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Pharmacobotanical Analysis and Regulatory Qualification of *Capsicum* Fruits and *Capsicum* Extracts — A Survey

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Additional information is available at the end of the chapter

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1. Introduction

Capsicum fruits contain *coloring pigments*, *pungent principles*, resin, protein, cellulose, pentosans, mineral elements and a small amount of *volatile oil*, while seeds contain *fixed (non-volatile) oil*. Besides these organic constituents *Capsicum* fruits also contain inorganic constituents, mostly potassium and sodium, calcium, phosphorus, iron, copper and manganese.

Paprikas derive their color in the ripe state mainly from *carotenoid pigments*, which range from bright red (capsanthin, capsorubin) to yellow (cucurbitene). The *pungent principles* capsaicin and its structurally closely related homologs (so-called *capsaicinoids*) and analogues, are contained only in small amounts, as low as 0.001 to 0.005% in "mild" and 0.1% in "hot" cultivars. The characteristic aroma and flavor of the fresh fruit is imparted by the *volatile oil* containing a range of alkylmethoxypyrazines and a structurally diverse group (alcohols, aldehydes, ketones, carboxylic acids, and esters of carboxylic acids) of oxygenated hydrocarbons. Apart from capsaicinoids, the taste of paprika is mostly due to the *fixed oil* which is comprised mainly of triglycerides of which linoleic, linolic, stearinic and other unsaturated fatty acids predominate.

The first part of the chapter summarizes the chemical characterization of the three classes of *Capsicum* ingredients (*coloring pigments*, *pungent principles*, and *volatile oils*), the most important analytical methods used in pharmacobotanical studies or acknowledged by different regulatory bodies.

Capsicum extracts are frequently used as active pharmaceutical ingredients (API) in manufacturing pharmaceutical products. For such applications *Capsicum* extract should be qualified not only on the basis of the amount of API(s) but on the basis of the amounts of possible impurities/contaminants as well. Among the most important impurities/contaminants (a) *toxic metals*, (b) *pesticides*, (c) *mycotoxins*, (d) *foreign organic matters,* and (e) *radioactivity* (if there is cause for concern) should be mentioned.

The second part of the chapter describes the Pharmacopoeial and other internationally recognized methods for determination of pesticides in *Capsicum* fruits and extracts.

2. Structure and biosynthetic pathways of the most important ingredients of *capsicum* fruits

Capsicum is a versatile plant used as vegetable, pungent food additive, colorant and raw material for pharmaceutical products. The genus *Capsicum*, which is commonly known as "chili", "red chili", "tabasco", "paprika", "cayenne", etc., is a member of the family *Solanaceae*, and closely related to eggplant, potato, petunia, tomato and tobacco. After much work by taxonomists concerning the classification of the presently domesticated species, they have been considered to belong to one of five species, namely *Capsicum annuum*, *Capsicum frutescens*, *Capsicum baccatum*, *Capsicum chinense* and *Capsicum pubescens* (Bosland, 1994).

Capsicum types are usually classified by fruit characteristics, i.e. *pungency*, *color*, *fruit shape*, as well as by their use. *Capsicum* species are commonly divided into two groups, *pungent* and *non-pungent*, also called hot and sweet.

The word "paprika" was borrowed from Hungarian (paprika). It entered a great number of languages, in many cases probably via German. In the end, also "paprika" is derived from a name of black pepper, in this case Serbian pepper. In most languages, "paprika" denotes the dried spice only, though in some (e.g., German) it is commonly used for the vegetable bell pepper.

Capsicum fruits contain *coloring pigments*, *pungent principles*, resin, protein, cellulose, pentosans, mineral elements and a small amount of *volatile oil*, while seeds contain *fixed* (non-volatile) *oil*. Besides these organic constituents *Capsicum* fruits also contain inorganic constituents, mostly potassium and sodium, calcium, phosphorus, iron, copper and manganese (Thresh, 1846; Brawer and Schoen, 1962; Brash et al., 1988; Pruthi, 2003).

The pungent principles capsaicin and its structurally closely related homologs (so-called *capsaicinoids*) and analogues, are contained only in small amounts, as low as 0.001 to 0.005% in "mild" and 0.1% in "hot" cultivars. Apart from capsaicin, the taste of paprika is mostly due to the *fixed oil* which is comprised mainly of triglycerides of which linoleic, linolic, stearinic and other unsaturated fatty acids predominate. The fixed oil content of the *Capsicum* seeds also play an important role in the visual sensing of the paprika powder since it can dissolve and homogeneously distribute the colored substances during grinding of the dried fruits. The characteristic aroma and flavor of the fresh fruit is imparted by the volatile oil containing a

range of alkylmethoxypyrazines (e.g., 2-methoxy-3-isobutylpyrazine, the "earthy" flavor) and a structurally diverse group (alcohols, aldehydes, ketones, carboxylic acids, and esters of carboxylic acids) of oxygenated hydrocarbons.

Furthermore, the fresh ripe paprika contains sizable amounts (0.1%) of vitamin C (ascorbic acid). It was the Hungarian biochemist Albert Szent-Györgyi who discovered that the Hungarian paprika is a rich source of vitamin C. Later (1937) he won the Nobel Prize "for his discoveries in connection with the biological combustion processes, with special reference to vitamin C and the catalysis of fumaric acid" (Encyclopedia Britannica).

Paprikas derive their color in the ripe state mainly from carotenoid pigments, which range from bright red (capsanthin, capsorubin) to yellow (cucurbitene); the total carotenoid content in dried paprika is 0.1 to 0.5%.

2.1. Volatiles

The characteristic flavor and aroma of the fresh fruits is due to their volatile oil content. The fruits of *Capsicum* species have relative low volatile oil content, ranging from about 0.1% to 2.6% in paprika. The total volatiles are generally isolated by steam distillation. In the case of heat-sensitive compounds present, vacuum distillation-continuous solvent extraction can be used. The pure volatile oil and concentrated extracts were analyzed by GC-MS methods. Most compounds of odor significance have been tentatively identified by their mass spectra, and the identification was confirmed by checking the retention time and mass spectra of authentic reference compounds. When Buttery et al. (1969a,b) identified 3-isobutyl-2-methoxypyrazine (1) (Fig 1) as a characteristic aroma compound, the alkylmethoxypyrazines aroused great interest among flavor chemists. The alkylmethoxypyrazines have been shown to be widely distributed in vegetables and with a greenish sweet smell that possibly plays a significant role in the aroma of salad vegetables (Murray, and Whitfield, 1975).

Volatiles have been identified in fresh, homogenized, cooked and stir-fried bell peppers and the effects of ripening and tissue disruption on the composition of volatiles have been determined (Wu and Liou, 1986; Whitfield and Last, 1991; Cremer and Eichner, 2000). About 60 volatile compounds have been identified in green California bell pepper using a vacuum Liken-Nickerson (Buttery et al., 1969a,b). Luning et al. (1994a,b) have identified 64 volatile compounds in fresh bell pepper at three ripening stages (green, turning, red) with dynamic headspace. The composition of volatile compounds indicated that the majority of green related odour volatile compounds decreased or even disappeared during maturation and fruity and sweet odour were higher at the turning and red stages.

There are common aroma compounds amongst the different species of the different fresh pepper, namely, 2,3-butanedione (caramel), 1-penten-3-one (pungent/spicy), hexanal (2) (grassy, herbal), 3-carene (red bell pepper, rubbery), *trans*-beta-ocimene (rancid), octanal (fruity), *trans*-2-hexenal (3) (sweet) and 2-isobutyl-3-methoxypyrazine (1) (green bell pepper). Keller et al. (1981) reported that volatiles of fresh red Jalapeno pepper extracts had a pleasant floral aroma (3-carene). Likewise, *trans*-2-hexenal (3) and *trans*-2-hexenol (4), which have an almond, fruity and spicy odour, were found to increase during maturation.

Study by Chitwood et al. (1983) suggested that *trans*-3-hexenol, 2-*sec*-butyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine are responsible for the frequent use of green descriptors in the aroma descriptive analysis of three *C. annuum* cultivar (Anaheim, Jalapeno and Fresno). Based on GC-MS and sensory analysis of volatiles of three cultivars of *C. annuum* – *Jalapeno*, *Fresno*, and *Anaheim* – twelve odor significant compounds were identified that had been found in one or the other of the earlier studies: 2,3-butadienone (caramel), 1-penten-3-one (pungent/ spicy), hexanal (grassy, herbal), 3-carene (red bell pepper, rubbery), *trans*-3-hexenol (4), *trans*-3-hexenyl isopentanoate (6) (associated with green and green-fruity odors); 3-isobutyl- (1) and 3-*sec*-butyl-2-methoxypyrazine (7) (with green vegetable and green bell capsicum odors); *beta*-ionone (5) (only in Jalapeno), linalool (both with floral character); and the aromatic compounds benzaldehyde (8) and methyl salicylate (9) (with sweetish, penetrating odors) (Chitwood et al., 1983).

The analysis of volatile compounds has been a challenge to many researchers. Many different analytical methods have been developed to determine fresh and processed chilli flavour, such as solvent extraction-simultaneous distillation extraction (Chitwood et al., 1983; Wu and Liou, 1986; Korany et al., 2002) and dynamic headspace (Luning et al., 1994a,b). However, these methods are time-consuming, expensive and likely to introduce artifact resulting from sample preparation and solvent interaction steps. Moreover they cannot represent the total composi- tion of volatile chemicals in equilibrium as found in the aroma of intact, fresh chilli. Solid phase microextraction (SPME) is a method that approaches the ideal extraction method and has been applied to the analysis of various aroma and flavour compound in samples (Peppard and Yang, 1994; Penton, 1996; Steffen and Pawliszyn, 1996; Ibanez et al., 1998; Sides et al., 2000). SPME has been recommended for the quantitative analysis of flavour and fragrance compounds (Zhang and Pawliszyn, 1993). The main advantages of SPME are simplicity, speed, solvent- free, high sensitivity, small sample volume, lower cost and simple automation (Kataoka et al., 2000).

2.2. Coloring pigments

The coloring pigments of red peppers are comprised of *carotenoids*. The term "carotenoid" encompasses not only the carotenes (C_{40} hydrocarbons), but also their oxygenated derivatives, the xanthophylls. Carotenoids are widely distributed groups of natural pigments, responsible for the yellow, orange, and red colors of fruits, roots, flowers, fish, invertebrates, and birds. Only bacteria, algae, fungi, and green plants can synthesize carotenoids, but humans incor- porate them from the diet. Especially bacterial carotenoids are most diverse. Carotenoid extracts and fruits rich in carotenoids are now being used in the food industry to color foods, thus such foods are also representing carotenoid sources of the human diet. The nutritional importance of carotenoids is mostly associated with the provitamin A activity of *beta*-carotene and others. Besides its well-established provitamin A activity, research is under way to study the relationship between *beta*-carotene intake and occurrence of atherosclerosis, cardiovascular diseases, in particular degree of LDL oxidation (Poppel and von Goldbochm, 1995; Hinds et al., 1997; Rodriguez-Amaya, 1997; Woodall et al., 1997; Maillard et al., 1998; Manirakiza et al., 1999, 2003).
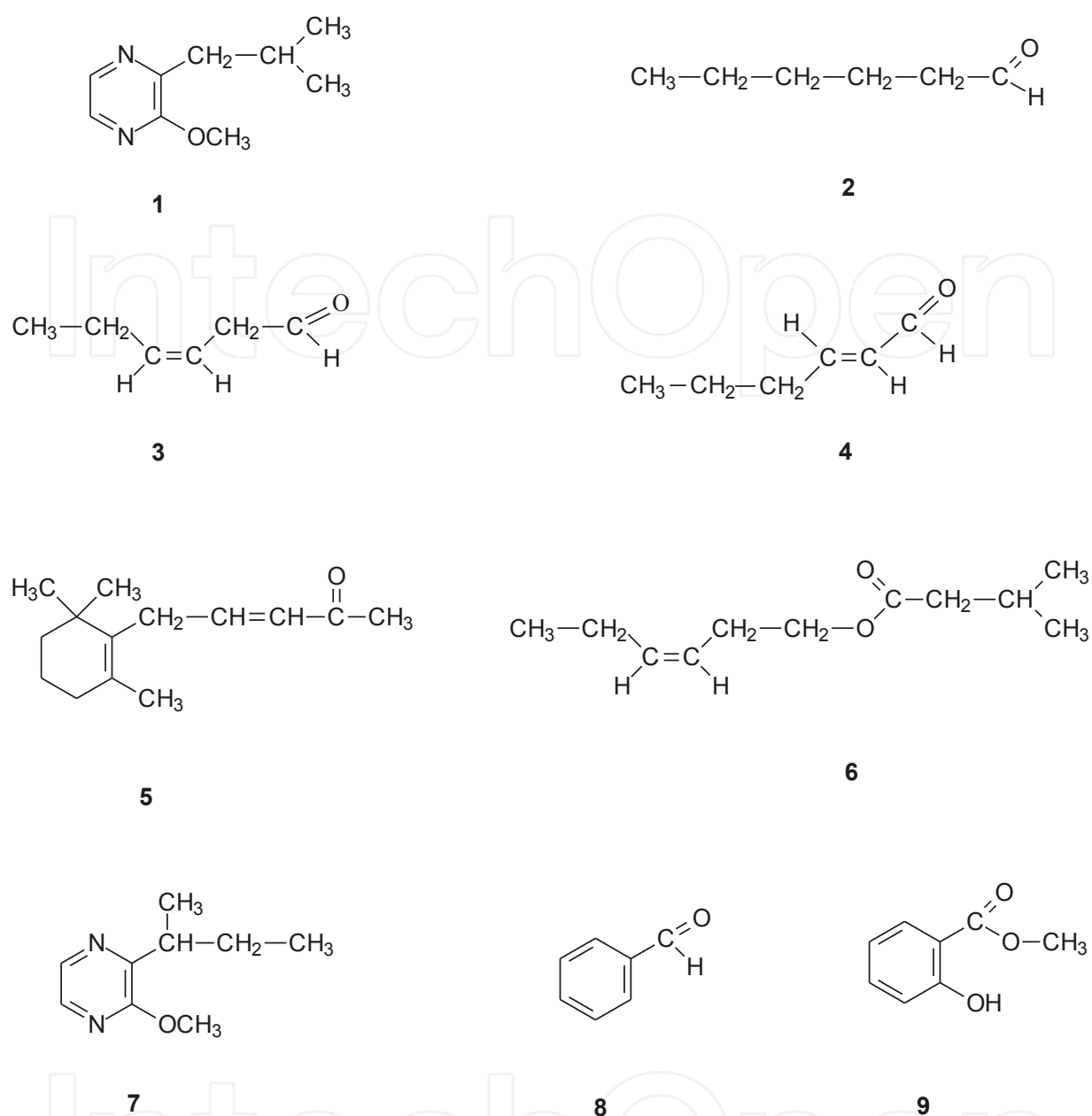
Figure 1. Main components of volatile oils of *Capsicum* furits.

