*-RSV (5% w/w based on the mass of dry nanofibers) was added to the PCL solution and mixed by Ultra Turrax T25 (Janke & Kunkel, IKA Labortechnik, Germany) for 5 minutes at 15,000 rpm to obtain a homogenous solution. The electrospinning was conducted at 15 kV, nozzle-to-planar collector distance 15 cm, and solution flow rate 1.3–2.5 mL/h resulted in nanofiber mats. The nanofiber morphology was examined using a 235 Supra 35VP-24-13 high-resolution scanning electron microscope (SEM) (Carl Zeiss,Germany) as described by Rošic [29].

Trans-RSV release was performed on a fully calibrated dissolution apparatus using the paddle method (USP Apparatus I, VanKel Dissolution Apparatus, model VK 7000, USA). 600 mL buffers with pH 1.2, 6.8, and 7.4 were used as dissolution media. To prevent nanofiber floating, 102 ± 2 mg mats were affixed to brackets that sank at the bottom of the vessel at the beginning of the experiment. The temperature was maintained at 37 ± 0.5 °C and vessel speed was kept at 50 rpm. At each sampling time point (0.25, 0.5, 1, 2, 3, 4, 8, and 12) 10 mL samples (not replaced) of the dissolution medium were withdrawn, filtered, and immediately analyzed.

2.8 Statistical analysis

All experiments were performed at least in triplicate and the data are expressed as mean ± standard deviation (S.D.). The results were statistically analyzed using repeated measured analysis of variance (ANOVA) and one-way ANOVA, followed by Turkey's post hoc test (PRISM software package, Version 5, Graphpad Software Inc., San Diego, USA).

3 RESULTS

3.1 Validation of analytical methods

Validation of UV/VIS, HPLC, and UPLC methods was performed to ensure accurate, specific, and reproducible results across the concentration range.

3.1.1 Specificity

The specificity of the selected analytical methods was evaluated on blank solution, standard solution, the mixture of both isomers and *trans*-RSV with degradation products. Blank solution

did not show any peaks or interference on the spectrum or on the chromatograms. Trans-RSV standard substance in solution eluted as a single peak at retention times 6.2 and 7.0 min for HPLC and UPLC, respectively (Fig 1). The spectrum of *trans*-RSV standard was the same in all three methods with the peak varying from 304 to 306 nm.

FIGURE 1 – 2 columns

The trans-RSV exposure to UV light resulted in isomerization. Its absorption maximum decreased at 306 nm and a new peak increased at 287 nm, representing the maximum of *cis*-RSV with time (Fig. 2A). The sample containing both isomers measured by all three methods is presented in Fig. 1. *Cis*-RSV eluted later than *trans*-RSV, with retention times 8.8 min in HPLC and 7.7 min in UPLC, due to its more lipophilic nature. The resolution between both peaks was 11.75 for HPLC and 7.5 for UPLC, which exceeded the required resolution (1.5). Extracted chromatographic peaks clearly showed that *cis*-RSV also absorbs at 306 nm. Therefore, the UV/VIS spectrum contained the sum of absorbances of both isomers, whereas the contribution absorbance of each isomer to the spectrum cannot be determined precisely.

FIGURE 2 – 1 column

By contract, the increased media pH caused trans-RSV degradation, and the process with time is presented in the UV/VIS spectra (Fig. 2B). When *trans*-RSV degraded, its maximum also decreased and absorbance increased at 265 nm, showing degradation compounds. The formation of degradation product was confirmed by HPLC and UPLC (Fig. 1). The two main degradation products had lower retention times due to more hydrophilic nature and absorbed at 268 and 274 nm for HPLC, and at 267 and 279 nm for UPLC, which is in line with new appearing peak in spectrum obtained by UV/VIS. However, as is seen in this spectrum, there was not possible to determine the contribution of degradation products in mixture with trans-RSV in absorption curve. Consequently, UV/VIS method was not enough specific, whereas both chromatographic methods match the required specificity (HPLC resolution was 3.5 and UPLC 2.7, required 1.5).

Although, more and better separated peaks were observed on the chromatograms of UPLC compared to HPLC. Thus, the UV/VIS method does not enable the separation of *trans*-RSV from *cis*-RSV and degradation products and due to their contribution to the absorption at 306 nm the HPLC or UPLC methods are advised to be used.

3.1.2 Linearity and sensitivity

Across the selected concentration range of *trans*-RSV in solution, a linear relationship between the absorbance or the peak area and *trans*-RSV concentration was obtained by all three methods with correlation coefficient (r) 1.0000 (Table 1), which is above the required value (1.0000>0.999) [27]. The UV/VIS method was the most sensitive (Table 1).

TABLE 1

3.1.3 Accuracy and precision

Accuracy, presented as % of recovery, and precision, as repeatability and intermediate precision in RSD, are shown in Table 2. Accuracy (98–102%), precision (< 1%), and intermediate precision (< 2%) are within the acceptable limits for all analytical methods as required [27, 30]. The precision is better at higher concentration.

TABLE 2

3.2 Stability of *trans*-RSV at various pH levels and temperature

As evident from Fig. 3, *trans*-RSV degradation varies tremendously in aqueous solutions with different pH levels. *Trans*-RSV was demonstrated to be relatively stable in acidic conditions, whereas its degradation rate exponentially increases in alkaline ones. Results obtained by all three analytical methods are similar only at pH 1.2, which means that most of *trans*-RSV is relatively stable up to 90 days. At all higher pH levels it is clearly seen that HPLC and UPLC show lower stability of *trans*-RSV than the UV/VIS, which reveals a smaller decrease of its concentration. However, the results of both chromatographic methods are well-matched.

FIGURE 3 – 1 column

The calculated data for kinetics of *trans*-RSV degradation obtained at various pH levels, correlation coefficients, and the half-lives are shown in Table 3. The *trans*-RSV degradation in the pH range 1.2–8.0 followed first-order kinetics with a correlation coefficient higher than 0.99. At pH 9.0 there was a combination of zero- and first-order kinetics, whereas at pH 10.0 *trans*-RSV was stable for the first 2 min and then rapidly degraded with zero order. It is important to point out that the half-life of *trans*-RSV is given in days for the pH range up to 7.4, in hours at pH 8.0, and in minutes at pH 9.0 (Table 3). For example, its half-life at pH 1.2 was 329 days, which decreased to 3.3 minutes at pH 10. *Trans*-RSV in buffer with pH 9.0 was totally degraded in less than 24 min and at pH 10.0 in less than 9 min (Fig. 3). The differences between the chromatographic and UV/VIS methods increased with time due to the increased amount of degradation products. For example, at after 8 days at pH 7.4 (Fig. 3) the concentrations obtained by UV/VIS were highly inaccurate being approximately 700% higher as compared to HPLC or UPLC. Consequently, the false UV/VIS data resulted in the lower calculated degradation constants and the longer half-life of *trans*-RSV (Table 3).

TABLE 3

The stability of *trans*-RSV was high in the acidic conditions, whereas in the alkaline pH range of 6.8–9.0 the degradation constant increased exponentially. In the latter case it was found that the pH dependence of the degradation rate constant k could be adequately described by an empirical relation in the form of equation: $\ln(k) = 3.882\text{pH} - 32.685$ ($r = 0.9998$) (Fig. 4).

