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3

Analytical Methods for Isolation, Separation and Identification of Selected Furanocoumarins in Plant Material

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1. Introduction

Coumarins are α–pyrone derivatives synthesized as secondary metabolites in plants. They occur as free compounds or glycosides in plants. They have been isolated from A. Vogel, since 1820, from the tonka beans (*Coumarouna odorata* Aubl. = *Dipteryx odorata* Will.) and they have been synthesized in 1868 from W. H. Perkin, through the famous Perkin reaction (Dewick, 2009).

Furanocoumarins are one of the coumarin derivatives. They can be grouped into the linear type, where the furan ring (dihydro) is attached at C(6) and C(7), and the angular type, carrying the substitution at C(7) and C(8).

The most abundant linear furanocoumarins are psolaren, xanthotoxin, bergapten and isopimpinellin, whereas the angular type is mostly represented by angelicin, sphondin, and pimpinellin. Some structures of furanocoumarins are presented in table 1. As was mentioned for the simple coumarins, numerous minor furanocoumarins have been described in the literature, for example bergamottin (5-geranoxy-psolaren) (Stanley & Vannier, 1967), which has received attention recently as a major grapefruit component interfering with drug metabolism by intestinal CYP3A4 (Bourgaud et al., 2006; Wen et al., 2008).

2. Distribution of furanocoumarins in plants

Linear furanocoumarins (syn. psolarens) are principally distributed in four angiosperm families: Apiaceae (Umbelliferae), Moraceae *(Brosimum, Dorstenia, Fatoua* and *Ficus),* Rutaceae and Leguminose (restricted to Psoralea and Coronilla generae). The angular (dihydro) furanocoumarins are less widely distributed and primarily confined to the Apiaceae and Leguminosae (Berenbaum et al., 1991; Bourgaud et al., 1995).

Moreover, furanocoumarins have been reported from Asteraceae (Compositae), Pittosporaceae, Rosaceae, Solanaceae and Thymelaeaceae (Milesi et al., 2001; Murray et al., 1982). Certain precursors to this group of compounds are found in the Cneoraceae (Murray, 1982).

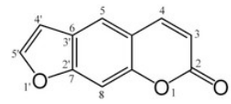
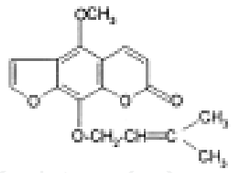
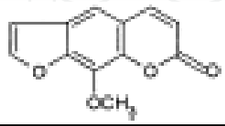
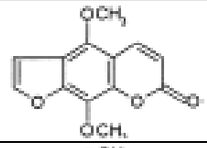
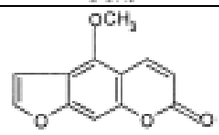
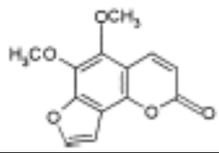
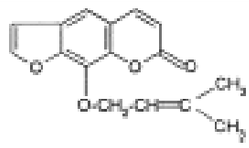
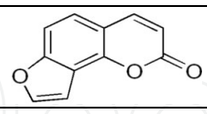
Name of compound	Structure
Psoralen	
Phellopterin	
Xanthotoxin	
Isopimpinellin	
Bergapten	
Pimpinellin	
Imperatorin	
Angelicin	

Table 1. The structure of some furanocoumarins

Coumarins are distributed across different parts of the plants, and they have specific histological locations in the tissues. Within the plant they are most abundant in fruits and roots. However, in flowers and leaves they are evident in fewer quantities. In some plant species coumarins were also found in the bark or stems (Głowniak, 1988).

The amount of particular furanocoumarins depends on the enzymes active in plants secondary metabolism. Plants with similar enzyme profiles contain comparable amount of secondary metabolites that are products of chemical reactions induced by these enzymes. Thus, furanocoumarins' content, in different species, varieties and forms may contribute to their better distinction, and better understanding of the taxonomy of genuses within which they are present.

Diawara et al. (1995) examined the relative distribution of furocoumarins in celery (*Apium graveolens* L. var. *dulce* Miller) plant parts and found that leaves of the outer petioles contained significantly higher levels of the three phototoxic constituents than did other plant parts, followed by leaves of the inner petioles.

On the other hand, levels of furanocoumarins observed in plants grown in the field are higher than those observed in plants grown in laboratory or greenhouse conditions and may fluctuate over the season (Trumbe et al., 1992; Diawara et al., 1995). In most studies, bergapten has been found to occur in highest concentrations, followed by xanthotoxin, but psoralen is often observed only in trace quantities (Trumbe et al., 1990; Trumbe et al., 1992; Diawara et al., 1993). However, other studies have found that xanthotoxin (Beier et al., 1983) or psoralen (Diawara et al., 1993; Trumble et al., 1990) is most abundant (Stanley-Horn, 1999).

Considering the histological location of furanocoumarins in plant tissues, they are arranged differently. For example, celery contains schizogenous canals scattered throughout the pericycle, which are secretory and are thought to extend through the stem and foliage (Maksymowych & Ledbetter, 1986). Furanocoumarins are thought to be restricted to schizogenous canals in seeds of celery (Berenbaum, 1991) and accumulate primarily in petiolar and foliar canals in cow-parsnip, *Heracleum lanatum* Michx. (Apiaceae). However, there is also evidence suggesting that this group of compounds occur in and on the surfaces of tissues as well. A study of several apiaceous and rutaoeous species by Zobel and Brown (1990) revealed that a large proportion of each furocoumarin was located on the leaf surface in most of the plants studied. Furanocoumarins of *Ruta graveolens* L. are present in the epidermal layer of both stems and leaves and in the mesophyll directly below the epidermis, while glands of leaves contain only traces of furanocoumarins. In fact, the cuticular layer contains 15.60% of the psoralens found in leaves (Zobel et al., 1989). The occurrence of bergapten and xanthotoxin in the surface wax of leaves of wild carrot, *Daucus carota* L., a plant containing only trace levels of furanocoumarins has also been reported (Ceska et al., 1986; Stanley-Horn, 1999).

The content of coumarins in plants is conditioned by the degree of the development of the plant and its vegetation stage, too. Concentrations of linear furanocoumarins increase dramatically with plant age between 8 and 18 weeks (Reitz et al., 1997) with a subsequent decline in bergapten concentrations in the last six to eight weeks before harvest (Trumble et al., 1992). Significant decreases in levels of furanocoumarins were also observed both in and on senescing leaves of *Ruta graveolens* (Zobel & Brown, 1991). The content of some furanocoumarins in *Apium graveolens* and *Petroselinum sativum* decreases in summer and in autumn increases (Kohlmünzer, 2010).

3. Biosynthesis of furanocoumarins

The biosynthesis of linear and angular furanocoumarins is still poorly understood at the molecular level. They are produced *via* the shikimic acid biosynthetic pathway beginning with the conversion of phenyloalanine to trans-cinnamic acid by phenylalanine ammonia lyase. Orto-hydroxylation of trans-cinnamic acid yields 2'-hydroxycinnamic acid, which is converted to its cis form, the precursor to coumarin, in the presence of UV light. Alternatively, trans-cinnamic acid may undergo parahydroxylation to yield p-coumaric

acid. P-coumaric acid may undergo 2'–hydroxylation followed by conversion by 4-coumarate: CoAligase to 4-coumaryl CoA. This compound is intermediate in the biosynthesis of both flavonoids and phenylpropanoids, including 7-hydroxycoumarin (umbelliferone). Umbelliferone is the precursor to both the angular and linear furanocoumarins. The production of the latter involves prenylation to form marmesin, followed by oxidative loss of the hydroxypropyl group in marmesin by 'psoralensynthase' to yield psoralen (Berenbaum, 1991; Stanley-Horn, 1999). A second cytochrome P-450-dependent monooxygenase enzyme then cleaves off the hydroxyisopropyl fragment (as acetone) from marmesin to give the furocoumarin psoralen. Psoralen can act as a precursor for the further substituted furanocoumarins bergapten, xanthotoxin and isopimpinellin. On the other hand, angular furanocoumarins, such an angelicin can arise by a similar sequence of reactions, but these involve initial dimethylallylation at the alternative position *ortho* to the phenol (Dewick, 2009).

4. Biological activities of furanocoumarins

Due to their biological activities, furanocoumarins are very interesting compounds and widely investigated. The various biological and pharmacological activities of coumarins, have been known for a long time.

They play the role of phytoalexin in plants (Szakiel, 1991), which can be synthesized as a result of elicitation by microorganisms, insects, fungi as well as abiotic elicitors such as UV radiation, environment pollutants and mechanical breakage. Defensive activity of furanocoumarins consists in their toxicity against phytopathogens (e.g. retardation of DNA synthesis) (Waksmundzka-Hajnos et al., 2004).

Linear furocoumarins can be troublesome to humans since they can cause photosensitization towards UV light, resulting in sunburn or serious blistering. Used medicinally, this effect may be valuable in promoting skin pigmentation and treating psoriasis. Plants containing psoralens have been used internally and externally to promote skin pigmentation and suntanning. Bergamot oil obtained from the peel of *Citrus aurantium* ssp. *bergamia* (Rutaceae) can contain up to 5% bergapten and is frequently used in external suntan preparations. The psoralen absorbs in near UV light and allows this radiation to stimulate formation of melanin pigments (Dewick, 2009).