The basic carotenoid structure is a symmetrical, linear, 40-carbon tetraterpene built from eight carbon isoprenoid units joined in such a way that the order is reversed at the center. Fig 2 shows the structure of *beta*-carotene (10), one of the most typical carotenoid components of *Capsicum* fruits.

Carotenoids were for a long time assumed being synthesized by the mevalonate pathway for isoprenoid biosynthesis. This view was prevalent up until the mid-nineties when it was discovered that the carotenoid precursor isopentenyl-pyrophosphate (IPP) was synthetized by two independent metabolic pathways in plants (Lichtenthaler et al., 1997).
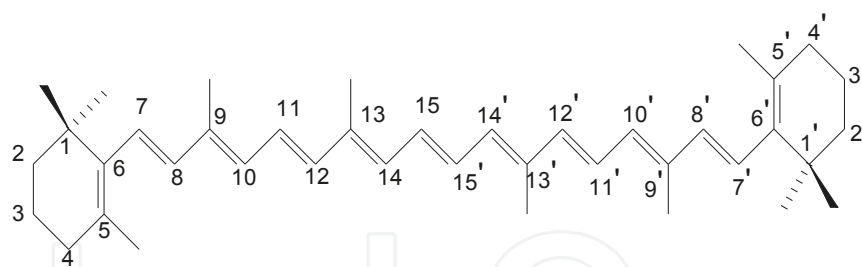
Figure 2. Structure of *beta*-carotene (10)

The first pathway occurs in the cytoplasmic compartment from mevalonic acid and gives rise to compounds such as sterols and cytokinins. In fungi, carotenoids are derived via the mevalonate biosynthetic pathway. The second pathway, effective in bacteria and plastids of plants, is responsible for biosynthesis of gibberellins, carotenoids, abscisic acid, and also contributes to the biosynthesis of tocopherols as well as chlorophyll A and B. This plastidial pathway of isoprenoid synthesis is named after its first metabolite 1-deoxyxylulose 5-phosphate (DOXP) and has pyruvate and glycerinaldehyde-3-phosphate as precursors. Further details can be obtained from a number of review articles, (Davies, 1980; Spurgeon, and Porter, 1983; Britton, 1991).

Carotenoids can be extracted from natural sources by lipid solvents. With fresh material, ethanol or acetone act both as dehydrating agent and extracting solvents. When lipids and esterified xantophylls are present (hydroxylated carotenoids generally occur as esters of fatty acids), the extracts are saponified and the free carotenoids extracted for analysis. Most carotenoids are unstable in oxygen atmosphere and light thus careful extractions and separations are generally carried out under inert atmosphere, subdued light and low temperature (Rodriguez-Amaya, 1997; Deli and Molnár, 2002). High performance liquid chromatography (HPLC) is the most powerful chromatographic technique to separate and – coupled with mass spectrometry (MS) – identify carotenoids. Recent reviews on the field provide up-to-date summary of carotenoid analysis (Rodriguez-Amaya, 1997; Wall and Bosland, 1998; Deli and Molnár, 2002; Felt et al., 2005).

The composition of carotenoid pigments produced by paprika has been investigated in detail. Some twenty carotenoids have been isolated so far with *capsanthin* (11) and *capsorubin* (12) (Figure 3) representing the most abundant (Deli and Molnár, 2002). The ripening process is marked by the disappearance of chlorophyll and a rapid rise in the colored carotenoids (Rodriguez-Amaya, 1997; Rahman, and Buckle, 1980; Hornero-Mendez et al., 2000; Gnayfeed et al., 2001; Deli and Molnár, 2002). A small number of cultivars do not produce significant amounts of carotenoids; when chlorophyll levels decrease in the last stages of ripening, these chilies develop a pale hue often referred to as "white". Due to small amounts of chlorophyll and/or yellow carotenoids, the "white" is, however, more precisely described as a pale greenish-yellow.

Some varieties of paprika contain pigments of anthocyanin type and develop dark purple, aubergine-coloured or almost black pods; in the last stage of ripening, however, the antho-

cyanins get decomposed, and the unusual darkness thus gives way to normal orange or red colors. The same anthocyanins cause the dark spots which are sometimes seen on unripe fruits or particularly the stems of paprika plants and which almost all paprika varieties can develop. In other *Capsicum* species, anthocyanin production is a rare phenomenon.
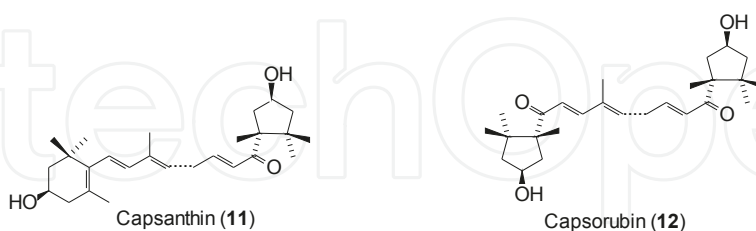

Figure 3. Structure of capsanthin (11) and capsorubin (12).

2.3. Capsaicinoids

Capsicum fruits have been valued for over a thousand of years for the piquant taste they added to the flavorless foods, as well as for the therapeutic effects as a stimulant and counter irritant. These effects have been related to the components stimulating pungency.

The degree of pungency (heat or bite) is determined by the amount of compounds called capsaicinoids. These substances produce the characteristic sensations associated with ingestion of spicy cuisine as well as the agents responsible for causing severe irritation, inflammation, erythema, and transient hypo-and hyperalgesia at sites exposed to paprika extracts. Capsaicinoids are particulary irritating to the eyes, skin, nose, tongue and respiratory tract.

The nature of the causal components in the spice has been established as a mixture of acid amides of vanillylamine and C_8 to C_{13} fatty acids, which are known generally as *capsaicinoids*. The major capsaicinoids in red peppers are *capsaicin* (13), *dihydrocapsaicin* (17) and *nordihydrocapsaicin* (16) (Table 1). In commercial *Capsicums*, *capsaicin* generally comprises 33-59%, *dihydrocapsaicin* accounts for 30-51%, *nordihidrocapsaicin* is 7-15% and the remainder is less than 5% of the capsaicinoids (Reineccius, 1994).

The seven homologous branched-chain alkyl vanillylamides are *capsaicin* (13), *homocapsaicin I* (14), *homocapsaicin II* (15), *nordihydrocapsaicin* (16), *dihydrocapsaicin* (17), *homodihydrocapsaicin I* (18) and *homodihydrocapsaicin II* (19). (Hoffman et al., 1983; Reilly et al., 2001a, Karnka et al., 2002). In addition, three straight-chain analogs, octanoyl vanillylamide (20), nonoyl vanillylamide (nonivamide) (21) and decyl vanillylamide (22), have also been shown to occur in *Capsicum* fruits (Govindarajan, 1986d).

Each capsaicinoid possesses a 3-hydroxy-4-methoxybenzylamide (vanilloid) pharmacophore, but differ from *capsaicin* from their hydrophobic alkyl side chain. Differences in the side chain moiety include saturation of the carbon-carbon double bond, deletion of a methyl group, and changes in the length of the hydrocarbon chain. The structures of capsaicin as well as its homologs and analogs are given in Table 1.

Name	Structure
capsaicin (13)	
homocapsaicin I (14)	
homocapsaicin II (15)	
nordihydrocapsaicin (16)	
dihydrocapsaicin (17)	
homodihydrocapsaicin I (18)	
homodihydrocapsaicin II (19)	
octanoyl vanillylamide (20)	
nonoyl vanillylamide (21)	
decyl vanillylamide (22)	

Table 1. Chemical structures of capsaicin homologs and analogs.

Capsaicin and its natural homologs are always found in the *trans* (*E*) form because in the *cis* (*Z*) isomer, the -CH(CH$_3$)$_2$ and the longer chain on the other side of the Δ6,7 carbon-carbon double bond will be too close to each other which causes strong repulsive force. This steric hindrance does not exist in the *trans* isomer, so the (*E*) form is a more stable arrangement than (*Z*) form.
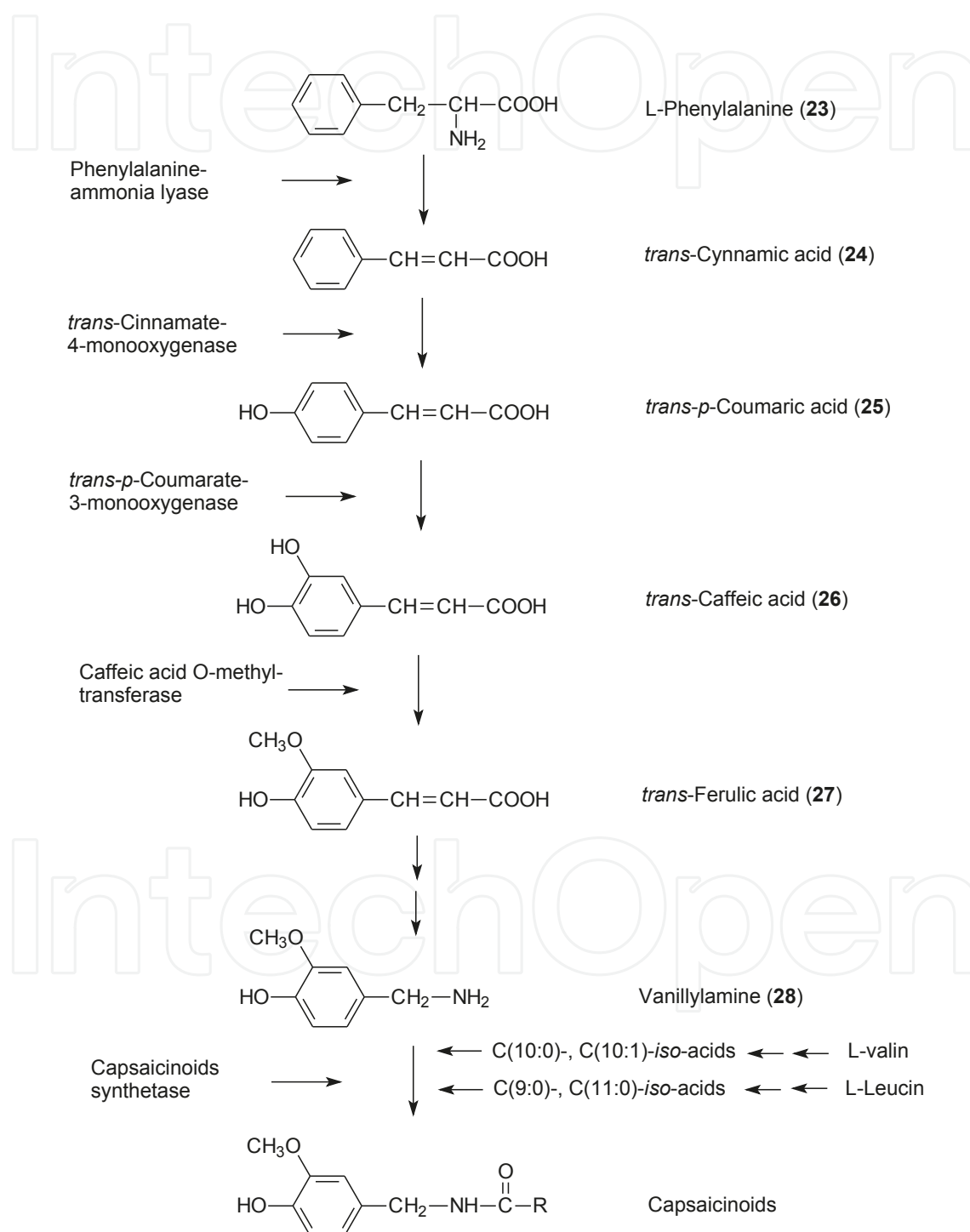

Figure 4. Simplifies biochemical pathway of synthesis of capsaicinoids.

