Inclusion Complexes of the Aurone Sulfuretin and the Chalcone Butein from *Cotinus coggygria* Wood in Two Cyclodextrin Types

First data on physico-chemical properties

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The quest for new drug leads derived from natural products is a mainstay of today's pharmaceutical research. Aurones and chalcones are flavonoid subclasses with high therapeutic potential but disadvantaged by poor water solubility. Cyclodextrins (CDs) are recognized drug carriers, able to accommodate hydrophobic drugs and enhance pharmacokinetic properties. The current research presents, as a first step, the extraction of two highly bioactive flavonoids, the aurone sulfuretin and the chalcone butein, from the wood of the European smoke tree (Cotinus coggygria Scop.). Subsequently, each of the flavonoids was included, for the first time, in two cyclodextrin types, resulting four types of complexes. Hydroxypropyl-β-CD and randomely methylated-β-CD were employed at a ratio of 1:2 = guest: host. Complex formation, stability and particle size were assessed, providing first physico-chemical data on these novel sulfuretin-CD and butein-CD entities.

Keywords: Sulfuretin, Butein, cyclodextrin complexation

Natural products provide highly diversified structures in chemical space [1]. Their molecules are tuned throughout evolution for optimal interactions with enzymes and receptors, explaining the success of natural compounds as drug leads. Among plant metabolites, flavonoids are one of the largest classes counting over 6000 phenolic structures [2]. In recent years, the attention of several research groups was attracted by *minor* flavonoids with emerging therapeutic potential, including aurones and chalcones [3,4]. Aurones are 2-benzylidene-coumaranone derivatives which distinguish themselves by a five-membered C-ring (fig. 1), while in most flavonoids this ring is six-membered. Aurones are mainly synthesized by plants as pigments of petals and fruits, but may occasionally be encountered in the heartwood of trees [5]. Their medicinal relevance includes antiviral, antimicrobial, antiparasitic, antioxidative, anti-inflammatory, anti-glycation and antitumor properties [6]. Among aurones, sulfuretin is a widespread molecule. It displays antineoplastic effects by induction of apoptosis [7], stimulation of tumor suppressor genes [8] and reduction of cellular invasion [9]. Furthermore, it protects neuronal cells against reactive oxygen species and beta-amyloid toxicity, demonstrating its potential in the management of Alzheimer's disease [10]. On the other hand, chalcones are 1,3-diaryl-2-propen-1-one derivatives, structurally related to flavones but displaying an opening of the C-ring (fig. 1). They are yellow to orange colored pigments [5]. Among them, butein displays an impressive array of biologic activities in experimental settings of cancer, inflammation, diabetes, nephritis and liver disorders [11].

Previously published biological studies on sulfuretin and butein are based on their extraction from the wood of the *Rhus verniciflua* Stokes, an Asian Anacardiaceae species [7,12-14]. However, these compounds are as well present in the European shrub *Cotinus coggygria* Scop. (smoke tree), belonging to the same family. The highest sulfuretin and butein content were determined in the heartwood of stems and branches with a diameter of over 7 cm [15].

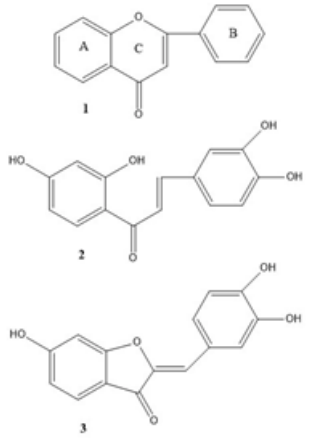

Fig. 1. Chemical structures. Flavone skeleton (1), butein (2) and sulfuretin (3)

A major drawback in the therapeutic exploitation of flavonoids is their low bioavailability, related to a poor water solubility. Cyclodextrins (CDs) are cyclic oligosaccharides with a cage-like supramolecular structure, able to accommodate hydrophobic drugs and enhance their physico-chemical and pharmacokinetic properties [16]. Their use as excipients in the pharmaceutical industry is well-established. Inclusion complexes of various flavonoid types (flavonols like quercetin, myricetin, fisetin and kaempferol; flavanones including naringenin; flavones like luteolin; isoflavones including daidzein and genistein) have previously been prepared and characterized [17-20]. Stable complexes of the isoflavone genistein with hydroxypropyl-β-CD (HPBCD) and randomly methylated-β-CD (RAMEB), with an increased anti-inflammatory activity compared to free genistein have been described [21]. Despite complexation in the CD cavity, the antioxidative activity of

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flavonoids may be maintained, as demonstrated by the oxygen radical absorbance capacity (ORAC) test [22].

