Determination of shelf life of *Chelidonium majus*, *Sambucus nigra*, *Thymus vulgaris* and *Thymus serpyllum* herbal tinctures by various stability-indicating tests

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Abstract

Stability testing of herbal preparations has recently been recognized as essential for quality control to support their shelf life. Various stability-indicating tests were assessed for their predictive power in herbal tincture stability testing and compared to reference quantitative determination of marker compounds. Herbs from Western herbal medicine with different active constituents were selected: *Chelidonium majus*, *Sambucus nigra* flowers, *Thymus serpyllum* and *Thymus vulgaris*. Their freshly prepared and commercially available tinctures were tested for stability under normal and accelerated conditions. Quantitative chromatographic assays were developed for chelidonine in *Chelidonium* tincture, for isoquercitrin, rutin and quercetin in *Sambucus* tinctures, and for thymol in *Thymus* tinctures. Additional procedures were assessed for their predictive power on tincture stability: chromatographic profiling, spectrophotometric evaluation of tincture colour, DPPH antioxidant assay. With the exception of the DPPH assay in *Sambucus* tincture, none of the assessed stability-indicating tests was satisfactory in comparison with reference determination. *Sambucus* tincture was stable for more than six months, while *Chelidonium* and *Thymus* tinctures were stable for less than 1.5 months with the decrease in marker compound >10 %. Compound borneol is proposed as a marker for the deterioration of *Thymus* tinctures. *Chelidonium* tincture was additionally tested for its stability on UV and visible light. Chelidonine degrades in the tincture under the visible light. The results of the present study implicate that the only assays suitable for stability testing of different tinctures are those that determine concentration of the active compounds or some active compound-linked property of the tincture.

Keywords: *Chelidonium majus*, *Sambucus nigra*, *Thymus vulgaris*, *Thymus serpyllum*, tincture stability, chromatography, antioxidant assay

1. INTRODUCTION

Stability testing for active substances is an important and mandatory aspect of quality assurance in the pharmaceutical industry, either in their pure form or in formulations. Protocols for stability testing are prescribed by an International Conference on Harmonization (ICH) in ICH Topic Q1A Stability Testing Guidelines (Anon., CPMP/ICH/380/95, 1998). For herbal preparations, stability testing has come under the spotlight only recently due to increasing evidence of public health risk from low grade herbal products. The Traditional Herbal Medicines Directive (Anon., Directive 2004/24/EC…, 2004) required each EU member state to put in a registration scheme for manufactured traditional herbal medicines. Application for a traditional use registration requires the applicant to compile a dossier broadly covering the proof of traditional use, safety and quality of which stability testing is a recent yet essential requisite. The guidelines for stability testing have been specifically adapted by European Medicines Agency - EMEA, Committee for Herbal Medicinal Products – HMPC (Anon., CPMP/QWP/2820/00 Rev 1…, 2006). Compared with pharmaceutical active compounds, there are important differences in the proposed procedures. Either constituents with known therapeutic activity or markers should be quantified during the stability testing, which should last at least six months and the limit is set to ±5 % of the initial value in the case of known active constituent and ±10 % of the initial value for the marker compound. However, an additional condition should be met: design and implementation of additional product-specific stability-indicating tests. They should be appropriate for detecting any changes in the product quality during its shelf-life (Anon., CPMP/QWP/2820/00 Rev 1…, 2006).

Currently, stability testing of herbal tinctures and other preparations is done predominantly by chromatographic fingerprinting and quantitative determination of active or marker constituents (Bilia et al., 2001, 2002a, 2002b, 2006, 2007; Bos et al., 1996). The possible drawback is the need to extract samples in order to minimize the influence of the interferences on determination. Various detectors are used, but by far the most specific is the

mass spectrometer. In order to avoid the loss of compounds in the sample preparation and chromatographic separation steps, more elaborate combination of mass spectrometry and nuclear magnetic resonance has been proposed for tincture testing, but it involves the use of elaborate and expensive equipment (Politi et al., 2009). Another interesting approach to stability testing is the application of the appropriate biochemical assays, e.g. in vitro immune assay to test the stability of *Echinacea* spp. preparations (Senchina et al., 2005).

The tinctures of commonly used Europaean medicinal plants (greater celandine - *Chelidonium majus* L. , Papaveraceae; garden thyme – *Thymus vulgaris* L. and wild thyme - *Thymus serpyllum* L., both Lamiaceae; elder – *Sambucus nigra* L., Adoxaceae) were chosen for the testing on the basis of chemically different active constituents, with the aim of evaluating the stability testing protocols for diverse possible herbal constituents. The chemical profiles of these medicinal plants have already been extensively studied. To the best of our efforts, we haven't found any studies regarding the stability testing of the tinctures made from these herbs. Kaack and Christensen (2010) followed the content of various phenolic compounds in dried elderflower herb packed in different materials over a period of 21 months. They concluded that phenolic compounds were stable under the applied storage conditions.

The active constituents of the greater celandine (*Chelidonium majus*) are most likely its alkaloids (El-Readi et al., 2013; Ernst and Schmidt, 2005; Gilca et al., 2010; Monavari et al., 2012; Yang et al., 2011). The plant is traditionally used in Western herbal medicine internally as a choleretic, cholagogue and hepatoprotective and externally as an antifungal and antiviral, specifically for common warts (verrucae) (Gilca et al., 2010; Monavari et al., 2012). Research has shown the effectiveness of its alkaloids against MRSA and *Mycobacterium tuberculosis* (Liang et al., 2011; Zuo et al., 2011) and against cancer cells (El-Readi et al., 2013). An effective anti-cancer drug Ukrain has been developed from its extract (Ernst and Schmidt, 2005). In animal models, it has also shown promising results in the treatment of atopic dermatitis (Yang et al., 2011). Other isolated compounds have shown interesting properties as well, e.g. a cysteine proteinase inhibitor might be responsible for the antiviral

activity of the sap (Rogelj et al., 1988), and chelidonic acid has shown anti-inflammatory properties (Shin et al., 2011), useful in the treatment of ulcerative colitis (Kim et al., 2012).

Both garden and wild thyme (*Thymus vulgaris* and *Th. serpyllum*, respectively) are traditionally used as expectorants and antimicrobials in upper respiratory infections because of their bronchoantispasmodic, expectorant and antibacterial activity (Blumenthal et al., 2000; Staszek et al., 2014). Antibacterial activity is attributed to its essential oil (Lakis et al., 2012; Sfeir et al., 2013), containing phenolic compounds thymol and carvacrol (Móricz et al., 2012; Staszek et al., 2014). Spasmolytic effect is attributed to the same, in synergy with the flavonoids (Engelbertz et al., 2012). In addition to the effect on the respiratory system, thyme preparations have also been shown to be effective spasmolytics in dysmenorrhaea (Direkvand-Moghadam and Khosravi, 2012). Many species of *Thymus* are used in the traditional medicine for the same purpose. Additionally, same species of thyme can show wide phenotypic variation, resulting in chemical polymorphism and different ratio of constituents (De Lisi et al., 2011; Staszek et al., 2014; Thompson et al., 2013). In spite of that, thymol and carvacrol have been proposed as markers for the authentication and quality control of thyme species (Sgorbini et al., 2015).

In elder (*Sambucus nigra*), both flowers and berries are used in the traditional medicine. Flowers (Sambuci flos) are used in colds (Blumenthal et al., 2000), in feverish conditions and as an anticatarrhal in allergic conditions. The active constituents are presumably hydroxycinnamic acids and flavonol glycosides (Dawidowicz et al., 2006; Mikulic-Petkovsek et al., 2015). One of the most abundant glycosides, isoquercitrin, has been shown to inhibit histamine release from mast cells (Matsumoto et al., 2009) and to exert an anti-inflammatory effect in an asthma model (Rogerio et al., 2007). Recently, acyl spermidines have been identified as constituents of elderflowers, possibly contributing to their cold-alleviating properties (Kite et al., 2013). Herb quality is assessed by determination of flavonoid content, calculated as isoquercitrin (Blumenthal et al., 2000).

In the present work, we aimed to assess the stability of freshly prepared and commercially available tinctures by comparing the established approach, i.e. monitoring of the active or

marker constituents with the appropriate chromatographic method, with more simple procedures as stability-indicating tests, thus meeting the EMEA requirements of additional testing (Anon., CPMP/QWP/2820/00 Rev 1…, 2006). The procedures should be simple in order to enable their rapid application and interpretation. Monitoring of colour change by spectrophotometric means and an antioxidant test with a stable DPPH radical (Brand-Williams et al., 1995) were evaluated for their predictive power in the stability testing. They were compared with the quantitative determination of at least one of the possible active constituents in each herb: alkaloid chelidonine in *Chelidonium majus*; phenolic compound thymol in *Thymus* sp.; flavonoids isoquercitrin, rutin and quercetin in *Sambucus nigra* flowers (Figure 1).

Figure 1

2. EXPERIMENTAL

2.1. Chemicals and materials

The following standard compounds were used: chelidonine (Sigma, USA, >97 %); thymol (Fluka, Germany, >99 %); quercetin (Sigma, USA, anhydrous); isoquercitrin (Fluka, Germany, >90 %); rutin (Sigma, USA, >94 %). Stable DPPH (2,2-diphenyl-picrylhydrazyl) radical was purchased from Sigma-Aldrich, USA. Solvents methanol, acetonitrile, *n*-propanol were HPLC grade from Sigma, USA. Deionised water was prepared by MilliQ system (Millipore Corp. USA). Other chemicals were *p.a.* grade from various producers.

Stock solutions of active compounds were prepared in methanol or *n*-propanol by dissolving accurately weighed solid standard to give a concentration of 0.5-1.0 mg/ml. They were stored in refrigerator and stable for several months. Calibration solutions were prepared by dilution of stock solutions in deionised water to the desired concentration as specified under individual methods.