The capsaicin biosynthetic pathway has two distinct branches, one of which utilizes L-phenylalanine (23) as the precursor of aromatic residue of capsaicinoids, presumably via *trans*-cinnamic acid (24) and its hydroxylated derivatives *trans*-caffeic acid (26) and *trans*-ferulic acid (27) following the well-established pathways in other plants (Ishikawa, 2003). Vanillylamine (28) as precursor showed a high level of incorporation into the capsaicinoids and possibly the immediate progenitor of natural capsaicinoids (Figure 4). The enzymes involved in the formation of the precursors, phenolics, and fatty acids, are similar to those studied for long in other biological systems. The second group of enzymes form the branched-chained fatty acids by elongation of deaminated valine (Figure 4). The *capsaicinoids synthetase*, however, has been found to have narrow specificity in accepting only the *iso*-C(9:0) to C(11:0) fatty acids and in the fruit system forming predominantly the vanillylamides of even-number branched fatty acids, capsaicin (13) and dihydrocapsaicin (17), in all the cultivated varieties of the *Capsicum* species (Ravishankar et al., 2003). In the isolated systems, however, the synthethase favors formation of capsaicin (13) and nordihydrocapsaicin (16), while the light induced activation of the synthase in *Capsicum annuum* cv. *grossum*, results in higher formation of nordihydrocapsaicin (16) and dihydrocapsaicin (17) (Govindarajan, 1986a,b). Synthesis of capsaicinoids by means of recent development of biotechnological methods has been reviewed in deatails (Ravishankar et al., 2003).

3. Quality control

Capsicum is now one of the two most widely used spices. Quality of food should fulfill the consumers' expectations – not necessarily the maximum of each attribute, but the optimal level and combination appropriate for each food. Thus a range of quality attributes is required to make different foods of optimal quality. Quality control which can be exercised through measurement of the physical and chemical properties of the component stimuli needs to be validated by a relationship with sensoryly perceived responses individually and in combination. It is obvious that the accuracy and reproducibility of any instrumental method meaningful for food quality measure is that which correlates with the sensoryly perceivable differences (Kramer, 1966).

Besides the sensory attributes, capsicum like other products used as foods and food additives, should also have certain functional properties for its optimal use in the industrial sectors, which also have to be considered. The standards of the importing countries are based on the requirements of the food processing industries and include additional emphasis on cleanliness, which progressively cover, in addition to insects and rodent parts, limits of chemical and microbiological contaminations, and freedom of health-hazard organisms. These specifications assure genuiness, purity and cleanliness, but they do not give information on the sensory attributes which the consumers require. In the case of some processed products, e.g., ground paprika and oleoresin, specifications for total color and capsaicinoid content are found in standards and manufacturer literature, these being the main selling factors in this increasing competitive market (Govindarajan, 1986c).

Herbs and fruits that are used as spice, active pharmaceutical ingredients of drugs, or constituents of food additives should also fulfill even more special requirements described by the Pharmacopoeias and/or other international organizations like EC, FAO, WHO, ISO, ASTA, etc., and national bodies, which guide the industry concerned in the respective activities such as manufacturing or trade. The standards are the results of conscientious efforts in standardization. In the lack of space, it is not possible to cover all such standards. Only those have been listed that are closely related to the quality of *Capsicum* and *Capsicum*-originated products that can be used for the pharmaceutical industry.

3.1. Capsicum fruits

3.1.1. Capsicum (Ph. Eur. 7.0)

The 2011 edition of the European Pharmacopoeia Edition 7.0 (Ph. Eur. 7.0) lists *Capsicum fruit* (Capsici fructus) and describes its *Definition*, *Identification*, *Nonivamid Test*, and *Assay* as follows.

DEFINITION: Dried ripe fruits of *Capsicum annuum* L. var. *minimum* (Miller) Heise and small-fruited varieties of *Capsicum frutescens* L.

CONTENT: Minimum 0,4 per cent of total capsaicinoids expressed as capsaicin ($C_{18}H_{27}NO_3$; M_r 305.4) (dried drug).

IDENTIFICATION

a. The fruit is yellowish-orange to reddish-brown, oblong conical with an obtuse apex, about 1 cm to 3 cm long and up to 1 cm in diameter at the widest part, occasionally attached to a 5-toothed inferior calyx and a straight pedicel. Pericarp somewhat shrivelled, glabrous, enclosing about 10 to 20 flat, reniform seeds 3 mm to 4 mm long, either loose or attached to a reddish dissepiment.

b. Reduce to a powder. The powder is orange. Examine under a microscope using *chloral hydrate solution R.* The powder shows the following diagnostic characters: fragments of the pericarp having an outer epicarp with cells often arranged in rows of 5 to 7, cotucle uniformly striated: parenchymatous cells frequently containing droplets of red oil, occasionally containing misrosphenoidal crystals of calcium oxalabe; endocarp with characteristic island groups of sclerenchymatous cells, the groups being separated by thin-walled parenchymatous cells. Fragments of the seeds having an episperm composed of large, greenish-yellow, sinuous-walled sclereids with thin outer walls and strongly and unevenly thickened radial and inner walls which are conspicuously pitted; endosperm parenchymatous cells with drops of fixed oil and aleurone grains 3 μm to 6 μm in diameter. Occassional fragments from the calyx having an outer epidermis with anisocytis stomata, inner epidermis with many trichomes but no stomata; trichomes glandular, with uniseriate stalks and multicellular heads; mesophyll with many idioblasts containing microsphenoidal crystals of calcium oxalate.

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13 crystals of calcium oxalabe; endocarp with characteristic island groups of sclerenchymatous cells, the groups being separated by thin-

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17 having an outer epidermis with anisocytis stomata, inner epidermis with many trichomes but no stomata; trichomes glandular, with

18 unicellular stalks and multicellular heads; mesophyll with many idioblasts containing microsphenoidal crystals of calcium oxalate.

19 C. Thin-layer chromatography.

20 *Test solution.* To 0.50 g of the powdered drug (500) add 5.0 ml of *ether R,* shake for 5 min and filter.

21 *Reference solution.* Dissolve 2 mg of *capsaicin R* and 2 mg of *dihidrocapsaicin R* in 5.0 ml of *ether R.*

22 Plate: *TLC octadecylsilyl silica gel plate R. Mobile phase: water R, methanol R* (20:80 V/V).

23 *Application:* 20 μl, as bands.

24 *Development:* over a path of 12 cm.

25 *Drying: in air.*

26 *Detection:* spray with a 5 g/l solution of *dichloroquinonechlorimide R* in *methanol R.*

27 Expose the plate to ammonia vapour until blue zones appear. Examine in daylight.

28 *Results:* see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

29 Furthermore, other zones may be present in the chromatogram obtained with the test solution (Figure 5).

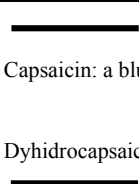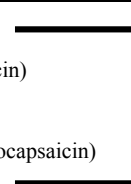30 **Figure 5**. Characterization of the top of the TLC plate (Ph. Eur. 6.0.)

Top of the plate	
▬▬▬▬	▬▬▬▬
Capsaicin: a blue zone	A blue zone (capsaicin)
Dyhidrocapsaicin: a blue zone	A blue zone (dihydrocapsaicin)
▬▬▬▬	▬▬▬▬
Reference Solution	**Test solution**

31

32 **Figure 5**. Characterization of the top of the TLC plate (Ph. Eur. 7.0)

33 **Nonivamide.** Liquid chromatography.

34 *Test solution.* To 2.5 g of the powdered drug add 100 ml of *methanol R.* Allow to macerate for 30 min. Place in an ultrasonic bath for

35 15 min. Filter into a 100 ml volumetric flask, rinse the flask and filter with *methanol R.* Dilute to 100,0 ml with *methanol R.*

36 *Reference solution.* Dissolve 20.0 mg of *capsaicin R* and 4.0 mg of *nonivamide R* in 100.0 ml of *methanol R.*

37 *Column: size:* l=0.25 m, ∅ =4.6 mm; *stationary phase:* phenylsilyl silica gel for chromatography R (5 μm); *temperature:* 30 ºC.

TESTS

Nonivamide. Liquid chromatography.

Test solution. To 2.5 g of the powdered drug add 100 ml of *methanol R.* Allow to macerate for 30 min. Place in an ultrasonic bath for 15 min. Filter into a 100 ml volumetric flask, rinse the flask and filter with *methanol R.* Dilute to 100,0 ml with *methanol R.*

Reference solution. Dissolve 20.0 mg of *capsaicin R* and 4.0 mg of *nonivamide R* in 100.0 ml of *methanol R.*

Column: *size*: l=0.25 m, ∅=4.6 mm; *stationary phase*: phenylsilyl silica gel for chromatography R (5 μm); *temperature*: 30 ºC.

Mobile phase: mixture of 40 volumes of *acetonitril R* and 60 volumes of a 1 g/l solution of *phosphoric acid R.*

Flow rate: 1.0 ml/min.; *Detection:* spectrophotometer at 225 nm.; *Injection:* 10 μl.

System suitability: reference solution:

• *resolution:* minimum 3.0 between the peaks due to capsaicin and nonivamide.

Limit: calculate the percentage content of nonivamide

• *nonivamide:* maximum 5.0 per cent of the total capsaicinoid content.

ASSAY: Liquid chromatography as described in the test for nonivamide.

3.2. Capsicum extracts

Presently, virtually all commercial spice extraction is carried out by one of two methods. One the methods is solvent extraction, wich involves treating a ground dry spice with an organic solvent such as hexane, acetone, methanol, ethanol or methylene chloride. Pursuant to this method, the spice extract is recovered by removal of the solvent, usually by distillation with heat under vacuum. The spice extract recovered in this way is known as an "Oleoresin" (Eisvale, 1981; Pruthi, 2003). In the case of oleoresin from *Capsicum*, the oleoresin is further treated with polar solvent, methanol, in order to separate the pungent component *Oleoresin Capsicum* from the color component *Oleoresin paprika*. Oleoresins are used almost exclusively by the food and pharmaceutical industries as a substitute of ground spices and spice tinctures.

The composition of an oleoresin is affected by the choice of organic solvent used in the extraction, but typically will include phospholipids, oils, waxes, sterols, resins, and a range of non-volatile and volatile compounds which make up much of the aroma and flavor of the original spice. In its use as food additive, the best oleoresin of *Capsicum* is that which contains the color and flavor components and that which truly recreates, when appropriately diluted in food formulations, the sensory qualities of fresh materials (Govindarajan, 1986c).

The other commercial method of spice extraction is the aqueous distillation of the whole or ground, fresh or dried spice using either boiling water or steam. This method recovers only the steam volatile components of the spice; i.e., the "*Essential oil*" which is high in aroma and flavor compounds (Simon, 1990). Many variations of these two methods are possible. The essential oil may be prepared by distillation from the original spice, or by distillation from a previously prepared solvent extracted oleoresin.

These traditional processes have a number of disadvantages. Most organic solvents are toxic, and government food regulations dictate that their residues must be reduced in the oleoresin to very small concentrations, generally in the range of 25-30 ppm or less (Pruthi, 2003). The distillation processes used to remove the solvents, or to recover essential oils, lowers the content of the very light volatiles which contribute to aroma and flavor. Of more importance is the growing consumer demand for food ingredients which are completely natural and free of contact with synthetic chemicals.

Extraction of spices with supercritical fluid carbon dioxide has been proposed as a means of eliminating the use of organic solvents and providing the prospect of simultaneous fractionation of the extract. The use of supercritical fluids (SCF) for extraction purposes was introduced in the late nineteenth century. A supercritical fluid is a substance at temperatures and pressures beyond its critical point at which the liquid phase of the substance will not exist. At these temperatures and pressures, the supercritical fluid has properties between gas-and liquid-phase characteristics. These properties make supercritical fluids efficient extraction solvents with high mass transfer characteristics (McHugh and Krukonis, 1986;; Krukonis, 1988). Consequently, supercritical fluids are often used to selectively extract or separate specific compounds from a mixture by varying fluid density through changes in pressure and temperature. In food technology, the use of supercritical fluids is essentially limited to supercritical carbon dioxide (SCF-CO$_2$) extraction since carbon dioxide has the advantages of being inexpensive and nontoxic and because its critical point is easily reached.

Oleoresins have several advantages over ground spices, e.g., elimination of microbial contamination, uniformity of color and flavor strenght and optimal utilization. The Essential Oil Association of America has detailed specifications for three types of *Capsicum* oleoresins (Table 2). *Oleoresin paprika* is mainly used as food coloring in meats, dairy products, soups, sauces and snacks. *Oleoresin red pepper* is used for both coloring and pungency, mainly in canned meats, sausages, in some snacks and in a dispersed form in some drinks such as ginger ale. *Oleoresin Capsicum* is the most pungent and is used for its counter-irritant properties in plasters and some pharmaceutical preparations.

Type of oleoresin Capsicum	Botanical source	Preparation	Color value	Color discription	Scoville Heat Units
Oleoresin Capsicum (EOA No. 244)	C. frutescens or C. annuum	Solvent extraction	4,000 max	Clear red, light amber, or dark red	480,000 min
Oleoresin red pepper (EOA No. 245)	C. annuum var. lognum	Solvent extraction	20,000 max	Deep red	240,000 min
Oleoresi paprika (EOA No. 239)	C. annuum	Solvent extraction	40,000-100,000	Deep red	Nil or negligible

Table 2. Nomenclature of oleoresins of Capsicums (Essential Oils Association, 1975).

3.2.1. Capsicum Oleoresin

The 2012 edition of the United States Pharmacopoeia-National Formulary (*USP36-NF31*) lists *Capsicum Oleoresin* and describes its *Capsicum Oleoresin* as follows.

3.2.1.1. Capsicum Oleoresin (USP30-NF25)

DEFINITION: *Capsicum Oleoresin* is an alcoholic extract of the dried ripe fruits of *Capsicum annuum* var. *minimum* and small fruited varieties of *C. frutescens* (Solanaceae). It contains not

less than 8.0 percent of total capsaicins [capsaicin ($C_{18}H_{27}NO_3$), dihydrocapsaicin ($C_{18}H_{29}NO_3$), and nordihydrocapsaicin ($C_{17}H_{27}NO_3$)].