Methoxsalen (xanthotoxin; 8-methoxypsoralen), a constituent of the fruit of *Ammi majus* (Umbelliferae/Apiaceae), is used medically to facilitate skin repigmentation where severe blemishes exist (vitiligo). An oral dose of methoxsalen is followed by long – wave UV irradiation, though such treatments must be very carefully regulated to minimize the risk of burning, cataract formation, and the possibility of causing skin cancer. The treatment is often referred to as PUVA (psoralen + UV-A). PUVA is also of value in the treatment of psoriasis, a widespread condition characterized by proliferation of skin cells. Similarly, methoxsalen is taken orally, prior to UV treatment. Reaction with psoralens inhibits DNA replication and reduces the rate of cell division. Because of their planar nature, psoralens intercalate into DNA, and this enables a UV – initiated cycloaddition reaction between pyrimidine bases (primarily thymine) in DNA and the furan ring of psoralens. A second cycloaddition can then occur, this time involving the pyrone ring, leading to interstrand cross – linking of the nucleic acid (Dewick, 2009; Żołek et al., 2003).

A troublesome extension of these effects can arise from the handling of plants which contain significant levels of furocoumarins. *Apium graveolens* (= celery; Umbelliferae/Apiaceae) is normally free of such compounds, but fungal infection with the natural parasite *Sclerotinia sclerotiorum* induces the synthesis of furanocoumarins as a response to the infection. Field workers handling these infected plants may become very sensitive to UV light and suffer from a form of sunburn termed photophytodermatitis. Infected parsley (*Petroselinum crispum*) can give similar effects. Handling of rue (*Ruta graveolens;* Rutaceae) or giant hogweed (*Heracleum mantegazzianum*; Umbelliferae/Apiaceae), which naturally contain significant amounts of psoralen, bergapten, and xanthotoxin, can cause similar unpleasant reactions, or more commonly rapid blistering by direct contact with the sap. The giant hogweed can be particularly dangerous. Individuals vary in their sensitivity towards furanocoumarins, some are unaffected, whilst others tend to become sensitized by an initial exposure and then develop the allergic response on subsequent exposures (Dewick, 2009).

Methoxsalen in combination with ultraviolet light is also used for antineoplastic effects and for treating certain skin disorders, including alopecia, cutaneos T-cell lymphoma, excema, lichen planus, mycosis fungoides and psoriaris. A recent report has found that this drug inhibits the enzyme, CYP2A6, which is responsible for the metabolism of nicotine. When 8-methoxypsoralen is taken with oral nicotine, this drug can reduce the number of cigarettes smoked by about one quarter and decrease overall levels of tobacco smoke exposure by almost half in tobacco dependent individuals (Lehr et al., 2003).

Xanthotoxin is used orally or topically in combination with controlled exposure to long wavelength ultraviolet radiation (UVA) or sunlight to repigment vitiliginous skin in patients with idiopathic vitiligo. Many studies have shown that naturally occurring furocoumarins, e.g. imperatorin and isopimpinellin, inhibit P450-mediated enzyme activities *in vitro*. Imperatorin and isopimpinellin have also the potential chemopreventive effects when administered in the diet. The stimulation of melanogenesis by bergapten is related to increased tyrosinase synthesis. In addition, bergapten stimulated TRP-1 synthesis and induced a dose-dependent decrease of DCT activity without modification of protein expression. Osthole could prevent postmenopausal osteoporosis. It can also delay aging, build up strength, enhance immune function, and adjust sex hormone levels (Chen et al., 2007).

Psoralen and bergapten exert their photosensibilising effects through a covalent interaction with DNA triggered by light of a specific wavelength (320-400 nm). The resulting complex blocks the DNA interaction with transcriptases and polymerases, avoiding cell replication. This mechanism consist of three steps, i.e., (1) drug intercalation between DNA nucleotide bases, (2) drug absorption of a UVA photon and covalent bond formation between the furan ring double bond and a thymine base (T2) of the DNA molecule, (3) absorption of a second photon (UVA) and covalent bonding between the lactone ring double bond and another thymine base (T1), which, in the end, results in a psoralen cross-linked DNA (da Silva et al., 2009; Panno et al., 2010; Cardoso et al., 2002). The same effects have been alternatively utilized for the treatment of human lymphoma and of autoimmune diseases through extracorporeal photochemotherapy (Panno et al., 2010).

Panno et al. (2010) investigated the pro-apoptotic effects induced by high doses of bergapten (methoxypsoralen; 5-MOP), in the absence of UV rays, in human breast cancer cells. The same authors examined the effects of bergapten, alone and in combination with UV light, on

the cellular growth of breast tumoral cells. Their study suggested that bergapten alone, or as a photoactivated product, could be used as an active molecule able to counteract effectively the survival and growth of breast hormone-responsive tumors.

Furanocoumarins isolated from fruits of *Heracleum sibiricum* L. inducing apoptosis by forming adducts with DNA. Bogucka – Kocka (2004) reported a visible influence of these compounds on the inhibition of the proliferation and on induction of apoptosis processes in the human HL-60 cell lines. Moreover, compounds isolated from *Angelica dahurica* (Apiaceae) were examined regarding their cytotoxic activity against L1210, HL-60, K562, and B16F10 tumor cells lines using the MMT cell assay. It was discovered that pangelin and oxypeucedanin hydrate acetonide exhibited the most cytotoxic activity against all selected tumor cell lines (Heinrich et al., 2004). Um and co-authors (2010) were isolated four furonocoumarins (bergapten, isopimpinellin, xanthotoxin and imperatorin) from *Glehnia littoralis* F. Schmidt ex Miquel (Apiaceae), which exhibited dose-dependent inhibitory effectson the cell proliferation. Their study demonstrated that *G. littoralis* has potent inhibitory effect on proliferation of HT-29 human colon cancer cells.

In addition, Oxypeucedanine (= prangolarin), which was isolated from *Prangos, Hippomarathrum, Angelica* and *Ferulago* (genera of Apiaceae) and *Ruta* genus of Rutaceae, has pharmacological and biological activities. It was reported to have antiarrhytmic, channel blocer and antiestrogenic activity. Razavi et al. (2010) studied phytotoxic, antibacterial, antifungal, antioxidant and cytotoxic effects of oxypeucedanin. Their results revealed that this compound exhibits considerable phytotoxic activity and might play an allelopathic role for plants. On the other hand, oxypeucedanin exhibits considerable cytotoxicity against Hela cell line (IC_{50} value of 314 μg/ml).

The ethanol extract of the *Cnidii* fructus and coumarins separated from it have growth-inhibitory effects on the tumor cells (Chen et al., 2007).

One of the major bioactive components of the fruits of *Cnidium monnieri* (L.) Cusson, bergapten, possesses antiinflammatory and analgesic activities. However, imperatorin exhibits strong cytotoxic activity on human leukemia, chemopreventive effects on hepatitis and skin tumor, and antiinflammatory activity (Li & Chen, 2004).

In addition of bergapten, this plant also contained numbers of others coumarins, such as xanthotoxin, isopimpinellin, bergapten, imperatorin and osthole. These constituents regarded for biological activity of this crude drug, which is used for treatment of pain in female genitalia, impotence and supportive (Chen et al., 2007).

Pharmacological studies have indicated that coumarins such as isoimperatorin, notopterol and bergapten possess anti-inflammatory, analgesic, anti-cancer and anti-coagulant activities (Qian et al., 2007).

Imperatorin (8-isopentenyloxypsoralen; 9-(3-methylbut-2-enyloxy)-7H-furo [3,2-g]chromen-7-one) is a bio-active furanocoumarin isolated e.g. from roots of *Angelica dahurica* and fruits of *Angelica archangelica* (Umbelliferae) (Baek et al., 2000). Experimental evidence indicates that imperatorin irreversibly inactivates γ-aminobutyric acid (GABA)-transaminase (the enzyme responsible for the degradation of GABA) and thus, increases the GABA content in the synaptic clefts of neurons and elevates the inhibitory neurotransmitter GABA level in the brain (Choi et al., 2005; Łuszczki et al., 2007).

Quite recently, it has been documented that imperatorin in a dose-dependent manner increased the threshold for maximal electro-convulsions in mice (Łuszczki et al., 2007a). The time–course and dose–response relationship analyses revealed that the time to peak of the maximum anticonvulsant effect for imperatorin was established at 30 min after its systemie (i.p.) administration in mice (Łuszczki et al., 2007a).

Recently results indicate that imperatorin administered at subthreshold doses enhanced the anticonvulsant effects of carbamazepine, phenytoin and phenobarbital, but not those of valproate against maximal electroshock-induced seizures in mice. It is important to note that the anti-seizure effects of carbamazepine combined with imperatorin were greater than those observed for the combinations of phenobarbital and phenytoin with imperatorin.

The difference in the anti-seizure effects of carbamazepine and phenytoin or phenobarbital in the maximal electroshock seizure test may be explained through pharmacokinetic interaction between imperatorin and carbamazepine. It was found that imperatorin significantly increased total brain carbamazepine concentrations, having no impact on the total brain phenytoin and phenobarbital concentrations in experimental animals. The selectivity in the increase in total brain carbamazepine concentration one can try to explain through the fact that imperatorin probably enhances the penetration of carbamazepine into the brain by modifying the blood–brain barrier permeability. On the other hand, it may be hypothesized that the selective increase in carbamazepine content in the brain tissue resulted from imperatorin-induced inhibition of multi-drug resistance proteins or P-glycoproteins that normal physiological activity is related to the removal of drugs from the brain tissue. Thus, inhibitors of these proteins may contribute to the accumulation of antiepileptic drugs in the brain (Brandt et al., 2006; Łuszczki et al., 2007).

Considering molecular mechanisms of the action of conventional antiepileptic drugs and imperatorin, one can ascertain that imperatorin-induced irreversible inactivation of GABA-transaminase and subsequent increases in GABA content in the brain, as well as, the enhanced GABA-mediated inhibitory neurotransmitter action through the interaction of imperatorin with benzodiazepine receptors. This may exhibit complementary potentials to the anticonvulsant activity of carbamazepine, phenytoin and phenobarbital shown in experimental animals testing. Noteworthy, the main anticonvulsant mechanism of the action of carbamazepine and phenytoin is related to the blockade of Na+ channels in certain neurons (Łuszczki et al., 2007).

