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Dynamics of the isoflavone metabolome of traditional preparations of *Trifolium pratense* L.

Gonzalo R. Malca-Garcia[a], **Daniel Zagal**[a], **James Graham**[b], **Dejan Nikolić**[a], **J. Brent Friesen**[b,c], **David C. Lankin**[a,b], **Shao-Nong Chen**[a,b], and **Guido F. Pauli**[a,b,*]

[a]UIC/NIH Center for Botanical Dietary Supplements Research

[b]Center for Natural Product Technologies (CENAPT), Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood St., Chicago, IL 60612, USA

[c]Physical Sciences Department, Rosary College of Arts and Sciences, Dominican University, River Forest, IL 60305, USA

Abstract

Ethnopharmacological relevance—The flowering tops of *Trifolium pratense* L., popularly known as red clover, are used in ethnic Western and Traditional Chinese medicine, in a variety of preparations, including infusions, decoctions and tinctures. Red clover has been reported to be helpful for treatment of menopausal symptoms, premenstrual syndrome, mastalgia, high cholesterol, and other conditions.

Aims of the study—The aims were to compare the chemical dynamics between traditional preparations of infusions, decoctions, and tinctures, as well as to identify the chemical variability over time in a traditional red clover tincture. For this purpose, eight isoflavone aglycones as well as two glucosides, ononin and sissotrin, were used as marker compounds.

Materials and methods—Quantitative NMR (qHNMR), LC-MS-MS, and UHPLC-UV methods were used to identify and quantitate the major phenolic compounds found within each extract.

Results—Infusions, decoctions and tinctures were shown to produce different chemical profiles. Biochanin A and formononetin were identified and quantified in infusion, decoction, and tinctures of red clover. Both infusion and decoction showed higher concentrations of isoflavonoid glucosides, such as ononin and sissotrin, than 45% ethanolic tinctures. Dynamic chemical

*Corresponding Author. Tel.: (312) 355-1949; Fax: (312) 355-2693. gfp@uic.edu.

Conflict of interest

The Authors declare no conflict of interest.

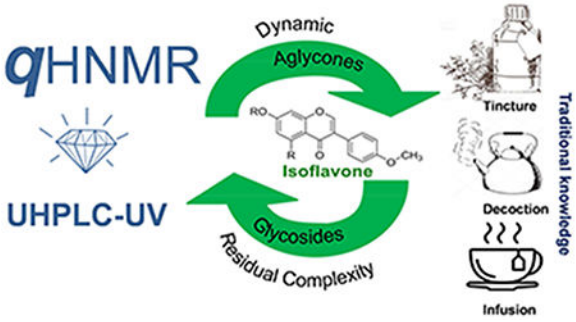
Dedication

Dedicated to James B. McAlpine on the occasion of his 80th birthday.

variability (“dynamic residual complexity”) of the red clover tincture was observed over time (one-month), with biochanin A and formononetin reaching peak concentrations at around six days.

Conclusions—Insight was gained into why different formulation methods (infusions, decoctions, and tinctures) are traditionally used to treat different health conditions. Moreover, the outcomes show that tinctures, taken over a period of time, are dynamic medicinal formulations that allow for time-controlled release of bioactive compounds.

Graphical Abstract



Keywords

Infusion; Decoction; Ethanolic tincture; qHNMR; Metabolomic investigation; Red clover; CPC; Formononetin; Biochanin A

1. Introduction

Trifolium pratense L. has been widely used as a food, dietary supplement or medicine in the treatment of a wide variety of ailments (Lim, 2014). These include cancer, heart problems, inflammatory diseases, epilepsy, high cholesterol, sexual transmitted diseases, and various “female complaints” including dysmenorrhea, premenstrual syndrome, mastosis, menopausal symptoms and mastalgia (Booth et al., 2006; Duke et al., 2002). Red clover has been shown to contain substantial amounts of isoflavonoids, especially biochanin A (1) and formononetin (2), along with their respective glucosides that constitute one of the major sources of isoflavonoids in western dietary supplements (Geller and Studee, 2006).

Dry red clover contains biochanin A (1) and formononetin (2) in concentrations ranging from 0.1% to 0.9% w/w. Minor isoflavonoids, typically well below 0.1% w/w, are genistein (3), daidzein (4), pratensein (11), pectolinarigenin, calycosin (8), trifoside, and pseudobaptigenin (7) (He et al., 1996). However, in isoflavonoid-enriched clinical red clover extract (RCE), isoflavonoid content can be much higher, reaching >10% levels of certain compounds such as 14.5% w/w of biochanin A (1) and 14.3% of formononetin (2), in addition to 0.23% of daidzein (4) and 0.41% of genistein (3), in the clinical extract investigated on our Botanical Center (Booth et al., 2006; Phansalkar et al., 2017). Biochanin A (1) and formononetin (2) are the 4’-*O*-methyl derivatives of genistein (3) and daidzein (4) (see Fig. 1), and are the predominant isoflavones found in a number of leguminous plants, including *Medicago sativa* L. (alfalfa), red clover, and *Cicer arietinum* L. (chickpeas) (Mazur et al., 1998). The bioactivity of red clover extracts, especially in the domain of women’s

health, is associated with estrogenic isoflavones that can undergo metabolism *in vivo.* Hepatic enzymes capable of demethylating the proestrogenic isoflavones, biochanin A (1) and formononetin (2), yield the more potent phytoestrogens, genistein (3) and daidzein (4) (Tolleson et al., 2002). In addition, prunetin (5) has also been reported as a "prodrug", because it can be converted metabolically into genistein (3) (Hu et al., 2003).

Red clover, when consumed orally in ethnomedicine, is typically taken in the form of infusions, decoctions, or tinctures (Barnes et al., 2007; Duke et al., 2002; Mamedev et al., 2015), and different preparations are used to treat a variety of ailments. Infusions are used for treating colds, as well as bronchitis and asthma. Decoctions of dry leaves and flowers are used to treat chest pain, malaria, chronic rheumatism, kidney problems, and inflammation of the bladder (Barnes et al., 2007; Duke et al., 2002; Zevin et al., 1997). Tinctures have been used traditionally for their broad estrogenic activity in a variety of women's health indications, as well as to increase resistance to viral and bacterial infections (Fitzgerald, 2014; Zevin et al., 1997).

Infusions are typically prepared by first boiling water and removing it from the heat before adding the plant material. Using this extraction method yields infusions that are generally relatively rich in glycosides and essential oil (Handa et al., 2008; Voon et al., 2012). In decoctions, plant material is boiled continuously in water for a period of time. Decoctions are reported to yield the highest amount of total glycosides, as compared with other extraction methods that may use organic solvents such as maceration, percolation, and Soxhlet extraction (Voon et al., 2012). In contrast, tinctures are alcoholic (ethanolic) extracts of plants made with varying percentages of alcohol. In traditional preparations, these are typically made with locally-available alcohol. In Peru, for example, the cane liquors "Canazo" or "Yonque" are often utilized (Bussmann and Sharon, 2015), whereas Russian traditional medicine uses vodka (Shikov et al., 2017) and Traditional Chinese Medicine (TCM) employs wine or 50% alcohol (Hong et al., 2015).

Aqueous plant-based remedies like infusions and decoctions are typically utilized immediately or within a short time of their preparation. Tinctures, on the other hand, can be prepared, stored and used over much longer periods of time. The effect of long periods of maceration on the chemical extractives, such as contained in tinctures, has been studied only marginally (Ieri et al., 2015). It is well known that plants produce a great diversity of chemicals (Owen et al., 2017). In addition to the differences in preparation, the complexities of harvest, dosage forms, and human metabolic variation multiply the overall complexity involved in understanding the totality of processes that affect therapeutic outcomes (Sumantran and Tillu, 2013).

In order to identify and quantify the chemical variation in the prepared extracts simultaneously and without the need for repeated sourcing of identical reference compounds, this study used quantitative ^{1}H nuclear magnetic resonance (qHNMR) spectroscopy, along with more classical analytical chemistry methods such as high pressure liquid chromatography (HPLC) coupled with UV and mass spectrometric detectors (UHPLC-UV, LC-MS). An advantage of qHNMR is its unbiased view of the sample composition and inherent avoidance compound-specific response factors (the ratio between a

signal produced by a particular instrument for a specific compound, and its quantity in the sample) that are unavoidable in almost all other analytical methods. Moreover, qHNMR has the ability to quantify multiple compounds simultaneously, making it a powerful method for metabolomic studies and quality control of complex natural samples such as foods, plants, herbal remedies, and biofluids (Pauli et al., 2012). Related to the dynamic aspect of the present studies, qHNMR has also been used to monitor metabolic changes in the fermentation or aging processes of a variety of food products such as red wine (Lopez-Rituerto et al., 2009) and soy sauce (Ko et al., 2009), as well as to provide for the analysis and quality control of herbal products (Napolitano et al., 2014).

This report describes the development and application of a qHNMR method for the investigation of *T. pratense* extracts typically used in traditional medicine. Serendipity, empirical innovation, and evolution are all relevant for understanding the dynamic nature of traditional preparations, as different preparation methods applied to the same botanical material can affect the concentrations of extracted components that have the potential to elicit different biological responses. This study evaluated the variability of ten isoflavonoids that represent bioactive marker compounds of *T. pratense* and were identified as well as quantified with qHNMR over one month in a traditional ethanolic extract of red clover.

2. Materials and methods

2.1. Processed T. Pratense L. samples

The aerial parts of *T. pratense* L. were purchased from Mountain Rose Herbs (Eugene, OR, USA) in April 2013. A voucher specimen (code: BC 719) is kept at the Botanical Center, Chicago, IL, USA. This plant material was powdered using a mill grinder (KitchenAid, St. Joseph, MI, USA). A tea sieve was used to obtain uniform size particles, then the powder was collected and stored at room temperature.

2.2. Chemicals and reagents

Chemical solvents and reagents were purchased from Fischer (Hanover Park, IL, USA) and Sigma Aldrich (St. Louis, MO, USA). Reference standards of biochanin A (1), formononetin (2), genistein (3), ononin (10), sissotrin (9), and daidzein (4) were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). Irilone (6) and prunetin (5) were purified from clinical red clover extract (RCE) using Centrifugal Partition Chromatography (CPC); the Supporting Information shows their ^{1}HNMR (400 MHz) spectra in Figs. S16 and S17. The external calibrants, dimethylsulfone (Code N. 048-33271; 100%) was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan) and caffeine (CAS-N. 5808-2; 100%) was purchased from Sigma Aldrich (St. Louis, MO, USA). The internal calibrant, 3,5-dinitrobenzoic acid (CAS-N. 99-34-3; 99.99%) was also purchased from Sigma Aldrich (St. Louis, MO, USA). Deuterated dimethyl sulfoxide (DMSO-d_6, 99.9% D) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA).