IDENTIFICATION: To about 0.5 g of it in a beaker add 5 mL of water and 10 mL of a mixture of water, 0.2 *M* potassium chloride, and 0.2 N hydrochloric acid, and mix. Add 5.0 mL of 0.5 *M* sodium nitrite and 5.0 mL of 0.02 *M* sodium tungstate, and mix. Heat at 55° to 60° for 15 minutes, allow to cool, and filter. To the filtrate add 10 mL of 1 N sodium hydroxide: a bright yellow color is produced (*presence of capsaicin*).

ASSAY

Mobile phase— Prepare a mixture of methanol and 2% acetic acid (56:44), filter through a 0.5-μm or finer porosity filter, and degas.

Standard preparation— Prepare a solution of *USP Capsaicin RS* in methanol having a known concentration of about 0.5 mg per mL. Filter a portion of this solution through a 0.2-μm porosity filter, and use the filtrate as the *Standard preparation.*

Assay preparation— Transfer about 1000 mg of *Capsicum Oleoresin*, accurately weighed, to a 100-mL volumetric flask, dissolve in and dilute with methanol to volume, and mix. Filter a portion of this solution through a 0.2-μm porosity filter, and use the filtrate as the *Assay preparation.*

Chromatographic system— The liquid chromatograph is equipped with a 280-nm detector and a 3.9-mm × 30-cm column that contains packing L1. The flow rate is about 2 mL per minute. Chromatograph the *Standard preparation*, and record the peak responses as directed for *Procedure:* the tailing factor is not more than 2.0; and the relative standard deviation for replicate injections is not more than 2.0%. Chromatograph the *Assay preparation*, and record the peak responses as directed for *Procedure:* the relative retention times are about 0.9 for nordihydro-capsaicin, 1.0 for capsaicin, and 1.6 for dihydrocapsaicin; and the resolution, *R*, between the nordihydrocapsaicin peak and the capsaicin peak is not less than 1.2.

Procedure— Separately inject equal volumes (about 10 μL) of the *Standard preparation* and the *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the three major peaks. Calculate the percentage of total capsaicins in the portion of *Capsicum Oleoresin* taken by the formula:

$$(CP\,/\,W)(r_U\,/\,r_S)$$

in which

C is the concentration, in mg per mL, of *USP Capsaicin RS* in the *Standard preparation*;

P is the designated purity, in percentage, of *USP Capsaicin RS,*

W is the weight, in mg, of *Capsicum Oleoresin* taken to prepare the *Assay preparation*;

r_U is the sum of the peak responses for nordihydrocapsaicin, capsaicin, and dihydrocapsaicin obtained from the *Assay preparation*; and

r_S is the peak response obtained from the *Standard preparation.*

3.3. Quantitation of capsicum pigments

Appearance and color, the first of the percieved attributes, directly provide a basis for a decision of appropriateness. Size, shape, and maximum percentage of defects are easily measured specifications are given in standards. Color of *Capsicum* fruits is basicly determined by the nature and distribution of the above described carotenoids of which can be hidden or modified by other pigments such as chlorophylls and anthocyanins. The major coloring pigments in paprika are capsanthin and capsorubin, comprising majority of the total carotenoids. Other pigments are *beta*-carotene, zeaxanthin, violaxanthin, neoxantin and lutein (Anu and Peter, 2000). It is also worth repatedly mentioning that the relative amounts of the colored pigments are changing during the ripening period according to a rather well investigated biochemical pathways (Rodriguez-Amaya, 1997; Rahman and Buckle, 1980; Hornero-Mendez et al., 2000; Gnayfeed et al., 2001; Deli and Molnár, 2002).

It is also worth mentioning that carotenoid research in the field of plant and food chemistry is a very extensive area. The interested readers can consult recent reviews to learn the analytical methods that are currently used to analyse plant and food samples for their carotenoid contents (Rodriguez-Amaya, 1997; Wall and Bosland, 1998; Deli and Molnár, 2002; Felt et al., 2005). Some of the methods to measure coloring parameters of paprika and oleoresins currently accepted as official are summarized below.

3.3.1. The color matching method

This early method for total pigments expressed as Nesslerimeter color value used in the industry was standardized and adopted by the Essential Oils Association of America (EOA) for *Oleoresin Capsicum* (Essential Oil Association (1975): Specification of oleoresin paprika – EOA No. 239, Oleoresin Capsicum – EOA No. 244, Oleoresin red pepper – EOA No. 245, Essential Oil Association, New York). The method is based on matching the color of the properly diluted oleoresin acetone solution with that of a potasium dichromate ($K_2Cr_2O_7$) and cobaltous chloride ($CoCl_2$ x $6H_2O$) containing reference solutions.

3.3.2. Spectrophotometric methods

The alternative method uses the spectrophotometer to measure the total carotenoid pigments.

3.3.2.1. The EOA method

(Essential Oil Association (1975): Specification of oleoresin paprika – EOA No. 239, Oleoresin Capsicum – EOA No. 244, Oleoresin red pepper – EOA No. 245, Essential Oil Association, New York)

The absorbance of a 0.01% acetone solution of oleoresin is measured at 458 nm. The absorbance value is multiplied by 61,000 (an empirical factor worked out to relate the data from the color matching method) gives the total pigment as the Nesslerimeter color value.

3.3.2.2. The American Spice Trade Association (ASTA) method

By the above spectrophotometric method, results from different laboratories were not directly comparable due to differences in the spectrophotometers. In the new ASTA 20.1 Method (ASTA, 1968) a reference solution of inorganic salts (potassium dichromate and cobaltous ammonium sulfate in 1.8 M sulfuric acid solution) absorbing in the same region as the carotenoids is used to calculate an instrument factor which makes interlaboratory comparison possible. In the ASTA Method 20.1 for extractable color (pigments) in *Capsicums* absorbance of acetone extract of ground paprika and other capsicums is measured at 460 nm. The color value (in ASTA) is calculated using the determined instrument correction factor.

A direct correlation between the earlier ASTA Method 19, measuring absorbance at 450 nm, and the new Method 20.1 can not be established, an empirical factor (16.4) in the formula to give values nearly equal to those obtained by the earlier Method No. 19.

3.3.2.3. The Hungarian standard method

In Hungary, where specified grades of ground paprika are produced (*csípős, csemege, édes-nemes*), the total pigments are determined by similar absorbtion measurements. Earlier, the total pigment concentration in ground capsicums or in oleoresins was calculated by using the extinction coefficient in benzene of the major pigment capsanthin ($^{1\%}E_{477nm}$=1826). The results were expressed in grams of capsanthin per kilograms of dry matter (Hungarian Standard. Examination of Ground Paprika Spice. Determination of Pigment Content, MSZ 9681/5-76.). At present, the measurements are performed using acetone extracts similar to the ASTA Method 20.1 (MSZ 9681-5:2002)

3.4. Quantitation of pungent principles

For the major portion of *Capsicum* species pruduced and traded, pungency is the important quality attribute. The nature of the causal components in the spice has been established as a mixture of seven homologous branched-chain alkyl vanillylamides, named *capsaicinoids*. Smal amounts of three straight-chain analogues have also been shown to occure. The chemistry of these compounds has been reviewed (Suzuki and Iwai, 1984). The structures are given in Table 1.

The average composition of these related vanillylamides in the videly traded chillis (*Capsicum annuum* var. *annuum*) varieties is capsaicin 33 to 59%; dihydrocapsaicin, 30 to 51%; nordihydrocapsaicin, 7 to 15%; and others, in the range of 0 to 5% each. Fruits of the species *Capsicum frutescens*, stimulating high pungency and mostly used in the pharmaceutical industry, have higher capsaicin (63 to 77%) and dihydrocapsaicin (20 to 32%) with other homologues and analogues making up around 10% (Jurenitsch et al., 1978). The total capsaicinoids varied greatly (0.001 to 0.01% in paprika and 0.1 to >1% in chillis), but the proportion of capsaicin and dihydrocapsain ranged from 77 to 90% int he fruits of the species *C. annuum* and from 89 to 98% in those from species *C. frutescens* (Govindarajan et al., 1987).

The pungency stimulated by the different alkyl acyl vanillylamides varied greatly, all much lower compared to capsaicin and dihydrocapsaicin, which were equal (Govindarajan et al.,

1987). Thus, the estimation of total capsaicinoids, reproducibly and accurately correlating with the determined pungency, should be sufficient for quality control. Where the minor capsaicin-related vanillylamides make up a larger portion (above 20%), however, their individual estimation could become necessary because they stimulate much lower pungency. It is also known for a long time that synthetic nonoyl vanillylamide (pelargonyl vanillylamide) has considearable pungency and heat (Kulka, 1967). and has been found in varying amounts in commercial oleoresins. Therefore, it was necessary to determine the upper limits of the straight chain analogs to determine adulteration.

3.4.1. Official methods for organoleptic determination of pungency

3.4.1.1. The Scoville method

A number of methods have been reported from time to time since 1912 for assaying the pungency or capsaicin content of *Capsicum* fruits and/or the processed fruits (Pruthi, 2003). The basic principle of pungency evaluation using an organoleptic method was eshtablished in 1912 by W. L. Scoville (Scoville, 1912). The method is based on sensory evaualtion deter-mining the amount of sugar to neutralize the heat from the pepper. A solution of the pepper extract is variably diluted with sugar solution are tested in increasing concentration. The highest dilution at wich pungency is just detected is taken as a measure of the heat value. The dilution value, in milliliters per gram has since then been called Scoville Heat Units (SHU). The SHU for pure capsaicin is reported as $16\text{-}17 \times 10^6$. The Scoville Heat Units of various chilli pepper varieties are shown in Table 3. The greatest weakness of the Scoville organoleptic test is its imprecision, because it relies on human subjectivity.

Type	Heat rating (in Scoville Heat Units)
Habanero	200,000-300,000
Tabasco	30,000-50,000
Cayenne	35,000
Serrano	7,000-25,000
Jalapeno	3,500-4,500
Anaheim	1,000-1,400
Bell & Pimento	0

Table 3. The Scoville Heat Units of various chilli pepper varieties.

3.4.1.2. The EOA method

This method (Essential Oil Association (1975): Specification of oleoresin paprika – EOA No. 239, Oleoresin Capsicum – EOA No. 244, Oleoresin red pepper – EOA No. 245, Essential Oil Association, New York) is the codification of the procedure that was in use by the spice processing industry to check the constancy of pungency of a usual trade variety and source.

The method, based on the approach of the original Scoville method, specified for oleoresins of capsicum as follows.

A standard solution for testing is made by diluting a stock alcoholic solution of the oleoresin and it is tested by five trained panelists. If three of the five on a panel agree on just perceptible pungency at the given dilution (which is equivalent to 240,000 ml/g), this value is called the SHU of pungency of the sample. If the pungency response is strong, this first diluted standard is further diluted and the panel testing is repeated to find the dilution at which three of the five judge the pungency just perceptible. The Scoville test run shows a rather high correlation to total capsaicinoids content (1,500,000 Scoville units=1% capsaicin).

3.4.1.3. The British standard method

The British Standard Institution adapted the above industry procedure except that dilutions were rationalized in more convenient volumes (British Standards Institution (1979): Methods for testing spices and condiments: Determination of Scoville index of chillies, BS 4548 (Part 7), BSI, London)

3.4.1.4. The International Standard Organization (ISO) method

The British Standard Method adopted and improved by the International Standards Organization (ISO) requires the testing of a series of dilutions around the anticipated value by individuals experienced in recognizing pungency (International Standards Organization (1997): Spices and condiments – chillies: Determination of Scoville index, ISO 3513:1977E, ISO, Geneva). Testing of dilutions should be done from the weekest to the strongest until a level at which three of five panelists agree on recognition pungency. There is no published reports on the extensive use and efficiency of these latter two methods, except a few which reported comparison of experimentally determined SHU values with that of calculated based on capsaicinoid content of oleoresin capsicum samples. (Suzuki et al., 1957).

3.4.1.5. The ASTA method

The ASTA adopted as early as 1968 Method No. 21.0 as an official method for pungency evaluation which took care of many variables that were to be controlled for good reproducibility (ASTA (1968): Method 21.0, Pungency of capsicum spices and oleoresins (Scoville heat test), In: Official Analytical Methods, ASTA, Englewood Cliffs, N.J.). The procedure followed in the industry and EOA was thoroughly revised by designing it as a general method applicable to samples of a large range of capsaicinoids content by careful steps for determining the recognition threshold using an ascending concentration series.