Given the high therapeutic interest of sulfuretin and butein, the aim of the present research was to obtain, for the first time, CD complexes hosting these flavonoids and to characterize them from a physico-chemical point of view. To our knowledge, CD complexes of either sulfuretin or butein have not been prepared until the present. Two types of CDs were employed: HPBCD and RAMEB. The two mentioned flavonoids sulfuretin were obtained through extraction from the wood of smoke tree, using a succession of chromatographic methods. The obtained complexes provide a stable alternative for biological tests evaluating the medicinal potential of these natural products.

Experimental part

Isolation of sulfuretin and butein from C. coggygria wood

Stems of *C. coggygria* were decorticated and the orange-colored heartwood (3.4 kg) was ground. The crude extract was obtained after exhaustive extraction with methanol at room temperature in an ultrasonic bath (8-fold repetition; plant:solvent ratio = 1:8 (m/m), followed by the evaporation of the solvent at reduced pressure in a rotary evaporator (extraction yield: 8.15%). A portion (225 g) of the crude extract was suspended in distilled water and subjected to partitioning using solvents of increasing polarities: petroleum ether, diethyl ether, ethyl acetate, and *n*-buthanol. The diethyl ether fraction (DEE, 87 g) was employed for the preparation of sulfuretin and butein; the presence of these compounds in the fraction could be ascertained by TLC. A portion of the DEE-soluble extract (80 g) was subjected to fractionation over RP-18 material (100 g, particle size 40-63 μm; Merck) by vacuum liquid chromatography. The mobile phase was a CH_3CN/water gradient (0:100 → 95:5, 19 steps; each 500 mL, with increasing CH_3CN content, 5 parts each time). The ninth subfraction, collected at CH_3CN/water ratio of 46/54, v/v (6.021 g) was further used for the isolation of butein and sulfuretin : 0.861 g were chromatographed over Sephadex LH-20 (Pharmacia Biotech, gel bed 80x3.8 cm) using acetone as an eluent. Butein eluted at volumes 390-430 mL, while sulfuretin was obtained from volumes 595-690 mL.

Preparation of cyclodextrin complexes was performed by the kneading method, using a 1:2 molar ratio (flavonoid:CD). HPBCD and RAMEB were purchased from Cyclolab (Hungary). A mixture of flavonoid and CD was triturated in a mortar, adding at the same time drops of a 50% ethanol solution [23]. The obtained paste was kneaded until the majority of the solvent evaporated; subsequently the mixture was dried at room temperature for 24h. Afterwards, the mixture was heated in an oven at 105°C for several hours, followed by pulverization and sieving through a 100 μm sieve.

Apparatus

NMR: 2D and 1D measured on a Bruker (Bruker Biospin, Rheinstetten, Germany) at 300 MHz (^{1}H) and 75 MHz (^{13}C); TMS as internal standard.

LC-MS data were obtained with an Esquire 3000plus (Bruker Daltonics, Bremen, Germany) mass spectrometer coupled to Agilent HPLC system type HP 1100. MS parameters: split, 1:5; ESI, alternative ion polarity mode; spray voltage, - 4.5 kV; interface temperature, 350°C; drying gas flow rate, 10.00 L/min; nebulizer gas, 40 psi; mode, full scan: *m/z* range, 100–1500.

Differential Scanning Calorimetry (DSC). The analysis was carried out with a Mettler-Toledo DSC1 instrument (Mettler-Toledo, Switzerland). Small portions of samples (4.1-4.3 mg) were placed in standard aluminum crucibles (40μL) with pierced caps and were heated between 40-280°C in an inert atmosphere (100 mL/min Ar) and a 5 degree/min heating speed. A reference material (empty aluminum crucible with pierced cap) was used simultaneously.