2.3. Instrumentation

The qHNMR experiments were performed on a JEOL Resonance Inc. (Akishima, Tokyo, Japan) 400 MHz YH NMR spectrometer, model JNM-ECZ400S/L1 at 25 °C (298 K)

equipped with a liquid N_2 Super COOL probe (NM-Z161331TH5SC). The NMR data were analyzed and processed with JEOL Delta v5.0.4.4 (Delta™ NMR Data Processing Software, Akishima, Tokyo, Japan) and/or MestReNova 11.0.4 software from Mestrelab Research S. L. (Santiago de Compostela, Spain). For UHPLC analysis, a Shimadzu (Kyoto, Japan) Nexera UHPLC-UV system equipped with a DAD and employed a Kinetex 1.7 μm XB-C18 100 Å column (50 mm × 2.1mm) and LabSolutions software for data analysis. The column oven, detector cell, and autosampler temperature were maintained at 40 °C, 40 °C and 4 °C, respectively, throughout the analysis.

LC-MS-MS analyses were carried out using a Waters 2695 (Milford, MA, USA) solvent delivery system connected to a Waters SYNAPT quadrupole/time-of-flight (Q/TOF) mass spectrometer operated in the positive ion electrospray mode.

2.4. T. pratense extract preparation

The diversity of dosage forms and preparations, along with therapeutic indications, for red clover were reported by Duke, Fitzgerald and Zevin, (Duke et al., 2002; Fitzgerald, 2014; Zevin et al., 1997). Extract preparations of red clover from literature sources were followed, with some modification; the methods utilized in extract preparation for this experiment are briefly described below.

2.4.1. Infusion—Dry plant material has been recommended for oral administration at ½ tablespoon of the infusion (4.0 g in ~200 mL of water) taken 3-4 times daily (Duke et al., 2002). Thus, the present experiments used 2.0 g of powdered red clover, adding to 100 mL of water at 100 °C. After 60 min at room temperature, the product was filtered, and the water was evaporated using a rotary evaporator at 40 °C *in vacuo*. Finally, the sample was freeze-dried to yield 152.9 mg (7.6%) of crude extract, which was subject to qHNMR and UHPLC-UV.

2.4.2. Decoction—The decoction of *T. pratense* is generally consumed in a dose of 250 mL (15.0 g of plant material in ~400 mL of water brought to a boil for 30 min or more), 3-4 times per day (Zevin et al., 1997). Thus, the present experiments used 4.0 g of red clover powder, added to 100 mL of water, and boiled for 30 min. The mixture was filtered and the water removed by rotary evaporation at 40 °C *in vacuo.* Samples were then freeze-dried, yielding mg (12.5%) of crude extract.

2.4.3. Tincture—Tinctures (10 g in 100 mL of 45% ethanol) are prescribed in literature for internal use at the dose of 1-2 mL, taken 3 times per day (Barnes et al., 2007; Duke et al., 2002; Zevin et al., 1997). The experiments with tinctures were performed in triplicate (A, B, and C; in Table S16) and simultaneously for a period of 1 month (tincture samples: 0.5, 1, 16, 24, 72, 96, 144, 240, 360, 480, 600, and 720 hours). Approximately 800 mg of powdered red clover was used in each vial, containing 20 mL of 45% ethanol at room temperature.

In order to evaluate changes in the chemical composition of the red clover tincture over time, a total of 12 identically processed samples were prepared; each was evaluated at a different time period, ranging from 30 minutes to one month of maceration. Each of these temporal preparations were filtered (Step 1, Fig. 2), producing two samples: the filtrate, representing a

45% ethanol tincture solution (ETS), and the spend plant material (SPM). The ETS was dried with a rotary evaporator (Step 3, Fig. 2), and the SPM was oven-dried at 37 °C for 3 days (Step 2, Fig. 2). This complex process involving the separation of the tincture from its residue was necessary, particularly in the earlier timeframes of the maceration process (prior to three days maceration), because the tincture at this time was found to contain a very complex mixture of isoflavonoid glycosides and their corresponding aglycones that were difficult to resolve by qHNMR. Dried SPM was macerated twice for 5 hours, each time using 150 mL of commercial 95% ethanol with stirring at room temperature, then filtered (Step 4, Fig. 2). The SPM filtrates were combined, and the solvent removed by rotary evaporation *in vacuo* followed by lyophilization (Step 5, Fig. 2). This SPM extract (SPME) was analyzed by qHNMR in order to identify and quantify its isoflavonoid content. After qHNMR analysis of the SPME, the ETS and SPME were combined (Step 6, Fig. 2), and the resulting mixture was lyophilized for further qHNMR and UHPLC-UV investigation, in order to get an accurate metabolomic profile of the red clover plant material. Table S16 presents the amounts of plant material used for the tincture experiments (Step 7, Fig. 2).

2.4.4. Red clover methanol extract (RCME)—Three control extracts were prepared for maceration simultaneously, using 100 mg of powdered plant material in 400 mL of methanol under stirring for two days at room temperature. Then the product was filtered and dried using a rotary evaporator. The procedure yielded a total of 14.64 mg (14.5%) of crude extract.

2.5. qHNMR sample preparation

Samples were analyzed by qHNMR in triplicate. Approximately 15 mg of dry extract was dissolved in commercial DMSO (1.0 mL) and filtered to remove insoluble materials. DMSO was then removed with a Speedvac (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C. Next, 1.0 mL of water was added to each sample and frozen at −5 °C prior to lyophilization. Finally, between 7.0 to 10.0 mg of each dried sample were weighed to the nearest 0.1 mg and used for qHNMR analysis.

Spiking experiments were performed by adding 50 μL of 1.47 mg/mL biochanin A (1) and 0.87 mg/mL formononetin (2) standards in DMSO-d_6 to previously prepared samples. Biochanin A (1) spiking solution was added to the 240 hours tincture sample (3mm NMR tube containing 4.64 mg in 200 μL of DMSO-d_6) and formononetin (2) spiking solution was added to the 720 hours sample (3mm NMR tube containing 4.62 mg in 200 μL of DMSO-d_6). See Supporting Information, Figs. S38 and S39.

2.6. qHNMR analysis

2.6.1. qHNMR measurement parameters—Prior to NMR analysis, each sample was dried under vacuum (<1 mbar) in a desiccator overnight for removal of residual solvents and water. Samples were dissolved in 250 μL DMSO-d_6, of which 200 μL were delivered into a 3 mm NMR tube (NORELL, Landisville, NJ, USA) using a 1000 μL precision syringe (Valco Instruments, Baton Rouge, LA, USA). NMR spectra were acquired at 298 K. The qHNMR spectra of all extracts were acquired using standard qHNMR conditions (Napolitano et al., 2014; Pauli, 2001; Pauli et al., 2007): a 60 s relaxation delay (D1), a

receiver gain (RG) of 46, 32 scans (NS), a calibrated 90° pulse (P1), and automatic gradient shimming. The data was processed with a Lorentzian-Gaussian window function (exponential factor - 0.3, Gaussian factor 0.05 in GF mode) and the baseline corrected by a fifth order polynomial function. The residual DMSO-d_5 signal at 2.500 ppm was used for chemical shift referencing.

2.6.2. $DMSO_2$, caffeine and 3,5-dinitrobenzoic acid standards—Dimethylsulfone ($DMSO_2$) is a universal calibrant for qHNMR analysis (Wells et al., 2004). A calibration curve was constructed with $DMSO_2$ concentrations of 1.05, 2.28 and 4.29 mg/mL in 200 μL of DMSO-d_6 using 3 mm NMR tubes. The experiments were conducted under the same parameters as were used for tinctures. The $DMSO_2$ signal appeared at δ_H 2.993 ppm (6H).

In previous investigations caffeine has been regarded as a highly accurate external calibrant for the quantitation of natural products (Burton et al., 2005; Pauli et al., 2012). A caffeine calibration curve was established using three different concentrations: 4.25, 8.50, and 16.95 mg/mL in 200 μL of DMSO-d_6 using 3 mm NMR tubes. Three signals of caffeine were chosen to establish the calibration curve: three methyl groups at δ_H 3.204 (singlet), 3.398 (singlet), and 3.867 (doublet) ppm. External calibration (EC) was performed for each experiment using identical qHNMR parameters. The data for the calibration curve of $DMSO_2$ and caffeine such as relaxation time (T_1) experiments are described in the Supporting Information, Figs. S18–S23.

3,5-Dinitrobenzoic acid as an internal calibrant (IC) has been successfully used for quantitation of natural products and purity determination of organic molecules (Phansalkar et al., 2017; Weber et al., 2014). The advantage of using 3,5-dinitrobenzoic acid instead of other ICs, is that the two signals at 8.794 and 8.904 ppm do not overlap with any signal in the extracts. The IC was weighed together with two lyophilized tincture extracts in order to evaluate the accuracy of EC experiments (see Supporting Information, Figs. S41 and S42).

2.7. Purity of the ECs

The purity of the $DMSO_2$ and caffeine reference materials was reported in the certificate of analysis as being 100.00% for both $DMSO_2$ and caffeine. These purity assignments were analyzed independently by the relative 100% qHNMR method (Pauli et al., 2014). Different concentrations of $DMSO_2$: 1.05, 2.28 and 4.29 mg/mL; and caffeine: 4.25, 8.50, and 16.95 mg/mL were analyzed (Supporting Information, Table S4) and the average purities ($DMSO_2$: 99.94% and caffeine: 99.89%) were used for absolute external calibration (EC) calculations of the infusions, decoctions, and tinctures.

2.8. Linearity of EC-based qHNMR quantitation

The linearity of EC-based qHNMR quantitation was obtained by plotting the absolute integrals of $DMSO_2$ (at δ_H 2.993 ppm [6H]) and caffeine (at δ_H 3.204 ppm [3H], 3.398 ppm [3H] and 3.867 ppm [3H]) versus concentration (in mmol/mL). Excellent linearity ($R^2 \geq 0.9991 – 0.9999$) was obtained for both $DMSO_2$ and caffeine, taking into consideration that the absolute integral (I) is directly proportional to the number of protons (n) integrated. The determined linear equations were $I = 24.548*[DMSO_2]-0.007$ (6H) and $I =$

23.949*[caffeine]+0.060 ($-CH_3$); I = 24.222*[caffeine]+0.0552 ($-CH_3$); I = 23.507*[caffeine]+0.0533 ($-CH_3$). The NMR data acquisition and processing methods were identical for all samples. Collectively, this demonstrated the effectiveness of the external calibration for quantitation of natural products.

2.9. Limit of quantitation (LOQ) and limit of detection (LOD)

The LOQ and LOD thresholds were determined using the signal-to-noise ratio (S/N) method. According to the International Council for Harmonization protocols, a S/N value of 3:1 is a generally considered an acceptable LOD threshold. For LOQ calculations in LC (Liquid Chromatography)-based methods, a typical S/N is 10:1 (Saito et al., 2009; Sugimoto et al., 2010). A ratio of LOQ = 3.3 × LOD was used for qHNMR validation in the present study (Gödecke et al., 2013). The S/N ratio calculator functions in MestReNova and NMR Delta Processing Software were used to determine the S/N of individual signals.

2.10. UHPLC-UV analysis

The tincture and other samples were analyzed using Shimadzu UHPLC-UV equipment. A UHPLC-UV method was developed for a total of 11 min. Solvents A and B were water and acetonitrile respectively, both containing 0.1% formic acid. The mobile phase gradient consisted of 20-26% B after 0.5 min, 26-31.5% B at 6.5 min, held isocratic at 31.5% B up to 6.7 min., re-equilibrated from 31.5-20% B at 8 min, and reconditioned at 20% B up to 11 min. The flow rate was 0.6 mL/min, and the injection volume was 1.0 μL. Tincture samples were dissolved in 1.0 mL methanol/water (1/1), and filtered with a 0.2 μm PTFE syringe filter before injection.