3.5. Quantitation of capsaicinoids

In addition to pungency, as a bulk characteristics of total capsaicinoids, estimation of individual level of each capsaicinoid is also an important quality attribute of *Capsicum* fruits. There are over a hundred papers published on the estimation of total capsaicinoids in *Capsicum* fruits,

the oleoresins and products containing their extracts. The methods can be grouped into four sets as follows:

3.5.1. Early direct and methods

As early as 1931, von Fodor used vanadium oxytrichloride or ammonium vanadate and hydrochloric acid to react with the phenolic hydroxyl group of capsaicinoids and measured the blue color formed. The accuracy of determinations based on color formation reactions of the phenolic moiety could be conveniently used when the color produced with the chromogenic reagent had absorption maxima far removed from the absorption range (300 to 550 nm) of the red and yellow carotenoids of *Capsicum* fruits. Thus the blue colors formed when the phenolic moiety of the capsaicinoids reacted with reagents such as vanadium oxychloride (Palacio, 1977) and phosphomolybdic or phosphotungstic reagents (Jentzsch et al., 1969) had their absorption maxima around 725 nm. On the other and, the chromophore produced by the more specific 2,6-dichloro-*p*-benzoquinone-4-chlorimide (Gibb's phenol reagent), depending on the reaction conditions, absorbed maximally at 590 or 615 nm (Jentzsch et al., 1969; Rajpoot and Govindarajan, 1981). The extinction coefficients for the blue colours using different reagents varied greatly and affected sensitivity. There are also reports using potassium ferricianide plus ferric chloride (Spanyar and Blazovich, 1969) sodium nitrite molybdate reagent (Bajaj, 1980) and Folin-Ciocalteu reagent (Kosuge and Inagaki, 1959) as chromogenic reagents for determination of total capsaicinoids. It is worth mentioning that the color reactions could also be applied for visualization of thin layer chromatography (TLC) spots of separated capsaicinoids.

3.5.2. Methods based on separation of capsaicinoids

The specificity and accuracy of determination of capsaicinoids were improved by a preliminary separation of interfering pigments and other substances. In the early methods separation of capsaicinoids from the pigments was accomplished by solvent partition. Several combination of partition system has been found in the literature (Spanya et al., 1957; Benedek, 1959; Chem. Abstr. 1963a; Tirimanna, 1972) none of the methods, however, was validated by pungency tests, without which the accuracy of the determination can not be ascertained.

There have been several methods developed for preliminary separation of capsaicinoids from the *Capsicum* pigments using short column clean-up methods. The purified capsaicinoids were quantitated by colorimetry after reacting with chromogenic reagents (Hollo et al., 1957, Chem. Abstr., 1958; Bajaj, 1980) or directly at the absorption maxima (282 nm) of pure capsaicin (Suzuki et al., 1957; Brawer and Schoen, 1962; Chem. Abstr., 1963b). Suzuki et al. determined pungency values for a number of chilli and oleoresin samples by threshold testing and validated the capsaicinoids values determined by the proposed method (Suzuki et al., 1957). After several reviews, the Joint committe (Pharmaceutical Society/Society of Analytical Chemistry) recommended the diethyl ether-alkali partition method for separation and the spectrophotometric difference method or the method using the Gibb's reagent for measurement (Joint Committee (PS/SAC) 1964). The method has been adopted by both the British

Standards (BS) and International Standards Institutions (ISO) for estimation of capsaicinoids (International Standards Organization, 1981).

3.5.2.1. Thin layer chromatography

As newer separation methods emerged (e.g., paper chromatography and TLC) which gave rapid and more efficient separation, they were quickly used in the determination of total and later the individual capsaicinoids. The main body of papers in the 1960s practically relegated the earlier solvent partition and column methods to the past and published reliable, rapid micromethods. Most of the early thin layer chromatography (TLC) methods used silica gel plates with a wide range of variations in the developing solvents. The versatility of TLC method for separation of complex mixture of compounds could be further improved, however, by using reversed-phase plates and polar developing solvents containing silver nitrate (Todd et al., 1975). Methods of visualization applied UV light or chromogenic reagents. The estimation method also varied: visual comparison of size and intensity of spots, direct densitometry on the plate, collection of the marked spot into a tube, development of color with a chromogenic reagent and absorption measurements. Quantitation was by reference to a standard curve using pure capsaicinoids treated under the same conditions. A comprehensive listing of the methods can be found in some reviews on capsaicinoids (Suzuki, and Iwai, 1984; Govindarajan et al., 1987).

3.5.2.2. Gas chromatography

Gas chromatography (GC) was early used to detect adulteration of capsaicinoids with synthetic vanillylamides and individual components in crystalline capsaicinoids through the analysis of methyl esters of fatty acids derived from them (see Table 2). As early as 1967, Morrison demonstrated that capsaicinoids can be analyzed by gas chromatography without derivatization (Morrison, 1967). In order to improve peak symmetry, prevent degradation of column and improve reproducibility of measurements, however, most of the GC methods need derivatization step to increase volatility of the capsaicinoids, and, furthermore, an efficient clean-up step is necessary (Todd et al., 1977; Iwai et al., 1979; Krajewska and Powers, 1987; Manirakiza et al., 1999). Two types of derivatization procedures have been reported: trimethylsilylation of capsaicinoids (Lee et al., 1976; Todd et al., 1977; Iwai et al., 1979; Fung et al., 1982) and hydrolysis of capsaicinoids to yield fatty acids and subsequent esterification (Jurenitsch et al., 1978; Jurenitsch and Leinmueller, 1980). To overcome the problem of tailing peaks and to avoid the use of derivatization step, Thomas et al. (Thomas et al., 1998) and Hawer et al. (Hawer et al., 1994) have recognized the use of polar capillary column for interaction with polar functional group of the molecules. In an earlier work Di Cecco also used stable polar analytical column (Carbowax-Teflon) to analyse column purified capsaicinoids from ground capsicum (Di Cecco, 1976). Furthermore, the use of a thermoionic selective detector (TSD) instead of flame ionization detection allowed the elimination of sample clean-up (Thomas et al., 1998)

Although direct analysis of capsaicinoids could be disandvantageous direct GC-MS analysis of commercially available natural capsainoids has been proved to be a proper method to

separate the main capsaicinoids and quantitate capsaicin and dihydrocapsaicin in *Capsicum* extracts as well (Kuzma et al., 2006) A typical gas chromatogram of a commercially available natural capsaicin is shown on Figure 6.
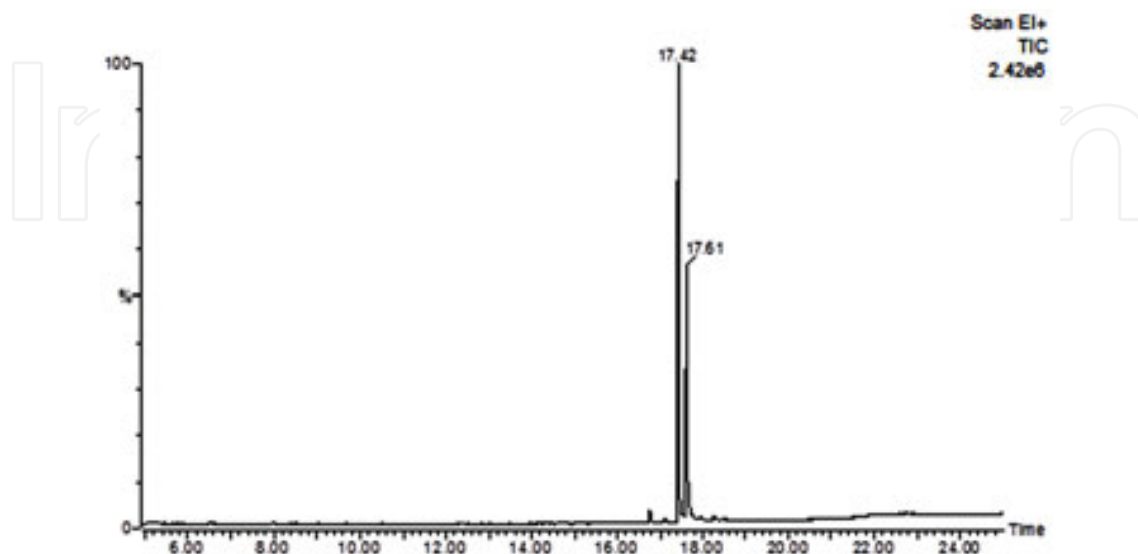

Figure 6. Gas chromatogram of a commercially available natural capsaicin preparation (Mózsik et al., 2009). Retention times: 16.8 min (not shown): nordihydrocapsaicin; 17.4 min capsaicin; 17.6 min: dihydrocapsaicin.

Lee et al. (Lee et al., 1976) used selective ion monitoring to identify and quantify individual capsaicinoids at the nanogram level in partially or fully separated and even mixed peaks from GC of trimethylsylilated capsaicinoids. Aliquots of fruit extracts were subjected to TLC or reversed phase (RP) HPLC to separate the capsaicinoids from other components of the extracts. Iwai et al. (Iwai et al, 1979) developed a similar method for determining all homologs in total extracts of *Capsicum* fruits. By this analysis, similar to other GC-MS methods (Fung et al., 1982; Reilly et al., 2001a, b) the straight-chain homologs (octanoyl, nonoyl and decyl vanilly-lamides – identified in minor amounts in other analyzes (Jurenitsch et al., 1978; Jurenitsch and Leinmueller, 1980) – were not found in any of the samples. This was the case because the analysis was based on selected *m/e* values but not on monitoring the mass of octanoyl vanil-lylamide, and the method could not differentiate between nonoyl vanillylamide and nordi-hydrocapsaicin eluting in the same area. Pena-Alvarez et al. (Pena-Alvarez et al., 2009) used solid phase microextraction (SPME)–gas chromatography–mass spectrometry for the analysis of capsaicin and dihydrocapsaicin in peppers and pepper sauces. In addition to capsaicin and dihydrocapsaicin, the method could differentiate several straight-chain analogs (e.g. nordi-hydrocapsaicin, nonoyl vanillylamide, homodihydrocapsaicin I and homodihydrocapsaicin II) in the different peppers and pepper sauces.

3.5.2.3. High performance liqiud chromatography

This chromatographic technique has superior and rapid separation capabilities arising from the use of very fine and highly uniform particles, newer solid phases, and high pressure to
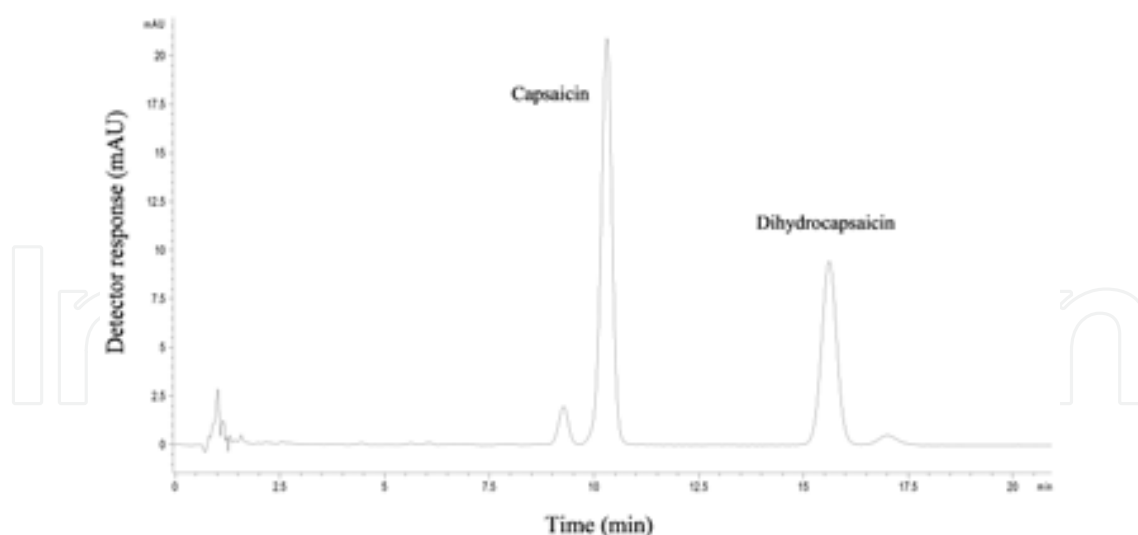
Figure 7. HPLC chromatogram of a commercially available capsaicin preparation (Mózsik et al., 2009). Retention times: 9.3 min: nordihydrocapsaicin; 10.3 min: capsaicin; 15.6 min: dihydrocapsaicin; 17.0 min: homocapsaicin.

move the eluting solvent and fractions. With all its advantages, high performance liquid chromatographic analysis is being increasingly used for routine analyses in both industrial and research laboratories. HPLC has superior separation capabilities for closely related compounds typically occuring in the case of extract of natural sources. Combined with additional operational parametres, e.g., reversed-phase systems, silver-ion complexing of olefinic compounds, optical as well as mass selective detectors, the separation efficiency, sensitivity, and quantification at submicrogram levels of capsaicinoids have been demonstrated in the recent years.

There has been published several HPLC methods for determination of capsaicin homologs and analogs. Since there is no space to summarize all the methods published so far, attention of interested readers is drawn to recent reviews to get a comprehensive knowledge on the field (e.g., Govindarajan et al., 1987; Wall and Bosland, 1998; Pruthi, 2003; Manirakiza et al., 2003). Here only selective papers are summarized with data obtained. A typical HPLC chromatogram of a commercially available natural capsaicin is shown on Figure 7.

Lee et al (Lee et al., 1976) and Iwai and colleagues (Iwai et al., 1979) early used HPLC for separation of capsaicinoids in one or two fractions from total extracts for the subsequents analysis by mass spectrometry. Sticher et al. (Sticher et al., 1978) reported separation of four homologs of capsaicin in purified capsaicinoids using a reversed-phase system. Jurenitsch at al. (Jurenitsch et al., 1979a,b) accomplished separation of the capsaicin homologs and analogs directly from ground fruit extracts on a reversed-phase system. Detection and quantitation were done by absorbance at 280 nm. Four samples of *Capsicum* fruits were analyzed by this HPLC method and also by the TMS-GC method earlier used by the group (Jurenitsch et al., 1978) for comparison.