Particle size measurement. The size and the stability of particles were evaluated using ethanol solutions (1%, w/v) and a Cordouan Zetasizer instrument (Cordouan Technol., France) consisting of a Vasco Particle Size Analyzer and a Wallis Zetapotential Analyzer. Vasco Particle Size Analyzer parameters were set at: sample volume (~50 μL), temperature (25°C), time interval (6μs), number of channels (450), laser power (100%), DTC position: UP, acquisition mode (continuous), and algorithms (Pade-Laplace, Cumulants). Wallis Zetapotential Analyzer parameters: sample volume (~1.2 mL), samples' *p*H at 25°C (~7.1), cuvette type (plastic, wavelength 380-780 nm), temperature (25°C), laser power (45%), applied field (automatic), resolution (medium, 0.8 Hz), 3 measures/sequence, and Henry function (Huckel).

Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR). Spectra were obtained in attenuated total reflectance (ATR) mode on a Perkin Elmer SPECTRUM 100 device. Spectra were collected in the 4000-600 cm^{-1} spectral range, with a resolution of 4 cm^{-1} and with 64 acquisitions as co-added scans. The samples were analyzed as received, without further preparation.

Results and discussions

Extraction of sulfuretin and butein

Butein and sulfuretin co-occur in several Anacardiaceae plants, making representatives of this botanical family particularly attractive for the obtainment of these compounds with recognized medicinal potential. In fact, chalcones and aurones are biosynthetically related, representing two metabolic stages of the flavonoid scaffold in woody plants [5]. The present research completes the previous work on *Cotinus coggygria* as a useful source of butein and sulfuretin [15], describing the isolation of both compounds during the same workflow – a gel-filtration on Sephadex LH-20 using acetone as a mobile phase. This provides an advantage over the already described method where butein was obtained by high-speed countercurrent chromatography, and sulfuretin after column chromatography on Sephadex LH-20 using methanol as a mobile phase.

Butein (E)-1-(2,4-dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)prop-2-en-1-one was isolated as orange crystals (41.22 mg) and displayed maximum UV wavelengths of λ=200, 260, 375 nm. Structure assignment was based on 1D-NMR (fig. 2) and 2D-NMR spectra, and spectroscopic data were compared with those reported by Tian et al [24]. Identity was further confirmed by LC-MS fragmentation patterns (fig. 3): ESI-MS (positive mode): m/z = 273.0 $[M + 1]^+$, ESI-MS (negative): m/z = 271.1 $[M - 1]^-$.

Sulfuretin, (2*Z*)-2-(3,4-dihydroxybenzylidene)-6-hydroxy-1-benzofuran-3(2*H*)-one, was obtained as a brown powder (263.75 mg) and displayed maximum UV wavelengths of λ=200, 250, 265, 390 nm. Structure assignment was based on proton NMR (fig.4) and 2D-NMR spectra, and comparison of spectroscopic data with those reported by Zhao et al [25]. Typical fragmentation ions were in ESI-MS (positive mode): m/z = 271.0 $[M + 1]^+$, and in ESI-MS (negative): m/z = 269.3 $[M - 1]^-$, 539.0 $[2M - 1]^-$ (fig. 5)

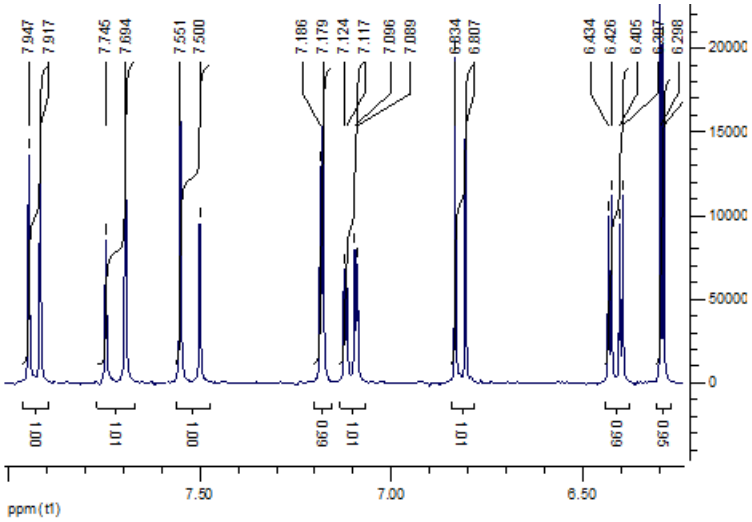

Fig. 2. ^{1}H NMR spectrum of butein obtained through extraction from *C. coggygria* wood (CD_3OD, 300.13 MHz)