Plant materials were collected in their appropriate season (greater celandine whole plant in July 2011; thyme aerial parts in June 2011; elderflowers in June 2011) in the south-western part of Slovenia. They were identified by the authors, who are trained medical herbalists. No voucher specimens were retained. Collected plants were dried and stored in paper bags in a cool, dark place, and were subjected to tincture preparation immediately before the beginning of the stability testing. Tinctures were prepared at the latest date six months after the collection of herbs by the cold percolation method in the mixture of tap water and food-grade ethanol in the same strength and solvent ratios as those used for commercial manufacture: *Chelidonium majus* 1:5 (45 % ethanol); *Thymus serpyllum* 1:3 (45 % ethanol); *Sambucus nigra* flowers 1:4 (25 % ethanol). The ratios (strength) of tinctures are given as mass divided by volume (*m/V*).

Commercial tinctures were purchased from Herbal Apothecary (Whitby, UK): *Thymus vulgaris* (Thyme herb, 1:3, 45 % ethanol; Batch No. N2129, DOM 14/10/11), herbal material originating from Morocco; *Sambucus nigra* Flos (Elder flowers, 1:4, 25 % ethanol; Batch No. N2024, DOM 16/08/11), herbal material originating from the Eastern Europe. They were prepared by cold percolation method six months or less previous to the beginning of the experiment. *Chelidonium majus* commercial tincture was not available at the time.

2.2. Determination of thymol by HS-SPME-GC-MS method

Calibration solutions (at least five) were prepared in deionised water in the range 0.5 – 5.0 mg/L. *Thymus* tinctures were diluted with deionised water 1:100 (*V/V*). Each calibration solution or tincture was subjected to the extraction and analytical method in triplicate. A 5 ml portion of the calibration solution or diluted tincture was measured into a 20-ml headspace vial, crimped and thermostated in a water bath for 10 min at 40 ^{o}C. An SPME fibre (DVB/CAR/PDMS coating, film thickness 50/30 μm, from Supelco, USA) was inserted in the headspace and the compounds were sampled for 8 min at 40 ^{o}C. The fibre was subsequently removed from the headspace, inserted into the injector port of a gas chromatograph and desorbed for 10 min at 250 ^{o}C.

Analysis was performed with a gas chromatograph with mass spectrometric detector (Agilent Technologies 7890A GC with MSD 5975C, Agilent Technologies, USA). The temperature program on column Rtx-20 (WCOT, dimensions 60 m x 0.25 mm (i.d.), film thickness 1.0 μm, from Restek, USA) was: 50 °C (5 min) - 10 °C/min - 180 °C (5 min) - 50 °C/min - 240 °C (10 min). Carrier gas was helium with flow rate 1.0 ml/min. Temperature of the injector was 250 °C, temperature of the GC-MS interface was 280 °C. Electron impact (EI) ionisation at 70 eV was used and the chromatograms were recorded in the total ion current (TIC) mode. Thymol was identified on the basis of its retention time by comparison with standard; other compounds were identified from their mass spectra using the searchable EI-MS spectra library (NIST05). The peak area for quantitation was measured in the TIC chromatogram.

2.3. Determination of chelidonine by HPLC-DAD method

Calibration solutions (at least five) were prepared in the deionised water in the range 0.5 - 50 mg/L. *Chelidonium* tincture was diluted with deionised water up to 1:5 (*V/V*). Each calibration solution and tincture was subjected to the analytical method in triplicate. Analysis was performed on a high-performance liquid chromatograph with diode-array spectrometric detector (Series 1100, Agilent Technologies, USA). The analytical column was Discovery HS C18 (dimensions 150 mm x 4.6 mm (i.d.), particle size 3 μm, from Supelco, USA). Gradient elution was applied using mobile phase A - acetonitrile and mobile phase B - 0.030 mol/L formic acid in deionised water. The gradient program was as follows: from 0 to 5 min 15 % A; from 5 to 20 min increase from 15 % to 90 % A; from 20 to 22 min 90 % A. The mobile phase flow rate was 0.7 ml/min. Analysis was performed at ambient temperature, injection volume was 20 μL. Chromatograms were recorded at 210 nm and 280 nm, wavelength 280 nm was used for quantitation purposes. UV spectra of compounds were recorded in the 200-400 nm range. Additional LC-MS analyses for the identification of other peaks in the chromatogram were performed at identical chromatographic conditions and with mass

spectrometer 3200Q TRAP LC-MS/MS System (MDS SCIEX, Canada), positive electrospray ionisation (ESI+).

2.4. Determination of isoquercitrin, rutin and quercetin by HPLC- DAD method

Calibration solutions (at least five) of all three compounds were prepared in the deionised water in the range 0.5 - 50 mg/L. Prepared Sambuci flos tincture was diluted with deionised water up to 1:10 (*V/V*), while the commercial tincture was analysed as obtained. Each calibration solution and tincture was subjected to the analytical method in triplicate. Analysis was performed on a high-performance liquid chromatograph with diode-array spectrometric detector (Series 1100, Agilent Technologies, USA). The analytical column was Discovery HS C18 (dimensions 150 mm x 4.6 mm (i.d.), particle size 3 μm, from Supelco, USA). Gradient elution was applied using mobile phase A - acetonitrile and mobile phase B - 2 % (*m/m*) acetic acid in deionised water. The gradient program was as follows: from 0 to 10 min 10 % A; from 10 to 25 min increase from 10 % to 40 % A; from 25 to 35 min increase from 40 % to 90 % A. The mobile phase flow rate was 0.7 ml/min. Analysis was performed at ambient temperature. Injection volume was 20 μL. Chromatograms were recorded at 210 nm, 260 nm and 360 nm, wavelength 360 nm was used for quantitation purposes. UV spectra of compounds were recorded in the 200-400 nm range.

2.5. Spectrophotometric colour evaluation

Tincture colour was evaluated by recording its absorption spectra in the ultraviolet and visual light range 200-750 nm versus blank (deionised water) with UV-Vis Spectrometer Lambda EZ 201 (Perkin Elmer, USA).

2.6. Antioxidant assay with a stable DPPH radical

Antioxidant capacity of the tinctures was evaluated by performing the assay with the stable DPPH radical as originally described in Brand-Williams et al. (1995). DPPH was prepared by dissolving an appropriate amount of accurately weighed solid standard in methanol. The

solution was further diluted with methanol to obtain the working DPPH solution with concentration $6 \cdot 10^{-5}$ mol/L. Appropriate amount of working DPPH solution was mixed with each tincture and absorbance at 515 nm was recorded after 30 min versus blank (tincture diluted with methanol) with UV-Vis Spectrometer Lambda EZ 201 (Perkin Elmer, USA). Relative antioxidant activity was calculated as percent of DPPH radical consumed in the reaction with antioxidant species from the tincture during the 30 min waiting time.

2.7. Stability testing experiment

Each tincture was measured (50 ml) into two glass bottles and tightly capped. One of the bottles was stored in a closed box at room temperature. The other bottle was placed into the laboratory oven kept at a constant temperature of 40 ± 1 ^{o}C. The control of air humidity in the storage area was not exerted due to the high water content (55 % or 75 %) of the tinctures. It was assumed that the gaseous phase in the closed bottles was saturated with water at the given temperature.

The appropriate volume of the tinctures needed to perform the chromatographic assay, the DPPH assay and absorption spectrum recording was removed from the bottles after 40, 100 and 200 days. They were analysed for their respective active or marker compounds. Their absorption spectra were recorded and DPPH assay was performed. Each of these assays was performed at time zero, after 40, 100 and 200 days, which roughly corresponds to 1.5, 3 and 6 months.

Additional testing for light stability was performed for *Chelidonium majus* tincture which was observed to fade in colour over the time. Tightly capped vials with 5 ml tincture diluted 1:25 (*V/V*) with deionised water were irradiated with UV lamp (254 nm; 100 W; Applied Photophysics LTD, GB). A portion of the solution was removed every 3 min (0–24 min) and analysed to monitor the time dependance of the photodegradation. To simulate the sunlight, Suntest CPS+ apparatus with xenon lamp (Heraeus Industrietechnik, Germany) was used. Tightly capped vials with 5 ml tincture diluted 1:25 (*V/V*) with deionised water were irradiated

in the apparatus. A portion of the solution was removed every 30 min (0–6 h) and analysed to monitor the time dependance of the photodegradation.

3. RESULTS AND DISCUSSION

3.1. Development of the chromatographic methods

The principal goal of chromatographic method development in tincture stability testing is to enable the separation and quantification of the constituents with as little sample handling as possible, which minimizes the possibility of any loss. Thus, tinctures with the constituents that are amenable to liquid chromatography were subjected to the analysis without any sample preparation except for the dilution. This was possible for chelidonine from *Chelidonium majus* tincture and flavonoids from *Sambucus nigra* flowers tinctures, but not for thymol from *Thymus* tinctures, which is a volatile compound more amenable to gas chromatography. Although LC analysis would be possible, many interferences and inadequate detection with DAD detector were the limiting factors in the latter case.

3.1.1. HPLC-DAD method for chelidonine in Chelidonium majus tincture

Chelidonium alkaloids are usually separated by HPLC (Suchomelová et al., 2007). A good separation of chelidonine (Figure 1) and other *Chelidonium* alkaloids was achieved by using a reversed phase (C18) stationary phase and for the mobile phase, a gradient of acetonitrile and deionised water acidified with formic acid. An example of the chromatogram obtained by direct injection of diluted *Chelidonium majus* tincture is shown in Figure 2. The calibration parameters for the quantified compound chelidonine are given in Table 1. Initial concentration of chelidonine in freshly prepared 1:5 tincture (*m/V*, 45 % ethanol) was 8.3 ± 1.0 g/L. Commercial tincture was not available at the time.