FIGURE 4 – 1 column

The next study, the effect of temperature on *trans*-RSV stability in aqueous solutions with various pH levels was monitored in the range of –22 to 37 °C for three months. At pH 7.4 *trans*-RSV rapidly degraded at 25 and 37 °C, whereas the degradation was slowed at 4 °C and prevented at –22 °C (Fig. 5). The kinetics of *trans*-RSV degradation at pH 7.4 and the

temperatures investigated followed first-order kinetics. The effect of temperature on the degradation rate of *trans*-RSV was expressed by a linear plot of Arrhenius equation $\log k_{obs}$ = 14.063 – 4425(1/T) (r=0.97). The calculated activation energy of *trans*-RSV was 84.7 kJ/mol. While not presented in detail, at different pH similar conclusions were derived. For instance, *trans*-RSV degradation rate at 6.8 was lower at lower temperatures, e.g. a 1.2-fold decrease at 25 °C and a 22-fold decrease at 4 °C as compared to 37 °C. To conclude, in acidic media in which *trans*-RSV was stable, temperature had the minimal impact on degradation. In contrast, at alkaline pH higher temperatures further accelerated the *trans*-RSV degradation rate.

FIGURE 5 – 1 column

3.3 *Trans*-RSV solubility at different pH levels

Solubility of *trans*-RSV at pH 1.2 was 64 µg/mL, at pH 6.8 61 µg/mL, and at 7.4 50 µg/mL after 72 h, measured by UPLC (Table 4). The one-way ANOVA statistical test did not show any significant difference between *trans*-RSV solubility at pH 1.2 and 6.8, but at pH 7.4 it was significantly lower ($p < 0.05$) irrespective of the analytical method used. These results clearly indicated that already weak alkaline pH affected *trans*-RSV solubility. The solubility data obtained by different methods in the same time interval did not significantly differ at pH 1.2 and 6.8, but solubility at pH 7.4 was markedly different. UV/VIS data were significantly ($p < 0.05$) higher for all three time points (by 10.3 %, 11.9 %, and 18.8 %, respectively) as compared to UPLC results. These results were not only the consequence of solubility but also the simultaneous degradation and lower specificity of the UV/VIS method.

TABLE 4

3.4 Release profiles of *trans*-RSV from nanofiber mats

Trans-RSV release profiles from nanofiber mats with 5% drug and an average nanofiber diameter of 510 ± 280 nm at various pH levels are presented in Fig. 6. Most of the *trans*-RSV was released in the first 4 h and the remainder in the following 8 h in a sustained manner. The release profiles

at pH 1.2 and 6.8 were not dependent on pH or on the analysis method used. In the medium with pH 7.4 different release profiles were obtained, because *trans*-RSV degradation took place immediately after its release from the nanofibers. This was confirmed by appearance of new peaks in the chromatograms. After 12 hours at pH 1.2 *trans*-RSV release reached 76.8%, at pH 6.8 80.1%, and at 7.4 pH it dropped to 48.6% (data obtained by UPLC). Comparison of release profiles obtained by different analytical methods showed that the results obtained by UV/VIS were found to be increasingly inaccurate the longer the experiment progressed.

FIGURE 6 – 2 columns

4 DISCUSSION

Previous studies have often reported the low stability and solubility of *trans*-RSV, but it was rarely measured and discussed systematically. While previous study has reported that *trans*-RSV is stable for months, except in high-pH buffers [12], our work shows that already at pH above 6.8 *trans*-RSV started to degrade significantly. Higher degradation rates were observed after 24 h in buffers with pH 7.4 at 37 °C. This was also reported by other authors [31, 32]. *Trans*-RSV was stable in acidic pH because its hydroxyl groups were protected from radical oxidation by positively charged H_3O^+ [10]. On the contrary, t*rans*-RSV was not stable in alkaline conditions. Its degradation followed first order kinetics with reaction rates that increased exponentially in the pH range 6.8–8.0 (Table 3). The degradation profiles above pH 8 were more complex and cannot be described by simple first order kinetics. At pH 9 the degradation profile seemed to be composed of two distinct regions with zero and first order kinetics and at pH 10 the degradation profile seemed like an autocatalytic degradation process where some small amount of induction time passes before the reaction started (Fig. 3 and Table 3). At pH 9.0 one hydroxyl group of *trans*-RSV was predominantly deprotonated (depending on the study, pK_{a1} varies from 8.1 to 8.8. [33]), whereas at pH 10.0 one hydroxyl group was fully deprotonated and another partially so (pK_{a2} is 9.7–9.9 [33]). This suggests that the degradation mechanism depended on the degree of dissociation of *trans*-RSV hydroxyl groups. The presence of phenate ions in the *trans*-RSV structure made it particularly susceptible to oxidation due to electrophilic attack on this ion, leading to the formation of the phenoxy radical [34]. *Trans*-RSV and its radical form led further to the formation of several secondary degradation products excluding *cis*-RSV (Fig. 1).

Additionally, Callemien et al. reported that the presence of oxygen in the medium promoted radical reactions leading to *trans*-RSV degradation [35]. The *trans*-RSV stability was also dependent on the temperature. Degradation process exhibited intermediate sensitivity to temperature, because its activation energy was within the range of 50–96 kJ/mol [36]. Therefore, at 37 °C degradation was fast, whereas the freezing completely stopped the reaction by the decreased molecular mobility. Consequently, its stability can be increased not only with a lower pH but also with a lower temperature, as our findings confirmed (Fig. 5). To summarize, the impact of individual factors appear in the following order (Table 5): UV light causes isomerization of *trans*-RSV in minutes (Fig 2), pH effects differently on its degradation rate (Table 3), and increased temperature enhance the degradation (Fig. 5). Thus the special attention has to be dedicated to the experimental and storage conditions due to the low stability of *trans*-RSV.

TABLE 5

Trans-RSV stability has been improved with special drug delivery systems or solid dosage forms. For example, *trans*-RSV encapsulation in a nanoemulsion prevented oxidative degradation at 4 °C but was not effective at higher temperatures [9], and the stability of *trans*-RSV suspension during 1 year of storage at pH 7.4 was improved by the addition of carboxymethylated (1,3/1,6)-β-D-glucan [37]. When Jensen et al. evaluated crystalline *trans*-RSV according to ICH guidelines for stability testing, they found that *trans*-RSV is stable for at least 3 months at accelerated and ambient conditions even when exposed to UV and florescent light [38]. Therefore, crystalline *trans*-RSV or solid delivery systems with incorporated *trans*-RSV are the most appropriate form from the stability point of view, as is also supported by our study using nanofibers as an attractive novel drug delivery system [39].

The effect of pH on *trans*-RSV solubility was less pronounced as on stability (Table 4). The solubility in buffers with pH 1.2 and 6.8 was approximately 60 μg/mL, whereas *trans*-RSV solubility at pH 7.4 was 14% lower. This effect was due to lower *trans*-RSV stability at pH 7.4, in which dissolution and degradation occurred at the same time, resulting in the increasing concentration of degraded products which decreased *trans*-RSV solubility, probably due to

formation of insoluble complexes between them, as has already been shown for other drug mixtures [40]. To the best of our knowledge, the reduction in *trans*-RSV solubility at pH 7.4 as compared to 1.2 or 6.8 has not been published before. The *trans*-RSV solubility was determined at 37 °C because this temperature is recommended for dissolution studies in sink conditions [25] and for *in vitro* experiments on cell lines [41]. However, most studies reported *trans*-RSV solubility at 25 °C to be 19.6–30 µg/mL [16, 42]. Solubility usually increases with temperature [43], which could explain higher solubility determined in the study. However, other authors reported *trans*-RSV solubility at 37 °C varying from 50 ng/mL to 20 µg/mL, which is not in line with our results. Solubility data serve as a base for design of dosage forms and for decisions regarding testing methodology. For example, the sink conditions for release studies cannot be determined without knowing the solubility of the drug. To overcome low solubility in case of some poorly soluble drug the addition of surfactants is sometimes necessary [25]. Das and Ng claimed that *trans*-RSV solubility in simulated intestinal fluid was too low to observe its release in standard dissolution apparatus even in the presence of a high amount of surfactant. Hence, they devised an alternative method in which they estimated the amount of *trans*-RSV remaining in their formulation after each time point instead of measuring the drug released in the medium [44]. On the contrary, our solubility data, release study, as well as results of several other researchers demonstrates that the release studies of *trans*-RSV in different formulations can be normally obtained in buffers without the addition of any surfactant [18, 20, 21, 45].