It is interesting to note that imperatorin did not potentiate the protective action of valproate against maximal electroshock-induced seizures. This apparent lack of effects of imperatorin on the antiseizure action of valproate, one can try to explain by the fact that valproate possesses a number of variousmechanisms of action that contribute to its anti-seizure activity in both rodents and humans (Łuszczki et al., 2007).

The evaluation of acute adverse effect potentials is exhibited within combinations of imperatorin with conventional antiepileptic drugs revealing that the combinations did not disturb long-term memory, impair motor co-ordination, or change neuromuscular grip-strength in experimental animals. Therefore, the investigated combinations seem to be secure and well tolerated by experimental animals (Łuszczki et al., 2007).

It was shown that imperatorin enhances the protective action of carbamazepine, phenytoin and phenobarbital, but not that of valproate against maxima electroshock-induced seizures

in mice. The lack of any changes in total brain phenytoin and phenobarbital concentrations suggested that the observed interactions of imperatorin with phenytoin and phenobarbital were pharmacodynamic in nature and thus, they deserve more attention from a preclinical viewpoint. If the results from the study of Łuszczki and co-authors (2007) can be extrapolated to clinical settings, a novel therapeutic option in the management of epilepsy may be created for epileptic patients.

Piao et al. (2004) assayed eleven furanocoumarins, isolated from *Angelica dahuricae* to determine its antioxidant activities. 9-hydroxy-4-methoxypsoralen inhibited DPPH formation by 50% at a concentration of 6.1 μg/ml (IC_{50}), and alloisoimperatorin 9.4 μg/ml, thus the other nine furanocoumarins (oxypeucedanin hydrate, byakangelicol, pabulenol, neobyakangelicol, byakangelicin, oxypeucedanin, imperatorin, phellotorin, and isoimperatorin), with an IC_{50} values higher than 200 μg/ml, showed only a little DPPH radical-scavenging activities.

Tosun et al. (2008) evaluated the anticonvulsant activity of the furanocoumarins among others compounds, obtained from the fruits of *Heracleum crenatifolium*. This activity was estimated against maximal electroshock seizures induced in mice. Among analyzed compounds, bergapten showed significant anticonvulsant activity.

Osthole, a coumarin derivative extracted from many plants, such as *Cnidium monnieri* and *Angelica pubescens*, has been showed to exhibit estrogen-like effects and prevent postmenopausal osteoporosis in overiectomized rats. The latest research suggested that this compound can alleviate hyperglycemia and could be potentially developed into a novel drug for treatment of diabetes mellitus (Liang et al., 2009).

Tang et al. (2008) have reported that imperatorin and bergapten induce osteoblast differentiation and maturation in primary osteoblasts. These compounds increased also BMP-2 (bone morphogenetic protein type 2) expression via p38 and ERK-dependent (extracellular signal-regulated protein) pathways. Long–term administration of imperatorin and bergapten into the tibia of young rats also increased the protein level of BMP-2 and bone volume of secondary spongiosa.

However, the toxic effects of furanocoumarins are also well known. Da Silva et al. (2009) at computational analysis of psoralen, bergapten and their predicted metabolites revealed the presence of six toxicophoric groups related to carcinogenicity, mutagenicity, photoallergenicity, hepatotoxicity and skin sensitization.

Numerous studies have indicated that furanocoumarins are carcinogenic, and their ability to intercalate into DNA in the presence of long wave UV light accounts for their mutagenicity. Linear furocoumarins have been shown to exhibit varying levels of phototoxicity. It must be stated that with isopimpinellin, it results in having the least photosensitizing activity (Lehr et al., 2003).

Moreover, coumarin derivatives in high doses can produce significant side effects. They may induce headaches, nausea, vomiting, sleepiness, and in extreme cases, serious liver damage with potential hemorrhages as a result of hypoprothrominemia (Lozhkin & Sakanyan, 2006).

5. Analytical methods of furanocoumarins isolation

5.1 Extraction from plant material

As furanocoumarins have wide applications in biology and have many therapeutic activities, the study of isolation and identification of these compounds is very important. In this part of our work, review of possible methods of isolation of furanocoumarins will be described as follows.

Coumarins typically appear as colorless or yellow crystalline substances, well soluble in organic solvents (chloroform, diethyl ether, ethyl alcohol), as well as in fats and fatty oils. Coumarin and its derivatives exhibit sublimation on heating to 100˚C (Lozhkin & Sakanyan, 2006).

In this process of quantitative analysis of plant secondary metabolites, preliminary treatment of the plant materials is one of the most time-consuming steps. The first problem is the extraction of the compounds from the plant material – usually performed by liquid – solid extraction (LSE).

In research of the content of pharmacologically active compounds in medicinal plants, the routine procedure of extraction from plant tissues is usually applied. The extraction from plant material is frequently carried out by means of "classic" solvent-based procedures, in Soxhlet apparatus, or more simply, in laboratory flask at the temperature of the solvent's boiling under reflux (de Castro & da Silva, 1997; Saim et al., 1997). The imperfection of the time and solvent-consuming methods consists of poor penetration of the tissues by the solvent and also possible destruction of thermolabile compounds. Advantages of conventional extraction methods result from basic, inexpensive and simple equipment to operate. In the Soxhlet extraction, the sample is repeatedly contacted with fresh portions of the solvent in relatively high temperature and with no filtration required after the leaching step (de Castro & da Silva, 1997; de Castro & Garcia-Ayuso, 1998). Recently, modern alternative extraction methods, applied in the environmental analysis and in phyto-chemistry, are sometimes reported: (1) ultrasonification (USAE) (maceration in ultrasonic bath at various temperatures) (de Castro & Garcia-Ayuso, 1998; Court et al. 1996; Saim et al., 1997); (2) microwave-assisted solvent extraction in closed and open systems (MASE) (de Castro & Garcia-Ayuso, 1998; Saim et al., 1997); (3) accelerated solvent extraction (ASE) (called also PLE, pressurized solvent extraction) (Boselli et al., 2001; de Castro & Garcia-Ayuso, 1998; Ong et al., 2000; Papagiannopoulos et al., 2002; Saim et al., 1997); and (4) supercritical fluid extraction (SFE) (Saim et al., 1997). The above methods give better penetration of solvents into plant tissues or other solid matrices that are rapid and solvent saving. ASE apart from this advantage is dynamic, fast and also enables automatization of extraction and analysis procedures (Waksmundzka-Hajnos et al., 2004; Waksmundzka-Hajnos et al., 2007).

Coumarins are usually isolated from plants by extraction with solvents such as ethanol, methanol, benzene, chloroform, diethyl and petroleum ethers, or their combinations (Lozhkin & Sakanyan, 2006). The most exhaustive extraction of coumarins is achieved with ethanol and its aqueous solutions, either in cold or on heating. The total dense extract obtained after the evaporation of extractant is purified by treatment with chloroform and diethyl or petroleum ethers (Lozhkin & Sakanyan, 2006).

Petroleum ether is the extractant usually used in selective extraction of furanocoumarin fraction from plant tissues (Głowniak, 1988), whereas more polar coumarins—hydroxyderivatives are extracted with methanol. Methanol, used after petroleum ether on the same plant material, extracts more hydrophylic coumarins, but also the residual of furanocoumarins.

Historically, exhaustive extraction with different solvents, which can be performed in Soxhlet apparatus, proved to be the most accurate method of isolation of these groups of compound (Głowniak, 1988; Hadacek et al., 1994). The extraction of the same plant material is usually continued with methanol. For example, peucedanin was successfully isolated using this type of extraction with methanol (Lozhkin & Sakanyan, 2006).

Waksmundzka-Hajnos et al. (2004) compared methods of extraction of furanocoumarins. Some of furanocoumarins from *Pastinaca sativa* fruits were extracted using exhaustive extraction with petroleum ether in Soxhlet apparatus, ultrasonification (USAE), accelerated solvent extraction (ASE) and microwave-assisted solvent extraction (MASE).

USAE was performed with petroleum ether in ultrasonic bath at an ambient temperature of 20˚C or at a temperature of 60˚C for 30 min three times.

In the ASE method, the plant material was mixed with neutral glass and placed into a stainless steel extraction cell. The application of neutral glass, playing the role of dispersion agent, is recommended to reduce the volume of the solvent used for the extraction (ASE 200, 1995). This extraction was performed with pure methanol or petroleum ether at the same pressure (60 bar).

MASE was also used in the isolation of furocoumarin fractions performing with 80% methanol in a water bath using a two-step extraction with results of 40% generator power during 1 min and by 60% generator power during 30 mins in open and closed systems.

In most cases of the Waksmundzka-Hajnos et al. (2004) experiment, exhaustive extraction in Soxhlet apparatus indicates low yields of furanocoumarins. For example, the use of ultrasonification at 60˚C gives, in most cases a higher yield than the exhaustive Soxhlet method. In some cases, this method gives the highest yield of extraction (for xanthotoxin and for isopimpinellin) in comparison to all methods used in experimentation. Also, the use of ASE gives, in most cases, higher yields than the Soxhlet extraction (compare yield of extraction of isopimpinellin, bergapten, imperatorin and phellopterin). In case of bergapten, imperatorin, and phellopterin the yield of extraction by ASE was highest in comparison to all extraction methods used in experiments.

Microwave-assisted solvent extraction gives fair extraction yield for more polar furanocoumarins, probably because of the necessary use of more polar extractant (80% MeOH in water). From the gathered data, it is seen that the extraction yield of phellopterin and imperatorin in pressurized MASE is distinctly lower than in open systems. It shows that in a closed system, the extracted compounds were changed by microwaves. Hence, pressurised MASE cannot be recommended as a leaching method of furanocoumarin fraction (Waksmundzka-Hajnos et al., 2004).