Figure 2

Table 1

3.1.2. HPLC-DAD method for isoquercitrin, quercetin and rutin in Sambucus nigra flowers tincture

The technique of choice for flavonoid compounds is HPLC with either spectrophotometric or mass-spectrometric detection (Dawidowicz et al., 2006; Mikulic-Petkovsek et al., 2015). Separation of the selected flavonoids (Figure 1) in *Sambucus* tincture was achieved on a reversed phase (C18) stationary phase with a gradient of acetonitrile and deionised water, acidified with acetic acid. Wavelength 360 nm was chosen as the most suitable for quantitation on the basis of UV spectra of the compounds and lower number of interfering compounds absorbing at that wavelength. The calibration parameters for the three quantified compounds are given in Table 1.

In Figure 3A, a chromatogram of directly injected diluted home-prepared *Sambucus* 1:4 (*m/V*) tincture (25 % ethanol) is shown. The most prominent was the peak for rutin (initial concentration 1.91 ± 0.70 g/L), there was also some isoquercitrin present (0.083 ± 0.010 g/L), but very little quercetin (0.022 ± 0.019 g/L).

Commercial *Sambucus* tincture of matching strength and ethanol content has had quite a different chromatographic profile (Figure 3B). There was a prominent peak for quercetin (initial concentration 0.070 ± 0.014 g/L), whilst peaks for rutin and isoquercitrin were comparatively small (initial concentrations 0.0022 ± 0.0010 g/L and 0.0090 ± 0.0013 g/L, respectively). Notably, concentrations of all three flavonoids were much lower in the commercial tincture compared to freshly prepared tincture, which had to be diluted 1:10 (*V/V*) with deionised water prior to chromatographic analysis in order to obtain peak areas of the quantified compounds within the calibration range.

Figure 3

3.1.3. GC-MS method for thymol in Thymus tinctures

Thymol (Figure 1) and other volatile components of the *Thymus* sp. are usually analysed by gas chromatography (GC) (De Lisi et al., 2011; Móricz et al., 2012; Sgorbini et al., 2015; Staszek et al., 2014; Thompson et al., 2013). Although direct injection of tincture into a gas chromatograph would be possible, its high water content would severely shorten the lifetime of the chromatographic column. Therefore, a preliminary extraction step was chosen and to avoid the interference of any extraction solvent, a solventless technique solid-phase microextraction (SPME) was used. It was performed from the headspace (HS) (Sgorbini et al., 2015) in order to avoid extraction of any other than volatile compounds from the tincture. The appropriate solid phase was chosen to maximize the amount of compounds of interest on the SPME fibre, which was subsequently thermally desorbed. This extraction technique offers excellent selectivity and low limits of detection but care must be taken to avoid discrimination of components and to take different selectivity into account when interpreting the composition of volatile phase (i.e. essential oils) on the basis of HS-SPME-GC chromatographic profile (Prosen and Zupančič-Kralj, 1999). The calibration parameters for the quantified compound thymol are given in Table 1. The upper limit of linear range was between 5.0 and 10.0 mg/L due to the limited capacity of the SPME fibre.

Figure 4

In Figure 4A, a chromatogram after HS-SPME extraction of freshly prepared tincture from *Thymus serpyllum* 1:3 (*m/V*, 45 % ethanol) is shown. Most prominent were peaks for thymol (initial concentration 0.40 ± 0.05 g/L) and carvacrol (estimated initial concentration on the basis of response factor RF=1: 0.23 g/L), the marker compounds of the essential oil (Sgorbini et al., 2015). Other constituents were identified on the basis of their electron impact mass

spectra and are listed in Table 2. The antibacterial compounds from the essential oil: thymol, carvacrol and linalool (Móricz et al., 2012) were present in this tincture.

Table 2

Commercial *Thymus vulgaris* 1:3 tincture (*m/V*, 45 % ethanol) yielded a different chromatographic profile (Figure 4B, Table 2) with the peaks for several compounds (borneol, camphene, and *p*-cymene) more prominent than peaks for the marker compounds (Sgorbini et al., 2015) thymol and carvacrol. Based on the literature data (Blumenthal et al., 2000; Lakis et al., 2012; Sgorbini et al., 2015), thymol should be the main component of the thyme essential oil. Although HS-SPME extraction does not necessarily reflect the actual composition of the essential oil, chemically similar compounds are extracted in approximately similar amounts (Prosen and Zupančič-Kralj, 1999) and this would be expected for the constituents of the volatile fraction of the thyme tincture. In the commercial tincture, a low amount of thymol (initial concentration 0.090 ± 0.009 g/L) and somewhat higher amount of carvacrol (estimated initial concentration, RF=1: 0.40 g/L) was found. Antibacterial compounds α-terpineol and linalool (Móricz et al., 2012) were also present. The reason for this discrepancy between the freshly prepared and commercial tincture could be in the different species and different origin of the herbal material. *Thymus* genus is known for its high chemical polymorphism even within the same species (De Lisi et al., 2011; Staszek et al., 2014; Thompson et al., 2013).

3.2. Stability of *Chelidonium majus* tincture

Four parameters were determined during the stability experiment at room temperature or at 40 °C: chelidonine concentration, chromatographic profile, antioxidant capacity (measured as DPPH consumed during the 30 min waiting time) and evaluation of UV-Vis spectrum. After 200 days, chelidonine concentration decreased to 70.8 % of the initial value at room temperature and to 61.3 % at 40 °C (Figure 5A). Time regression analysis (quadratic

function) for both temperatures also indicated differences in the trend. Antioxidant capacity interestingly decreased to 52.4 % of the initial value at room temperature and to 61.2 % at 40 ^{o}C (Figure 5B). The shelf life of this tincture, according to EMEA-HMPC guidelines, is thus estimated below 1.5 months as the concentration of marker compound had already reduced by at least 10 % in 40 days.

Figure 5

However, chromatographic profile of the tincture remained the same in terms of chromatographic peaks and the ratio of their heights. In the UV-Vis spectrum of the tincture, an absorption peak at 660-700 nm was significantly diminished after storage for six months at 40 ^{o}C, but not at room temperature. Compared to concentration determination of the active/marker compound, chromatographic profile performed inferiorly as it failed to detect differences in tincture composition. UV-Vis spectrum indicated changes in tincture composition at accelerated conditions, but not at room temperature. DPPH assay indicated the deterioration of the tincture, but the decrease in the antioxidant capacity was more pronounced at room temperature. To probe the correlation between the results for chelidonine concentration and DPPH consumption, chelidonin was tested for its antioxidant capacity and shown to be practically free from it. Therefore, antioxidant capacity of *Chelidonium majus* tincture is due to the presence of other constituents, presumably flavonoid and phenolic compounds, and not alkaloids. The value of DPPH test is therefore questionable in the evaluation of the stability of *Chelidonium majus* tinctures and presumably other tinctures with alkaloids as active costituents.

Additional testing for the light stability of the *Chelidonium majus* tincture was performed, as it was observed that the tincture exposed to daylight gradually lost its colour. Diluted tincture and chelidonine standard solution were irradiated with mercury lamp with emission wavelength 254 nm, and with xenon lamp with the emission spectrum similar to solar light. Results in Table 3 show an interesting pattern: chelidonine is rapidly degraded under

mercury lamp in the standard solution, but significantly slower in the tincture. Under xenon lamp, whose spectrum is practically devoid of the UV component, chelidonine in standard solution is not degraded at all, but its concentration is markedly decreased in the tincture. Degradation or absence of it in the colourless standard solution under UV and visible light, respectively, can be readily explained by the absorption spectrum of chelidonine (insert in Figure 2) with prominent absorption in the UV range and none in the visible range. In the tincture, molecules of chelidonine are shielded from the UV light by other absorbing compounds, therefore the degradation is slower. The tincture contains many coloured compounds which obviously absorb visible light; these may transform into activated species and react with or transfer the energy to other non-absorbing constituents, such as chelidonine. This phenomenon of photosensitization is well known from the field of photochemistry (Gilbert and Baggott, 1991). Changes in antioxidant capacity of the tincture upon irradiation correspond very well with chelidonine concentration changes (Table 3). The instability of active constituent chelidonine in tincture exposed to visible light has important practical consequences. Tinctures should be stored in containers impenetrable to light. Brown glass bottles should be tested for the purpose, but as they absorb only a part of visible spectrum, they would not necessarily prevent degradation.

Table 3

3.3. Stability of *Sambucus nigra* flowers tincture

In the freshly prepared tincture, rutin was present in the highest concentration and was rapidly decreasing with time (Figure 6A): difference after 200 days was –44.7 % (room temperature) or –48.6 % (40 ^{o}C); the difference between the two is within the RSD (Table 1). However, isoquercitrin concentration in the same tincture (Figure 6B) was actually increased during the stability experiment, possibly as a result of rutin degradation, which can easily hydrolyse both to isoquercitrin and to quercetin (Bilia et al., 2001). After initial rise, isoquercitrin concentration started to decrease at accelerated testing conditions (40 ^{o}C),

implying the possible role of temperature in its degradation. However, its concentration at both storage temperatures after 6 months remained higher than the initial value: by +128 % at room temperature and by +33.2 % at 40 °C. Quercetin concentration remained low throughout the experiment and was somewhat decreased after 6 months (Figure 6B): –30.4 % at room temperature and –80.5 % at 40 °C. The time regression (quadratic function) equations also indicated a significant difference. Therefore, an influence of temperature on quercetin degradation was observed. In the commercial tincture, quercetin was initially present in the highest concentration, suggesting degradation of its glycosides rutin and isoquercitrin before the time of purchase. Additional evidence for this is obtained from the stability experiment on fresh tincture, in which rutin was not stable. Quercetin concentration further decreased during the stability experiment by –97.0 % at room temperature and by –98.7 % at 40 °C; the difference is within the experimental error. The initially low concentrations of isoquercitrin and rutin further decreased during the experiment by –63 % to –79 %. The results for room temperature and accelerated conditions for both compounds are again within the experimental error. Therefore, the influence of temperature on the degradation of the three flavonoids could not be confirmed on commercial tincture.