2.11. LC-MS-MS analysis

LC-MS-MS separations were carried out using a Waters YMC AQ C18 column (2×10 mm, 3 μm particle size). The mobile phase consisted of 0.1% FA (solvent A) and acetonitrile (solvent B) with a linear gradient from 10% to 95% B in 30 min. The flow rate was 0.2 mL/min, and the column temperature was set at 30 °C. High-resolution accurate mass measurements were performed at the resolving power of 10 000 FWHM using Leuenkephaline as the lock mass. Product ion spectra were recorded at 15 or 25 eV using argon as the collision gas. For identification of compounds, molecular compositions and tandem mass spectra were compared with the standard spectra from public and in-house generated databases as well as with spectra published in the primary literature.

3. Results and discussion

3.1. Characterization of red clover methanol extract (RCME)

A control red clover methanol extract (RCME) was prepared, as described in the materials and methods section. This was done in order to record the baseline chemical profile of red clover before chemical changes caused by high temperature, enzymatic modification or chemical hydrolysis that may occur during preparation of traditional extracts, such as infusions, decoctions, and tinctures (Matsuura et al., 1995; Sun et al., 2018; Wilding et al., 2015). Only formononetin (2) was quantified in this extract as 0.18% w/w (Biochanin A (1) was found below LOQ S/N=2:1) by qHNMR. UHPLC-UV reported 0.08% biochanin A (1)

and 0.22% formononetin (2) w/w, respectively (see Supporting Information, Figs. S27 and S28).

Red clover crude extracts are known to contain several types of glycoside congeners. The three major ononin congeners are formononetin-7-O-β-D-glucoside (ononin), formononetin-7-O-β-D-glucoside-6”-O-malonate, and formononetin-7-O-β-D-glucoside-6″-O-acetate, while the three major sissotrin congeners are biochanin A-7-O-β-D-glucoside (sissotrin), biochanin A-7-O-β-D-glucoside-6″-O-malonate, and biochanin A-7-O-β-D-glucoside-6″-O-acetate (Klejdus et al., 2001).

The ^{1}H NMR spectrum of the RCME reveals a complex chemical profile, here briefly described for a few critical spectral regions. The bottom of Fig. 3 shows the ^{1}H NMR spectra of the control extract, above it are the reference spectra of the monoglucosides, sissotrin (9) and ononin (10). In Fig. 3, different spectral regions are outlined, corresponding to proton signals H-2, the characteristic singlet of isoflavonoid, H-5 (A-ring), H-2′/H-6′ (AA’XX’ spin system in the B-ring), H-8/H-6 (coupling in A-ring), and H-37H-5′ (AA’XX’ in the B-ring). The RCME showed the presence of three sissotrin and three ononin congeners. Due to signal crowding, with more than eight overlapping signals for H-2, six signals for H-5, twelve signals for both H-2′/H-6′ and H-3′/H-5′ regions, and broad signals for A-ring protons H-8/H-6, the exact identity of each of these congener signals could not be assigned. Instead, LC-MS-MS analysis was used to tentatively identify (Level 2b) (Schymanski et al., 2014) these ononin and sissotrin congeners (Supporting Information, Fig. S29).

3.2. Characterization of infusion and decoction

The next step consisted of collecting spectroscopic data for red clover infusion and decoction preparations. Because both infusion and decoction utilize hot water, thermally labile compounds in the plant were expected to be affected by the high temperature during preparation (Fueki et al., 2016; Zhang et al., 2018), generating derivatives or products of decomposition (Sun et al., 2018). We observed a number of quantitative differences between infusion and decoction preparations using ^{1}H NMR and UHPLC-UV.

For the infusion, the concentrations of biochanin A (1) and formononetin (2) were below the LOQ (10:1) and LOD (3:1) (Godecke et al., 2013) (see Supporting Information Figs. S3 and S4). Based on UHPLC-UV profiles, the infusion reports only traces of biochanin A (1) and formononetin (2) (see Supporting Information Fig. S5). Sissotrin (9) and ononin (10) were quantified in infusion preparations by qHNMR according to the areas of the H-2 signals at chemical shifts 8.480 ppm for sissotrin (9) and 8.445 ppm for ononin (10) (Lv et al., 2009; Vitor et al., 2004), yielding values of 0.53% and 0.15% w/w, respectively (see Supporting Information Fig. S6).

The concentrations of biochanin A (1) and formononetin (2) in the decoction were determined by EC qHNMR as 0.27% and 0.51% w/w, respectively (see Supporting Information Figs. S9 and S10). Similar quantities of biochanin A (1) and formononetin (2) were observed by UHPLC-UV (see Supporting Information Fig. S11). The content of

sissotrin (9) in decoction was 0.25% w/w and ononin (10) was found below the LOQ (see Supporting Information Figs. S12 and S13).

The infusion only report traces of biochanin A (1) and formononetin (2) while the decoction extract shows greater amounts of the same two compounds (Fig. 4). This is consistent with the hypothesis that, during the decoction process, the extraction itself is altered and/or thermolabile compounds may degrade (Fig. 4) (Sun et al., 2018). Because plant material typically contains a complex mixture of phytochemical ingredients, extract preparations utilizing heat can alter the phytochemical profile of an extract. The choice of solvent can also greatly affect the extraction efficiency. Infusions and decoctions employ high temperatures and water, and are typically consumed within a short timeframe. On the other hand, tinctures represent extractions made with lower temperatures and non-aqueous solvents, and the products can be used over a much longer timeframe.

3.3. Dynamic chemical composition of the tincture over time

The present experiment evaluated a traditional tincture preparation involving maceration of a botanical for a period of one month. Some literature sources indicate that tinctures may be macerated over six weeks (Fitzgerald, 2014). Observed between 0.5 and 720 hours, total extraction yields were in the range 249.7-260.6 mg. During this period, it is perceivable that different percentages of biochanin A (1) and formononetin (2) are the result of differences in extraction efficiency, whereas during the same period enzymes could potentially hydrolyze or synthesize isoflavone glycosides. By monitoring the dynamic transformation of certain metabolites in the tincture, this study showed that a patient using such a traditional medicine consumes different concentrations of secondary metabolites depending on the day of consumption.

This study also established profiles of the dynamic variability of red clover metabolites in a 45% ethanolic tincture. Samples were prepared by adding 800 mg of red clover plant material to 20 mL of 45% ethanol. At designated intervals, individual samples were filtered and the filtrate ethanolic tincture solution (ETS) was dried. The spent plant material (SPM) was dried and extracted with 95% ethanol. Both ETS and the SPM extract (SPME) were analyzed separately and in combination.

Accurate plant material weight was necessary to ensure reproducibility of the extraction process and yields. Table S16 summarized the statistical evaluation of plant material weight before starting the tincture experiment. Moreover, the concentration of compounds needed to be uniform throughout the qHNMR analyses (Table S17). Yield variation of 45% ethanol (ETS) and tinctures (ETS+SPME) is shown in Fig 5. Samples were identified based on the time at which they were collected. Triplicate samples are represented by 3 bars, which show high reproducibility (average weight and standard deviation, as shown in Supporting Information, Tables S5 and S6). Total extract yields were in the range between 249.7 to 260.6 mg (31.2 to 32.6%) which is noticeable higher than RCME, infusion and decoction.

Fig. 5 shows that the high mass of the ETS compared to the SPM extract it is due the presence of housekeeping metabolites such as saccharides and fatty acids esters which are components of NADES, Natural Deep Eutectic Solvents (Liu et al., 2016). NADES are

hydrophilic, and formulated as a dynamic matrix, these species display remarkable solubilizing and stabilizing abilities for a broad polarity range of natural products (Dai et al., 2013; Liu et al., 2018).

3.3.1 UHPLC-UV analysis of tinctures (ETS+SPME)—UHPLC-UV experiments were conducted (in triplicate) on tinctures (ETS+SPME) for 12 sample timeframes (0.5, 1, 16, 24, 72, 96, 144, 240, 360, 480, 600, and 720 hours). Comparison of UHPLC-UV profiles for tinctures (ETS+SPME), red clover clinical extract (Phansalkar et al., 2017), and standards of biochanin A (1), formononetin (2), genistein (3), and daidzein (4) were used to verify the identity of each compound in these extracts (see Fig. S31 and Table S15, Supporting Information).

For the temporal analysis of chemical changes taking place within the tinctures (ETS +SPME), we observed numerous changes in the phytochemical profile taking place over one month. These included alterations in the isoflavone marker concentrations of biochanin A (1) and formononetin (2). Quantitative changes in isoflavone marker concentrations by UHPLC-UV are illustrated in Fig. 6. The curve clearly shows a bimodal distribution of isoflavone concentration which occurs over time, with concentrations dropping after 1 hour, peaking at six days (144 hours), and stabilizing after 240 hours. The calculated concentrations of the marker compounds over time are listed in the Supporting Information (Table S11 and S12).

3.3.2 qHNMR analysis of tinctures (ETS+SPME)—The qHNMR experiments were conducted in triplicate for the tinctures (ETS+SPME) using 12 sample timeframes from 0.5-720 hours. The kinetic curves for biochanin A (1) and formononetin (2) concentration changes over time were constructed using the absolute integral of the combination of ETS +SPME (Tables S7–S9). Using external calibrants (EC) $DMSO_2$ and caffeine (see the Materials and Methods section). Both external calibrants produced similar curves for the two target compounds (Fig. 7 and Fig. S25). Additionally, an internal calibrant (IC) was used with two samples to confirm the accuracy of the external calibration method for the quantification of biochanin A (1) and formononetin (2). The difference in percent calculated by IC and EC was 0.05% for biochanin A (1) and 0.03% for formononetin (2), indicating that the EC method was consistent with the IC method. As in the UHPLC-UV analyses, the qHNMR experiments also reflected a bimodal distribution curve. The details can be found in Tables S9 and S10 of the Supporting Information.

Importantly, the concentration curves for the isoflavone markers over time show the interplay of chemical and enzymatic modifications that contribute to the dynamic residual complexity (Chen et al., 2009; Simmler et al., 2013). The formation and cleavage of glycosidic bonds can involve many different enzymes, including glycosyltransferase and glucosidase enzyme families (Chuankhayan et al., 2007; Hofer, 2016). Presumably, the activity of certain enzymes was not (entirely) inhibited by ethanol in our experiment (Kaur et al., 2007; Michlmayr et al., 2010; Toebes et al., 2005), but single-enzyme kinetic models are unlikely suitable to explain the dynamics at work here and, hence, were not investigated further.

3.3.3. Quantitation and identification of isoflavones—

Figs. 8, 9, and 12 illustrate the NMR spectra of the extracts over time. The signals of biochanin A (1), formononetin (2), genistein (3), daidzein (4), prunetin (5), irilone (6), pseudobaptigenin (7), and calycosin (8) were readily assigned, and quantified by comparing qHNMR data (Phansalkar et al., 2017; Yoon et al., 2004). Overlapping H-2 isoflavonoid protons were quantified using Global Spectral Deconvolution (GSD) with one specific ^{1}H NMR signal for quantitation (Çiçek et al., 2018; Phansalkar et al., 2017).