These results are similar to those obtained from the same authors in previous investigations, in which they isolated furanocoumarins from *Archangelica officinalis* fruits. This study indicated the highest yield of psoralens by ASE, using methanol or petroleum ether as the extractant. It was also reported that microwave-assisted solvent extraction in the closed system probably causing the change of analytes (Waksmundzka-Hajnos et al., 2004a).

Soxhlet extraction, ultrasound-assisted extraction and microwaves-assisted extraction in the closed system have been investigated to determine the content of coumarins in flowering tops of *Melilotus officinalis*. Soxhlet extraction was performed in a Soxhlet apparatus equipped with cellulose extraction thimbles. Extraction was performed with ethanol (85˚C). Ultrasound-assisted extraction was conducted with 50% (v/v) aq. ethanol, in an ultrasonic bath, and MASE with 50% (v/v) aq. ethanol was performed using a closed-vessel system (Martino et al., 2006).

Soxhlet extraction was used in the isolation of oxypeucedanin from *Prangos uloptera*. Dried and powdered leaves were extracted with n-hexane, dichloromethane and methanol (Razavi et al., 2010).

Celeghini et al. (2001) studied the extraction conditions for coumarin analysis in hydroalcoholic extracts of *Mikania glomerata* Spreng leaves. Maceration, maceration under sonication, infusion and supercritical fluid extraction (SFE) were compared. In SFE method, the solvent extraction system was pressurized in the high pressure vessel with the aid of a nitrogen cylinder. Several solvent mixtures were used including CO_2:EtOH (95:5), (90:10), (85:15) and CO_2:EtOH:H_2O (95:2.5:2.5). The experiment was conducted at the same pressure and temperature. The evaluation of these methods showed that maceration under sonication had the best results.

Kozyra & Głowniak (2006) examined the influence of using solvent in the isolation of furanocoumarins. They carried out extraction techniques with different eluents such n-heptane, dichloromethane and methanol. These extractions were performed on a water bath with boiling eluent and on an ultrasonic bath, for 12 and 24 hrs. The more efficient for bergapten was extraction with dichloromethane.

In another study, six solvents (n-hexane, chloroform, ethyl acetate, ethanol, acetonitrile and water) were used to extract *Cnidii Fructus* in order to evaluate their efficiency in extracting osthole. A comparative evaluation showed that aqueous alcoholic solvent was the most efficient solvent (100%) (Yu et al., 2002).

The furanocoumarin determination from air-dried plant material was also performed using 75% methanol in an ultrasonic ice–water bath (Yang et al., 2010), with 100% methanol (Cardoso et al., 2000; Ojala, 2000), 70% methanol (Chen & Sheu, 1995), with hot (70˚C) pure methanol on a water bath (Bartnik & Głowniak, 2007), with pure ethanol in heated reflux (Yu et al., 2002), with 95% ethanol at 80˚C (Wang et al., 2007; Zheng et al., 2010), with acetone at room temperature (Taniguchi et al., 2011), with ether at 40˚C (Liu et al., 2004a), with dichloromethane at room temperature (Um et al., 2010), with petroleum ether at room temperature (Tosun et al., 2008), with chloroform in a sonic bath (Cardoso et al., 2000).

The extraction with all solvents was usually done 2–5 times, obtaining solutions that were filtered and evaporated under reduced pressure. Frequently, residuals after methanol/ethanol extractions were suspended in water and portioned a few times with chloroform or petroleum ether (Wang et al., 2007; Zheng et al., 2010).

5.2 Sample purification

The next step in sample preparations is the purification of the crude extract. Plant extracts contain much ballast material, both non-polar (chlorophylls, waxes) and polar such as

tannins or sugars. Most often liquid-liquid extraction (LLE) is used, which takes advantages of solubility differences of hydrophobic substances, which have affinity for non-polar solvents, and hydrophobic substances, which have an affinity for aqueous solutions. Although the analyses can be easily obtained by evaporation of the solvent, the method has many disadvantages – for example emulsions can be formed and the process is time-consuming. Purification can also be achieved by solid-phase-extraction (SPE). This method uses a variety of adsorbents and ion-exchangers and is widely used for a variety of purposes (Fritz & Macha, 2000; Hennion, 1999; Nilsson, 2000; Snyder et al., 1997; Waksmundzka-Hajnos et al., 2007).

The SPE method is very often used in sample pre-treatment for HPLC. This method has been developed for the purification of furanocoumarins from *Peucedanum tautaricum* Bieb. In the first step, aqueous methanol (50%; v/v) solutions of the samples were passed through conditioned microcolumns to adsorb furanocoumarins on the adsorbent bed. The microcolumns were washed with 50% methanol (Zgórka & Głowniak, 1999), and the compounds of interest group were separated from fatty components and chlorophyll by use of SPE microcolumns (LiChrolut RP-18 E; 500 mg, 3 mL). In the next step, the absorbed furanocoumarins were eluted at a flow-rate of 0.5 mL min^{-1} with 80% methanol into vials previously calibrated with a pipette (Bartnik & Głowniak, 2007).

Sidwa-Gorycka et al. (2003) used SPE for purification furanocoumaric fractions obtained from *Ammi majus* L. and *Ruta graveolens* L. methanolic (30%) extracts. They were loaded into octadecyl-SPE microcolumns activated previously with 100% methanol, followed by the selective elution of compounds. The cartridges were washed with 20 ml of 60% methanol to elute the coumarins. The eluting solvents were passed through the sorbent beds at a flow rate of 0.5 ml min^{-1}.

In addition, the SPE has been developed for purification of furanocoumarin fractions from creams and pomades. The obtained samples were cleaned-up using two methods. Each extracted sample was re-dissolved in chloroform and fractionated on cartridges, which were previously conditioned with chloroform and sequentially eluted with chloroform (first fraction), chloroform:methanol (90:10; v/v) (second fraction, furanocoumarins), chloroform:methanol (1:1; v/v) (third fraction) and methanol (fourth fraction). Next, each sample extracted above was re-dissolved in methanol in a sonic bath and fractionated on cartridges, which were previously conditioned with methanol and sequentially eluted with methanol (first fraction), methanol:chloroform (80:20; v/v) (second fraction, furanocoumarins), methanol:chloroform (1:1; v/v) (third fraction) and chloroform (fourth fraction). All fractions were evaporated to dryness in a stream of nitrogen (Cardoso et al., 2000).

5.3 Chromatographic methods in the analysis of furanocoumarins

5.3.1 Column Chromatography (CC)

The good results for purification, separation of the total furanocoumarins and the isolation of individual compounds give column chromatography (CC) a significant advantage of the use of various sorbents and solvent systems.

Furanocoumarins can be fractionated on an aluminum oxide column eluted with petroleum ether, petroleum ether-chloroform (2:1), chloroform, and chloroform-ethanol (9:1; 4:1; 2:1)

mixtures or on silica gel column eluted sequentially with hexane-chloroform and chloroform-ethanol systems with increased proportion of a more hydrophilic component (Lozhkin & Sakanyan, 2006).

Separation of the psoralens from *Heracleum sibiricum* L. (Apiaceae) fruits was performed by gravitation column chromatography. Glass columns were filled with silica gel (230-400 mesh) and run, under UV-lamp control, in the following eluents: 1) benzene-ethyl acetate; mixtures of increasing polarity (12.5 to 77.5%); 2) benzene-ethyl acetate (17:3); 3) benzene-chloroform-ethyl acetate (1:1, v/v, 5%). Chromatographically pure clean compounds, in this study, were crystallized from 96% ethanol (Bogucka-Kocka, 1999).

In another investigation, the coumarin mixture from fruits of *Heracleum crenatifolium* was subjected to CC on silica gel and eluted successively with an n-hexane-ethyl acetate solvent system, with increasing polarity (99:1 to 80:20). The collected fractions were applied to preparative-TLC on silica gel plates and pure furanocoumarins were obtained. After chromatography with the use of n-hexane-ethyl acetate (3:1), isobergapten and pimpinellin were obtained. Fractions, which were chromatographed with n-hexane-dichloromethane-ethyl acetate (4:4:2) resulted in a production of bergapten, and fractions, after chromatography using toluene-ethyl acetate (9:1), resulted in a production of yielded isopimpinellin, sphondin and byak-angelicol (Tosun et al., 2008).

On silica-gel column chromatography was also subjected to chloroform residue from roots of *Angelica dahurica*. The furanocoumarins were eluted stepwise with petroleum ether-acetone mixtures (Wang et al., 2007).

Similar techniques were performed for furanocoumarins from the roots *Angelicae dahuricae*. The methylene chloride soluble was chromatographed using column chromatography over silica gel. In this study, a stepwise gradient solution with hexane-ethyl acetate (5:1 to 0:1) was used. Repeated column chromatography of obtained fractions produced an isoimperatorin, imperatorin, oxypeucedanin, phellotorin, byakangelicol, neobyakangelicol, alloisoimperatorin, pabulenol, byakangelicin, and 9-hydroxy-4-methoxypsoralen (Piao et al., 2004).

A vacuum liquid chromatography on silica gel was developed for the isolation of oxypeucedanin from the leaves of *Prangos uloptera.* Hexane extract was subjected, starting with 100% hexane, followed by step gradient of ethyl acetate mixtures (1:99; 5:95; 10:90; 20:80; 40:60; 60:40; 80:20; 100) and finally methanol. The obtained fractions were purified by preparative-TLC on silica gel using $(CH_3)_2CO$-$CHCl_3$, 5:95 as the mobile phase to yield oxypeucedanine (Razavi et al., 2010).