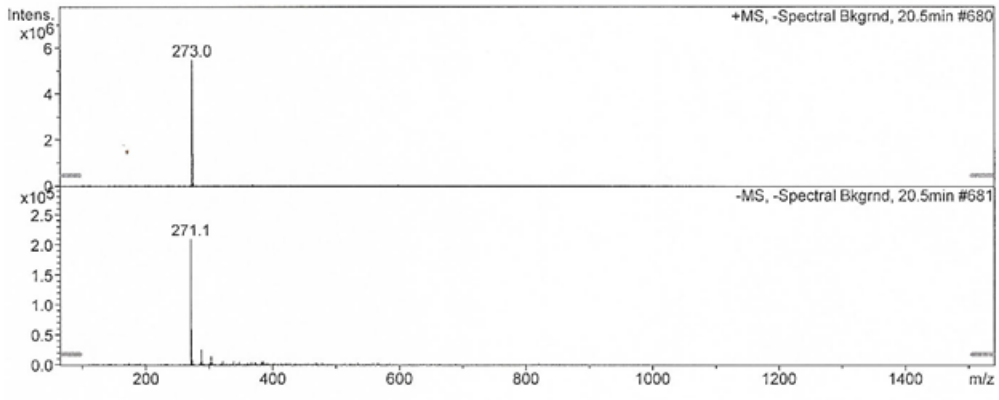

Fig. 3. LC-MS fragmentation patterns of butein

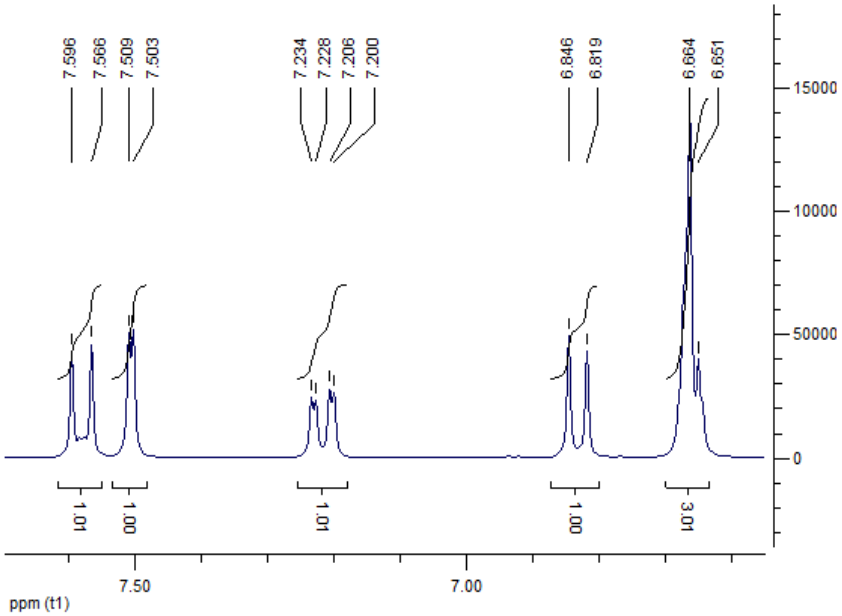

Fig.4. ^{1}H NMR spectrum of sulfuretin obtained through extraction from *C. coggygria* wood (CD_3OD, 300.13 MHz)