ETS+SPME: Thorough analysis of the qHNMR spectra of each tincture provided identification and quantitation of eight isoflavone marker compounds. Figs. 8 and 9 correspond to the same qHNMR experiment downfield and upfield, respectively. The NMR spectra were divided into five regions of interest. The first (upfield) region between 0.5 to 2.4 ppm, as shown in Fig. 9, revealed fatty acids. Region 2 between 2.7 to 5.6 ppm, as shown also in Fig. 9, revealed methoxy group signals linked to the B-ring C-4′ or A-ring C-7 in prunetin (5). In addition, in this region, it was also possible to identify the doublet from anomeric hydrogens with a chemical shifts of 5.18 ppm (*d*, 3.736 Hz) corresponding to isoflavonoid glycosides, and the anomeric hydrogen signal from free sugars at 4.90 ppm (*d*, 3.548 Hz). Region 3 between 6.0 and 6.7 ppm contained signals of the A-ring hydrogens H-5, H-6, and H-8. The methylenedioxy moieties of irilone (6), and pseudobaptigenin (7) were also found in this region at 6.181, and 6.042 ppm, respectively. Region 4 between 6.8 and 8.1 ppm showed the typical signals of isoflavone B-rings with AA'XX' or AMX patterns. The signal assignments in this region were the most challenging because of peak overlap. However, this region also provided evidence for the presence of phenolic rings from minor components throughout the tinctures. The downfield region 5, between 8.1 and 8.5 ppm showed the C-ring H-2 singlets common to the 4-pyrone moiety of isoflavonoids (Fig. 8).

Further analysis of the qHNMR spectra in Fig. 8 showed that the H-2 singlets corresponding to genistein (3) (8.319 ppm), daidzein (4) (8.283 ppm), and calycosin (8) (8.275 ppm) appear already after 0.5 hours of tincture maceration. However, the analogous prunetin (5) and irilone (6) H-2 hydrogens resonating between 8.40 and 8.45 ppm, overlapped with those of isoflavonoid glycosides in the spectra of tinctures from 0.5 until 72 hours. Deconvolution of genistein (3), daidzein (4), and calycosin (8) signals in tinctures (ETS+SPME) was not feasible because these extracts contained very low concentrations of these isoflavonoids. For the identification of these low concentration compounds in the tinctures, it was necessary to investigate the isoflavonoid-enriched SPME (Table 1).

The boxes in Fig. 8 highlighted peaks that changed over the course of the experiment, i.e., the purple box (8.00 to 8.10 ppm) demonstrates how ononin and its congeners are completely hydrolyzed after three days. On the other hand, the range from 7.03 to 7.45 ppm (red box), 6.70 to 6.88 ppm (black box) and 6.277 ppm (blue box) showed the generation of some signals as well as the disappearance of others after 480 hours. The data highlighted in the red, black and blue boxes illustrate the dynamic residual complexity in the tincture extracts.

The region from 5.60 to 2.60 ppm in Fig. 9 showed the signals of the anomeric hydrogen of sugars that are linked to isoflavonoids by a hemi-acetal bond, giving rise to a doublet around 5.18 ppm, and free and hydrolyzed sugars at 4.90 ppm, also as a doublet. Methoxy group resonances were distributed between 4.44 to ~ 3.50 ppm, and those of fatty acids were found in the region from 3.00 to 0.60 ppm.

SPME: The yields of enriched isoflavonoid extracts were in the range 2.9 to 5.6%. For the SPM extracts, processing of all qHNMR data was performed in Mnova. Due to the complexity of the spectra, it was necessary to employ a peak-fitting (PF) technique (Global Spectral Deconvolution (Phansalkar et al., 2017)) in order to accurately quantify overlapping peaks (Fig. 10; PF-qHNMR). The Gaussian and Lorentzian window function [Exponential: −0.10 Hz; Gaussian: 0.1; GF: (0,1)] afforded the most suitable conditions for the deconvolution process and subsequent quantification using the H-2 singlet of pseudobaptigenin (7, 8.328 ppm), genistein (3, 8.303 ppm), calycosin (8, 8.275 ppm), daidzein (4, 8.272 ppm), and one unidentified isoflavonoid (11, 8.309 ppm).

The SPME consistently yielded high concentration of biochanin A (1) and formononetin (2). However, the SPME still retained hydrolyzed sugars as shown in the upfield region of the qHNMR spectra (Fig. S30). These remaining free sugars interfere with the accurate quantification of the percent isoflavonoids content, due to solubility issues.

Using the EC qHNMR method, yields were calculated for biochanin A (1) and formononetin (2) and are shown in Fig. 11 as a percentage of these two target compounds. The percentages of biochanin A (1) and formononetin (2) production followed a similar trend as Figs. 6 and 7. Interestingly, after 0.5 hour, the percentage of biochanin A (1) (3.89% w/w) and formononetin (2) (2.65% w/w) increased, and within one hour reached 8.43 and 11.32% w/w, respectively; after 16 hours the values decreased to 4.99% of and 6.52% w/w, respectively. It then produced the maximum percentage of these aglycones after 144 hours, at 11.64% and 14.64% w/w for 1 and 2, respectively. After this period of time, the yields leveled off in the range between 11.25 and 14.26% w/w for biochanin A (1) and formononetin (2), respectively.

Apparently, in the SPME isoflavone glycosides are being cleaved, enzymatically or hydrolytically, after 0.5 hour, generating the aglycones: genistein (3), daidzein (4), prunetin (5), irilone (6), pseudobaptigenin (7), and calycosin (8). Some of these compounds could not be observed during the investigation of tinctures (ETS+SPME), because the remaining isoflavonoid glycosides show extensive NMR signal overlap. However, analyzing the SPME alone would not have provided insights as to which congeners were hydrolyzed (completely) over time.

Interestingly, aglycones such as prunetin (5), irilone (6), pseudobaptigenin (7), and calycosin (8) reached their highest abundance after 24 hours (Table 1). The percentage of 3 was reduced after 1 hour. Likely due to the complexity of the mixture and the interactions between molecules, the quantities of 4, 5, 6, 7 and 11 showed considerable variation. Most of them followed the bimodal distribution like biochanin A (1) and formononetin (2).

Fig. 13 demonstrates how pseudobaptigenrn (7) and irilone (6) were identified using two specific ^{1}HNMR signals. Irilone (6) gives rise to a singlet at 8.425 ppm (H-2) with a normalized integral of 100, and a 2H singlet at 6.176 ppm (methylenedioxy hydrogens) with a normalized integral of 197 (1.5% deviation; Fig. 13). Similarly, pseudobaptigenin (7) features its singlet at 8.336 ppm with a normalized integral to 89 and the methylenedioxy 2H singlet at 6.042 ppm with an integral of 183 (2.8% deviation; Fig. 13). During other investigations of isoflavones ongoing in our laboratory (data not shown), the chemical shift of the H-2 singlet of pseudobaptigenin (7) was found to be in the range 8.344 to 8.324 ppm. This is consistent with literature reports of this chemical shift being located at 8.32 ppm (Yoon et al., 2004). The signals at 6.042 ppm and 6.176 ppm correspond to methylenedioxy moieties (R″-O-CH_2-O-R') present in both compounds. The 1:2 ratio of the integrals between the H-2 and the methylenedioxy signals are characteristic for each compound and helped confirm their identities.

ETS qHNMR: Analyzing two tincture extracts (after 24 and 72 hours) by qHNMR showed a low concentration of biochanin A (1) (0.24-0.40%) and formononetin (2) (0.39-0.55%) (Supporting Information, Fig. S26). Additionally, the qHNMR profile of ETS shows a high concentration of free saccharides products of the hydrolysis of isoflavonoids glycosides. In the RCME, the anomeric hydrogens corresponding to isoflavone glycosides (doublets around 5.180 ppm), are much more abundant than the free saccharide anomeric hydrogen doublets around 4.898 ppm. On the other hand, in the ETS samples the opposite was observed (See Supporting Information Figs. S26 and S27).

4. Conclusions

This study investigated the content of isoflavonoids and congeners in three different ethnomedical red clover extracts: infusion, decoction, and 45% ethanolic tincture. According to UHPLC-UV and qHNMR analyses of the bioactive isoflavone marker compounds, these three preparations exhibited markedly different chemical profiles, statically and at different time points. The outcomes also showed that a “microbiologically preserved” preparation, that can be kept for a period of time, such as an ethanolic tincture, can still undergo substantial change over time, thereby featuring a dynamic residual complexity (https://go.uic.edu/residualcomplexity) caused by endogenous reactions. The ratio of glycosides to aglycones in such a preparation increased and decreased in a bimodal pattern.

In terms of analytical methodology, qHNMR analysis was included for a number of reasons: it not only (at least) matched UHPLC-UV in accuracy, but importantly avoided the repeated need to source identical reference materials for quantification. This was particularly advantageous for low-level constituents that are rare chemicals and difficult to purify, such as the minor isoflavones investigated here. Another advantage of the qHNMR method was that, by employing external calibration (EC-qHNMR), the investigated samples were not contaminated with calibrants, thus allowing for subsequent biological testing. Comparing the internal (IC) and external calibration (EC) for two experiments, both provided percentages of error in the range between 5.81 – 3.96% for biochanin A (1) and 2.72 – 2.79% for formononetin (2), demonstrating, that our EC quantitation method was accurate (See Supporting Information, Figs. S41–S42). But as noted earlier, (Pauli et al., 2014) HPLC

quantitation generally reports a higher purity value than qHNMR because HPLC is not necessarily detecting every component in the mixture.

It is well established that the composition of botanical extracts is a result of the chemical variation of the source plant material. The present study showed that different ethnomedical preparation methods also impact extract composition and, thus, the dosage of bioactive constituents in a major fashion: each preparation type has a distinct metabolomic profile and certain dynamic properties (dynamic residual complexity profile). Collectively, this also translates plausibly into various ethnomedical preparation having their own value in treating specific ailments. For the *T. pratense* metabolites analyzed here, there are likely also variations in compound classes other than the isoflavonoids. While assessing the possible role of highly bioactive, low concentration compounds in the overall bioactivity of these preparations was beyond the scope of this study, it is a reasonable working hypothesis for future investigations.

This study provides evidence for the actual presence of dynamic changes over time in isoflavone concentration in an ethanolic tincture of red clover. While the existence of such a variation over time was generally expected to play a role, but possibly less so for the chemically rather stabile isoflavones, the NMR and LC-based analytical data provide clear evidence. The potential biological role of these dynamic changes and their therapeutic impact remain to be evaluated. However, in the tincture, the outcomes showed that hydrolysis played an important role in the temporal degradation of glycosides, even in alcoholic extracts, i.e., despite the alcohol content. This process can exert a significant effect on the chemical profile of tinctures and, thus, potentially their therapeutic outcomes, depending on when the tincture is administered. Additionally, the presence of NADES species, which undoubtedly are present in *T. pratense* and its ethnomedical preparation, very likely influenced the solubilization properties and stability/behavior of the dynamic residual complexity relationship between glycosides and aglycones, thereby altering the equilibrium of the reactions. The complex processes and chemical modifications in the tincture precede the equally complex metabolic transformations that occur once the medicine is ingested. Collectively, the results helped to explain why in alternative medicine, medicinal plants sometimes work slowly and provide relief gradually, when compared to drugs (API) as used in modern medicine (Chugh et al., 2018).