The optimization of electrospinning parameters enabled preparation of PCL nanofibers with successfully incorporated *trans*-RSV without visible any crystals on the surface. The dominated mechanism of *trans*-RSV release from PCL nanofibers was desorption from the polymer surface, while the PCL nanofibers are not degrading at an appreciable rate, as was shown in other drug-loaded PCL nanofibers [46]. The *trans*-RSV release was not affected by its degradation in acidic medium. However, we determined significant difference at pH 7.4 and 37 °C after three hours (Fig. 6), when the *trans*-RSV degradation started to become substantial (Fig. 3). To the best of our knowledge, there are no published data reporting the problem of *trans*-RSV degradation during release studies. For example, *trans*-RSV release profiles always increased with time with no degradation effects exhibited in various published articles [18, 45, 47]. Only Sessa et al. 2014 reported degradation due to other reasons, including dissolved oxygen, temperature, and light [21]. The possible explanation could be that the release rate dominated over the degradation rate,

as can be seen from our results. In the first 4 h, release was the dominant process, which can be observed by the increased slope in the release profile. The subsequent sustained release was slower compared to degradation kinetics resulting in the decreased *trans*-RSV concentration in the medium (Fig. 6). *Trans*-RSV degradation can be an alternative interpretation to the reasons as to why the release profiles of formulations did not reach 100% of released *trans*-RSV [45, 47]. Importantly, using the UV/VIS method for long releases at pH 7.4 and 37 °C results in falsely higher concentrations and therefore in faster release profiles, which may mask *trans*-RSV degradation. Consequently, published results obtained at these conditions may not present the actual release profile [18].

Awareness of the high *trans*-RSV degradation rate in buffers with pH 7.4 is important also in several other methods for *in vitro* evaluation of drug delivery systems. For example, cell cultures are one of the most often used for studying the effect of *trans*-RSV on cells. Effect of *trans*-RSV on cell proliferation assay can often be 48 hours long [48] or in some cases even 72 h [49]. During this time, more than half of *trans*-RSV at 37 °C and pH 7.4 would be degraded. There are several other cell assays including a 24-hour or longer incubation of cells with free or *trans*-RSV-loaded drug delivery systems for a purpose of evaluating its effect on the cell differentiation, morphology, cytotoxicity, survival under stress conditions, antioxidant activity, and intracellular reactive oxygen species levels [5, 41, 50]. Therefore, it is vital to realize that in the assays conducted under these conditions the effect of *trans*-RSV would not be evaluated at its predetermined concentration, but at its lower concentration and in the presence of degradation products. This warning is also supported by findings from Long et al. and Yang et al. [31, 32], where both confirmed the severe degradation of *trans*-RSV in a cell-buffered medium.

5 CONCLUSION

Validated UPLC method was developed and compared with HPLC and UV/VIS. Comparison of the method specificity for *trans*-RSV and it's degradation products followed in the order UPLC, HPLC, and UV/VIS. Chromatographic methods are necessary for determining *trans*-RSV in conditions under which it is not stable, because UV/VIS provides falsely higher concentrations due low specificity. Our findings reveal that *trans*-RSV in a water solution was stable at room or body temperature only under acidic conditions, whereas it degraded exponentially if the pH became alkaline. Thus the stability of *trans*-RSV in liquid dosage form can be improved by

reducing the pH and temperature, and limiting exposure to oxygen and light. It is important to keep in mind that *trans*-RSV dissolution and release studies done at pH 7.4 and 37 °C (as well as other experiments done under similar conditions) could be accompanied by the presence of significant *trans*-RSV degradation. This effect is more pronounced the longer the experiment. In our case it was evident already after three hours. In this regard, it is worthwhile to note that special attention has to be paid to *trans*-RSV dissolution testing or biological assays on cell lines in order to get the relevant data. This study is of interest because it draws attention to the importance of experimental conditions and their influence on the chemical stability of *trans*-RSV and the implications this has for formulation development, storage, and analysis.

Acknowledgements

This research was financially supported by the Slovenian Research Agency (Program P1-0189 and Research Projects J1-6749).

6 REFERENCES

[1] J.A. Baur, D.A. Sinclair, Therapeutic potential of resveratrol: the in vivo evidence, Nat. Rev. Drug Discov., 5 (2006) 493-506.

[2] A.R. Neves, M. Lucio, J.L. Lima, S. Reis, Resveratrol in medicinal chemistry: a critical review of its pharmacokinetics, drug-delivery, and membrane interactions, Curr. Med. Chem., 19 (2012) 1663-1681.

[3] J.M. Smoliga, O. Blanchard, Enhancing the delivery of resveratrol in humans: if low bioavailability is the problem, what is the solution?, Molecules, 19 (2014) 17154-17172.

[4] A. Amri, J.C. Chaumeil, S. Sfar, C. Charrueau, Administration of resveratrol: What formulation solutions to bioavailability limitations?, J. Control. Release, 158 (2012) 182-193.

[5] A. Amri, S. Le Clanche, P. Thérond, D. Bonnefont-Rousselot, D. Borderie, R. Lai-Kuen, J.-C. Chaumeil, S. Sfar, C. Charrueau, Resveratrol self-emulsifying system increases the uptake by endothelial cells and improves protection against oxidative stress-mediated death, Eur. J. Pharm. Biopharm., 86 (2014) 418-426.

[6] V. Sanna, A.M. Roggio, S. Siliani, M. Piccinini, S. Marceddu, A. Mariani, M. Sechi, Development of novel cationic chitosan-and anionic alginate-coated poly(D,L-lactide-co-

glycolide) nanoparticles for controlled release and light protection of resveratrol, Int. J. Nanomedicine, 7 (2012) 5501-5516.

[7] K. Coradini, F.O. Lima, C.M. Oliveira, P.S. Chaves, M.L. Athayde, L.M. Carvalho, R.C.R. Beck, Co-encapsulation of resveratrol and curcumin in lipid-core nanocapsules improves their in vitro antioxidant effects, Eur. J. Pharm. Biopharm., 88 (2014) 178-185.

[8] E. Antignac, G.J. Nohynek, T. Re, J. Clouzeau, H. Toutain, Safety of botanical ingredients in personal care products/cosmetics, Food Chem. Toxicol., 49 (2011) 324-341.

[9] M. Sessa, R. Tsao, R. Liu, G. Ferrari, F. Donsi, Evaluation of the stability and antioxidant activity of nanoencapsulated resveratrol during in vitro digestion, J. Agric. Food Chem., 59 (2011) 12352-12360.

[10] A. Francioso, P. Mastromarino, A. Masci, M. d'Erme, L. Mosca, Chemistry, stability and bioavailability of resveratrol, Med. Chem., 10 (2014) 237-245.