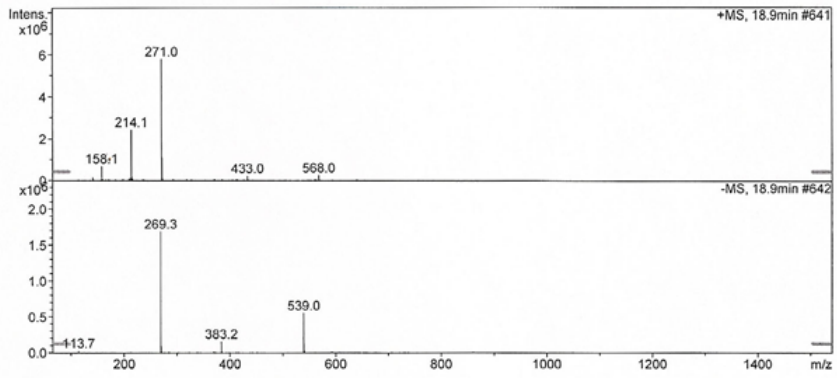

Fig.5. LC-MS fragmentation patterns of sulfuretin

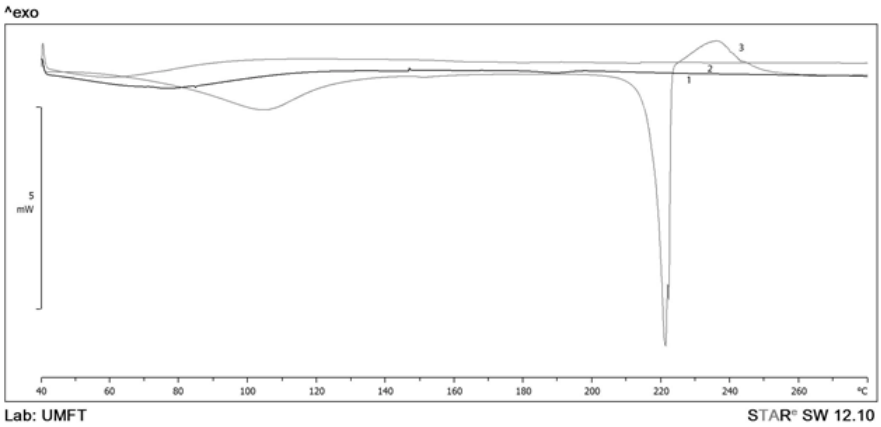

Fig. 6. DSC curves of butein-HPBCD (1), butein-RAMEB (2) and butein (3)

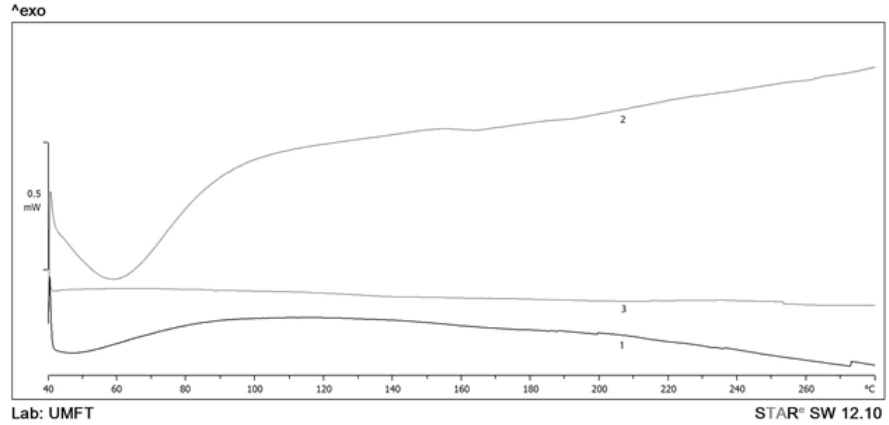

Fig. 7. DSC curves of sulfuretin-HPBCD (1), sulfuretin-RAMEB (2) and sulfuretin (3)