This study also demonstrated that efforts to find rational approaches toward promotion of traditional medicines in global healthcare systems can benefit from the application of modern technologies (including analytical methods), particularly concerning the efficacy, safety, and stability of botanicals used in traditional preparations. Ancestral knowledge forms are the basis for a number of drugs on the market today (Fabricant and Farnsworth, 2001); if past is prologue, ethnopharmacological expertise represents a valid component in the search for new medicines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at TBD. The original NMR data (FIDs) are made available at DOI: https://doi.org/10.7910/DVN/I7UPMA [link activation pending manuscript acceptance]

This paper represents part 34 of the series on Residual Complexity and Bioactivity (see http://go.uic.edu/residualcomplexity).

Abbreviations:

SPME	Spent plant material extract
ETS	Ethanol tincture solution
$DMSO_2$	Dimethylsulfone
qHNMR	Quantitative ^1H Nuclear Magnetic Resonance
EC	External calibrant
LC-MS	Liquid Chromatography-Mass Spectrometry
RCME	Red clover methanol extract
LOQ	Limit of quantitation
LOD	Limit of detection
HPLC	High-performance liquid chromatography
IC	Internal calibrant
DMSO	Dimethylsulfoxide
CPC	Centrifugal Partition Chromatography
RCE	Red Clover Extract

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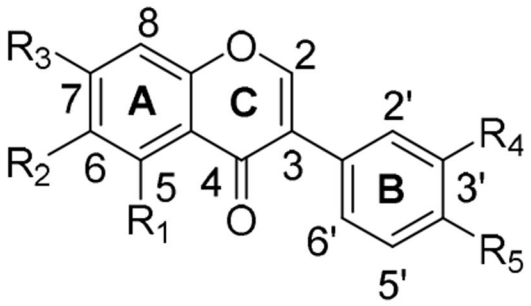

	R_1	R_2	R_3	R_4	R_5	H-2 δ (ppm)
Biochanin A (1)	OH	H	OH	H	OCH_3	8.357
Formononetin (2)	H	H	OH	H	OCH_3	8.328
Genistein (3)	OH	H	OH	H	OH	8.309
Daidzein (4)	H	H	OH	H	OH	8.274
Prunetin (5)	OH	H	OCH_3	H	OH	8.407
Irilone (6)	OH	-O-CH_2-O-		H	OH	8.434
Pseudobaptigenin (7)	OH	H	OH	-O-CH_2-O-		8.314
Calycosin (8)	H	H	OH	OH	OCH_3	8.277
Sissotrin (9)	OH	H	Glu	H	OCH_3	8.478
Ononin (10)	H	H	Glu	H	OCH_3	8.447

Fig. 1.
Bioactive marker compounds identified and quantified in the studied red clover preparation

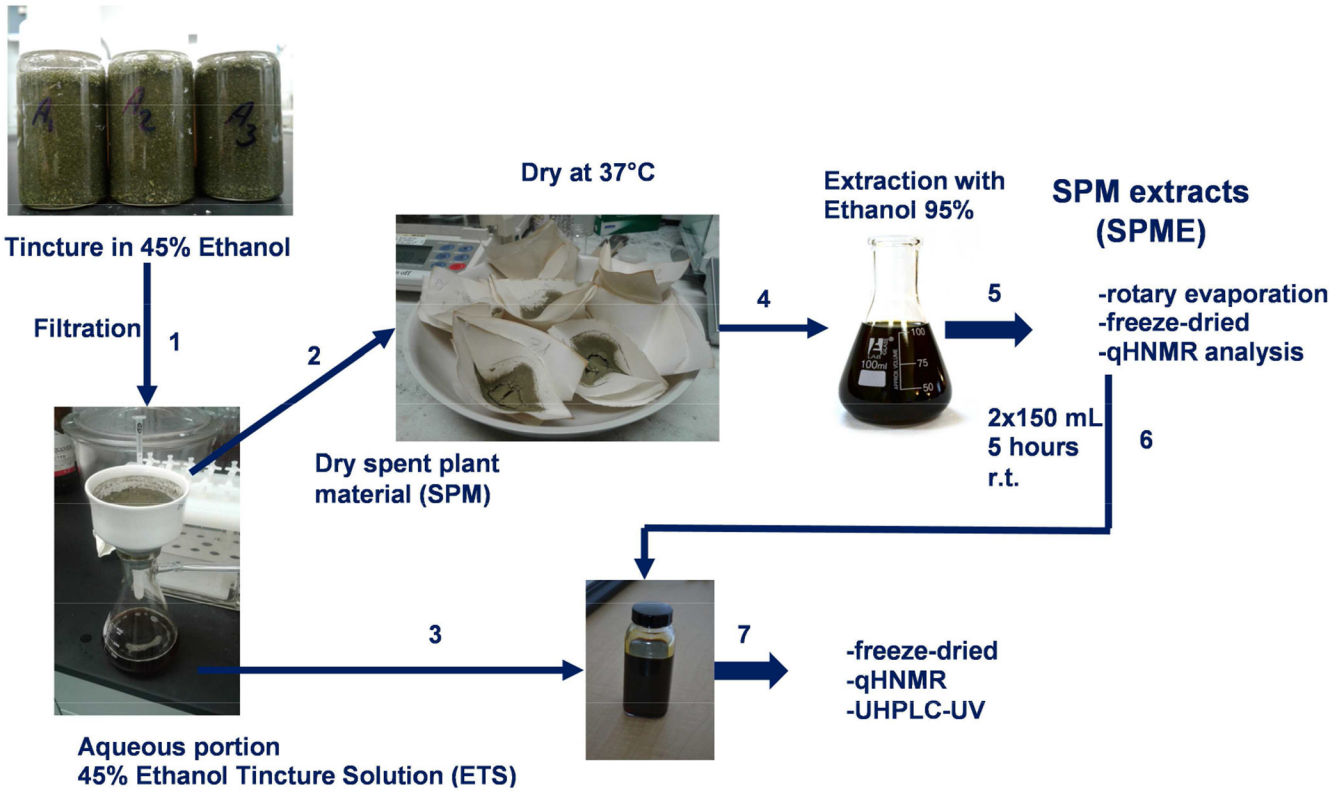

Fig. 2.

Seven steps of the preparation of tincture extracts for qHNMR and UHPLC: 1) Filter of tincture. 2) Dry spent plant material at 37°C. 3) Stop hydrolysis by adding 80 mL of methanol, then dry aqueous portion by using a rotary evaporator. 4) Prepare extracts of the spent plant material using 95% ethanol. 5) Dry extract for qHNMR. 6) and 7) Combine, reconstitute and dry each extract for qHNMR and UHPLC-UV analysis.

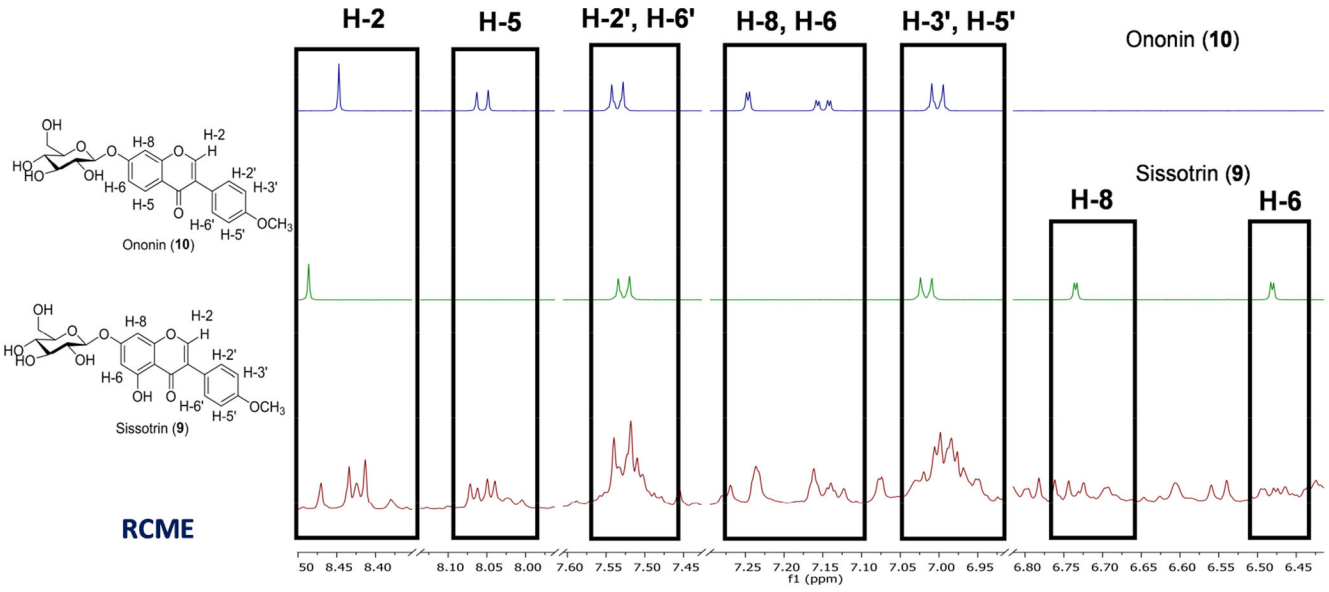

Fig. 3.

Downfield region of the ^{1}H NMR (400 MHz) spectra of the control red clover methanol extract (RCME) versus ononin (**10**) and sissotrin (**9**).