The CC technique was used to separate furanocoumarins from roots of several *Dorstenia* species. The afforded hexane residues were chromatographed on silica gel (230-400 mesh) eluting with hexane-chloroform mixtures (1:1) (gives psoralen) or with hexane-ethyl acetate mixtures of an increasing polarity to give bergapten, 4-[3-(4,5-Dihydro-5.5-dimethyl-4-oxo-2-furanyl)-butoxy]=7H-furo[3,2-g][1]benzopyran-7-one, psoralen and 7-hydroxycoumarin. The chloroform extracts were eluted using hexane-chloroform mixtures to give psoralen, 7-hydroxycoumarin and 4-[3-(4.5-Dihydro-5.5-dimethyl-4-oxo-2-furanyl)-butoxy]=7H-furo[3.2-g][1]benzopyran-7-one or hexane-ethyl acetate of increasing polarity to give psoralen, psoralen dimer and 7-hydroxycoumarin. The polar fractions from the methanol

extract were acetylated using pyridine and Ac_2O to give (2'S, 1''S)-2.3-dihydro-2(2-acetoxy-1-hydroxymethylethyl)-7H-furo [3.2-g][1]benzopyran-7-one (Rojas-Lima et al., 1999).

Another useful adsorbent for column chromatography is Florisil (100-200 mesh), which was used to fractionate furanocoumarins obtained from fruits of *Peucedanum alsaticum* L. and *P. cervaria* (L.) Lap. Concentrated petroleum ether extracts were fractionated on this sorbent with a dichloromethane-ethyl acetate (0-50%) gradient, then ethyl acetate and methanol as mobile phases. After CC separation, the fractions richest in coumarins were analyzed by preparative-TLC on silica gel. Separated zones of selected furocoumarins were eluted from the plates (Skalicka-Woźniak et al., 2009).

The Florisil was also used in an investigation performed by Suzuki et al. (1979). Bergamot oil was eluted on this column with methylene chloride and ethyl acetate. The ethyl acetate fractions were re-chromatographed with methylene chloride. The obtained residue was analyzed by preparative-TLC on silica gel using cyclohexane-tetrahydrofuran (1:1) as eluent. The bergapten zone was scraped and eluted with acetone.

Isolation of the furanocoumarins from grapefruit juice was accomplished by preparative thin layer chromatography. The obtained fractions were applied to tapered silica gel GF TLC plates with a fluorescent indicator. Resolution of compounds was accomplished by using solvent systems consisting of hexane:ethyl acetate (3:1 to 2:3; v/v), chloroform, chloroform/methanol (95:5), and benzene: acetone (9:1). The zones containing furanocoumarins were scraped and extracted with acetone (Manthey et al., 2006).

5.3.2 Thin Layer Chromatography (TLC)

The physicochemical properties of coumarins depend upon their chemical structure, specifically, the presence and position of functional hydroxy or methoxy groups, and methyl or other alkyl chains. As a result of these differences, group separation of the all groups of coumarins does not cause any difficulties (Jerzmanowska, 1967; Waksmundzka-Hajnos et al., 2006). Separation of individual compounds in each group – structural analogs, i.e. closely related compounds – is, however, a difficult task.

Several analytical methods for the quality control of furanocoumarins in plant materials, such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), high performance liquid chromatography–mass spectrometry (HPLC–MS), high-speed counter-current chromatography (HSCCC), gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), capillary electrophoresis (CE)), and pressurized capillary electrochromatography (pCEC), has been reported (Chen et al., 2009; Wang et et., 2007).

The oldest publications recommended one- or two- thin layer chromatography for separation and identification of furanocoumarins. This method provides a quite rapid separation of components in a sample mixture. Fractions obtained from column chromatography were usually checked with the use of TLC technique.

Several adsorbents have been applied for the chromatographic analysis of furanocoumarins, e.g. silica gel, C18 layers, alumina, poliamide, Florisil, etc. (Cieśla & Waksmundzka-Hajnos, 2009). Analyzed fractions of studied compounds are eluted using several solvent systems. Borkowski (1973) proposed the following eluents: 1) benzene-acetone (90:10, v/v); 2) toluene-acetone (95:5, v/v); 3) benzene-ethyl acetate (9:1, v/v); 4) benzene-ethylic ether-

methanol-chloroform (20:1:1:1, v/v); 5) chloroform, and 6) ethyl acetate-hexane (25:75, v/v) for analysis of coumarins.

The spots of coumarins on thin-layer and paper chromatograms are usually revealed by UV fluorescence at certain characteristic wavelengths, before or after the treatment with an aqueous-ethanol solution of potassium hydroxide or with ammonia vapor, or using some other color reactions. The fluorescent color does not provide accurate identification of the structure of coumarins; nevertheless, sometimes it is possible to determine the type of functional groups (Celeghini et al., 2001; Lozhkin & Sakanyan, 2006).

Joint TLC – colorimetric methods based on the azo-addition reaction with TLC separation on an aluminum oxide layer eluted in the hexane – benzene – methanol (5:4:1) system were developed for the quantitative determination of peucedanin in *Peucedanum morrissonii* (Bess.) and for the analysis of beroxan, pastinacin, and psoralen preparations (Lozhkin & Sakanyan, 2006). Colorimetric determination of xanthotoxin, imperatorin, and bergapten in *Ammi majus* (L.) fruits can be performed after TLC separation on silica gel impregnated with formamide and eluted in dibutyl ether. In order to determine psoralen alone and together with bergapten in *Ficus carica* (L.) leaves, the extract was purified from ballast substances and chromatographed in a thin layer of aluminum oxide in diethyl ether (Lozhkin & Sakanyan, 2006).

Thin layer chromatographic analyses were made by Celeghini and co-authors (2001) on silica gel 60G. As eluent a mixture of toluene:ethyl ether (1:1) saturated with 10% acetic acid was used. The plates were sprayed with an ethanolic solution (5% v/v) of KOH and examined under UV light at 366 nm.

In the other investigation, purification of the eluted furanocoumarins from leaves of *Conium maculatum* was also carried out on TLC plates (Si gel 60, f-254). The chromatograms were developed at room temperature using one of the following solvent systems: chloroform:ethyl acetate (2:1), or chloroform, or toluene:ethyl acetate (1:1) (Al-Barwani & Eltayeb, 2004).

Bogucka-Kocka (1999) analyzed furanocoumarins in fruits of *Heracleum sibiricum* L., after silica gel column chromatography, using 2D-TLC. Two-dimensional thin-layer chromatography was conducted of silica gel plates and run in the following phases: 1) benzene-chloroform-acetonitrile (1:1, v/v, 5%) or 2) benzene-chloroform-ethyl acetate (1:1, v/v, 5%) (first direction); 3) benzene-ethyl acetate (1:1) (second direction). The chromatograms were analyzed using UV light and daylight, after spaying with one of the derivativers: 1) 0.5% I dissolvent in KI; 2) Dragendorf's reagent or 25% $SbCl_5$ in CCl_4.

Unfortunately, in furocoumarin' group, these substances have comparable polarity and similar chemical structures. As a result, multi-dimensional separations are required in such cases.

Thin-layer chromatography gives the possibility of performing multi-dimensional separation – two-dimensional separation with the use of the same stationary phase, with different mobile phases (Gadzikowska et al., 2005; Härmäla et al., 1990; Waksmundzka-Hajnos et al., 2006), or by using a stationary phase gradient (Glensk et al., 2002; Waksmundzka-Hajnos et al., 2006). In TLC, there are almost no limits as far as mobile phases are concerned, because they can be easily evaporated from the layer after the

development in the first dimension. Both methods, use of the same layer and different mobile phases or two different layers developed with two mobile phases, make use of different selectivity to achieve complete separation in the two-dimensional process. The largest differences are obtained with a normalphase system, with an adsorption mechanism of separation, and a reversed-phase system, with a partition mechanism of separation, are applied for two-dimensional separations (Nyiredy, 2001). Two-dimensional thin-layer chromatography with adsorbent gradient is an effective method for the separation of large group of substances present in natural mixtures, e.g. plant extracts.

Silica gel is the most popular adsorbent, thus it has been widely used in different chromatographic methods. However, in case of two-dimensional separations of coumarins, it has been rarely applied as it is difficult to select solvent systems which are complementary in selectivity. Härmälä et al. (1990) proposed a very interesting method for the separation of 16 coumarins from the genus *Angelica* with the use of silica gel as an adsorbent. The application of two-dimensional over-pressured layer chromatography enabled complete resolution of the analyzed substances. The authors described a very useful procedure of choosing complementary systems that can be applied in the analysis of complex mixtures. It turned out that the systems, I direction – 100% $CHCl_3$ and II direction – AcOEt/*n*-hexane (30:70, v/v) provided excellent separation of all coumarins, although having only the fourth poorest correlation value.

Due to the possibility of the application of normal- and reversed – phase systems, polar bonded phases have been often a choice for two-dimensional separations. In the case of coumarins, the use of diol- and cyanopropyl-silica have been reported.

Waksmundzka-Hajnos et al. (2006) reported the use of diol-silica for the separation of 10 furanocoumarin standards. Firstly, the compounds were chromatographed with the use of 100% diisopropyl ether (double development), then in the perpendicular direction: 10% MeOH/H_2O (v/v) containing 1% HCOOH. The use of the first direction eluent caused the separation of analyzed substances into three main groups, which is useful for group separation of natural mixtures of coumarins. Chromatography in reversed-phase system enabled the complete resolution of all tested standards. The disadvantage of the applied reversed-phase system is the fact that it has low efficiency, and most of the substances, especially those containing hydroxyl groups are tailing. Diol-silica is similar in its properties to deactivated silica, thus the application of aqueous eluent may be responsible for tailing, which was only slightly reduced after the addition of formic acid.