Nonoyl vanillyamide content has assumed importance since more than 3 to 4% of this analog in a natural sample is considered adulteration unless declared. The method developed by

Jurenitsch et al. (1979a,b) was modified with the inclusion of silver nitrate in the mobile phase to selectively shorten the retention time of capsaicin, thus separating it from the coeluting nonoyl vanillylamide (Jurenitsch and Kampelmuehler, 1980). Constant and coworkers (Constant et al., 1995) also used complexation chromatography ($AgNO_3$) to separate norcapsaicin, zucapsaicin (civamide), capsaicin, nordihydrocapsaicin, nonivamide, homocapsaicin, dihydrocapsaicin and homodihydrocapsaicin-I.

Kawada et al. performed microdetermination of capsaicin by high-performance liquid chromatography with electrochemical detection (Kawada et al., 1985). Karnka et al. has reported an optimized HPLC method for sample preparation, separation, detection and identification of the major capsaicinoid compounds in various capsicum samples (Karnka et al., 2002). Isocratic reversed phase HPLC analysis performed in the author's laboratory allowed separation of five main capsaicinoids of a commercially available capsaicin preparation. The validated analytical method has been successfully applied to quantitate capsaicin and dihydrocapsaicin in industrial *Capsicum* extracts (Kuzma et al., 2014). Isocratic HPLC method with fluorimetric detection was used to determine capsaicin in rat tissues after acetone extraction (Saria et al., 1981).

HPLC analysis has made possible the accurate determination of the homologs and analogs of capsaicin and, combined with mass spectral analysis, has led to identification of structural isomers of some minor components (Govindarajan, 1986b; Reilly et al., 2001; Schweiggert et al., 2006; Singh et al., 2009; González-Zamora et al., 2013) and has made possible determination of nanogram levels of the individual capsaicinoids as is required in biosynthetic and metabolic studies (Reilly et al., 2002; Kozukue et al., 2005; Thompson et al., 2005; Zhang et al., 2010). The use of HPLC-MS (Reilly et al., 2001a) has been reported to differentiate nonivamide and capsaicin by mass-to-charge (*m/e*) ratio. The same authors have reported the use of LC-MS-MS with electrospray ionization source operating at selective ion monitoring mode (Reilly et al., 2001b). The quantification of capsaicinoids using LC-MS-MS was more sensitive (in the ng/ml range) and exhibited greater accuracy, even at low analyte concentrations. HPLC coupled with atmospheric pressure chemical ionization masss spectrometry has been reported to be a method of choice for separation and identification of the three groups of capsaicinoids: capsaicins possessing a methyl branched acyl residue with a carbon-carbon double bond, dihydrocapsaicins analogous to the previous class, but beeing saturated compounds, and capsaicin analogs (N-vanillyl-N-acylamides) composed of saturated, unbranched alkyl chains (Schweiggert et al., 2006).

In summary, HPLC analysis for total capsaicinoids or individual capsaicinoids is certainly rapid, reproducible, sensitive, and convenient for analysis of capsaicinoids in various capsaicinoid containing matrices.

3.5.3. Official methods for determination of capsaicinoids

3.5.3.1. The ASTA method for determination of capsaicinoids

In the 1980's it became clear that a more accurate and reproducible method of determining "heat" in peppers and pepper products was necessary. Under the auspices of the American

Spice Trade Association (ASTA), a new High Pressure Liquid Chromatography (HPLC) Method was adopted as ASTA Method 21.1. The HPLC measurement of capsaicin has evolved over the years as new and better instrumentation has allowed of greater accuracy of analysis. In 1996 AOAC issued Method 995.03: Capsaicinoids in Capsicums and their Extractives. In 1998, in a collaborative effort with AOAC, ASTA issued a revised method of analysis, ASTA Method 21.3 (HPLC Method). Using the revised method, the accepted pungency of pure capsaicin was re-stated from 15,000,000 to 16,000,000 SHU. AOAC revised its method to coincided with ASTA Method 21.3, in 1999, and in 2003 AOAC revised the method once more.

3.5.3.2. The United States Pharmacopoeia (USP) method

The 2012 edition of the USP36-NF31 lists *Capsaicin* and describes its Definition, Identification, Melting range, and Content of capsaicin, dihydrocapsaicin, and other capsaicinoids as follows.

Chemical name: 6-Nonenamide, (*E*)-N-[(4-Hydroxy-3-methoxy-phenyl)methyl]-8-methyl

Formula: $C_{18}H_{27}NO_3$

Molecular weight: 305.41

(*E*)-8-Methyl-N-vanillyl-6-nonenamide CAS-number: 404-86-4

Capsaicin contains not less than 90.0 percent and not more than 110.0 percent of the labeled percentage of total capsaicinoids. The content of capsaicin ($C_{18}H_{27}NO_3$) is not less than 55 percent, and the sum of the contents of capsaicin and dihydrocapsaicin ($C_{18}H_{27}NO_3$) is not less than 75 percent, and the content of other capsaicinoids is not more than 15 percent, all calculated on the dried basis.

Packaging and storage— Preserve in tight containers, protected from light, and store in a cool place

Caution—Handle *Capsaicin* with care. Prevent inhalation of particles of it and prevent its contact with any part of the body.

Solubility – It does not dissolve in water. It well dissolves in alcohols (methanol, ethanol 96%), ethylacetate and acetonitrile.

IDENTIFICATION: Prepare a test solution of *Capsaicin* in methanol containing 1 mg per mL. Prepare a Standard solution of *USP Capsaicin RS* in methanol containing 1 mg per mL. Separately apply 10-µL portions of the test solution and the Standard solution to a thin-layer chromatographic plate coated with a 0.25-mm layer of chromatographic silica gel mixture. Develop the chromatograms in a solvent system consisting of a mixture of ether and methanol (19:1) until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and allow it to air-dry. Spray the plate with a 0.5% solution of 2,6-dibromoquinone-chlorimide in methanol, allow to stand in a chamber containing ammonia fumes, and examine the chromatograms: the blue color and the R_f value of the principal spot obtained from the test solution correspond to those properties of the principal spot obtained from the Standard solution.

Melting range: between 57° and 66°, but the range between beginning and end of melting does not exceed 5°.

3.5.3.3. Content of capsaicin, dihydrocapsaicin and other capsaicinoids

Mobile phase— Prepare a mixture of diluted phosphoric acid (1 in 1000) and acetonitrile (600:400). Filter through a filter having a porosity of 0.5 µm or finer, and degas.

Standard capsaicin solution— Dissolve an accurately weighed quantity of *USP Capsaicin RS* quantitatively in methanol to obtain a solution having a known concentration of about 0.1 mg per mL.

Standard dihydrocapsaicin solution— Dissolve an accurately weighed quantity of *USP Dihydro-capsaicin RS* quantitatively in methanol to obtain a solution having a known concentration of about 0.025 mg per mL.

Test solution— Transfer about 25 mg of *Capsaicin*, accurately weighed, to a 250-mL volumetric flask, dilute with methanol to volume, and mix.

Chromatographic system— The liquid chromatograph is equipped with a 281-nm detector and a 4.6-mm × 25-cm column that contains 5-µm packing L11 and is maintained at a constant temperature of about 30°. Adjust the flow rate to obtain a retention time of about 20 minutes for the main capsaicin peak. Chromatograph the *Standard capsaicin solution*, and record the peak responses as directed for *Procedure:* the relative standard deviation for replicate injections is not more than 2%.

Procedure— Separately inject equal volumes (about 20 µL) of the *Standard capsaicin solution*, the *Standard dihydrocapsaicin solution*, and the *Test solution* into the chromatograph, record the chromatogram for a period of time that is twice that of the retention time of capsaicin, and measure the areas of the responses for all of the peaks.

Calculate the percentage of capsaicin ($C_{18}H_{27}NO_3$) in the portion of *Capsaicin* taken by the formula:

$$25{,}000(C \, / \, W)(r_U \, / \, r_S)$$

in which

C is the concentration, in mg per mL, of *USP Capsaicin RS* in the *Standard capsaicin solution*,

W is the weight, in mg, of *Capsaicin* taken to prepare the *Test solution*, and

r_U and r_S are the capsaicin peak responses obtained from the *Test solution* and the *Standard capsaicin solution*, respectively.

Not less than 55% is found.

Calculate the percentage of dihydrocapsaicin ($C_{18}H_{29}NO_3$) in the portion of *Capsaicin* taken by the formula:

$$25{,}000(C \, / \, W)(r_U \, / \, r_S)$$

in which

C is the concentration, in mg per mL, of *USP Dihydrocapsaicin RS* in the *Standard capsaicin solution*,

W is the weight, in mg, of Capsaicin taken to prepare the *Test solution*, and

r_U and r_S are the dihydrocapsaicin peak responses obtained from the *Test solution* and the *Standard dihydrocapsaicin solution*, respectively.

The sum of the percentage of capsaicin found and of the percentage of dihydrocapsaicin found is not less than 75%. Using the chromatograms obtained from the *Standard capsaicin solution* and the *Test solution*, calculate the percentage of other capsaicinoids in the portion of *Capsaicin* taken by the formula

$$25,000(C \, / \, W)(r_T \, / \, r_S)$$

in which

C is the concentration, in mg per mL, of *USP Capsaicin RS* in the *Standard capsaicin solution*,

W is the weight, in mg, of *Capsaicin* taken to prepare the *Test solution*,

r_T is the sum of the peak responses of the capsaicinoids other than capsaicin and dihydrocapsaicin in the chromatogram obtained from the *Test solution*, and

r_S is the capsaicin peak response obtained from the *Standard capsaicin solution*.

Not more than 15% of other capsaicinoids is found.

4. Pesticide control

Quality is a mandatory requirement in the materials to accomplish the Pharmaceutical Good Manufacturing Practices. Nowadays, the presence of pesticides in animal and vegetal (herbal) commodities is a topic of public concern for the potential health hazards derived from them (WHO, 1998). The presence of pesticide residues in vegetal raw materials can be originated in agricultural practices, environmental contamination or cross contamination.

Table 1 shows examples of potentially hazardous contaminants and residues that may occur in herbal products (WHO, 1998). The summary table includes information on possible sources of contaminants and residues, as well as the manufacturing stages at which they may be detectable. Some of them are considered as unavoidable contaminants or residues of herbal medicines.

4.1. Classification of contaminants

Contaminants in herbal medicines are classified into *chemical contaminants* and *biological contaminants*. A variety of *agrochemical agents* and some *organic solvents* may be important residues in herbal medicines (WHO, 1998).

4.1.1. Chemical contaminants

4.1.1.1. Toxic metals and non-metals

Contamination of herbal materials with toxic substances such as arsenic can be attributed to many causes. These include environmental pollution (i.e. contaminated emissions from factories and leaded petrol and contaminated water including runoff water which finds its way into rivers, lakes and the sea, and some pesticides), soil composition and fertilizers. This contamination of the herbal material leads to contamination of the products during various stages of the manufacturing process.

4.1.1.2. Persistent organic pollutants

Persistent organic pollutants include organic chemicals, such as the synthetic aromatic chlorinated hydrocarbons, which are only slightly soluble in water and are persistent or stable in the presence of sunlight, moisture, air and heat. In the past, they were extensively used in agriculture as pesticides. The use of persistent pesticides, such as DDT and benzene hexa-chloride (BHC), in agriculture has been banned for many years in many countries. However they are still found in the areas where they were previously used and often contaminate medicinal plants growing nearby.

4.1.1.3. Radioactive contamination

A certain amount of exposure to ionizing radiation is unavoidable because many sources, including of radionuclides occur naturally in the ground and the atmosphere. Dangerous contamination may be the consequence of a nuclear accident or may arise from other sources. WHO, in close collaboration with several other international organizations, has developed guidelines for use in the event of widespread contamination by radionuclides resulting from a major nuclear accident.

4.1.1.4. Mycotoxins

The presence of mycotoxins in plant material can pose both acute and chronic risks to health.. Mycotoxins produced by species of fungi including *Aspergillus*, *Fusarium* and *Penicillium* are the most commonly reported. Mycotoxins comprise four main groups, namely, *aflatoxins*, *ochratoxins*, *fumonisins* and *tricothecenes*, all of which have toxic effects. Aflatoxins have been extensively studied and are classifi ed as Group 1 human carcinogens by the International Agency for Research on Cancer.

4.1.2. Biological contaminants

4.1.2.1. Microbiological contaminants

Herbs and herbal materials normally carry a large number of bacteria and moulds, often originating in soil or derived from manure. While a large range of bacteria and fungi form the naturally occurring microflora of medicinal plants, aerobic spore-forming bacteria frequently

predominate.. The presence of Escherichia coli, Salmonella spp. and moulds may indicate poor quality of production and harvesting practices.

4.1.2.2. Parasitic contamination

Parasites such as protozoa and nematoda, and their ova, may be introduced during cultivation and may cause zoonosis, especially if uncomposted animal excreta are used. Contamination with parasites may also arise during processing and manufacturing if the personnel carrying out these processes have not taken appropriate personal hygiene measures.

4.1.3. Agrochemical residues

The main agrochemical residues in herbal medicines are derived from pesticides and fumigants.

Pesticides may be classified on the basis of their intended use, for example as follows:

- insecticides;

- fungicides and nematocides;

- herbicides; and

- other pesticides (e.g. ascaricides, molluscicides and rodenticides).

Examples of fumigants include ethylene oxide, ethylene chlorohydrin, methyl bromide and sulfur dioxide.

4.1.3.1. Pesticide residues

Medicinal plant materials may contain pesticide residues, which accumulate as a result of agricultural practices, such as spraying, treatment of soils during cultivation and administration of fumigants during storage. It is therefore recommended that every country producing medicinal plant materials should have at least one control laboratory capable of performing the determination of pesticides using a suitable method.