Figure 6

The antioxidant capacity of elder extracts is correlated to the total phenolic content (Mikulic-Petkvsek et al., 2015), but not necessarily to the flavonoids concentration (Dawidowicz et al., 2006). Nevertheless, similar trends were obtained during the stability experiments – depicted for freshly prepared diluted (1:5, *V/V*) tincture in Figure 6C. Antioxidant capacity was in diluted prepared tincure diminished by –19.0 % and –7.6 % at room temperature and 40 °C, respectively; and by –33.4 % (room temperature) and –52.7 % (40 °C) in undiluted commercial tincture. Better stability of freshly prepared tincture is attributed to at least two reasons: firstly, the initial antioxidant capacity was very high and the tincture had to be diluted in order to perform the measurements. If the degradation of compounds proceeds

with a constant rate with time, smaller differences would be seen at higher concentrations. Secondly, the most abundant constituent of the prepared tincture was rutin, which was degraded to isoquercitrin and quercetin. All three compounds are prominent antioxidants and their interconversion probably does not affect the overall antioxidant capacity of the tincture.

Isoquercitrin is one of the main flavonoids in the *Sambucus* flowers preparations (Dawidowicz et al., 2006) and possibly one of the active compounds (Blumenthal et al., 2000; Matsumoto et al., 2009; Rogerio et al., 2007). Its concentration in freshly prepared tincture actually increases during the storage. It is thus concluded, that the freshly prepared *Sambucus* flowers tincture is stable for at least 6 months at both normal and accelerated stability testing conditions. Prolonged testing would be needed to determine its shelf life, but it can be concluded that it is more than six months.

Chromatographic profiles of both tested tinctures were changed during the experiment, mainly reflecting the relative changes in the concentration of the three flavonoids. No further information could be elicited from their comparison. Finally, comparison of absorption spectra (200-750 nm) before, during and after the experiment revealed no changes.

3.4. Stability of *Thymus vulgaris* and *Thymus serpyllum* tinctures

Freshly prepared tincture of *Thymus serpyllum* was analysed immediately after preparation and had a high concentration of the active compound thymol (Figure 4A; Table 2). Its concentration had already significantly decreased after 40 days of storage both at room temperature and at 40 ^{o}C and was still decreasing on subsequent testing after 100 and 200 days, respectively (Figure 7A). The observed difference after 200 days was for normal storage conditions –73.5 % and –71.5 % for accelerated conditions. The slight difference at both conditions is within the experimental error of the method (Table 1). Time regression analysis (quadratic function) indicated the same trend at room temperature and at 40 ^{o}C. In the commercial tincture, thymol concentration decreased as well during the experiment, although to a lesser extent: –34.1 % under normal and –28.4 % under accelerated storage conditions. Again, time regression analysis data were very similar at both temperatures. In

both tinctures, the observed decrease in active component thymol concentration after 6 months is significantly higher than the set limit of ±5 % of the initial assay value. The concentration decrease exceeds the set limit already after 40 days of storage. The shelf life of these tinctures, according to EMEA-HMPC guidelines, is thus estimated below 1.5 months as the concentration of marker compound had already reduced by at least 10 %.

Figure 7

As Figure 7B shows, in 40 days the antioxidant activities of both tinctures has already diminished by more than 10 % during the stability experiment. Spasmolytic effects of *Thymus* sp. are currently attributed to the flavonoids and to a lesser extent to phenolics (Blumenthal et al., 2000; Direkvand-Moghadam and Khosravi, 2012; Engelbertz et al., 2012). Thus, diminished antioxidant activity of the tinctures could possibly signify decreased concentration of flavonoids and consequently diminished spasmolytic activity.

Chromatographic profiles of both tinctures have not significantly changed during storage except for the peaks getting lower. In the prepared *Thymus serpyllum* tincture, compound borneol, not present initially (Table 2), appeared after 6 months of storage at both conditions. In the commercial *Thymus vulgaris* tincture, this compound was present initially as the most abundant peak in the chromatogram (Figure 4B; Table 2). Therefore, it is proposed that it could be used as a marker of *Thymus* sp. tincture deterioration. One possible explanation for the increase in borneol content during storage would be the chemical transformation of some other compounds from the tincture, but additional experiments would be needed to confirm this hypothesis.

The absorption spectra of the *Thymus* tinctures revealed some changes, such as the decrease in the absorption maximum at approximately 670 nm. However, these changes do not necessarily correspond to the change in tincture efficacy. Active volatile compounds present in the *Thymus* sp. are colourless and do not contribute to the observed maximum at 670 nm.

4. CONCLUSIONS

The stability of freshly prepared and commercially available tinctures was assessed according to EMEA-HMPC guidelines by monitoring the concentration of their active or marker constituents with the appropriate chromatographic method. Stability testing was conducted for six months under both normal and accelerated conditions.

Tinctures of different herbs used in traditional Western herbal medicine were chosen on the basis of different active constituents. At least one of them was quantitatively determined: alkaloid chelidonine in *Chelidonium majus* tincture; phenolic compound thymol in *Thymus vulgaris* and *Thymus serpyllum* tinctures; flavonoids isoquercitrin, rutin and quercetin in *Sambucus nigra* flowers tinctures.

The predictive power of different simple procedures for the assessment of the tincture stability was tested and compared to the reference quantitative determination of the active/marker constituent: chromatographic fingerprinting, spectrophotometric monitoring of the tincture colour and DPPH antioxidant assay. All three performed inferiorly and failed to detect significant changes in tincture composition, except for the antioxidant assay, which was in accordance with the reference determination in the case of *Sambucus nigra* tinctures, where the quantified compounds have an antioxidant activity.

In the *Chelidonium majus* and *Thymus* sp. tinctures, the concentration of marker compounds after 40 days had already reduced by at least 10 %. The shelf life of these tinctures, according to EMEA-HMPC guidelines, was thus estimated below 1.5 months. However, freshly prepared *Sambucus nigra* flowers tincture had a satisfactory content of the active compound isoquercitrin after six months of testing. Prolonged testing would be needed to determine its shelf life, but it can be concluded that it is more than six months.

The results of our study imply that only assays directly linked to the content of active or marker compounds should be used for stability testing of the herbal tinctures. Other tests

incorrectly predict the stability of the tincture, which is correlated to its effectiveness and thus concentration of active compounds.

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H. Prosen and B. Pendry:

Determination of shelf life of *Chelidonium majus*, *Sambucus nigra*, *Thymus vulgaris* and *Thymus serpyllum* herbal tinctures by various stability-indicating tests

FIGURE CAPTIONS

Figure 1: Structures of the quantified compounds.

Figure 2: HPLC-DAD (280 nm) chromatogram of *Chelidonium majus* tincture (1:5 *m/V*, 45 % ethanol) diluted 1:5 (*V/V*) with deionised water. Insert: UV-Vis spectrum of chelidonine in methanol.

Figure 3: HPLC-DAD (360 nm) chromatograms of (**A**) freshly prepared, diluted 1:10 (*V/V*) with deionised water, and (**B**) commercial, non-diluted, *Sambucus nigra* flowers tincture (1:4 *m/V*, 25 % ethanol).

Figure 4: GC-MS (TIC) chromatogram of (**A**) *Thymus serpyllum* prepared tincture and of (**B**) *Thymus vulgaris* commercial tincture (both 1:3 *m/V*, 45 % ethanol) after HS-SPME extraction. Peak assignments are given in Table 2.

Figure 5: Stability of *Chelidonium majus* tincture 1:5 (*m/V*) at room temperature (**rT**) and at 40 ^{o}C (**40**). (**A**) chelidonine concentration; (**B**) antioxidant capacity as DPPH assay.

Figure 6: Stability of prepared *Sambucus nigra* tincture at room temperature (**rT**) and at 40 ^{o}C (**40**). (**A**) Concentration of rutin (**R**); (**B**) concentration of isoquercitrin (**IQ**) and quercetin (**Q**); (**C**) antioxidant capacity as DPPH assay.

Figure 7: Stability of prepared *Thymus serpyllum* tincture (**Ts**) and commercial *Thymus vulgaris* tincture (**Tv**) at room temperature (**rT**) and at 40 ^{o}C (**40**). (**A**) thymol concentration; (**B**) antioxidant capacity as DPPH assay.