Better results were obtained after the application of CN-silica. In this case, coumarin standards were firstly chromatographed with the use of normal-phase, then in reversed-phase system. The plate was triple developed in the first direction to improve separation of strongly retained polar coumarins.

The authors also investigated the use of multiphase plates for identification purposes. Coumarins were firstly chromatographed on a RP-18W strip with 55% MeOH/H_2O (v/v), and then in a perpendicular direction they were triple-developed with: 35% AcOEt/*n*-heptane (v/v). The use of reversed-phase system caused the separation of investigated coumarins into two groups: coumarins containing hydroxyl group, and furanocoumarins. The separation, according to the differences in polarity, is even greater than that observed on diol-silica. This system was then applied for separation of the furanocoumarin fraction

from fruits of *H. sibiricum*, where seven compounds were identified in the extract (Cieśla & Waksmundzka-Hajnos, 2009; Waksmundzka-Hajnos et al., 2006).

The use of graft thin-layer chromatography of coumarins was also reported (Cieśla et al., 2008; Cieśla et al., 2008a; Cieśla et al., 2008b). The authors applied two combinations of adsorbents: silica + RP-18W, and CN-silica + silica gel. In the first stage of this experiment, plates pre-coated with CN-silica were developed in one dimension by unidimensional multiple development. The same mobile phase (35% ethyl acetate in n-heptane) was used, over the same distance, and the same direction of the development. Plates were triple-developed with careful drying of the plate after each run. Unidimensional multiple development (UMD) results in increased resolution of neighboring spots (Poole et al., 1989). After chromatography the plates were linearly scanned at 366 nm with slit dimensions 5 mm × 0.2 mm. This chromatographic system was not suitable for separation of structural analogs. Isopimpinellin and byacangelicol are coeluted and phellopterin and bergapten also have very similar retention behavior. The isopimpinellin and byacangelicol molecules have two medium polarity groups in positions 5 and 8, which have similar physicochemical properties. Therefore, it was also easily noticeable that different non-polar substituents did not cause significant difference in retention behavior. Compounds with polar substituents – hydroxyl groups in simple coumarins are more strongly retained on CN-silica layer in normal-phase systems.

When other systems, for example silica with AcOEt–n-heptane and RP 18W with 55% MeOH in water, were used only partial separation of standards was achieved. This results from the similar structures and physicochemical properties of the compounds. On silica layers only polar aesculetin and umbelliferone are more strongly retained. Phellopterin with a long chain in the 8 position (with a shielding effect on neighboring oxygen) is weakly retained. These differences cause the aforementioned coumarins to be completely separated from other standards. Byacangelicol and umbelliferone, and bergapten, isopimpinellin, and xanthotoxin, with only slight differences in number and position of medium-polarity methoxy groups, are eluted together. More significant resolution of the investigated compounds was obtained on RP-18 plates, eluted with aqueous mobile phases. The differences in number, length, and position of medium-polarity and non-polar substituents cause differences in retention behavior of the analytes. These differences result in good separation of bergapten, xanthotoxin, and phellopterin by reversed-phase systems.

In the next step Cieśla et al. (2008b) investigated the search for orthogonal systems, which would ensure better separation selectivity for the coumarins, was conducted. To achieve this, graft TLC, with two distinct layers, was applied. The authors experimentally chose two pairs of orthogonal TLC systems:

- first dimension, CN-silica with 30% ACN + H_2O (three developments); second dimension, SiO_2 with 35% AcOEt + n-heptane (three developments);
- first dimension, SiO_2 with 35% AcOEt + n-heptane (three developments); second dimension, RP-18 with 55% MeOH + H_2O.

An application of multiple development technique (UMD) in the first dimension results in partly separated spots, which are transferred to the second layer with methanol. Use of methanol causes narrowing of starting bands, similarly to the effect of a preconcentrating zone. The preconcentration is responsible for symmetric and well separated spots being

obtained after development of the plate in the second dimension. This makes the densitometric estimation easier.

In the last step of Cieśla and co-authors (2008b) investigations, the separation of furanocoumarin fractions from *Archangelica officinalis, Heracleum sphondylium,* and *Pastinaca sativa* fruits was performed by the use of grafted plates SiO_2 with RP-18W and CN with SiO_2, with appropriate mobile phases. The identity of the extract components was confirmed by comparing retardation factors and UV spectra with the R_f values and spectra obtained for the standards.

Graft TLC in orthogonal systems characterized by different separation selectivity enables complete separation of structural analogs such as furanocoumarins. The use of two different TLC systems enables complete separation and identification of some furanocoumarins present in extracts obtained from *Archangelica officinalis, Heracleum sphondylium*, and *Pastinaca sativa* fruits (Cieśla et al., 2008b).

The graft-TLC system silica + RP-18W were successfully applied for construction of chromatographic fingerprints of different plants from the *Heracleum* genus.

Two-dimensional chromatography has also been applied for quantitative analysis of furanocoumarins in plant extracts (Cieśla et al., 2008b). In order to obtain reproducible results, all investigated compounds should be completely separated. Graft-TLC with the use of adsorbents silica + RP-18W was proven to be the most suitable for quantitative analysis. Resolution of compounds was insufficient in case of 2D-TLC on one adsorbent (CN-silica), as the standards had to be divided into two separate groups for an accurate estimation of peak surface area.

Quantitative analysis is difficult to perform after two-dimensional chromatographic run, as densitometers are not adjusted to scan two-dimensional chromatograms. This problem may be overcome if small steps between scans are used. In the proposed method, the authors scanned the plate with the slit of a dimension 5 mm×0.2 mm, operated at λ= 366 nm, obtaining 36 tracks that were not overlapping. This wavelength was chosen to get rid of intensive baseline noise, observed at lower wavelengths. Peak areas were measured with the use of the method called "peak approximation" (Cieśla et al., 2008b; Cieśla & Waksmundzka-Hajnos, 2009).

5.3.3 High Performance Liquid Chromatography (HPLC)

Furanocoumarins are also examined by means of high performance liquid chromatography (HPLC). This technique has shown to be a very efficient system for separation of this group of compounds. HPLC methods have been reported for the determination of psoralens in callus cultures, vitro culture, serum, dermis, plants, citrus essential oils, phytomedicines, but only the most recently published methods has reported assay validation (Cardoso et al., 2000; Dugo et al., 2000; Markowski & Czapińska, 1997; Pires et al., 2004).

Linear furanocoumarins, such as psoralen, bergapten, xanthotoxin, and isopimpinellin isolated from three varieties of *Apium graveolens* were examined by normal-phase HPLC equipped with a variable wavelength detector set at 250 nm. The mobile phase consisted of a mixture of ethyl acetate (0.1%) and formic acid (0.1%) in chloroform (Waksmundzka-Hajnos & Sherma, 2011).

In most recent applications, reversed-phase HPLC is used to evaluate furanocoumarins quantitatively.

For example, the quantitative analysis of some furanocoumarins from *Pastinaca sativa* fruits was performed by RP-HPLC in system C18/methanol + water in gradient elution. The authors used the following gradient: 0-10 min, 45% MeOH; 10-20 min, 45-55% MeOH; 20-30 min, 55-70% MeOH, and 30-40 min, 70% MeOH in bidistilled water (Waksmundzka-Hajnos et al., 2004).

The determination of two furocoumarins (bergapten and bergamottin) in bergamot fruits, was carried out by the HPLC system equipped with a diode array detector. C18 column and the mobile phase consisted of methanol and 5% (v/v) acetic acid aqueous solution in the following gradient: 5-20% (0-13 min), 20-100% (13-25 min), 100-5% (20-30 min), were used in this investigation (Giannetti et al., 2010).

The optimized HPLC-UV method was used to evaluate the quality of 21 samples of Radix *Angelica dahurica* from different parts of China. Bergapten, imperatorin and cnidilin were separated on C18 column; the mobile phase was 66:34 (v/v) methanol-water (Wang et al., 2007).

The HPLC technique was ensued for analyses of psoralen and bergapten. HPLC separation of the psoralens was performed using a Shimadzu octadecyl Shim-pack CLC-ODS reversed-phase column with a small pre-column containing the same packing. Elution was carried with acetonitrile-water 55:45 (v/v) and detections of the peaks were recording at 223 nm (Cardoso et al., 2002). The same conditions were used for determination of furanocoumarins in three oral solutions by Pires et al. (2004).

A rapid and sensitive reversed-phase HPLC method has been used for the determination of furanocoumarins in methanolic extracts of *Peucedanum tautaricum* Bieb. Compounds were separated on stainless-steel column packed with 5μm particle Hypersil ODS C18. The mobile phase was methanol-water gradient used following: 0-5 mins, isocratic elution with 60% (v/v) methanol; 5–20 mins, linear gradient from 60 to 80% methanol; 20 to 30 mins, linear gradient from 80 to 60% methanol; 30–40 mins isocratic elution with 60% methanol. An acetonitrile–water mobile phase gradient was also used (0–8 mins, isocratic elution with 50% acetonitrile; 8–25 mins, linear gradient from 50 to 70% acetonitrile; 25–28 mins, linear gradient from 70 to 50% acetonitrile; 28–40 mins isocratic elution with 50% acetonitrile) (Bartnik & Głowniak, 2007).

The mobile phase consisted of water with orthophosphoric acid 1:10000 (solvent A), methanol (solvent B) and acetonitrile (solvent C) was used for analysis of coumarins from *Melilotus officinalis* (L.) Pallas. The starting mixture (80% A, 5% B and 15% C) was modified as follows: within 20 mins the mobile phase composition became 65% A, 20% B, 15% C and was kept constant for 10 mins; in the following 10 mins the mixture composition came back to the initial eluting system (Martino et al., 2006).