[11] C.G. Silva, J. Monteiro, R.R. Marques, A.M. Silva, C. Martinez, M. Canle, J.L. Faria, Photochemical and photocatalytic degradation of trans-resveratrol, Photochem. Photobiol. Sci., 12 (2013) 638-644.

[12] B.C. Trela, A.L. Waterhouse, Resveratrol: Isomeric Molar Absorptivities and Stability, J. Agric. Food Chem., 44 (1996) 1253-1257.

[13] L. Camont, C.H. Cottart, Y. Rhayem, V. Nivet-Antoine, R. Djelidi, F. Collin, J.L. Beaudeux, D. Bonnefont-Rousselot, Simple spectrophotometric assessment of the trans-/cis-resveratrol ratio in aqueous solutions, Anal. Chim. Acta, 634 (2009) 121-128.

[14] A. Liazid, M. Palma, J. Brigui, C.G. Barroso, Investigation on phenolic compounds stability during microwave-assisted extraction, J. Chromatogr. A, 1140 (2007) 29-34.

[15] C.F. Hung, Y.K. Lin, Z.R. Huang, J.Y. Fang, Delivery of resveratrol, a red wine polyphenol, from solutions and hydrogels via the skin, Biol. Pharm. Bull., 31 (2008) 955-962.

[16] G. Shi, L. Rao, H. Yu, H. Xiang, H. Yang, R. Ji, Stabilization and encapsulation of photosensitive resveratrol within yeast cell, Int. J. Pharm., 349 (2008) 83-93.

[17] P.G. Cadena, M.A. Pereira, R.B. Cordeiro, I.M. Cavalcanti, B. Barros Neto, C. Pimentel Mdo, J.L. Lima Filho, V.L. Silva, N.S. Santos-Magalhaes, Nanoencapsulation of quercetin and resveratrol into elastic liposomes, Biochim. Biophys. Acta, 1828 (2013) 309-316.

[18] H. Singh, E. James, H. Kan, L.S. Nair, Fabrication and Evaluation of Resveratrol Loaded Polymeric Nanofibers, J. Biomater. Tissue. Eng., 2 (2012) 228-235.

[19] K. Teskac, J. Kristl, The evidence for solid lipid nanoparticles mediated cell uptake of resveratrol, Int. J. Pharm., 390 (2010) 61-69.

[20] K. Bolko, A. Zvonar, M. Gašperlin, Mixed lipid phase SMEDDS as an innovative approach to enhance resveratrol solubility, Drug Dev. Ind. Pharm., 40 (2014) 102-109.

[21] M. Sessa, M.L. Balestrieri, G. Ferrari, L. Servillo, D. Castaldo, N. D'Onofrio, F. Donsi, R. Tsao, Bioavailability of encapsulated resveratrol into nanoemulsion-based delivery systems, Food Chem., 147 (2014) 42-50.

[22] G. da Rocha Lindner, N.M. Khalil, R.M. Mainardes, Resveratrol-loaded polymeric nanoparticles: validation of an HPLC-PDA method to determine the drug entrapment and evaluation of its antioxidant activity, ScientificWorldJournal, 2013 (2013) 506083.

[23] T. Nunes, L. Almeida, J.F. Rocha, A. Falcao, C. Fernandes-Lopes, A.I. Loureiro, L. Wright, M. Vaz-da-Silva, P. Soares-da-Silva, Pharmacokinetics of trans-resveratrol following repeated administration in healthy elderly and young subjects, J. Clin. Pharmacol., 49 (2009) 1477-1482.

[24] L. Minuti, R.M. Pellegrino, I. Tesei, Simple extraction method and gas chromatography–mass spectrometry in the selective ion monitoring mode for the determination of phenols in wine, J. Chromatogr. A, 1114 (2006) 263-268.

[25] C.o. Europe, E.D.f.t.Q.o.M.a. Healthcare, European pharmacopoeia 8.0 : published in accordance with the Convention on the Elaboration of a European Pharmacopoeia, 8th ed., European Directorate for the Quality of Medicines & HealthCare, Strasbourg, 2013.

[26] *ICH*, Validation of analytical precedures: Text and methodology Q2(R1), 2005.

[27] U.F.a.D. Administration, Guidance for Industry: Q2B Validation of Analytical Procedures: Methodology, 1996.

[28] A.N. Martin, Physical Pharmacy: Physical Chemical Principles in the Pharmaceutical Sciences, in, Lippincott Williams & Wilkins, Philadelphia, 1993, pp. 284-323.

[29] R. Rošic, J. Pelipenko, J. Kristl, P. Kocbek, M. Bešter-Rogač, S. Baumgartner, Physical characteristics of poly (vinyl alcohol) solutions in relation to electrospun nanofiber formation, Eur. Polym. J., 49 (2013) 290-298.

[30] G.A. Shabir, Validation of high-performance liquid chromatography methods for pharmaceutical analysis. Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization, J. Chromatogr. A, 987 (2003) 57-66.

[31] N.C. Yang, C.H. Lee, T.Y. Song, Evaluation of resveratrol oxidation in vitro and the crucial role of bicarbonate ions, Biosci. Biotechnol. Biochem., 74 (2010) 63-68.

[32] L.H. Long, A. Hoi, B. Halliwell, Instability of, and generation of hydrogen peroxide by, phenolic compounds in cell culture media, Arch. Biochem. Biophys., 501 (2010) 162-169.

[33] J.M. Lopez-Nicolas, F. Garcia-Carmona, Aggregation state and pKa values of (E)-resveratrol as determined by fluorescence spectroscopy and UV-visible absorption, J. Agric. Food Chem., 56 (2008) 7600-7605.

[34] N. Graham, C.C. Jiang, X.Z. Li, J.Q. Jiang, J. Ma, The influence of pH on the degradation of phenol and chlorophenols by potassium ferrate, Chemosphere, 56 (2004) 949-956.

[35] D. Callemien, V. Jerkovic, R. Rozenberg, S. Collin, Hop as an interesting source of resveratrol for brewers: optimization of the extraction and quantitative study by liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry, J. Agric. Food Chem., 53 (2005) 424-429.

[36] C. Wu, X. Xu, C. Feng, Y. Shi, W. Liu, X. Zhu, J. Zhang, Degradation kinetics study of cabozantinib by a novel stability-indicating LC method and identification of its major degradation products by LC/TOF-MS and LC-MS/MS, J. Pharm. Biomed. Anal., 98 (2014) 356-363.

[37] A. Francioso, P. Mastromarino, R. Restignoli, A. Boffi, M. d'Erme, L. Mosca, Improved stability of trans-resveratrol in aqueous solutions by carboxymethylated (1,3/1,6)-beta-D-glucan, J. Agric. Food Chem., 62 (2014) 1520-1525.

[38] J.S. Jensen, C.F. Wertz, V.A. O'Neill, Preformulation stability of trans-resveratrol and trans-resveratrol glucoside (Piceid), J. Agric. Food Chem., 58 (2010) 1685-1690.

[39] J. Pelipenko, P. Kocbek, J. Kristl, Critical attributes of nanofibers: Preparation, drug loading, and tissue regeneration, Int. J. Pharm., 484 (2015) 57-74.

[40] B. Fritz, J.L. Lach, L.D. Bighley, Solubility analysis of multicomponent systems capable of interacting in solution, J. Pharm. Sci., 60 (1971) 1617-1619.

[41] J. Kristl, K. Teskač, C. Caddeo, Z. Abramovic, M. Sentjurc, Improvements of cellular stress response on resveratrol in liposomes, Eur. J. Pharm. Biopharm., 73 (2009) 253-259.