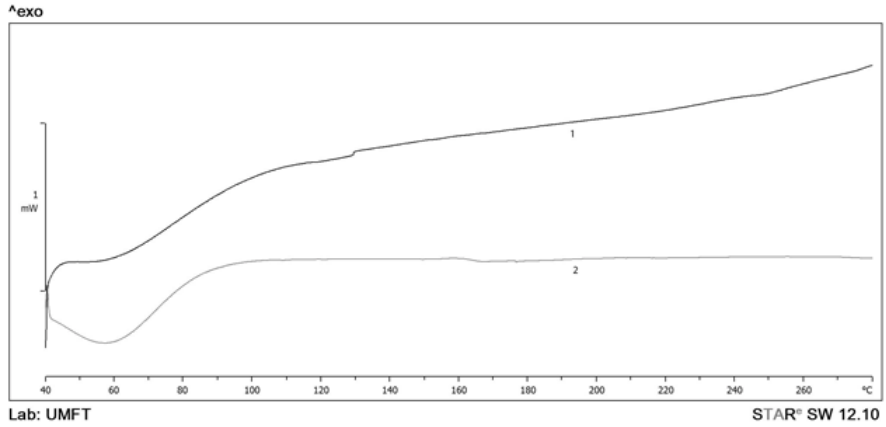

Fig. 8. DSC curves: (1) HPBCD; (2) RAMEB

Analysis of cyclodextrin-flavonoid complexes

The thermal analysis of CD complexes can provide qualitative and quantitative data about the physico-chemical state of the guest inside the CD cavity. Inclusion of the guest in to CD cavity may cause disappearance/ appearance of endothermic peaks, broadening/shifting to different temperature indicating a change in the crystal lattice, melting, boiling or sublimation points [26].

The thermograms of CD complexes with butein (fig. 6) show that dehydration of butein-HPBCD occurs through a temperature range from 68 to 149°C with the maximum at 107°C, while the dehydration of butein-RAMEB appears between 41 and 85°C, with a maximum at 59°C. The melting point of butein was observed at 221°C.

The thermograms of CD complexes with sulfuretin (fig. 7) indicate a slightly dehydration of sulfuretin-HPBCD occurring between 40 and 93°C with the maximum at 44°C, and an important dehydration of sulfuretin-RAMEB which appears between 41 and 101°C, with a maximum at 58°C. The melting of sulfuretin was not recorded inside the studied range of temperature (40-280°C).

The thermograms of pure CDs (fig. 8) show characteristic endothermic peaks at 56-58°C associated to crystal water losses (8-21%) from CD cavities. Dehydration of HPBCD occurs through a temperature range from 47 to 65 C with the maximum at 58°C, while the dehydration of RAMEB appears between 41 and 96, with a maximum at 56°C. An exothermic effect nearing 160°C is related to the cold crystallization of RAMEB, while an endothermic effect nearing 280°C is related to thermal destruction of HPBCD.

The measurement of size and stability is based on the Dynamic Light Scattering technique [27]. The sizes and stability of the obtained particles are presented in table 1.

Microparticles with sizes between 458 and 467 nm were obtained in the case of HPBCD complexes. Larger microparticles (635-667 nm) were synthesized as complexes of RAMEB with sulfuretin. Due to a higher value of zeta potential and lower particle size, inclusion complexes obtained with HPBCD are more stable in a colloidal suspension, in comparison with those obtained with RAMEB which have a higher tendency to cluster [28]. It is important to mention that polydispersity index shows a high degree of homogeneity.

The FTIR analysis of sulfuretin, butein and their complexes.

The successful encapsulation of guest molecules in CD may be pointed out by the shifting or disappearance of

Sample code	Particle size (nm)		Zeta Potential (mV) Mean ± SD
	Mean ± SD	Polydispersity index	
Butein-HPBCD	467 ± 19	0.2	19.11 ± 0.23
Butein-RAMEB	635 ± 24	0.3	13.87 ± 0.97
Sulfuretin-HPBCD	458 ± 10	0.2	19.46 ± 0.57
Sulfuretin-RAMEB	667 ± 21	0.2	13.65 ± 1.05
HPBCD	490 ± 12	0.1	18.96 ± 0.09
RAMEB	676 ± 16	0.2	12.64 ± 1.17

Table 1
THE ZETASIZER CHARACTERIZATION OF PARTICLES CONTAINING CYCLODEXTRINS

bands which are characteristic for the included molecule. In exchange, it can be expected that the spectrum of a CD complex resembles with the one of the pure CD.

Analysis of the IR spectrum of sulfuretin and butein.