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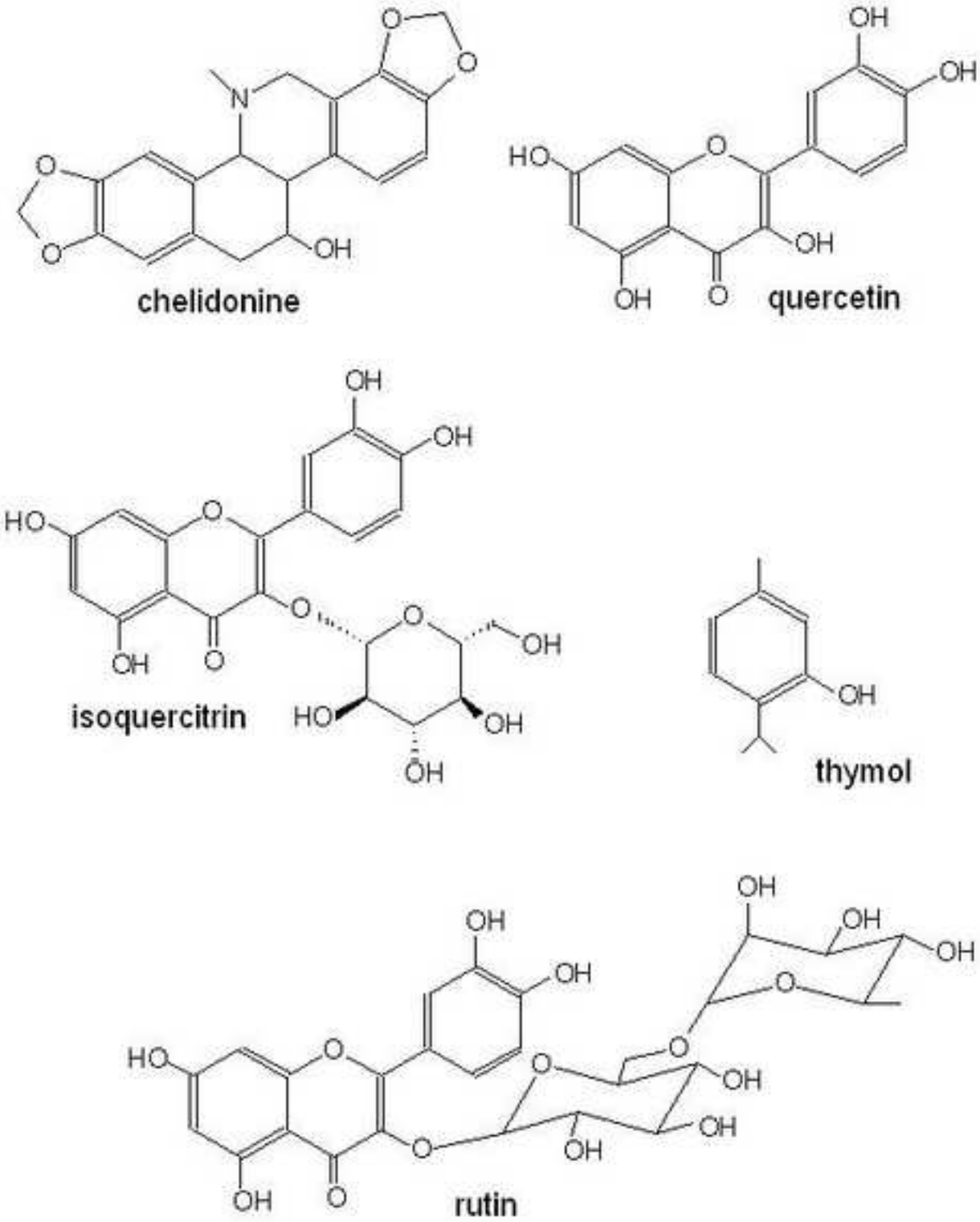

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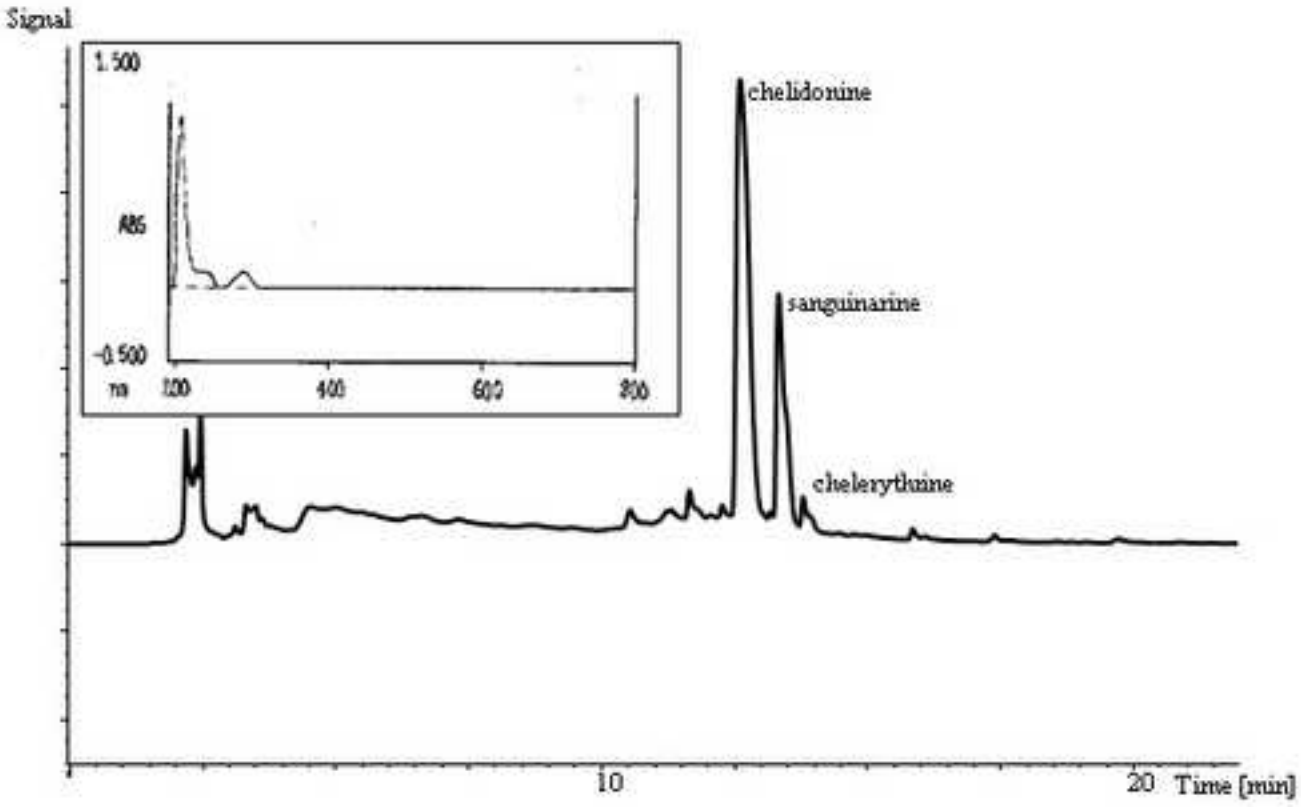

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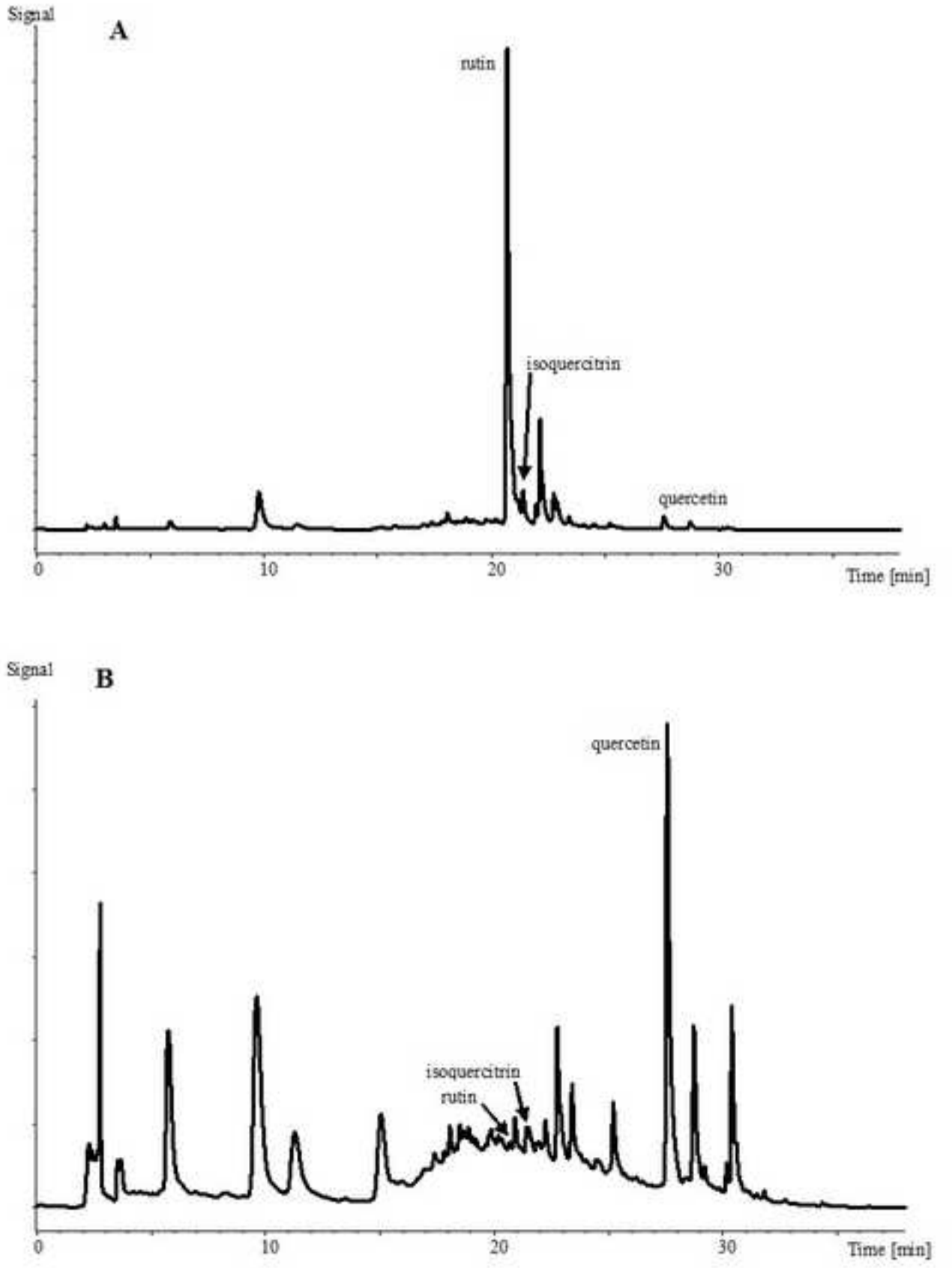