[42] G. Davidov-Pardo, D.J. McClements, Resveratrol encapsulation: Designing delivery systems to overcome solubility, stability and bioavailability issues, Trends Food Sci. Technol., 38 (2014) 88-103.

[43] V. Filip, M. Plocková, J. Šmidrkal, Z. Špičková, K. Melzoch, Š. Schmidt, Resveratrol and its antioxidant and antimicrobial effectiveness, Food Chem., 83 (2003) 585-593.

[44] S. Das, K.Y. Ng, Resveratrol-loaded calcium-pectinate beads: effects of formulation parameters on drug release and bead characteristics, J. Pharm. Sci., 99 (2010) 840-860.

[45] J. Shao, X. Li, X. Lu, C. Jiang, Y. Hu, Q. Li, Y. You, Z. Fu, Enhanced growth inhibition effect of resveratrol incorporated into biodegradable nanoparticles against glioma cells is mediated by the induction of intracellular reactive oxygen species levels, Colloids Surf. B. Biointerfaces, 72 (2009) 40-47.

[46] R. Srikar, A.L. Yarin, C.M. Megaridis, A.V. Bazilevsky, E. Kelley, Desorption-limited mechanism of release from polymer nanofibers, Langmuir, 24 (2008) 965-974.

[47] X. Lu, C. Ji, H. Xu, X. Li, H. Ding, M. Ye, Z. Zhu, D. Ding, X. Jiang, X. Ding, X. Guo, Resveratrol-loaded polymeric micelles protect cells from Abeta-induced oxidative stress, Int. J. Pharm., 375 (2009) 89-96.

[48] P.R. Pandey, H. Okuda, M. Watabe, S.K. Pai, W. Liu, A. Kobayashi, F. Xing, K. Fukuda, S. Hirota, T. Sugai, G. Wakabayashi, K. Koeda, M. Kashiwaba, K. Suzuki, T. Chiba, M. Endo, T. Fujioka, S. Tanji, Y.Y. Mo, D. Cao, A.C. Wilber, K. Watabe, Resveratrol suppresses growth of cancer stem-like cells by inhibiting fatty acid synthase, Breast Cancer Res. Treat., 130 (2011) 387-398.

[49] Š. Zupančič, P. Kocbek, M.G. Zariwala, D. Renshaw, M.O. Gul, Z. Elsaid, K.M. Taylor, S. Somavarapu, Design and development of novel mitochondrial targeted nanocarriers, DQAsomes for curcumin inhalation, Mol. Pharm., 11 (2014) 2334-2345.

[50] C. Caddeo, K. Teskač, C. Sinico, J. Kristl, Effect of resveratrol incorporated in liposomes on proliferation and UV-B protection of cells, Int. J. Pharm., 363 (2008) 183-191.

CAPTIONS

Fig. 1: UV/VIS, HPLC, and UPLC spectra and chromatograms of *trans*-RSV standard and *trans*-RSV exposed to light or pH 9 resulting in isomerization and degradation, respectively. The chromatograms of HPLC and UPLC were obtained at 306 nm. The spectra of two main degradation products (red and green), *trans*-RSV (violet), and *cis*-RSV (orange) were extracted from their retention times. (AU – absorbance units).

Fig. 2: The UV/VIS absorption spectra of (A) isomerization of *trans*-RSV to *cis*-RSV with UV light at 366 nm after 0, 1, 2, 3, 4, 5, 7.5, and 15 min at room temperature and (B) degradation of *trans*-RSV in buffer with pH 7.4 after 0, 1.5, 2.4, 8, 11, 21.5, 30.8, and 46.2 h at 37 °C.

Fig. 3: Kinetics of *trans*-RSV degradation dependent on pH levels. Concentrations of *trans*-RSV at different time points are presented by symbols whereas curves present fitted degradation order according to Table 3. Each point represents the mean of at least three measurements ± S.D., which is small; in many cases within the size of the symbols.

Fig. 4: Relationship between degradation kinetics log(*k*) of *trans*-RSV and pH aqueous solution at 37 °C.

Fig. 5: *Trans*-RSV stability in an aqueous solution with pH 7.4 stored at different temperatures in darkness for 3 months measured by UV/VIS, HPLC, and UPLC. Each point represents the mean of at least three measurements ± S.D., which is small, in many cases within the size of the symbols.

Fig. 6: SEM image of 5% *trans*-RSV-loaded nanofibers and its release profiles in buffer with pH 1.2, 6.8, and 7.4 at 37 °C using UV/VIS, HPLC, and UPLC methods.

TABLES

Table 1: Detection limit (DL), quantitation limit (QL), and linearity parameters for UV/VIS, HPLC, and UPLC methods.

Method	DL (µg/mL)	QL (µg/mL)	Linear response range (µg/mL)	Linearity	
				Slope	r
UV/VIS	0.09	0.26	0.5–15	0.1268	1.00000
HPLC	0.25	0.77	0.5–15	105.0	0.99998
UPLC	0.14	0.43	0.5–15	216119	0.99996

Table 2: Accuracy and precision for *trans*-RSV determined by UV/VIS, HPLC, and UPLC.

Method	Nominal conc. (µg/mL)	Accuracy (%)	Precision	
			Repeatability (%)	Intermediate precision (%)
UV/VIS	1	99.3	0.85	1.36
	5	99.5	0.47	1.11
	15	99.8	0.26	1.03
HPLC	1	100.9	0.75	2.00
	5	99.1	0.40	1.31
	15	98.7	0.33	0.53
UPLC	1	101.2	0.58	1.27
	5	101.0	0.17	0.08
	15	100.8	0.05	0.48

Table 3: The kinetics of *trans*-RSV degradation obtained at various pH levels with UV/VIS, HPLC, and UPLC (*k* - degradation constant, R - Pearson's correlation coefficient, $t_{1/2}$ - half-life).