Sulfuretin displays following characteristic FTIR bands: a broad band in the 3573-2493 cm^{-1} spectral range corresponding to stretching vibrations of the phenolic hydroxyl groups [29]. The presence of sharp peaks on the broad band is due to the fact that the sulfuretin molecules exist both as *free molecules* (sharp O-H stretching vibrations at 3293 and 3490 cm^{-1}), but as well as intermolecular H-bonded molecules (the broad band), due to the presence of O-H groups, and as well the H-bonding with the ketonic oxygen. The stretching of C-H from phenyl groups and benzylidene is represented by the bands at 3140 and 3074 cm^{-1}. The intense band at 1681 cm^{-1} is represented by the C=O vibration and is an intense band, characteristic for this functional moiety. This considerable shifting of the C=O vibration of ketone group (which usually appear around 1750 cm^{-1}) is due to the C=C and C=O conjugation. The existence of one or more aromatic rings in a structure is normally readily determined from the C-H and C=C-C ring-related vibrations. Some characteristic bands for the 1,2,4-substitution mode of the benzene ring is sustained by the medium bands in the spectral ranges 798-834 and 963-985 cm^{-1}, respectively. The benzofurane ring is represented by skeletal vibrations between 1349-1406 cm^{-1} and 985 cm^{-1}, respectively.

The FTIR spectrum of butein reflects some essential differences in comparison to sulfuretin, i.e. the lack of the benzofurane moiety and the acyclic nature of the ketone group.

The broad bands in the 3614-2910 cm-1 spectral range appear due to the presence of the O-H bonds and are associated with the stretching vibrations. The sharp peaks at 3538, 3496 and 3277 cm^{-1} are due to the presence of the unbounded O-H groups. The stretching of C-H bonds are dramatically diminished and appear around 3138 and 3093 cm^{-1} (are visible after deconvolution of the spectrum). The band at 1636 cm^{-1} (sharp and intense) is represented by the C=O vibration, characteristic for this functional moiety. This considerable shifting of the C=O vibration of ketone group (which usually appear around 1750 cm^{-1}) is due to the C=C and C=O conjugation. The shifting of the wavenumber comparative to the spectrum of sulfuretin is due to the different nature of this ketone group, which is an aliphatic one. The characteristic bands for the 1,2,4-substitution mode of the benzene ring is similar to the ones in sulfuretin appear in the same spectral ranges: 802-836 and 966-990 cm^{-1}, respectively. The characteristic skeletal vibrations of benzofurane ring are also missing, which is in good agreement with the chemical structure of the compound.

Analysis of HPBCD. FTIR spectrum for pure HPBCD shows characteristic bands attributable to different group vibrations: the broad absorption band in the spectral range 3658-3011 cm^{-1} (peak at 3342 cm^{-1}) correspond to the presence of the O-H group both from the bonded water in the CD cavity, but as well to the presence of the grafted hydroxyl groups on the polysaccharide structure. The C-H stretching vibrations appear as sharp peaks at 2965 and 2930 cm^{-1}, while the C-O stretching vibrations appear at 1152, 1080 and 1023 cm^{-1}. These peaks are however overlapped with the characteristic saccharide-structure bands in the 1200–1000 cm^{-1} spectral range. The presence of water in the HPBCD cavity is also sustained by the bent of the O-H groups from water at 1641 cm^{-1}.

Analysis of RAMEB. Randomly methylated-â-cyclodextrin (RMBCD) presents similar FTIR spectrum to HPBCD. Some characteristic peaks were noticed in the 3654–3013 cm^{-1} due to the O–H group stretching from contained water molecules (peak at 3399 cm^{-1}). The bands at 2928 cm^{-1} and 2967 cm^{-1} are due to C–H asymmetric/ symmetric stretching vibrations. In addition, a peak at 1641 cm^{-1} represented the H–O–H deformation bands of water present in RMBCD. Peaks at 1154 and 1024 cm^{-1} indicated C–H overtone stretching and that at 1080 cm^{-1} the O-C-H stretching. Absorption of the C–O–C vibration was seen at 1154 cm^{-1}.

Analysis of butein-cyclodextrin complexes. The FTIR spectra of inclusion complexes of the two flavonoids with HPBCD and RAMEB were found to be quite similar to spectra of pure CDs, because of the superimposion of the absorption of both the host and guest molecule in most of the important spectral regions. The characteristic bands and peaks of the guest molecules are masked by the bands of the CDs. However, some shifts of the wavenumbers were observed. The most important band is in all cases considered the ketone. In the case of the complex formation, this band is shifted to 1630 cm^{-1}, due to interaction with the HPBCD. The characteristic peaks of butein disappeared, shifted or decreased in intensities (especially the absorption bands from 3538, 3496 and 3277 cm^{-1}). Based on these results, we conclude that OH moieties of butein are trapped within the HPBCD, demonstrating the successful formation of HPBCD–But inclusion complexes.