The search for better conditions for application of HPLC has led to development of UPLC (Ultra Performance Liquid Chromatography), a relatively new liquid chromatography technique enabling faster analysis, consumption of less solvent and better sensitivity. The UPLC method enables a reduction of analysis time by up to a factor of nine compared with conventional HPLC without loss of quality of the analytical data generated. Another very

important advantage is high column efficiency which increases the possibility of compound identification and results in better quantitative analysis. UPLC is more efficient and therefore has greater resolving power than traditional HPLC (Novakova et al., 2006; Skalicka-Woźniak et al., 2009; Wren & Tchelitcheff, 2006).

The quantitative analysis by UPLC was performed for the furocoumarins in *Peucedanum alsaticum* and *P. cervia* (Skalicka-Woźniak et al., 2009). The optimalization of the RP-UPLC separation of the coumarins was achieved by the use of DryLab. The investigation was performed with an Acquity Ultra Performance LC (Waters, Milford, MA, USA) coupled with a DAD detector. Compounds were separated on a stainless-steel column packed with 1.7 μm BEH C18. Two linear mobile phase gradients from 5 to 100% of acetonitrile with gradient times of 10 and 20 min were used. Detection was at 320 nm.

A paper by Desmortreux et al. (2009) reports separation of furocoumarins of essential oils (lemon residue) by supercritical fluid chromatography (SFE). The authors studied many types of stationary phases and the effects of numerous analytical parameters. Amongst the numerous tested columns, good separation of analyzed furanocoumarins was obtained on a pentafluorophenyl (PFP) phase (Discovery HS F5), based on an aromatic ring substituted by five fluorine atoms. The mobile phase used was CO_2-EtOH 90:10 (v/v). Amongst the standard compounds, bergapten was well separated being eluted after the other furocoumarins in the lemon residue sample. The results obtained in this study show that SFC is a perfectly suited method to investigate the psoralens in essential oil composition, because of the great number of compounds separated in a reduced analysis time, and with a very short time for re-equilibration of the system at the end of the gradient analysis. Because of the absence of water in the mobile phase in SFC, the stationary phase can establish more varied interactions than in HPLC, making the stationary phase choice highly significant.

5.3.4 Hyphenated HPLC techniques

A hyphenated, HPLC-TLC procedure for the separation of couamrins, has been proposed by Hawrył et al. (2000). A mixture of 12 coumarins from *Archangelica officinalis* was completely separated as a result of the different selectivities of the two combined chromatographic techniques, RP-HPLC and NP-TLC. Firstly, the analyzed compounds were separated by means of RP-HPLC. The optimal eluent: 60% MeOH in water was chosen with the use of DryLab program. All HPLC fractions were collected, evaporated and finally developed in normal-phase system, on silica gel, with the use of a solvent mixture: 40% AcOEt (v/v) in dichloromethane/heptane (1:1). All fractions were completely separated. The combination of these methods gave successful results, although both methods, if used separately, failed to give good resolution. This procedure may be useful for micropreparative separation of coumarins (Cieśla & Waksmundzka-Hajnos, 2009).

The liquid chromatography coupled with mass spectrometry (LC-MS) technique is becoming increasingly popular, in particular, the introduction of atmospheric pressure chemical ionization (APCI) has dramatically influenced the possibilities for analyzing poorly ionizable compounds. The use of hyphenated techniques such as LC-MS provides great information about the content and nature of constituents of complex natural matrices prior to fractioning and carrying out biological assays. Moreover, MS presents a great advantage not only in its ability to measure accurate ion masses but also in its use in structure elucidation (Chaudhary et al., 1985; Dugo et al., Waksmundzka-Hajnos & Sherma, 2011).

Coumarins can be detected in both positive and negative ion modes. Whereas, the positive ion mode often generates higher yields, the noise level is lower in the negative ion mode, thus improving the quality of the signals. So, preliminary investigations regarding the polarity used are very important.

The main problem of working with LC-MS of natural products is the choice of the ionisation technique. Particle beam (PB) and thermospray (TSP) interfaces are the most commonly used for natural component analysis. Both of them exhibit many drawbacks, such as the difficulty to optimize ionisation conditions and the lack of sensitivity. Electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) techniques, which operate under atmospheric pressure, seem to be very promising. These ionisations differ in the way they generate ions, but show many similarities: both operate at atmospheric pressure, giving molecular weight information and additional structural information. Many classes of compounds can be analyzed by both APCI and ESI. However, ESI is the technique of choice for polar and higher molecular weight compounds, while APCI is suitable for less polar compounds and of lower molecular weight than ESI (Dugo et al., 2000).

A sensitive, specific and rapid LC–MS method has been developed and validated for the simultaneous determination of xanthotoxin (8-methoxypsoralen), psoralen, isoimpinellin (5,8-dimethoxypsoralen) and bergapten (5-methoxypsoralen) in plasma samples from rats after oral administration of *Radix Glehniae* extract using pimpinellin as an internal standard. A chromatographic separation was performed on a C18 column with a mobile phase composed of 1mmol ammonium acetate and methanol (30:70, v/v). The detection was accomplished by multiple-reaction monitoring (MRM), scanning via electrospray ionization (ESI) source operating in the positive ionization mode. The optimized mass transition ion-pairs (m/z) for quantitation were 217.1/202.1 for xanthotoxin, 187.1/131.1 for psoralen, 247.1/217.0 for isoimpinellin, 217.1/202.1 for bergapten, and 247.1/231.1 for pimpinellin (Yang et al., 2010).

A paper by Zheng et al. (2010) reports the quantitation of eleven coumarins including furocoumarins in *Radix Angelicae dahuricae*. By using this HPLC–ESI-MS/MS method, all coumarins were separated and determined within 10 min. These compounds were detected by ESI ionization method and quantified by multiple-reaction monitoring (MRM). The mass spectral conditions were optimized in both positive- and negative-ion modes, and the positive-ion mode was found to be more sensitive. The all coumarins exhibited their quasi-molecular ions $[M^+H]^+$, $[M^+Na]^+$, $[M^+NH_4]^+$, $[M^+K]^+$ and fragment ions $[M^+H-CO]^+$, $[M^+H-C_5H_9O]^+$, $[M^+H-C_5H_8]^+$, $[M^+H-C_5H_8-CO]^+$, $[M^+H-C_5H_8-CO_2]^+$, $[M^+H-CH_3]^+$.

Yang et al. (2010a) proposed a practical method for the characterization of coumarins, i.e. linear furanocoumarins, in *Radix Glehniae* by LC–MS. They described in details over 40 derivatives of psoralens. First, 10 coumarin standards were studied, and mass spectrometry fragmentation patterns and elution time rules for the coumarins were found. Then, an extract of *Radix Glehniae* was analyzed by the combination of two scan modes, i.e., multiple ion monitoring-information-dependent acquisition-enhanced product ionmode (MIM-IDA-EPI) and precursor scan information-dependent acquisition-enhanced product ionmode (PREC-IDA-EPI) on a hybrid triple quadrupole-linear ion trap mass spectrometer. This study has demonstrated the unprecedented advantage of the combination of these two scan modes. The MIM-IDA-EPI mode is sensitive, and no pre-acquisition of MS/MS spectra of

the parent ion is required due to the same precursor ion and product ion. A PREC-IDA-EPI mode was used to provide information on the parent ions, fragment ions and retention times of specified ions so the molecular weights of unknown coumarins and their glycosides could be identified. The information on the fragment ions from the MIM-IDA-EPI mode could be supplemented, and the retention time could be verified. Therefore, the characterization of trace furanocoumarins has become very easy and accurate by the combined use of the two modes and may play an important role in controlling the quality of medicinal herbs.

A high performance liquid chromatography–diode array detection–electrospray ionization tandem mass spectrometry (HPLC/DAD/ESI-MS^n) method was used for the chromatographic fingerprint analysis and characterization of furocoumarins in the roots of *Angelica dahurica* (Kang et al., 2008). The HPLC fingerprint technique has been considered as a useful method in identification and quality evaluation of herbs and their related finished products in recent years, because the HPLC fingerprint could systematically and comprehensively exhibit the types and quantification of the components in the herbal medicines (Drasar & Moravcova, 2004; Kang et al., 2008; Wang et al., 2007). Kang and co-authors (2008) showed that the samples from different batches had similar HPLC fingerprints, and the method could be applied for the quality control of the roots of *Angelica dahurica*. In addition, they identified a total of 20 furocoumarins by HPLC/DAD/ESI-MS^n technique, and their fragmentation patterns in an electrospray ion trap mass spectrometer were also summarized.

Recently, high-speed counter-current chromatography (HSCCC) equipped with a HPLC system for separation and purification of furanocoumarins from crude extracts of plant materials, was also described.

High-speed counter-current chromatography (HSCCC), which was first invented by Y. Ito (1981), is a kind of liquid–liquid partition chromatography. The stationary phase of this method is also a liquid. It is retained in the separation column by centrifugal force. Because no solid support is used in the separation column, HSCCC successfully eliminates irreversible adsorption loss of samples onto the solid support used in conventional chromatographic columns (Ito, 1986). As an advanced separation technique, it offers various advantages including high sample recovery, high-purity of fractions, and high-loading capacity (Ma et al., 1994). In the past 30 years, HSCCC has made great progress in the preparation of various reference standards for pharmacological studies and good manufacturing practice, such as coumarins, alkaloids, flavonoids, hydroxyanthraquiones (Liu et al., 2004b).