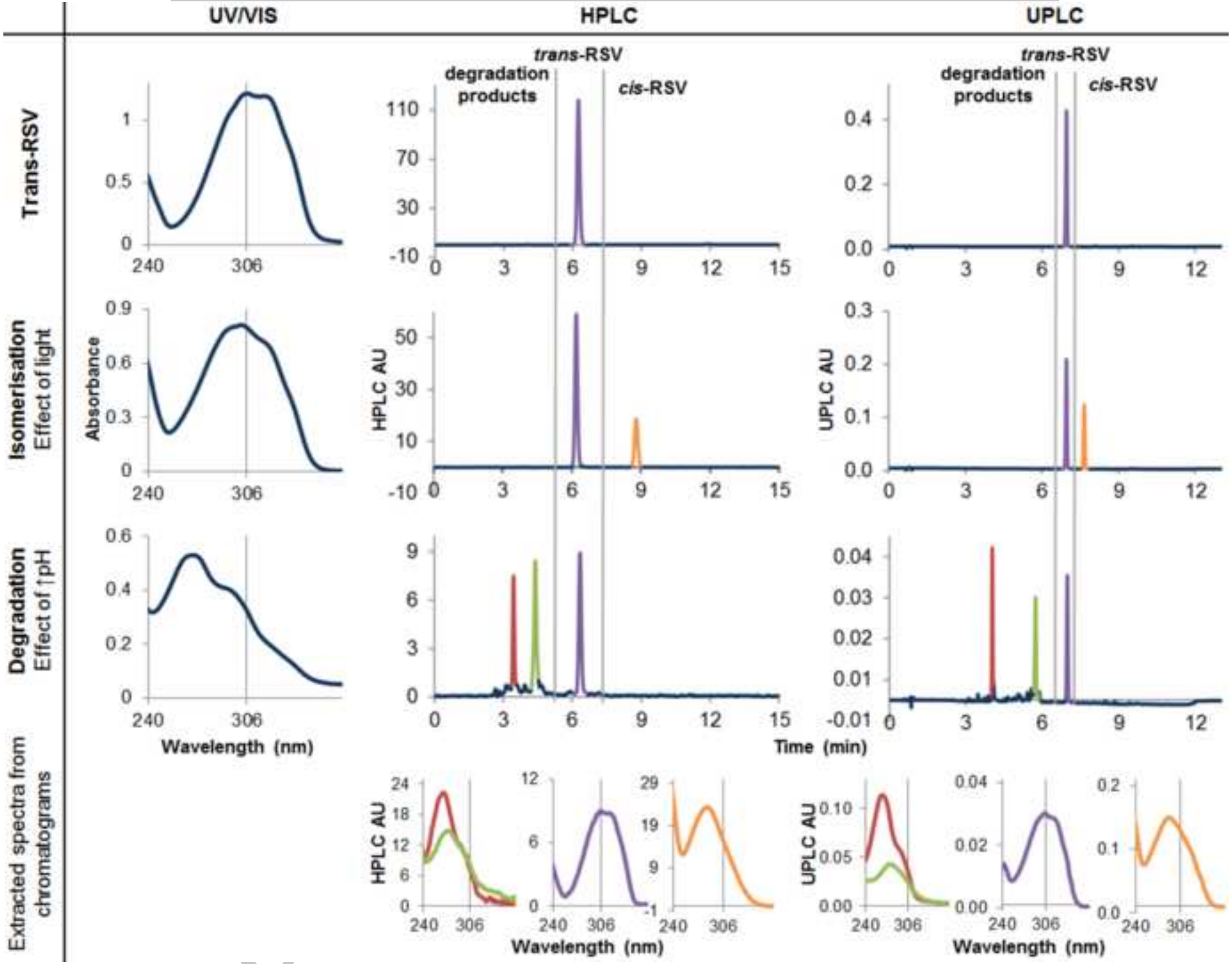
pH	Reaction order	*k* (µM/h (zero order), /h (first order))			R			$t_{1/2}$		
		UV/VIS	HPLC	UPLC	UV/VIS	HPLC	UPLC	UV/VIS	HPLC	UPLC
1.2	first	-0.000532	-0.000863	-0.000977	0.8093	0.9876	0.9227	441 days	303 days	329 days
6.8	first	-0.00125	-0.00192	-0.00199	0.9987	0.9994	0.9992	23.2 days	15.1 days	14.5 days
7.4	first	-0.0099	-0.0178	-0.0179	0.9822	0.9969	0.9961	2.9 days	1.6 day	1.6 day
8.0	first	-0.103	-0.176	-0.186	0.9955	0.9993	0.9991	6.7 h	3.9 h	3.7 h
9.0	zero	-246	-315	-328	0.9983	0.9998	0.9997	10.1 min	7.7 min	7.7 min
	first	-3.82	-10.12	-9.95	0.9926	0.9952	0.9966	10.9 min	4.1 min	4.2 min
10.0	zero after 2 min	-537	-675	-716	0.9955	0.9998	1.0000	4.3 min	3.3 min	3.3 min

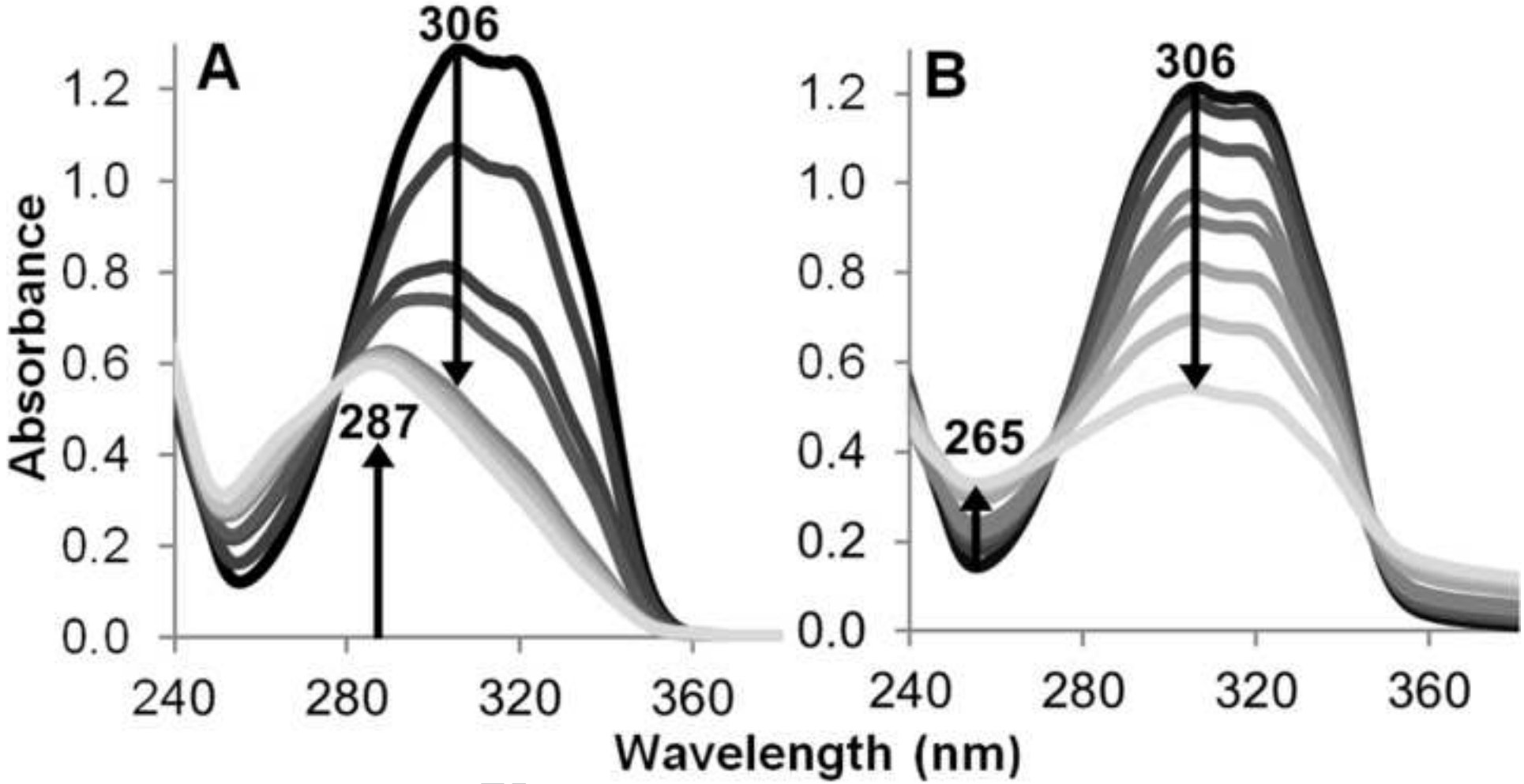
Table 4: *Trans*-RSV solubility (µg/mL) in buffers with pH 1.2, 6.8, and 7.4 at 37° C measured by UV/VIS, HPLC and UPLC.