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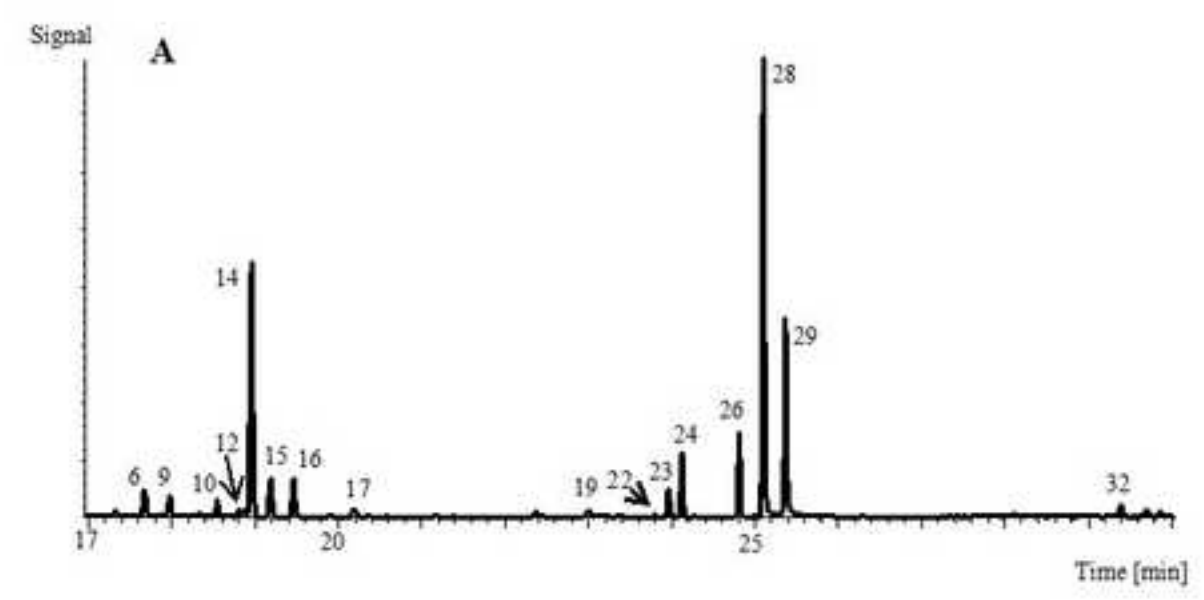

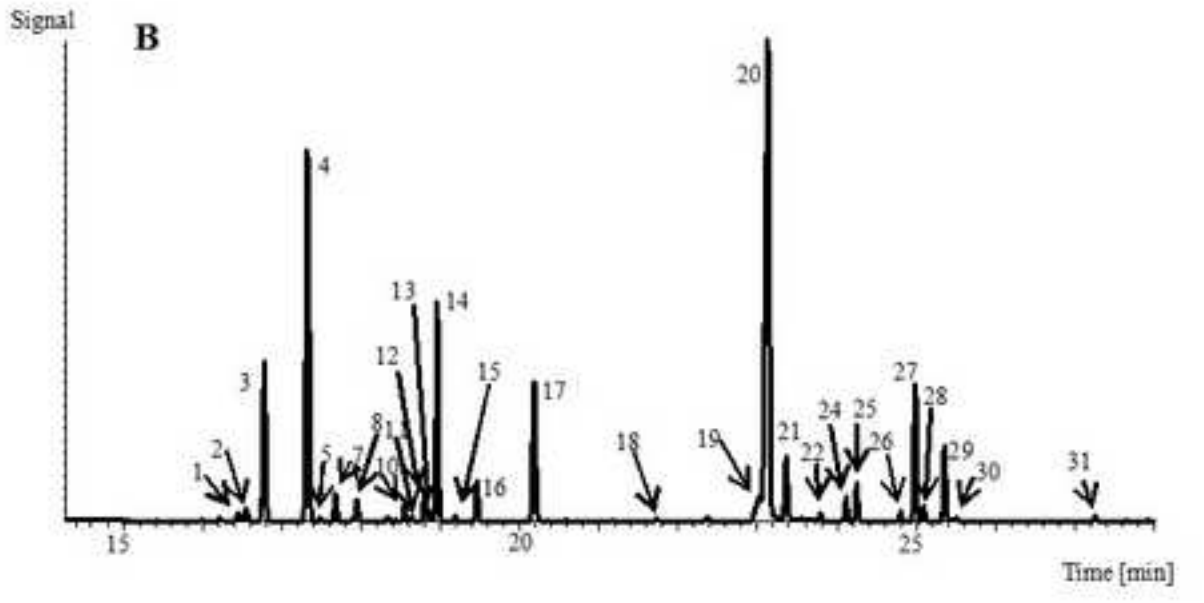

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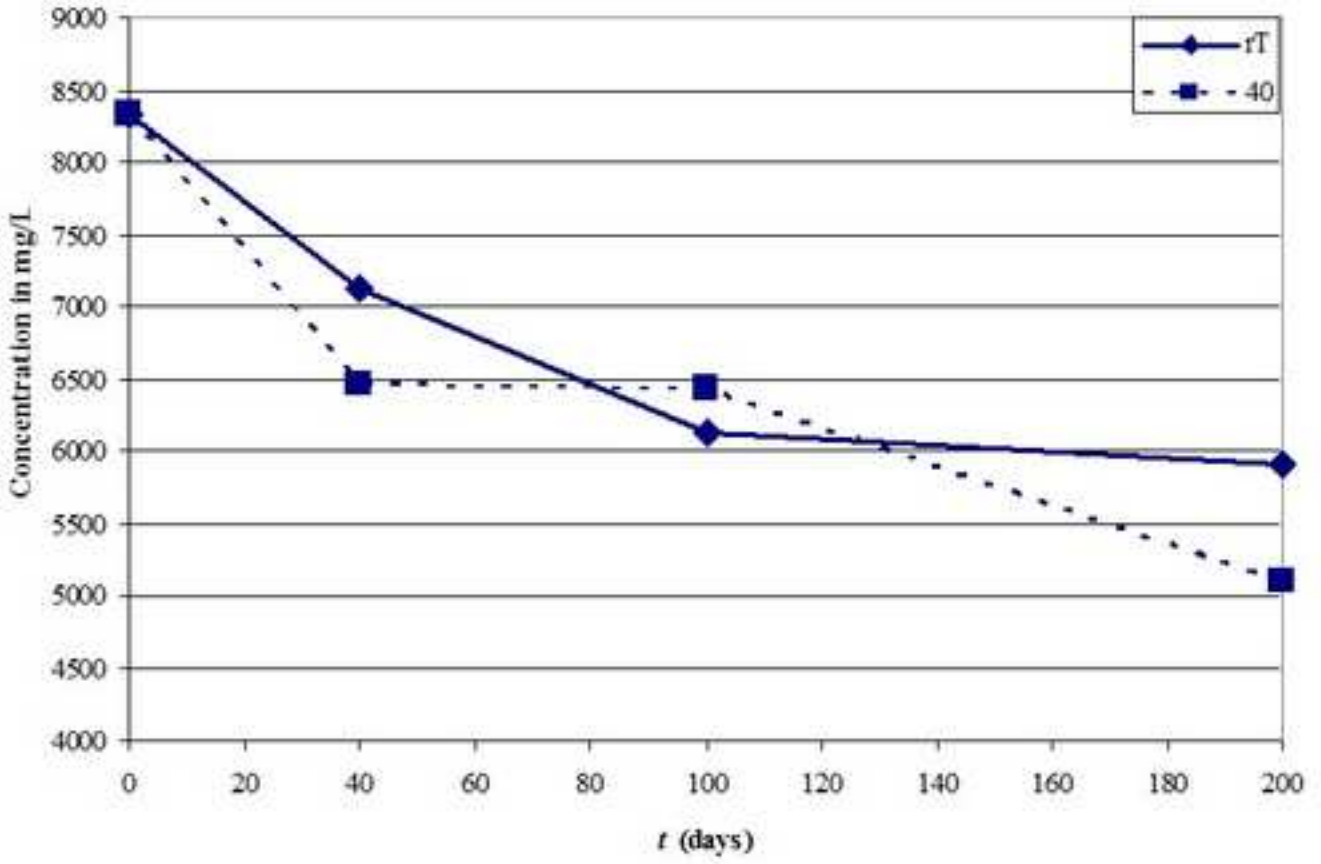