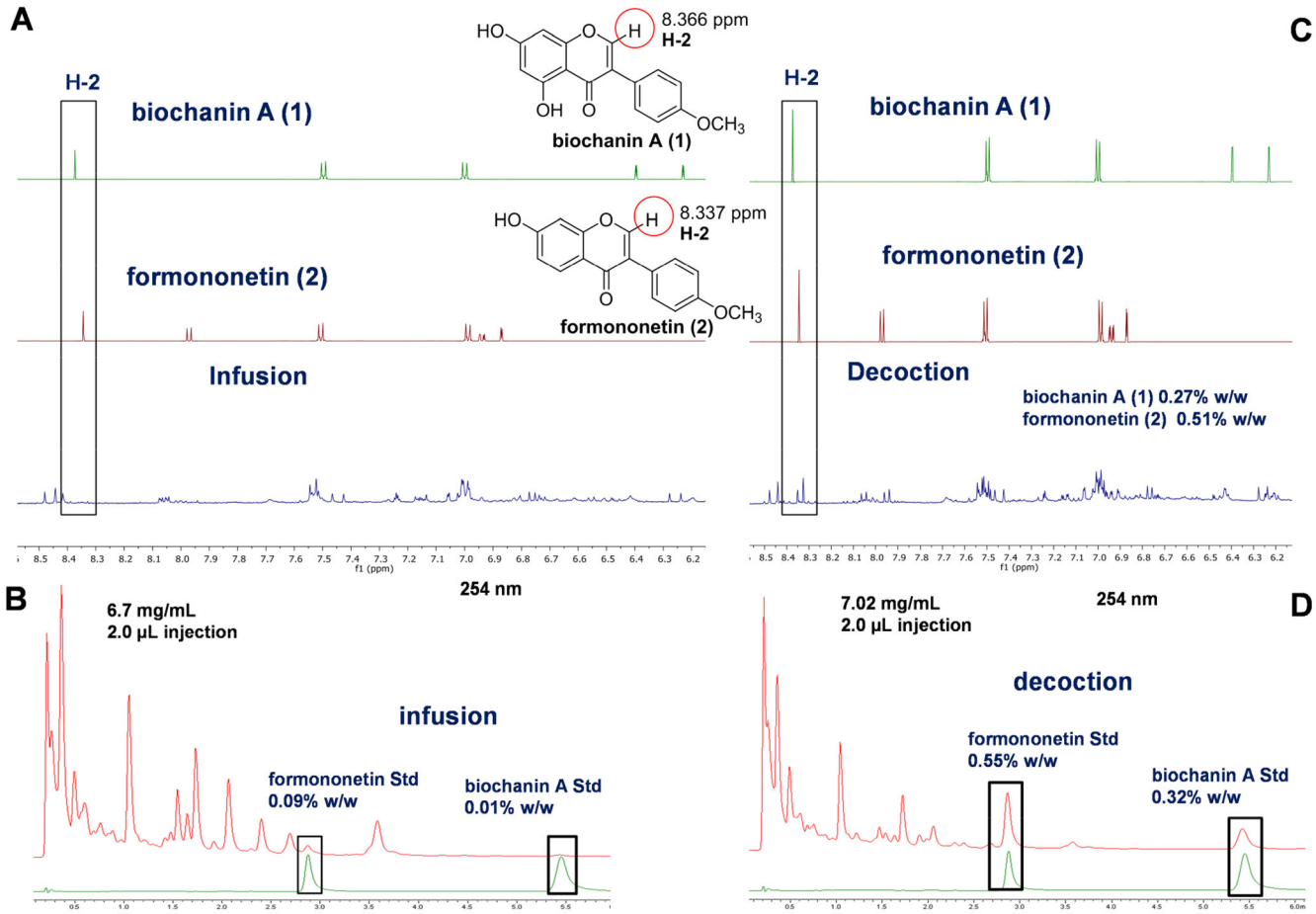

Fig. 4.

The ^{1}H NMR spectra (DMSO-d_6) and UHPLC-UV chromatograms of infusion (A and B, resp.) and decoction (C and D, resp.) compared with those of the reference standards, biochanin A (1) and formononetin (2).

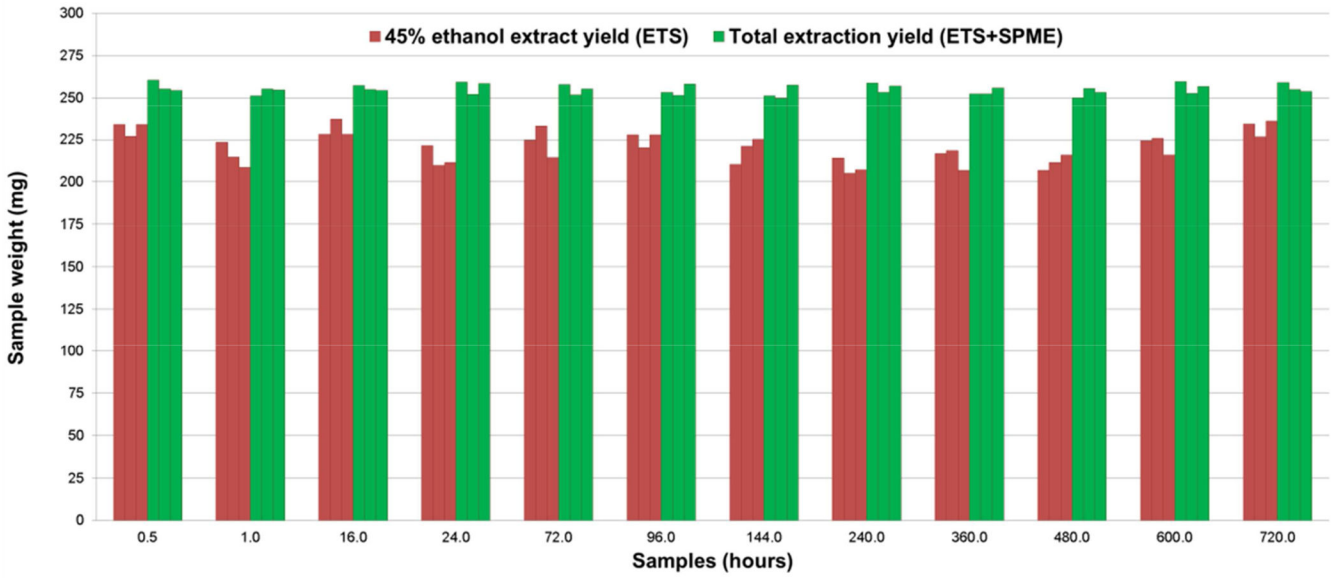

Fig. 5.

Extraction variability of the 45% ethanol (ETS, teal bars) and tincture (ETS+SPME, green bars).

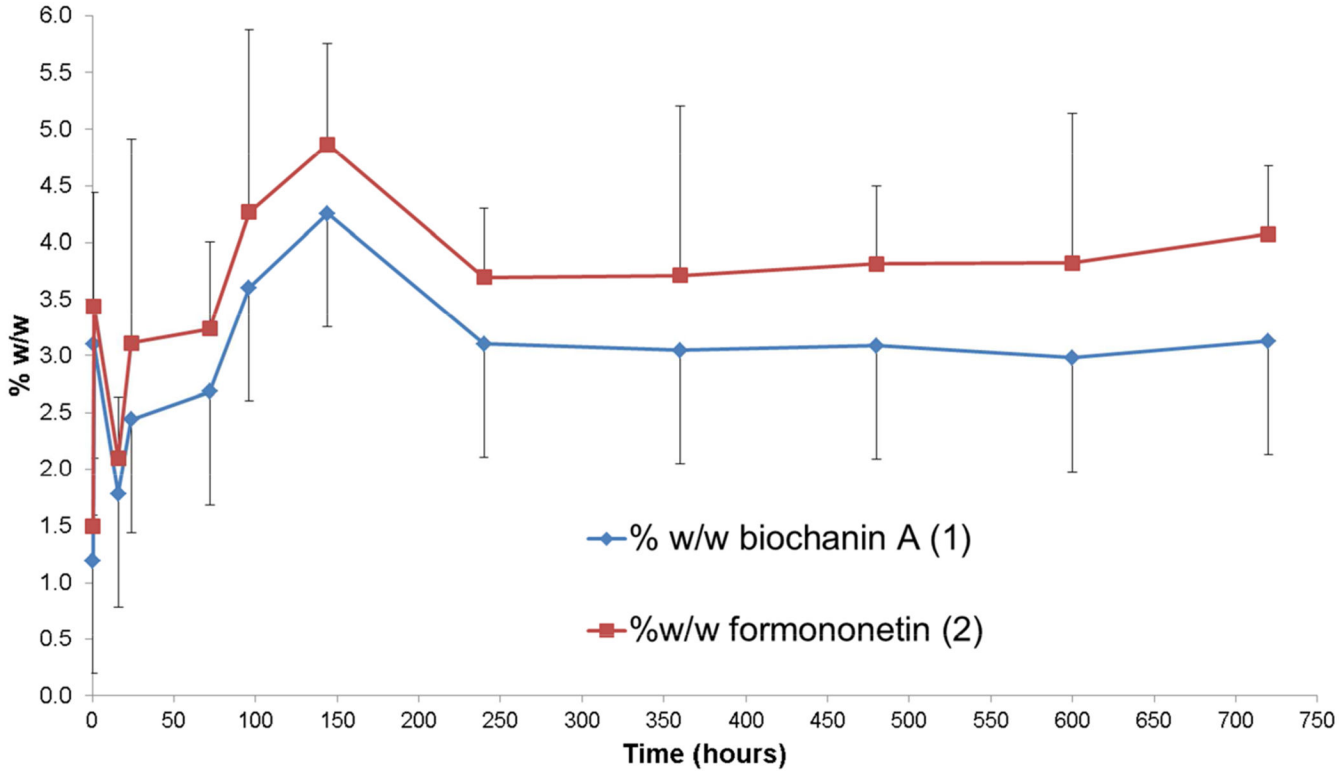

Fig. 6.

Biochanin A (1) and formononetin (2) content in red clover tinctures from 0.5 to 720 hours by UHPLC-UV analysis (%SD for (0.20-2.78%) biochanin A (1) and (0.10-1.80%) formononetin (2); Table S12 Supporting Information).

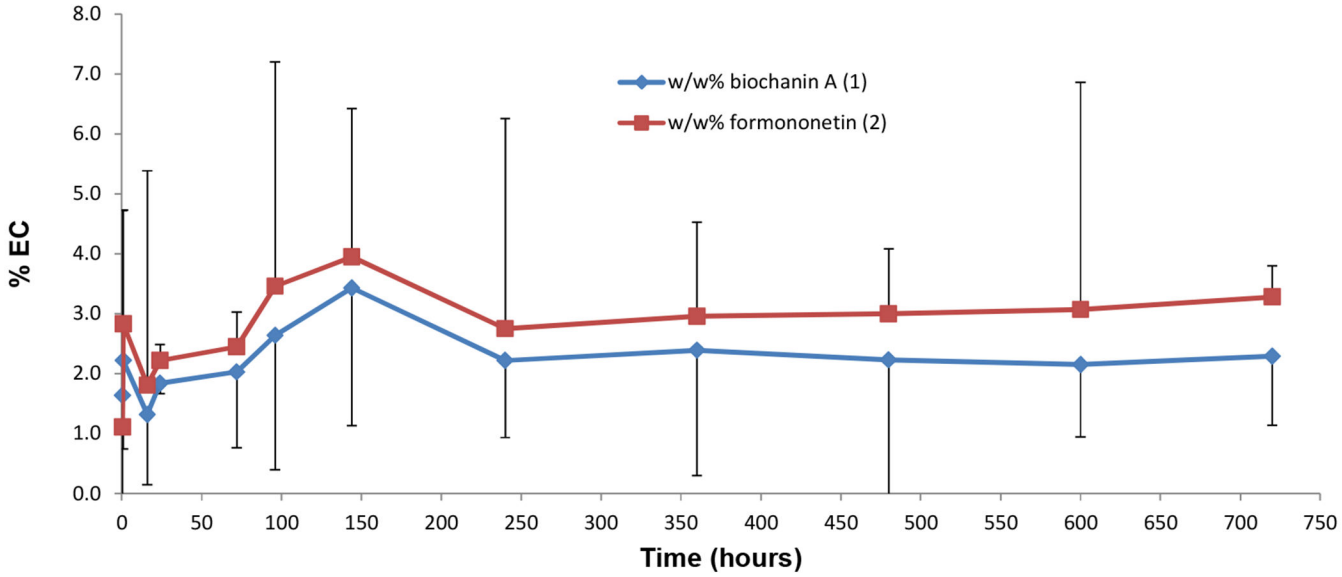

Fig. 7.