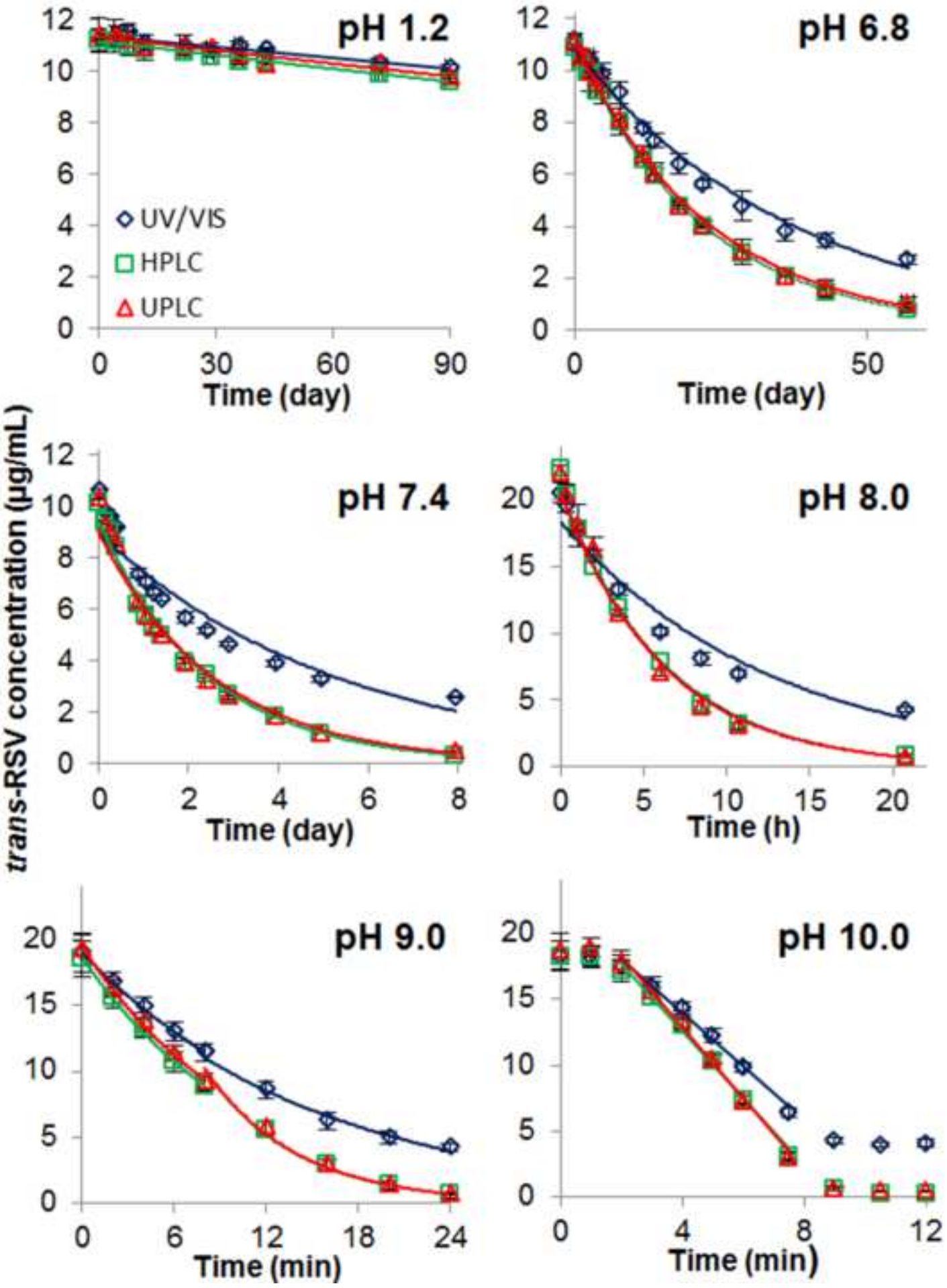
pH	Time (h)	Solubility (µg/mL)		
		UV/VIS	HPLC	UPLC
1.2	24	60.8 ± 1.0	60.2 ± 1.2	61.2 ± 1.8
	48	61.9 ± 2.2	62.6 ± 2.2	62.7 ± 1.2
	72	64.2 ± 1.2	63.7 ± 1.1	64.3 ± 1.4
6.8	24	61.9 ± 0.4	61.4 ± 0.3	61.0 ± 1.3
	48	62.2 ± 0.2	61.9 ± 0.3	62.3 ± 1.1
	72	62.4 ± 1.1	60.8 ± 1.4	61.2 ± 2.1
7.4	24	58.9 ± 0.9	54.1 ± 0.7	53.4 ± 1.5
	48	58.8 ± 0.9	52.8 ± 1.5	52.5 ± 1.1
	72	59.5 ± 1.4	51.0 ± 1.1	50.1 ± 0.7

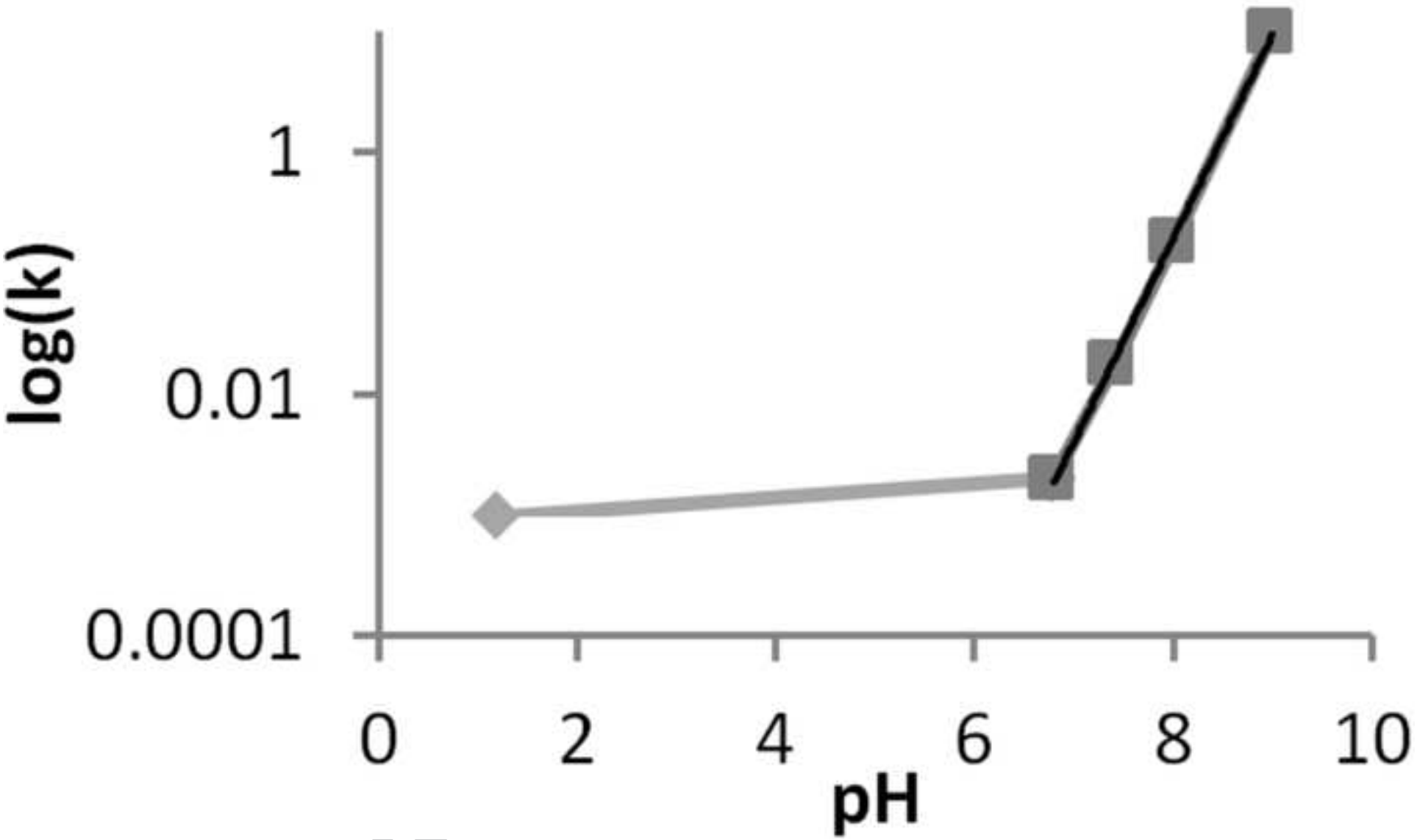
Table 5: The impact of the individual factors to *trans*-RSV isomerization or degradation to its half-life.