4.1.3.2. Classification of pesticides

Different classifications of pesticides exist. A classification based on the chemical composition or structure of the pesticide is the most useful for analytical chemists, for example:

- *chlorinated hydrocarbons* and related pesticides: hexachlorocyclohexane (HCH) or benzene hexachloride (BHC), lindane, methoxychlor

- *chlorinated phenoxyalkanoic acid* herbicides: 2,4-D, 2,4,5-T

- *organophosphorus* pesticides: carbophenothion (carbofenotion), chlorpyrifos and methyl-chlorpyrifos, coumaphos (coumafos), demeton, dichlorvos, dimethoate, ethion, fenchlorphos (fenclofos), malathion, methyl parathion, parathion

- *carbamate* insecticides: carbaryl (carbaril)

- *carbamoyl benzimidazoles*: benomyl, carbendazim

- *dithiocarbamate* fungicides: ferbam, maneb, nabam, thiram, zineb, ziram

- *amino acid* herbicides: glyphosate

- *inorganic* pesticides: aluminium phosphide, calcium arsenate

- *miscellaneous*: bromopropylate, chloropicrin, ethylene dibromide, ethylene oxide, methyl bromide, sulfur dioxide

- pesticides of plant origin: tobacco leaf extract, pyrethrum flower, and pyrethrum extract; derris and Lonchocarpus root and rotenoids.

Only the chlorinated hydrocarbons and related pesticides (e.g. HCH) and a few organophosphorus pesticides (e.g. carbophenothion) have a long residual action. Although the use of many persistent pesticides has been widely discontinued, residues may still remain in the environment (e.g. DDT). Thus the recording of all pesticide usage in countries should be strongly encouraged so as to enable cost-effective quality control of medicinal plants and of their products. The Stockholm Convention on Persistent Organic Pollutants currently includes DDT and 11 other POPs including dioxin (a potent carcinogen), aldrin, chlordane, dieldrin, endrin, heptachlor, mirex, toxaphene and hexachlorobenzene.

Most other pesticides have very short residual actions. Therefore it is suggested that, where the length of exposure to pesticides is unknown, the herbal materials should be tested for the presence of organically bound chlorine and phosphorus as a preliminary screening method which can be useful in predicting where a pesticide might be used.

4.1.4. Residual solvents

A range of organic solvents are used for manufacturing herbal medicines, and can be detected as residues of such processing in herbal preparations and finished herbal products. They should be controlled through GMP and quality control.

Solvents are classifi ed by ICH (Q3C (R5), according to their potential risk, into:

- class 1 (solvents to be avoided such as benzene);

- class 2 (limited toxic potential such as methanol or hexane); and

- class 3 (low toxic potential such as ethanol).

4.2. Pesticide analysis

One area of chemical testing of growing concern is pesticide analysis. Pesticides and their degradation products migrate from their intended point of application and spread through the air, water, soil, plants, microorganisms, and animals, including birds and fish. While many of the chemicals used in pesticide formulations are harmless, others may have toxic properties or could even form toxic by-products, potentially causing risks to human and animal health and/or environmental damage. There are over 1,000 pesticides available for use, many of which

are regulated by government agencies. As a result, powerful and rapid analytical methods are needed to detect very low concentrations of pesticides and their degradation products in diverse sample matrices.

4.2.1. Analytical advances

Scientific advances in the field of application of natural products with pharmaceutical relevance has been focused on analysis of (a) the actice constituents, and (b) the potentially hazardous contaminats. Methods for quantitative determination of the above mentioned contaminations varies for different classes of contaminants. Comprehensive review of the different methodologies is out of scope ofthe present monograph. Since pesticide residue analysis has been appeared as the most seriuus issue in connection with our *Capsicum extracts*, here we concentrate on regulatory requirements on this class of possible hasardous contaminants of herbal products.

Several modern *multiresidue precedures* employing different (a) *extraction methods*, (b) *clean-up techniques* and (c) a variety of *determination methods* have been reported for quantitation of pesticide residues in natural products with pharmaceutical applications. One of the cruitial point of these methods is to extract and isolate the target pesticides from the matrix. The optimal sample treatment hevealy depends on the complexicity of the matrix.

Not going into details, the most imortant ad frequntly used extraction techniques as follows:

- Liquid phase extraction,
- Solid phase extraction,
- Solid phase micro extraction,
- Microvawe assisted solvent extraction, and
- Supercritical fluid extraction

During extraction, the solvent comes in contact with the substrate matrix, to enable extraction of the pesticide along with some of the constituents of the substrate matrix also get solubilized. The extract not only contains pesticide residues but also other constituents, which are called co-extractives. The removal of interfering co-extractives from extract is called *clean up*. After removal of moisture, the other coextractives are removed by using various separation techniques.

The most imortant ad frequntly used clean-up techniques as follows:

- Liquid-liquid partitioning
- Chemical treatments
- Chromatographic techniques
- Thin layer chromatography
- Ion exchange chromatography

- Gel permeation chromatography, and

- Adsorption column chromatography

In accordance to the official methodologies, capillary gas-liquid chromatography (GC) is the most used separation technique in residue analysis of non-polar and semi-polar pesticides. Majos attantion has paid for determination of organochlorine, organophosphorus and pyrethroid pesticides. Most reports focuses on selective detection of pesticied using electron capture (ECD), nitrogen-phosphorous (NPD), thermal conductivity (TCD) and flame photometric (FPD) detectors. In spite of the increased selectivity and sensitivity of the GC-coupled detectors, the use of mass spectrometry is being compulsory induced in order to obtain relaible identification and confirmation of residues.

As a result of searching for non-persistent and biodegradable pesticides, which kill both detrimental and beneficial insects, introduction of more polar (and less volatile) agrochemical has been recognized. Such compounds have promted the use of high presuure liquid chromatography coupled with mass spectrometry (HPLC-MS), which at the moment is a widely accepted technique for monitoring of polar and most semipolar pesticeds as well as for regulatory isuues.

Recent advances of modern multiresidue precedures including application of different GC- and HPLC-based methodologies have been summarized in several review articles. Readers interested in the field can consult this literature (Hirahara et al., 2005, Abd El-Moneim et al., 2010, Perez-Parada et al., 2011, Niell et al., 2014).

4.2.2. Regulatory methods for pestice analysis of herbal products of pharmaceutical use

4.2.2.1. The European Pharmacopoeia (Ph.Eur.)

In the seventh edition of the European Pharmacopoeia (Ph. Eur. 7.0) pesticide residues are described under chapter 02 „Methods of analysis". The Ph.Eur. Monograph „2.8.13. Pesticide residues" contains „Definition", „Limits", „Sampling" and „Qualitative and quantitative analysis of pesticide residues" of herbal drugs. The requirements for "Herbal Drugs" and "Extracts", as well as "Herbal Drugs for Homoeopathic Preparations" and "Mother Tinctures for Homoeopathic Preparations" are refered within the scope of their monographs in chapter 05 „General texts":

For the purposes of the European Pharmacopoeia Pharmacopoeia, a pesticide is any substance or mixture of substances intended for preventing, destroying or controlling any pest, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of vegetable drugs. The item includes substances intended for use as growth-regulators, defoliants or desiccants and any substance applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport.

4.2.2.1.1. Limits

Unless otherwise indicated in the monograph, the drug to be examined at least complies with the limits indicated in Table 4. The limits applying to pesticides that are not listed in the table and whose presence is suspected for any reason comply with the limits set by European Community directives 76/895 and 90/642, including their annexes and successive updates. Limits for pesticides that are not listed in Table 4 nor in EC directives are calculated using the following expression:

$$\frac{ADI \ x \ M}{MDD \ x \ 100}$$

where

ADI=acceptable daily intake, as published by FAO-WHO, in milligrams per kilogram of body mass,

M=body mass in kilograms (60 kg),

MDD=daily dose of the drug, in kilograms.

If the drug is intended for the preparation of extracts, tinctures or other pharmaceutical forms whose preparation method modifies the content of pesticides in the finished product, the limits are calculated using the following expression:

$$\frac{ADI \ x \ M \ x \ E}{MDD \ x \ 100}$$

where

E=extraction factor of the method of preparation, determined experimentally, and

ADI, *M*, and *MDD* are as defined above.

Higher limits can also be authorised, in exceptional cases, especially when a plant requires a particular cultivation method or has a metabolism or a structure that gives rise to a higher than normal content of pesticides. The competent authority may grant total or partial exemption from the test when the complete history (nature and quantity of the pesticides used, date of each treatment during cultivation and after the harvest) of the treatment of the batch is known and can be checked precisely.

Substance	Limit (mg/kg)
Alachlor	0.02
Aldrin and Dieldrin (sum of)	0.05
Azinphos-methyl	1.0
Bromopropylate	3.0
Chlordane (sum of *cis*-, *trans*- and Oxythlordane)	0.05

Substance	Limit (mg/kg)
Chlorfenvinphos	0.5
Chlorpyrifos	0.2
Chlorpyrofos-methyl	0.1
Cypermethrin (and isomers)	1.0
DDT (sum of p,p'-DDT, o,p'-DDT, p,p'-DDE and p,p'-TDE)	1.0
Deltamethrin	0.5
Diazinon	0.5
Dichlorovos	1.0
Dithiocarbamates (as CS_2)	2.0
Endosulfan (sum of isomers and Endosulfan sulphate)	3.0
Endrin	0.05
Ethion	2.0
Fenitrothion	0.5
Fenvalerate	1.5
Fenofos	0.05
Heptachlor (sum of Heptachlor and Heptachlorepoxide)	0.05
Hexachlorobenzene	0.1
Hexachlorocyclohexane isomers (other than γ)	0.3
Lindane (γ-Hexachlorocyclohexane)	0.6
Malathion	1.0
Methidathion	0.2
Parathion	0.5
Parathion-methyl	0.2
Permethrin	1.0
Phosalone	0.1
Piperonyl butoxide	3.0
Pirimiphos-methyl	4.0
Pyrethrins (sum of)	3.0
Quintozene (sum of quintozene, pentachloroaniline and methyl pentachlorophenyl sulphide)	1.0

Table 4. Limits for pesticide residues (Ph. Eur. 7.0).

4.2.2.1.2. Qualitative and quantitative analysis of pesticide residues

The analytical procedures used are validated according to the regulations in force. In particular, they satisfy the following criteria:

- the chosen method, especially the purification steps, are suitable for the combination pesticide residue/substance to be analysed, and not susceptible to interference from co-extractives; the limits of detection and quantification are measured for each pesticide-matrix combination to be analysed,

- between 70 per cent to 110 per cent of each pesticide is recovered,

- the repeatability of the method is not less than the values indicated in Table 5,

- the reproducibility of the method is not less than the values indicated in Table 5,

- the concentration of test and reference solutions and the setting of the apparatus are such that a linear response is obtained from the analytical detector.

Concentration of the pesticide (mg/kg)	Repeatability (difference, ± mg/kg)	Reproducibility (difference, ± mg/kg)
0.010	0.005	0.01
0.100	0.025	0.05
1.000	0.125	0.25

Table 5. Repeatibility and reproducibility limits of determination of pesticides (Ph. Eur. 7.0).

Detailed description of qualitative and quantitative determination of organochlorine, organophosphorus and pyrethroid insecticides is divided into three sections:

- Extraction;

- Purification; and

- Quantitative analysis.

4.2.2.1.3. Extraction

To 10 g of the substance being examined, coarsely powdered, add 100 ml of *acetone R* and allow to stand for 20 min. Add 1 ml of a solution containing 1.8 µg/ml of *carbophenothion R* in *toluene R*. Homogenise using a high-speed blender for 3 min. Filter and wash the filter cake with two quantities, each of 25 ml, of *acetone R*. Combine the filtrate and the washings and heat using a rotary evaporator at a temperature not exceeding 40 °C until the solvent has almost completely evaporated. To the residue add a few millilitres of *toluene R* and heat again until the acetone is completely removed. Dissolve the residue in 8 ml of *toluene R*. Filter through a membrane filter (45 µm), rinse the flask and the filter with *toluene R* and dilute to 10.0 ml with the same solvent (solution A).

4.2.2.1.4. Purification

4.2.2.1.4.1. Organochlorine, organophosphorus and pyrethroid insecticides

Purificationis to be accomplished by means of size-exclusion chromatography.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.30 m long and 7.8 mm in internal diameter packed with *styrene-divinylbenzene copolymer R* (5 μm),

- as mobilephase *toluene R* at a flow rate of 1 ml/min.

Performance of the column. Inject 100 μl of a solution containing 0.5 g/l of *methyl red R* and 0.5 g/l of *oracet blue2R R* in *toluene R* and proceed with the chromatography. The column is not suitable unless the colour of the eluate changes from orange to blue at an elution volume of about 10.3 ml. If necessary calibrate the column, using a solution containing, in *toluene R*, at a suitable concentration, the insecticide to be analysed with the lowest molecular mass (for example, dichlorvos) and that with the highest molecular mass (for example, deltamethrin). Determine which fraction of the eluate contains both insecticides.

Purification of the test solution. Inject a suitable volume of solution A (100 μl to 500 μl) and proceed with the chromatography. Collect the fraction as determined above (solution B). Organophosphorus insecticides are usually eluted between 8.8 ml and 10.9 ml. Organochlorine and pyrethroid insecticides are usually eluted between 8.5 ml and 10.3 ml.