The same FTIR profile was observed in the case of the inclusion complex between butein and RAMEB. The spectral range 4000-1800 cm^{-1} of the complex is identical to the one of RMBCD, suggesting the encapsulation process. Also, the shifting of C=O wavenumber from 1636 cm^{-1} to 1630 cm^{-1} also proves the encapsulation of the compound.

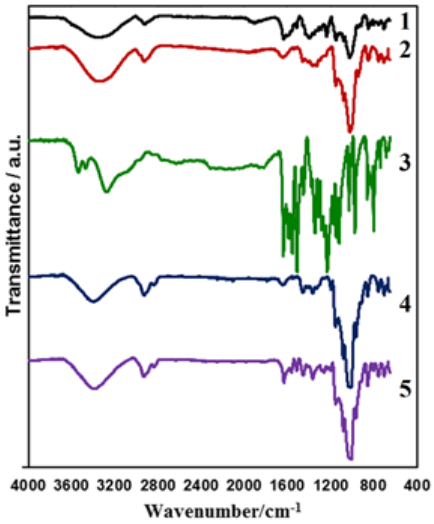

Fig. 9. ATR-FTIR spectra for butein-HPBCD complex (1), HPBCD (2), butein (3), RAMEB (4), and sulfuretin-butein-RAMEB complex (5)

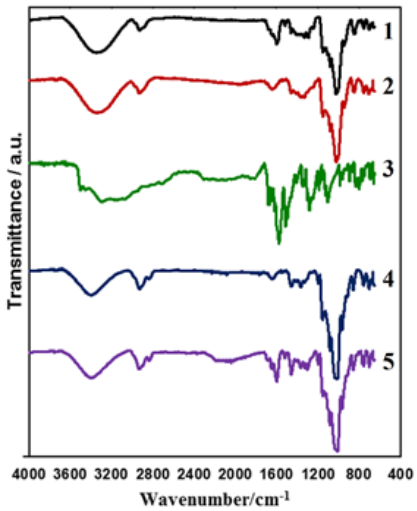

Fig.10. ATR-FTIR spectra for sulfuretin-HPBCD complex (1), HPBCD (2), sulfuretin (3), RAMEB (4), sulfuretin-RAMEB complex (5)

Analysis of sulfuretin-cyclodextrin complexes. As expected, the FTIR spectra of the inclusion complexes showed high resemblance with the ones of pure CDs. All the sharp peaks belonging to CD were observed, but the characteristic peaks of sulfuretin disappeared or shifted, especially the absorption band at 1681 cm^{-1} corresponding to C=O vibrations. In the case of sulfuretin-HPBCD the band is shifted to 1639 cm^{-1}, while in the case of sulfuretin-RAMEB, it is shifted to 1686 cm^{-1}. The benzene rings C-H bands are also suppressed, suggesting their encapsulation. Also, the characteristic bands of CDs are visible in the spectrum of the binary compound, like the 1365, 1194, 1154, 1083, 1008, 965 cm^{-1} (for RAMEB) and 1641, 1332, 1152, 1080, 1023, 948, 757 cm^{-1} (for HPBCD), suggesting the encapsulation of the aurone in the internal cavity and the modification of the vibrational behavior.

Conclusions

The aurone sulfuretin and the chalcone butein could successfully be isolated from *C. coggygria* wood in a three-step extraction procedure. Novel cyclodextrin inclusion complexes of sulfuretin and butein were synthesized, using for each compound two CDX types. Preparation was performed at a host-guest ratio of 2:1. Complex formation was demonstrated by FTIR, with the disappearance/shifting of characteristic bands of the guest molecules in spectra of the CD complexes. The thermal analysis by DSC substantiates the high yield of encapsulation, revealing important differences between the thermograms of flavonoids vs. the thermograms of cyclodextrin complexes. Stable microparticles based on cyclodextrin complexes with sizes between 458 and 676 nm were obtained. Inclusion complexes of flavonoids with HPBCD displayed a higher stability in colloidal suspension, as demonstrated by higher zeta potential value and lower particle size.

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