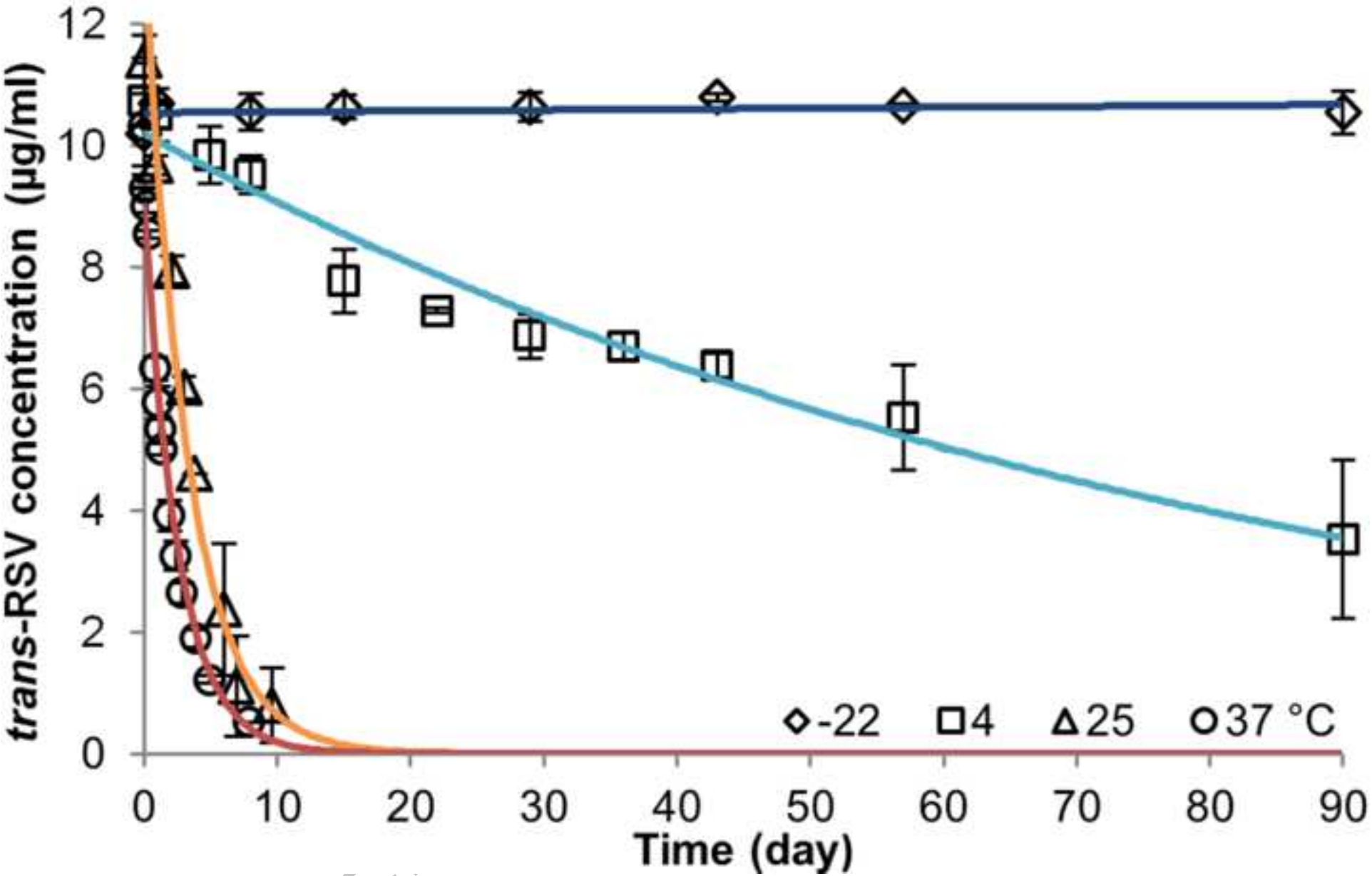
Factor	Process	Degradation time	Reference
UV light (366 nm)	isomerization	a few minutes	Fig. 2
pH 9 or 10	degradation	a few minutes	Fig. 3, Table 3
pH 8	degradation	hours	Fig. 3, Table 3
sunlight	isomerization	hours	[13]
pH 7.4	degradation	a few days	Fig. 3, Table 3
pH 6.8	degradation	more days	Fig. 3, Table 3
↓temperature	degradation	decrease degradation rate	Fig. 5
pH 1.2	degradation	almost a year	Fig. 3, Table 3

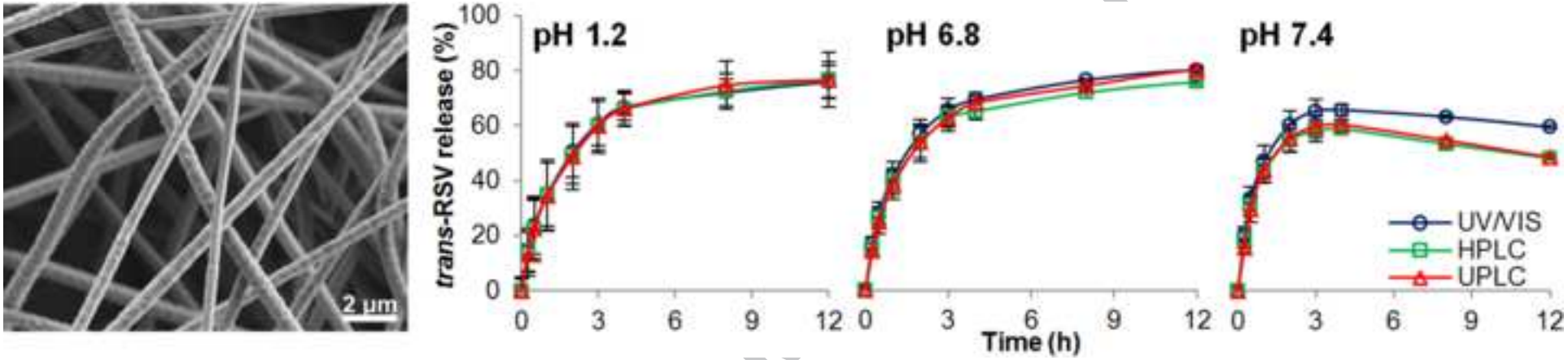





pH 1.2
pH 6.8
pH 7.4
pH 8.0
pH 9.0
pH 10.0
UV/VIS
HPLC
UPLC
trans-RSV concentration (µg/mL)
Time (day)
Time (h)
Time (min)




trans-RSV concentration (µg/ml)
12
10
8
6
4
2
0
0
10
20
30
40
50
60
70
80
90
Time (day)
-22
4
25
37 °C



Graphical abstract