Liu and co-authors (2004b) isolated and purified psoralen and isopsoralen from *Psoralea corylifolia* using HSCCC technique. In their investigation, they utilized TBE-300A HSCCC instrument with three multilayer coil separation column connected in series. The two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water was used for HSCCC separation. Each solvent was added to a separatory funnel and roughly equilibrated at room temperature. The upper phase (stationary phase) and the lower phase (mobile phase) of the two-phase solvent system were pumped into the column with the volume ratio of 60:40. When the column was totally filled with the two phases, the lower phase was pumped, and at the same time, the HSCCC apparatus was run at a revolution speed of 900 rmp. After

hydrodynamic equilibrium was reached, the sample solution containing the crude extract was injected into the separation coil tube through the injection valve. Each peak fraction was collected according to the chromatogram and evaporated under reduced pressure. The results of HSCCC tests indicated that n-hexane-ethyl acetate-methanol-water (5:5:4.5:5.5, v/v) was the best solvent system for the separation of psoralen and isopsoralen (Liu et al., 2004b).

In another investigations, the same authors had used the same HSCCC technique to induce preparative isolation and purification of furanocoumarins from *Angelica dahurica* (Fisch. ex Hoffm) Benth, et Hook. f (Liu et al., 2004) and from *Cnidium monnieri* (L.) Cusson (Liu et al., 2004a). The results of the first study (Liu et al., 2004) indicated that the best separation of imperatorin, oxypeucedanin and isoimperatorin was when the lower phase of n-hexane-methanol-water (5:5:5, v/v) and n-hexane-methanol-water (5:7:3, v/v) were used in gradient elution. The following gradient was used: 0-150 min, only the lower phase of n-hexane-methanol-water (5:5:5, v/v); 150-300 min, the volume ratio of the lower phase of n-hexane-methanol-water (5:7:3, v/v) changed from 0 to 100%.

A HSCCC method for separation and purification of psoralens from *C. monnieri* was developed by using with a pair of two-phase solvent system composed of light petroleum-ethyl acetate-methanol-water at volume ratios of 5:5:5:5, 5:5:6:4 and 5:5:6.5:3.5 (Liu et al., 2004a).

In described cases, the crude extracts obtained by HSCCC technique were analyzed by HPLC method. The column used was a reversed-phase symmetry C18 column. The mobile phase was methanol-water (68:32, v/v) (analysis of extract from *A. dahurica*), methanol-water (40:60, v/v) (analysis of extract from *P. corylifolia*), or methanol-acetonitrile-water system in gradient mode as follows: 30:30:40 to 50:30:20 in 30 min (analysis of extract from *C.monnieri*) (Liu et al., 2004, 2004a, 2004b). Bergapten and imperatorin obtained by HSCCC method from *Cnidium monnieri* were also analyzed by high-performance liquid chromatography. The column used was a reversed-phase symmetry C18 column, and the mobile phase adopted was methanol (solvent A) – water (solvent B) in the gradient mode as follows: 0-5 min, 60% A; 5-14 min, 60-80% A; 14-15 min, 80-60% A (Li & Chen, 2004).

5.3.5 Capillary electrophoresis

In some cases, capillary electrophoresis was chosen to determine quantities of furanocoumarins. For example Ochocka et al. (1995) used this method for separating psoralens from roots and aerial parts of *Chrysanthemum segetum* L. The analyses were performed with electrophoresis apparatus with UV detection at 280 nm. The best overall separation was obtained on uncoated silica capillary with 7-s pneumatic injection using a buffer solution of 0.2 M boric acid-0.05 M of borax in water (11:9, v/v) (pH 8.5). In another example, micellar electrokinetic capillary chromatography (MEKC) was used in the separation of coumarins contained in *Angelicae Tuhou Radix* (Chen & Sheu, 1995). In this investigation, the electrolyte was buffer solution [20 mM sodium dodecyl sulfate (SDS) – 15 mM sodium borate – 15 mM sodium dihydrogenphosphate (pH 8.26)] – acetonitrile (24:1).

The pressurized capillary electrochromatography (pCEC) was utilized for the separation and determination of coumarins in *Fructus cnidii* extracts from 12 different regions (Chen et al., 2009). Capillary electrochromatography (CEC), as a novel microcolumn separation

technology, couples the high efficiency of capillary electrophoresis with high selectivity of HPLC. The CEC analytes separation is usually achieved in capillaries containing packed stationary phases by an electroosmotic flow (EOF) generated by a high electric field. The experiments were performed in an in-house packed column with a monolithic outlet frit under the optimal conditions: pH 4.0 ammonium acetate buffer at 10 mM containing 50% acetonitrile at −6 kV applied voltage. This analytical method, with use of the novel column, gives good results in the determination of coumarins.

5.3.6 Gas chromatography

In the recent decade, tasks related to the isolation of furocoumarins and the quality control of related preparations were most frequently solved using GC techniques. Gas chromatography was predominantly used for the identification and quantitative analysis of furocoumarins in preparations and raw plant materials. Investigations of the chromatographic behavior (retention times) of substituted furocoumarins revealed the following general laws: 1) on passage from hydroxy- to methoxycoumarins, the retention time decreases (because of reduced adsorption via hydrogen bonds); 2) furocoumarins with O-alkyl substituents at C5 are eluted after 8-hydroxy isomers; 3) the logarithm of the relative retention time is a linear function of the molecular weight. This GC data can be used for determining the structure and estimating the retention time of analogous coumarins (Lozhkin & Sakanyan, 2006). A number of methods have been described for the analysis of furanocoumarins using capillary gas chromatography (GC) (Beier et al., 1994; Wawrzynowicz & Waksmundzka-Hajnos, 1990).

Gas chromatographic method was used to determine osthole content in *Cnidii Fructus* extract. The analytical conditions are the following: nitrogen as the carrier gas, the flow rate of 40 mL/min; the split ratio of 120:1. The column used was DBTM-5 (30 m × 0.53 mm I.D., 1.5 μm) equipped with a flame ionization detector (FID). The initial oven temperature was programmed to be at 135°C for 12 minutes. The temperature was then raised to 215°C at a rate of 12°C/min for 20 minutes. Caffeine anhydrous was used as the internal standard (Yu et al., 2002).

In another example, GC-FID was used to analyze of psoralen, bergapten, pimpinellin and isopimpinellin present in phytomedicines (creams and pomades) employed in the treatment of vitiligo in Brazil. The GC-FID assay method present here is rapid, sensitive and robust and can be applied to the determination of furanocoumarins in routine analysis of creams, pomades and other lipophilic phytocosmetics. These analyses were performed in a VARIAN 3400 gas chromatograph equipped with a capillary fused silica LM-5 and with a flame ionization detector (FID). H_2 was used as carrier gas at a flow rate 0.8 ml min^{-1} and the injection split ratio was 1:20. The injection temperature was 280°C. Column temperature was programmed from 150 to 240°C with a linear increase of 10°C min^{-1}, then 240–280°C with a linear increase of 5°C min^{-1} and was then held for 15 mins. The detector temperature was 280°C (Cardoso et al., 2000).

5.4 Structural analysis

For the structural identification and characterizing of the psoralen compounds, especially if they are novel, instrumental techniques such as nuclear magnetic resonance (NMR)

spectroscopy and infrared spectroscopy (IR) are used. NMR spectroscopy is an invaluable technique for the structural determination of all furanocoumarins. As well as providing information on the chemical environment of each proton or carbon nucleus in the molecule, the technique can be employed to determine linkages amongst nearby nuclei, often enabling a complete structure to be assembled (Rice-Evans & Packer, 2003).

Dimethyl sulfoxide (DMSO-d_6) and methanol (CD_3OD) are both suitable solvents for furanocoumarins.

The reader is referred to Rojas-Lima et al., 1999; Um et al., 2010; Taniguchi et al., 2011, and Tesso et al., 2005 publications, for details of the principles of NMR and general interpretation of NMR spectra.

6. Conclusions

As furanocoumarins have a lactone structure, they have a wide range of biological activity. Bergapten and the other furanocoumarins are used to treat dermatological diseases (psoriaris, vitiligo). As a result, their photosensitizing properties are playing an important role (Bhatnagar et al., 2007; Trott et al., 2008). Their ability to covalently modify nucleic acids is used in process called "extracorporeal photopheresis" that is medically necessary for either of the following clinical indications: erythrodermic variants of cutaneous T-cell lymphoma (e.g. mycosis fungioides, Sezary's syndrome) or chronic graft-versus-host disease, refractory to standard immunosuppressive therapy (Hotlick et al., 2008; Lee et al., 2007).

The aim of the present chapter was to present an overview of techniques of isolation, separations and identification of furanocoumarins in plant materials. Various analytical approaches exist for detection of coumarins and the analytical techniques should meet the following prerequisites: short time, relatively inexpensive, highly accurate, and precise for a variety of applications. This review may be helpful in the choice of the method of furanocoumarin compounds analysis.

7. References

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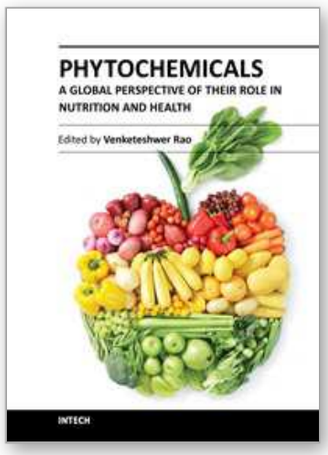

Phytochemicals - A Global Perspective of Their Role in Nutrition and Health

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Phytochemicals are biologically active compounds present in plants used for food and medicine. A great deal of interest has been generated recently in the isolation, characterization and biological activity of these phytochemicals. This book is in response to the need for more current and global scope of phytochemicals. It contains chapters written by internationally recognized authors. The topics covered in the book range from their occurrence, chemical and physical characteristics, analytical procedures, biological activity, safety and industrial applications. The book has been planned to meet the needs of the researchers, health professionals, government regulatory agencies and industries. This book will serve as a standard reference book in this important and fast growing area of phytochemicals, human nutrition and health.

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