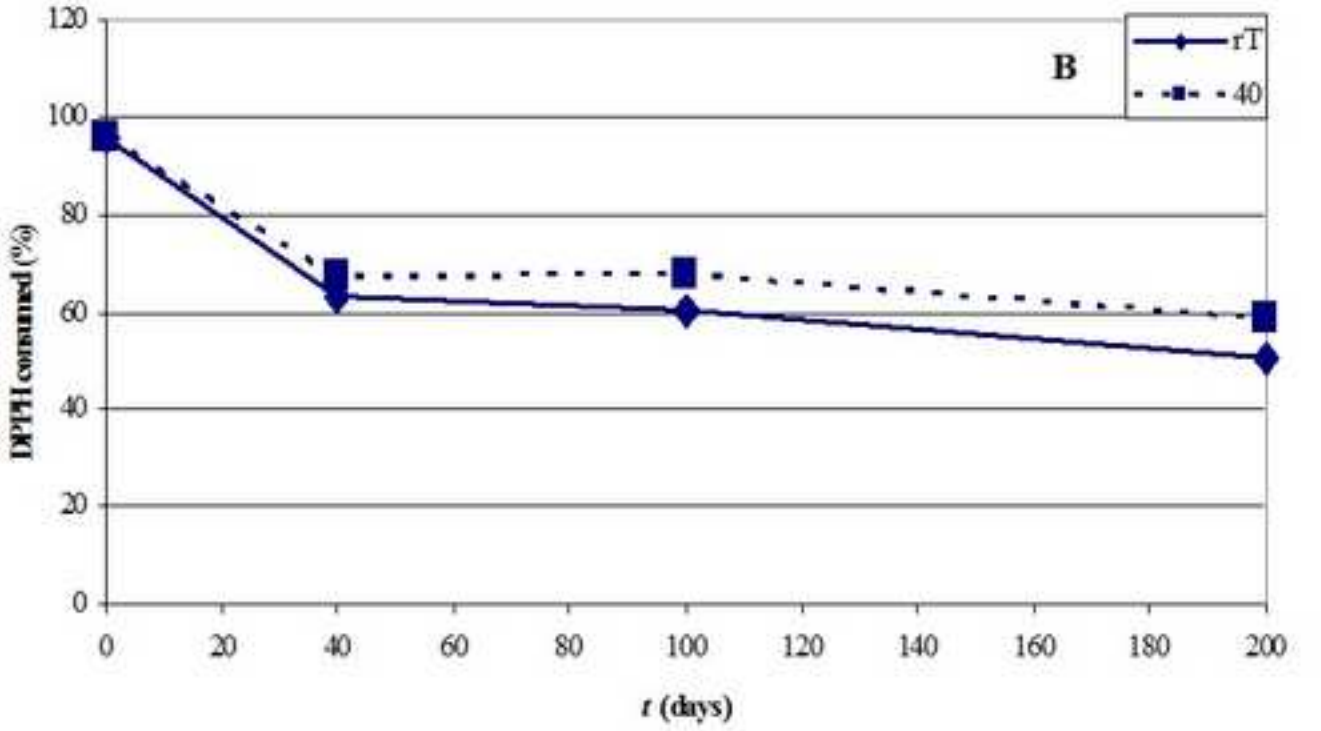

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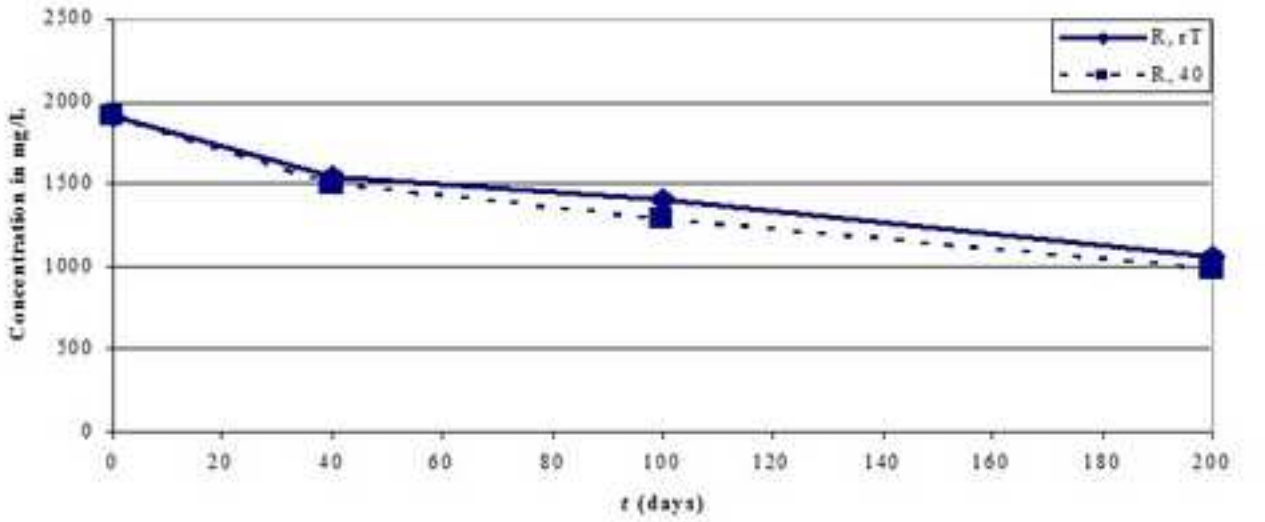