Biochanin A (1) and formononetin (2) content in red clover tincture (ETS+SPME) from 0.5 to 720 hours, as determined by qHNMR analysis (%SD for (0.17-3.28%) biochanin A (1) and (0.27-3.79%) formononetin (2); Table S9 Supporting Information) using $DMSO_2$ as external calibrant (EC).

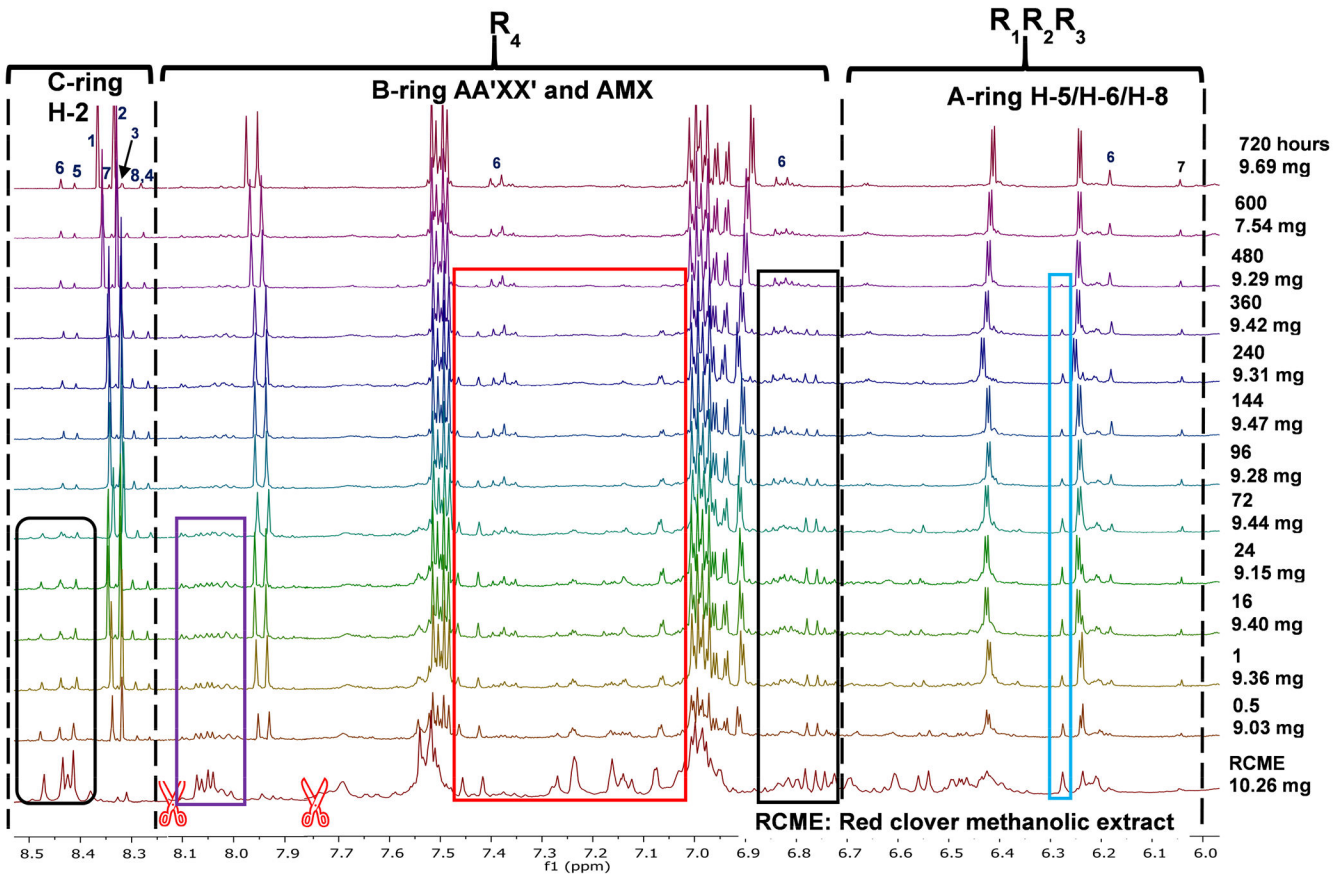

Fig. 8.

Downfield portion of the qHNMR (400 MHz) spectra of tinctures (ETS+SPME) investigated for one month.

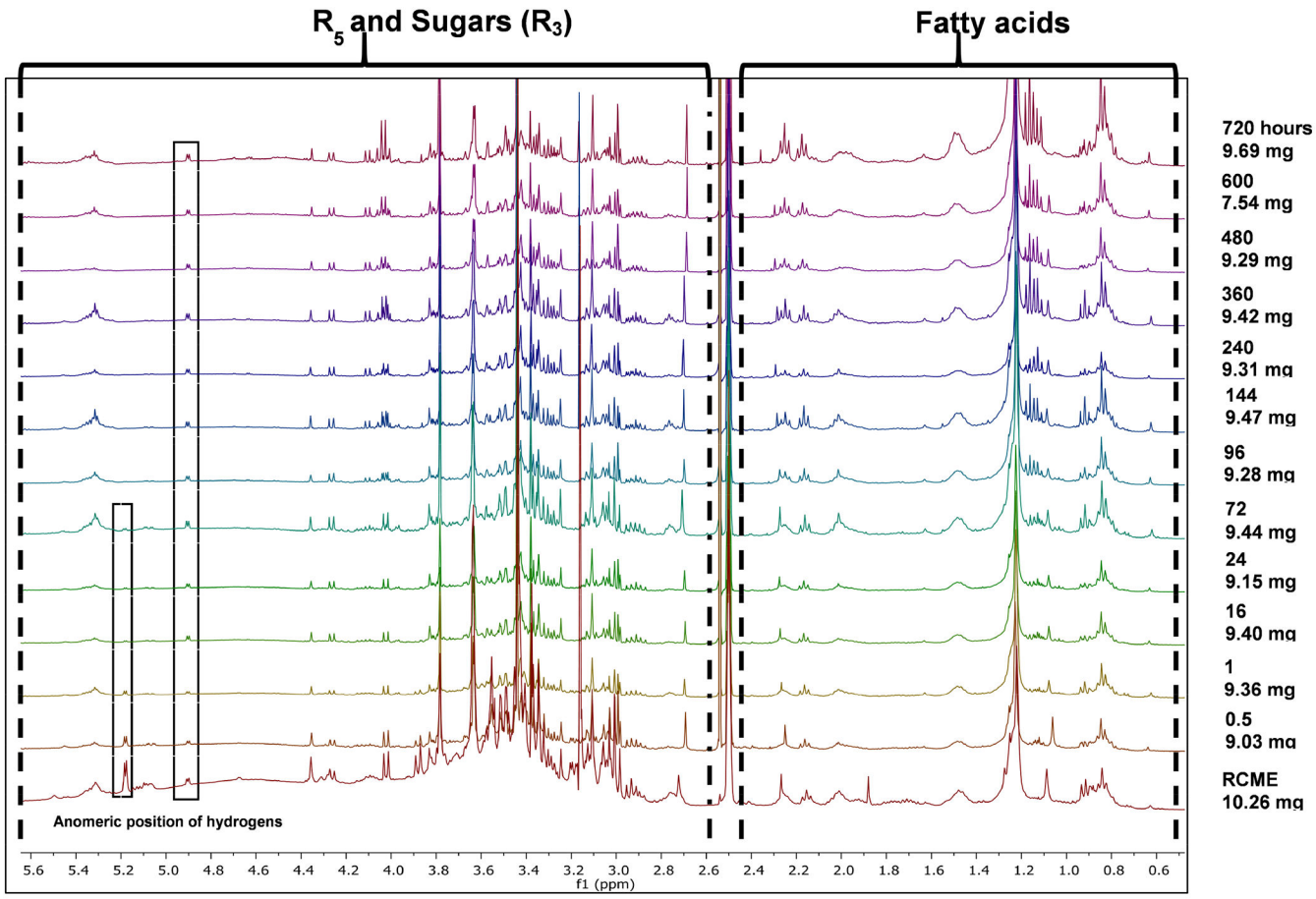

Fig. 9.

Upfield portion of the qHNMR (400 MHz) spectra of tinctures (ETS+SPME) investigated for one month.

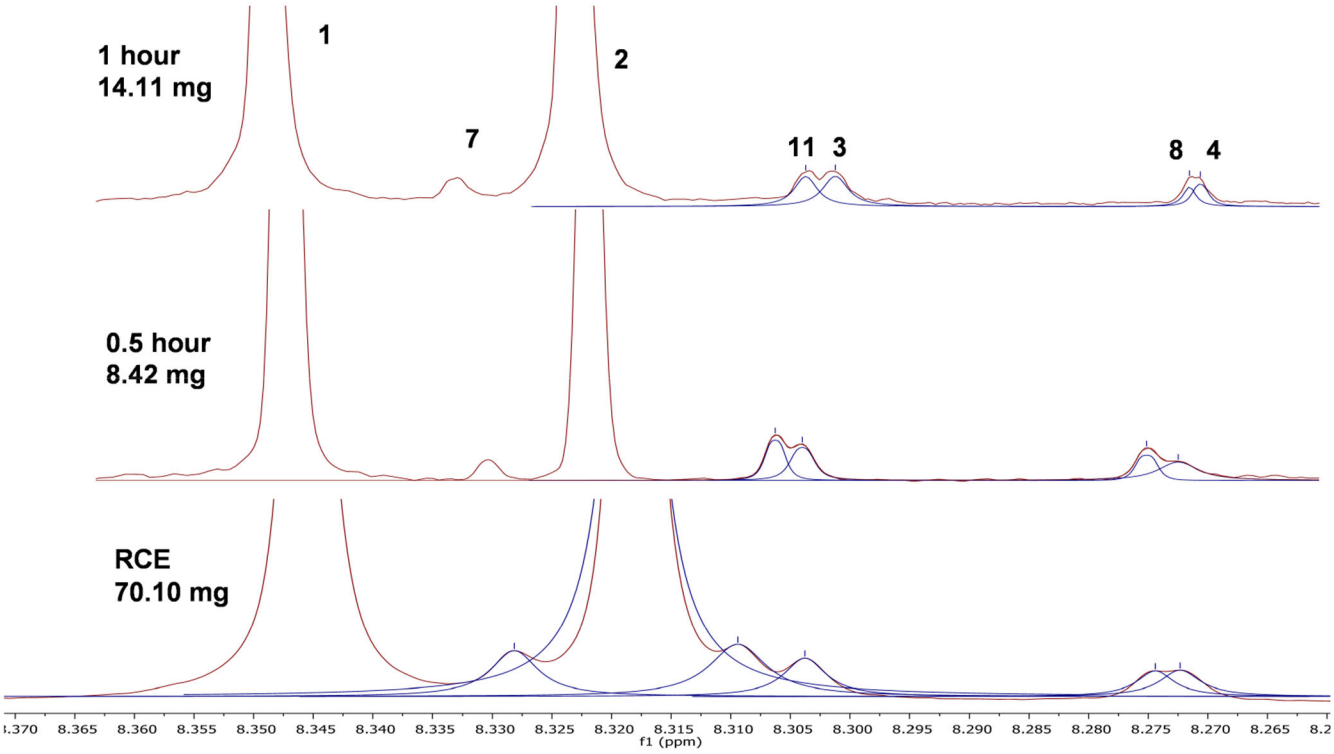

Fig. 10.