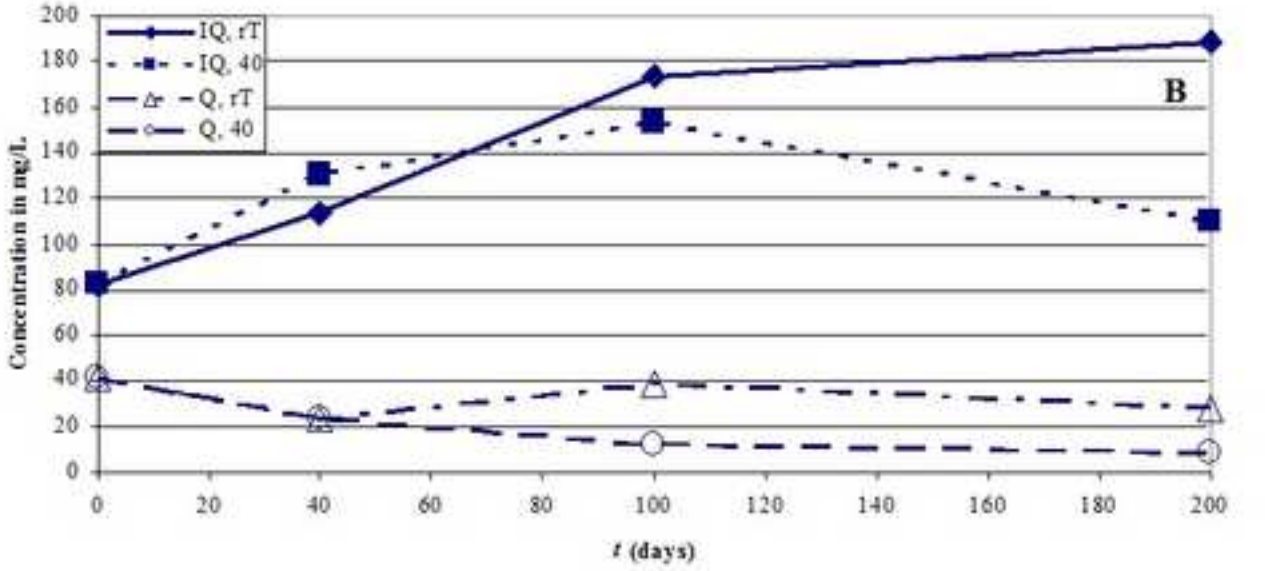

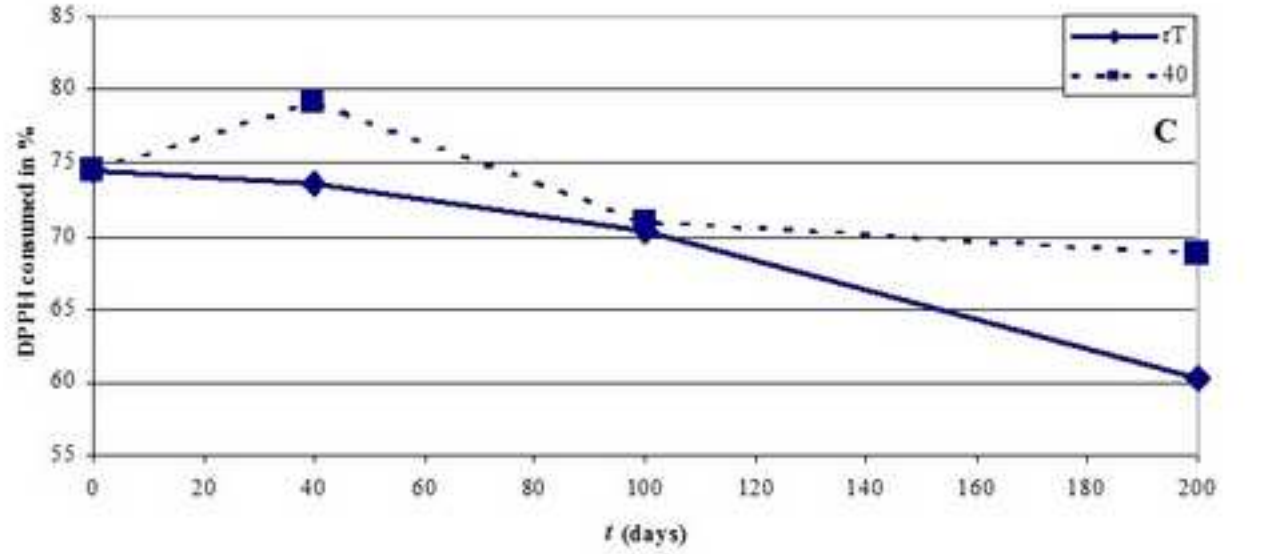

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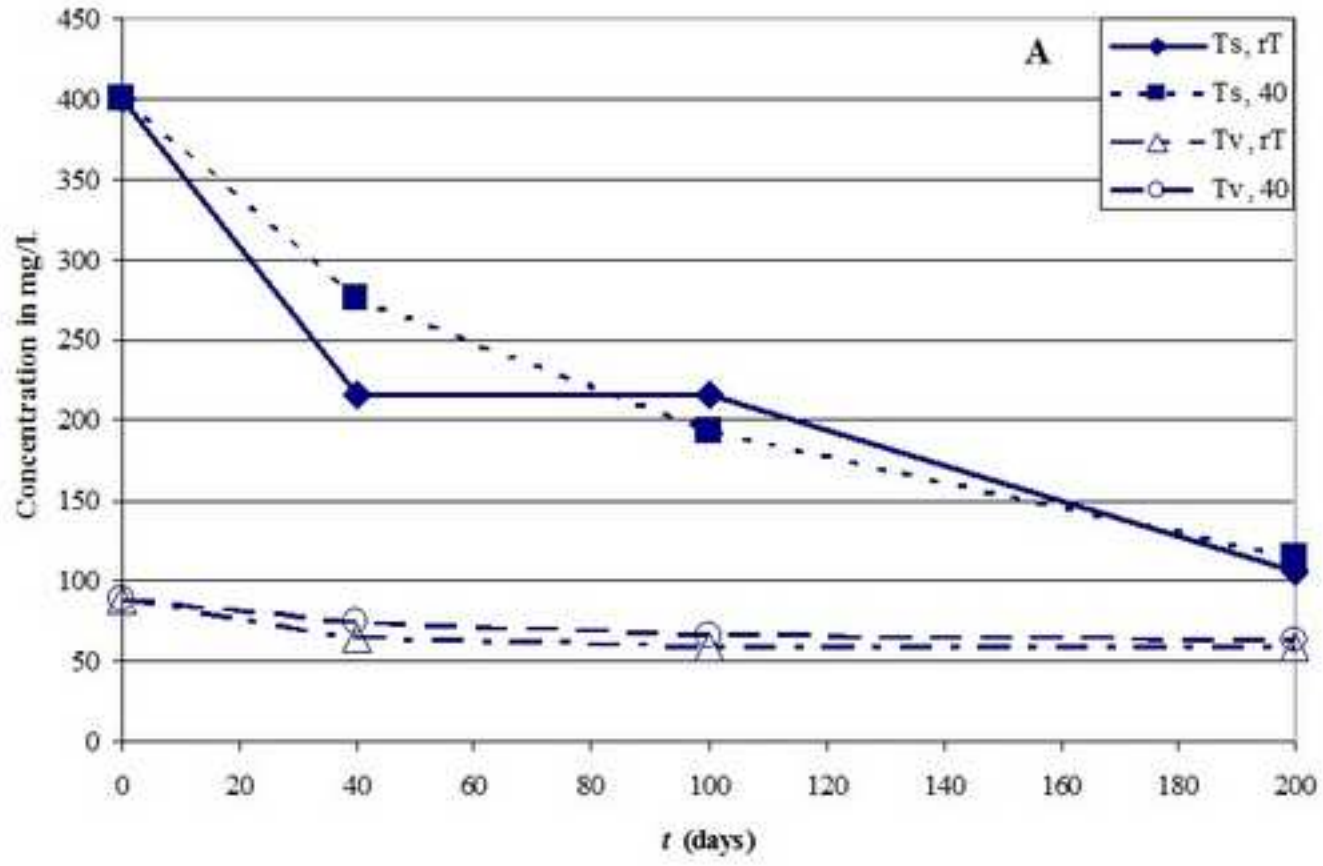

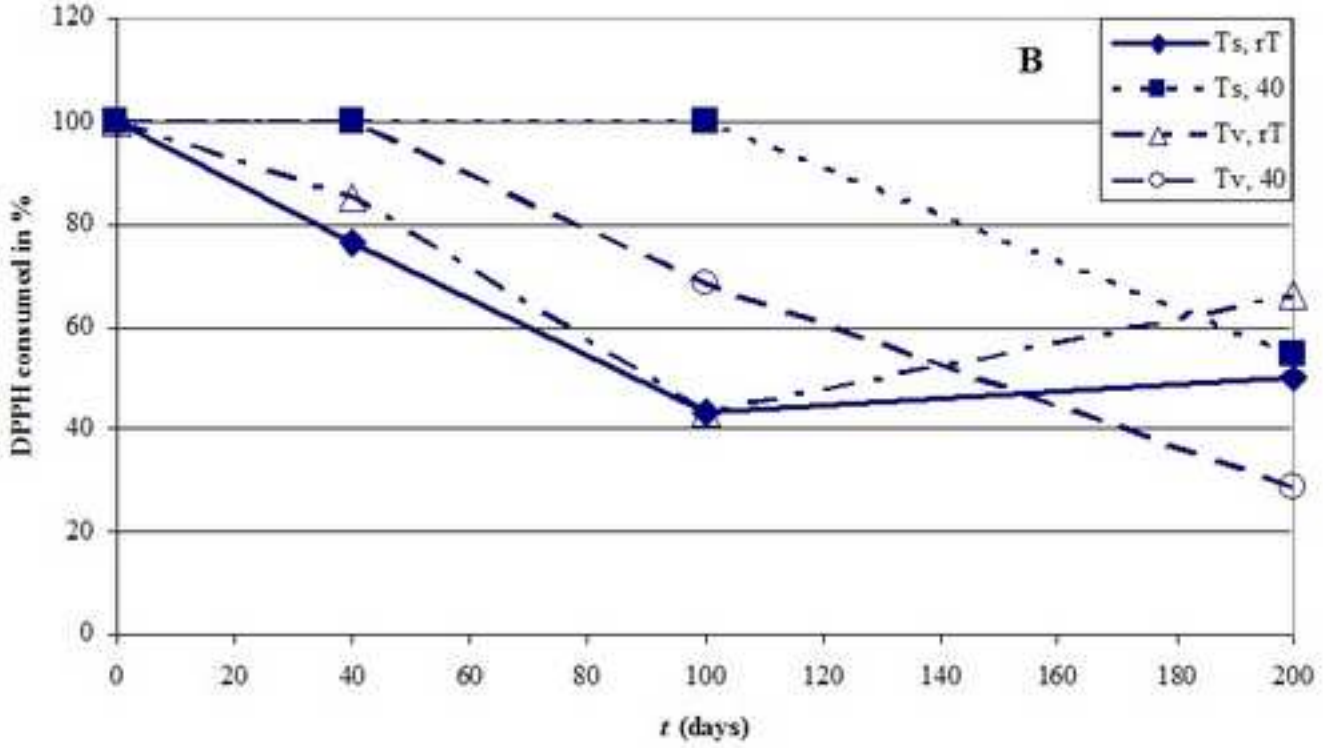

Table 1: Calibration parameters for the quantified compounds (standard solutions): chelidonine by HPLC-DAD method (peak areas at 280 nm); isoquercitrin, quercetin and rutin by HPLC-DAD method (peak areas at 360 nm); thymol by GC-MS (TIC) method. LLOQ (lower limit of quantitation) estimated as $10 \cdot s_{blank}$. RSD from at least three repeats. Ar - peak area.

	t_R [min]	calibration range	linear regression	R^2	RSD(peak area) [%]	estim. LLOQ
chelidonine	12.6	0.5 – 50.0 mg/L	$Ar = 17.01 \cdot c - 1.90$	0.9971	12.6	0.2 mg/L
isoquercitrin	21.3	0.5 – 50.0 mg/L	$Ar = 55.08 \cdot c - 21.13$	0.9984	9.4	0.4 mg/L
quercetin	27.5	0.6 – 50.0 mg/L	$Ar = 102.73 \cdot c - 62.39$	0.9999	8.4	0.6 mg/L
rutin	20.6	0.5 – 50.0 mg/L	$Ar = 47.07 \cdot c - 11.54$	0.9998	7.4	0.3 mg/L
thymol	25.1	0.5 – 5.0 mg/L	$Ar = 36.34 \cdot c - 8.28$	0.9547	3.6	0.2 mg/L

Table 2: Compounds identified in (**A**) the prepared *Thymus serpyllum* tincture and in (**B**) the commercial *Thymus vulgaris* tincture (both 1:3 (*m/V*), 45 % ethanol), on the basis of their mass spectra, by comparison with spectra from the NIST05 spectral library. Relative area is the peak area normalized to the most prominent peak in the chromatogram (relative area 100). Peak No. refers to the peaks in the Figures 4A and 4B.

Peak No.	t_R [min]	Compound	Rel. area (A)	Rel. area (B)	Peak No.	t_R [min]	Compound	Rel. area (A)	Rel. area (B)
1	16.45	origanene	n.d.	1.1	17	20.19	β-linalool	2.8	20.9
2	16.55	tricyclene	n.d.	2.0	18	21.72	α-campholenal	n.d.	0.5
3	16.77	α-pinene	n.d.	22.6	19	23.02	4-terpineol	2.3	6.2
4	17.32	camphene	n.d.	53.5	20	23.12	borneol	n.d.	100
5	17.49	α-thujene	n.d.	0.5	21	23.36	α-terpineol	n.d.	11.0
6	17.67	matsuka alcohol	6.9	n.d.	22	23.79	*trans*-dihydrocarvone	0.7	1.2
7	17.68	β-myrcene	n.d.	3.4	23	23.79	thymol-methylether (isomere)	6.4	n.d.
8	17.95	β-pinene	n.d.	3.2	24	24.11	thymol-methylether	14.0	3.0
9	17.98	3-octanone	5.1	n.d.	25	24.25	bornyl formate	n.d.	4.6
10	18.55	α-terpinene	4.0	2.3	26	24.80	thymoquinone	18.4	1.2
11	18.65	β-*trans*-ocimene	n.d.	0.7	27	24.99	bornyl acetate	n.d.	16.6
12	18.80	D-limonene	1.6	4.2	28	25.09	thymol	100	2.0
13	18.87	β-*cis*-ocimene	n.d.	1.3	29	25.37	carvacrol	48.2	9.0
14	18.96	*p*-cymene	63.4	27.8	30	25.50	α-terpinyl acetate	n.d.	0.4
15	19.20	eucalyptol	11.0	0.9	31	27.25	caryophyllene	n.d.	0.6
16	19.46	crithmene	10.3	5.3	32	29.40	isopropyl laureate	3.6	n.d.

n.d…. not detected

Table 3: Stability of chelidonine in standard solution and *Chelidonium majus* tincture (diluted 1:25 (*V/V*) with deionised water) on irradiation with UV or visible light; antioxidant capacity of irradiated tincture (diluted 1:25, *V/V*).

	remaining chelidonine (%)		remaining antioxidant capacity (%)
Sample	stand. solution	tincture	tincture
UV lamp 24 min	0	68	70
Xe lamp 6 h	100	46	37