Identification of isoflavonoids in RCE (See Material and Methods section 2.2) and two SPME enriched isoflavonoid extracts.

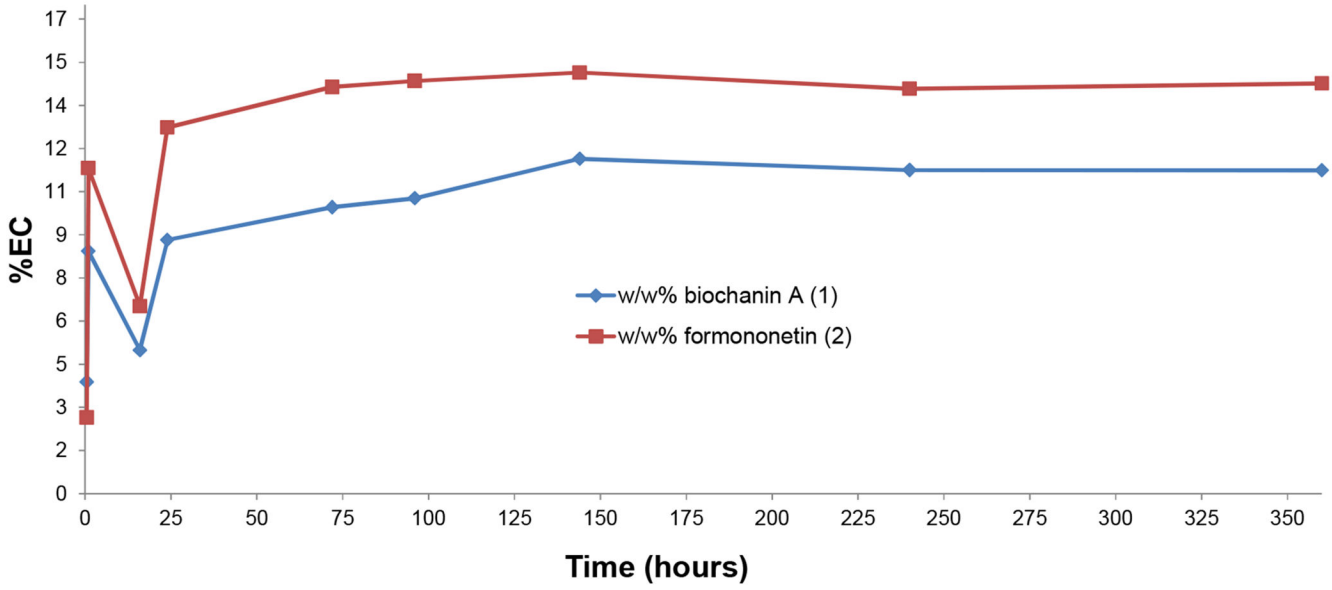

Fig. 11.

Biochanin A (1) and formononetin (2) content in SPME from 0.5 to 360 hours, as determined by qHNMR analysis.

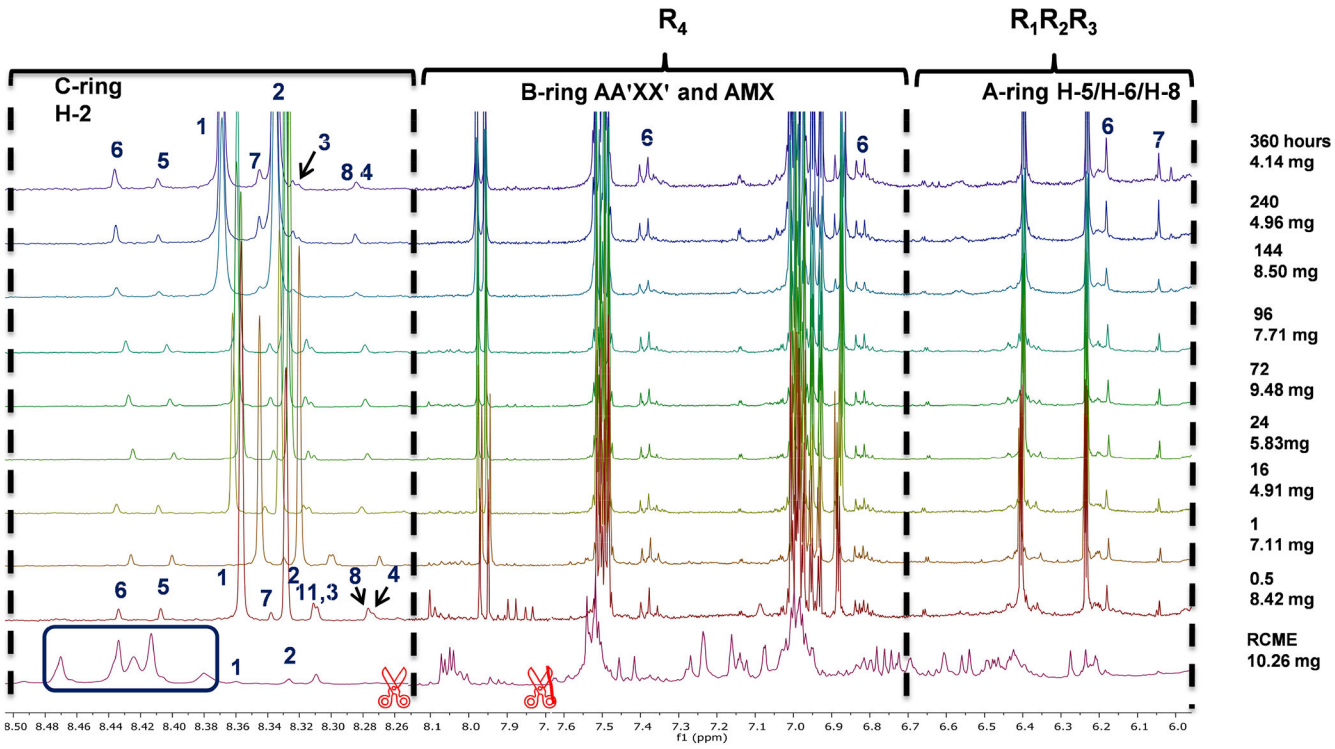

Fig. 12.
The qHNMR (400 MHz) spectra of the SPME (downfield).

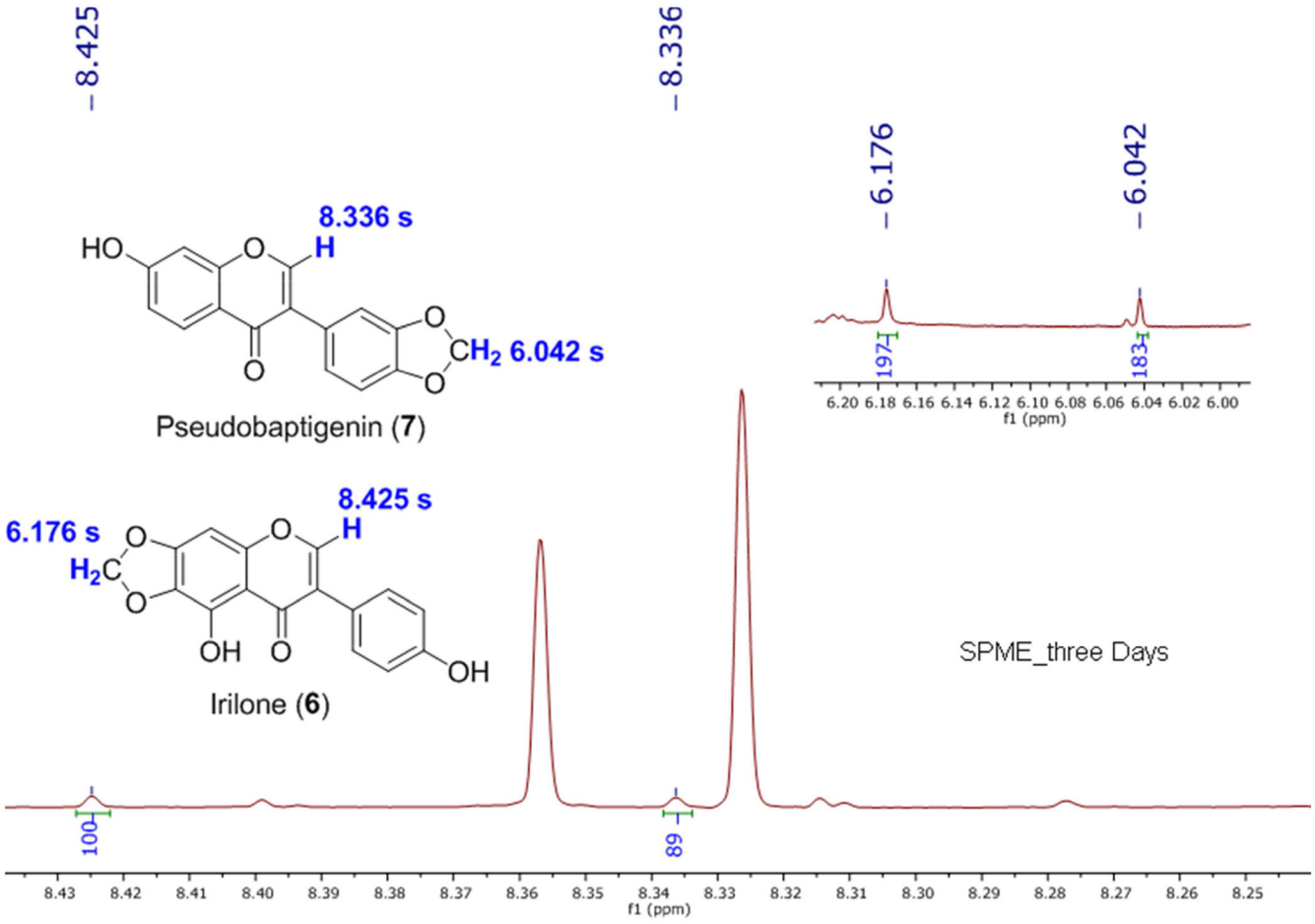

Fig. 13.

Example of the qHNMR-based identification and quantification (Table 1) of irilone (**6**) and pseudobaptigenin (**7**) in SPME after three days.

Table 1.

Percentages of isoflavonoids identified and quantified in SPME.

	% w/w of aglycones						
Samples	**3**	**4**	**5**	**6**	**7**	**8**	**11**
0.5	0.14	0.13	0.10	0.09	0.07	0.09	0.15
1	0.26	0.12	0.22	0.27	0.25	0.10	0.32
16	0.11	0.13	0.14	0.20	0.16	0.10	0.16
24	0.12	0.11	0.24	0.49	0.53	0.12	0.39
72	0.08	0.09	0.16	0.26	0.20	0.12	0.21
96	0.10	0.11	0.13	0.23	0.22	0.05	0.30
144	0.09	0.10	0.12	0.32	0.17	0.05	0.12
240	0.08	0.13	0.25	0.27	0.39	0.08	0.15
360	0.09	0.10	0.23	0.40	0.35	0.09	0.25