4.2.2.1.4.2. Organochlorine and pyrethroid insecticides

In a chromatography column, 0.10 m long and 5 mm in internal diameter, introduce a piece of defatted cotton and 0.5 g of silica gel treated as follows : heat *silica gel for chromatography R* in an oven at 150 °C for at least 4 h. Allow to cool and add dropwise a quantity of *water R* corresponding to 1.5 per cent of the mass of silica gel used; shake vigorously until agglomerates have disappeared and continue shaking for 2 h using a mechanical shaker. Condition the column using 1.5 ml of *hexane R*. Prepacked columns containing about 0.50 g of a suitable silica gel may also be used provided they are previously validated. Concentrate solution B in a current of *helium for chromatography R* or *oxygen-free nitrogen R* almost to dryness and dilute to a suitable volume with *toluene R* (200 μl to 1 ml according to the volume injected in the preparation of solution B). Transfer quantitatively onto the column and proceed with the chromatography using 1.8 ml of *toluene R* as the mobile phase. Collect the eluate (solution C).

4.2.2.1.5. Quantitative analysis

4.2.2.1.5.1. Organophosphorus insecticides

Quantitative determination to be accomplished by means of gas chromatography, using *carbophenothion R* as internal standard. It may be necessary to use a second internal standard to identify possible interference with the peak corresponding to carbophenothion.

Test solution. Concentrate solution B in a current of *helium for chromatography R* almost to dryness and dilute to 100 μl with *toluene R*.

Reference solution. Prepare at least three solutions in *toluene R* containing the insecticides to be determined and carbophenothion at concentrations suitable for plotting a calibration curve.

The chromatographic procedure may be carried out using:

• a fused-silica column 30 m long and 0.32 mm in internal diameter the internal wall of which is covered with a layer 0.25 μm thick of *poly(dimethyl)siloxane R*,

• *hydrogen for chromatography R* as the carrier gas. Other gases such as *helium for chromatography R* or *nitrogen for chromatography R* may also be used provided the chromatography is suitably validated.

• a phosphorus-nitrogen flame-ionisation detector or a atomic emission spectrometry detector, maintaining the temperature of the column at 80 °C for 1 min, then raising it at a rate of 30 °C/min to 150 °C, maintaining at 150 °C for 3 min, then raising the temperature at a rate of 4 °C/min to 280 °C and maintaining at this temperature for 1 min, and maintaining the temperature of the injector port at 250 °C and that of the detector at 275 °C. Inject the chosen volume of each solution. Calculate the content of each insecticide from the peak areas and the concentrations of the solutions.

4.2.2.1.5.2. Organochlorine and pyrethroid insecticides

Quantitative determination to be accomplished by means of gas chromatography, using *carbophenothion R* as internal standard. It may be necessary to use a second internal standard to identify possible interference with the peak corresponding to carbophenothion.

Test solution. Concentrate solution C in a current of *helium for chromatography R* or *oxygen-free nitrogen R* almost to dryness and dilute to 500 μl with *toluene R*.

Reference solution. Prepare at least three solutions in *toluene R* containing the insecticides to be determined and carbophenothion at concentrations suitable for plotting a calibration curve

The chromatographic procedure may be carried out using:

• a fused silica column 30 m long and 0.32 mm in internal diameter the internal wall of which is covered with a layer 0.25 μm thick of *poly(dimethyl)(diphenyl)siloxane R*,

• *hydrogen for chromatography R* as the carrier gas. Other gases such as *helium for chromatography R* or *nitrogen for chromatography R* may also be used, provided the chromatography is suitably validated,

• an electron-capture detector,

• a device allowing direct cold on-column injection, maintaining the temperature of the column at 80 °C for 1 min, then raising it at a rate of 30 °C/min to 150 °C, maintaining at 150 °C for 3 min, then raising the temperature at a rate of 4 °C/min to 280 °C and maintaining at this temperature for 1 min, and maintaining the temperature of the injector port at 250 °C and that of the detector at 275 °C. Inject the chosen volume of each solution. Calculate the content of each insecticide from the peak areas and the concentrations of the solutions.

4.2.2.2. United States Pharmacopoea-National Formulary (USP-NF)

In the 2012 edition of the United States Pharmacopoea-National Formulary (USP36–NF31) pesticide residues are described under General Chapters „<561> Articles of Botanical Origin USP". The „General Method for Pesticide Residues Analysys" section of the USP Monograph „„„<561> Articles of Botanical Origin USP" contains „Definition", „Limits", „Qualitative and quantitative analysis of pesticide residues" and „Test for Pesticides" of herbal drugs.

For the purposes of the United States Pharmacopoea the designation pesticide applies to any substance or mixture of substances intended to prevent, destroy, or control any pest, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport, or marketing of pure articles. The designation includes substances intended for use as growth regulators, defoliants, or desiccants, and any substance applied to crops before or after harvest to protect the product from deterioration during storage and transport.

4.2.2.2.1. Limits

Within the United States, many botanicals are treated as dietary supplements and are subject to the statutory provisions that govern foods but not drugs in the Federal Food, Drug, and Cosmetic Act. Limits for pesticides for foods are determined by the Environmental Protection Agency (EPA) as indicated in the Code of Federal Regulations (40 CFR Part 180) or the Federal Register (FR). For pesticide chemicals without EPA-established tolerance levels, the limits should be below the detection limit of the specified method. Result less than the EPA detection limits are considered zero values. The limits contained in the USP, therefore, are not applicable in the United States when articles of botanical origins are labeled for food purposes. The limits, however, may be applicable in other countries where the presence of pesticide residues is permitted.

Unless otherwise indicated in the monograph, the article to be examined complies with the limits indicated in Table 6. The limits for suspected pesticides that are not listed in Table 6 must comply with the regulations of the EPA. For instances in which a pesticide is not listed in Table 6 or in EPA regulations, calculate the limit by the formula:

$$\frac{A \times M}{100 \times B}$$

where

A is the acceptable daily intake (ADI), as published by FAO-WHO, in mg/kg of body weight;

M is body weight, in kg (60 kg); and

B is the daily dose of the article, in kg.

If the article is intended for the preparation of extracts, tinctures, or other pharmaceutical forms of which the preparation method modifies the content of pesticides in the finished product, calculate the limits by the formula:

$$\frac{A \times M \times E}{100 \times B}$$

where

E is the extraction factor of the preparation method, determined experimentally; and

A, *M*, and *B* are as defined above.

A total or partial exemption from the test may be granted when the complete history (nature and quantity of the pesticides used, date of each treatment during cultivation and after harvest) of the treatment of the batch is known and can be checked precisely according to good agricultural and collection practice (GACP).

Substance	Limit(mg/kg)
Aceohate	0.1
Alachlor	0.05
Aldrin and dieldrin (sum of)	0.05
Azinphos-ethyl	0.1
Azinphos-methyl	1
Bromide, inorganic (calculated as bromide ion)	50
Bromophos-ethyl	0.05
Bromophos-methyl	0.05
Bromopropylate	3
Chlordane (sum of *cis*-, *trans*-, and oxychlordane)	0.05
Chlorfenvinphos	0.5
Chlorpyriphos-ethyl	0.2
Chlorpyriphos-methyl	0.1
Chlorthal-dimethyl	0.01
Cyfluthrin (sum of)	0.1
λ-Cyhalothrin	1
Cypermethrin and isomers (sum of)	1
DDT (sum of *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDT, *p,p'*-DDT, *o,p'*-TDE, and *p,p'*-TDE)	1
Deltamethrin	0.5
Diazinon	0.5
Dichlorofluanid	0.1
Dichlorovos	1
Dicofol	0.5
Dimethoate and omethoate (sum of)	0.1
Dithiocarbamates (expressed as CS_2)	2

Substance	Limit(mg/kg)
Endosulfan (sum of isomers and endosulfan sulphate)	3
Endrin	0.05
Ethion	2
Etrimphos	0.05
Fenchlorophos (sum of fenchlorophos and fenchlorophos-oxon)	0.1
Fenitrothion	0.5
Fenpropathrin	0.03
Fensulfothion(sum of fensulfothion, fensulfothion-oxon, fensulfothion-oxonsulfon, and fensulfothion-sulfon)	0.05
Fenthion (sum of fenthion, fenthion-oxon, fenthion-oxon-sulfon, fenthion-oxon-sulfoxid, fenthion-sulfon, and fenthion-sulfoxid)	0.05
Fenvalerate	1.5
Flucytrinate	0.05
τ-Fluvalinate	0.05
Fonophos	0.05
Heptachlor (sum of heptachlor, cis-heptachloroepoxide, and trans-heptachloroepoxide)	0.05
Hexachlorobenzene	0.1
Hexachlorocyclohexane (sum of isomers α-, β-, δ-, and ε-)	0.3
Lindan (γ-hexachlorocyclohexane)	0.6
Malathion and malaoxon (sum of)	1
Mecarbam	0.05
Methacriphos	0.05
Methamidophos	0.05
Methidathion	0.2
Methoxychlor	0.05
Mirex	0.01
Monocrotophos	0.1
Parathion-ethyl and Paraoxon-ethyl (sum of)	0.5
Parathion-methyl and Paraoxon-methyl (sum of)	0.2
Pendimethalin	0.1
Pentachloranisol	0.01
Permethrin and isomers(sum of)	1
Phosalone	0.1

Substance	Limit(mg/kg)
Phosmet	0.05
Piperonyl butoxide	3
Pirimiphos-ethyl	0.05
Pirimiphos-methyl (sum os pirimiphos-methyl and N-desethyl-pirimiphos-methyl)	4
Procymidone	0.1
Profenophos	0.1
Prithiophos	0.05
Pyrethrum (sum of cinerin I, cinerin II, jasmolin I, jasmolin II, pyrethrin I, and pyrethrin II)	3
Quinalphos	0.05
Quintozene (sum of quintozene, pentachloraniline, and methyl pentachlorphenyl sulfide)	1
S-421	0.02
Tecnazene	0.05
Tetradifon	0.3
Vinclozolin	0.4

Table 6. Limits for pesticide residues (USP36–NF31).

4.2.2.2.2. Qualitative and quantitative analysis of pesticide residues

Use validated analytical procedures (e.g., FDA Pesticide Analytical Manual (PAM) [http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006955.htm], or other analytical procedures validated in accordance with EU guideline [Document No. SANCO/10684/2009, http://ec.europa.eu/food/plant/protection/resources/qualcontrol_en.pdf] or the USP Validation of Compendial Procedures <1225>) that satisfy the following criteria:

- the method, especially with respect to its purification steps, is suitable for the combination of pesticide residue and substance under test, and is not susceptible to interference from co-extractives;

- the limits of detection and quantification for each pesticide matrix combination to be analyzed;

- the method is shown to recover between 70% and 110% of each pesticide;

- the repeatability and reproducibility of the method are NLT the appropriate values indicated in Table 7; and

- the concentrations of test and reference solutions and the setting of the apparatus are such that a linear response in obtained from the analytical detector.

Concentration Range of the Pesticide (mg/kg)	Repeatability(RSD) (%)	Reproducibility(RSD) (%)
0.001-0.01	30	60
>0.01-0.1	20	40
>0.1-1	15	30
>1	10	20

Table 7. Repeatibility and reproducibility limits of determination of pesticides (USP36–NF31).

4.2.2.2.3. Test for pesticides

Unless otherwise specified in the individual monograph, the following methods may be used for the analysis of pesticides. Depending on the substance being examined, it may be necessary to modify, sometimes extensively, the procedure described hereafter. Additionally, it may be necessary to perform another method with another (e.g. mass spectrometry), or a different method (e.g. immunochemical method) to confirm the results.

Description of qualitative and quantitative determination of organochlorine, organophosphorus and pyrethroid insecticides is divided into three sections:

- Extraction;

- Purification; and

- Quantitative analysis.

4.2.2.2.4. Extraction

To 10 g of the coarsely powdered substance under test add 100 ml of acetone, and allow to stand for 20 min. add 1 ml of a solution in toluene containing 1.8 µg of carbophenothion per ml. Mix in a high-speed blender for 3 min. Filter this solution, and wash the residue with two 25-ml portions of acetone. Combine the filtrate and the washings, and heat, in a rotary evaporator, maintaining the temperature of the bath below 40° until the solvent has almost completely removed. Dissolve the residue in 8 ml of toluene. Pass through a membrane filter of 45 µm pore size, rinse the flask and the filter with toluene, dilute with toluene to 10 ml (*Solution A*), and mix. [NOTE – Use the above procedure for the analysis of samples of articles having a water content of less than 15%. Samples having a higher water content may be dried, provided that the drying procedure does not significantly affect the pesticide content.]

4.2.2.2.5. Purification

4.2.2.2.5.1. Organochlorine, organophosphorus, and pyrethroid

Purification is to be accomplished by means of size-exclusion chromatography. The size-exclusion chromatograph is equipped with a 7.8 mm x 30 cm stainless steel column containing 5 µm packing L21. Toluene is used as the mobile phase at a flow rate of about 1 ml/min.

Performance of the Column – Inject 100 µl of a solution in toluene containing, in each ml, 0.5 mg of methyl red and 0.5 mg of oracet blue or equivalent. The column is not suitable unless the color of the eluate changes from orange to blue at an elution volume of about 10.3 ml. If necessary, calibrate the column, using a solution in toluene containing suitable concentrations of the pesticide of interest having the lowest molecular weight (for example, dichlorvos) and that having the highest molecular weight (for example, deltamethrin). Determine which fraction of the eluate contains both pesticides.

Purification of the Test Solution – Inject a suitable volume (100 to 500 µl) of *Solution A* into the chromatograph. Collect the fraction (*Solution B*) as determined above under *Performance of the Column*. Organophosphorus pesticide elute between 8.8 and 10.9 ml. Organochlorine and pyrethroid pesticides elute between 8.5 and 10.3 ml.

Organochlorine and Pyrethroid Insecticides – Into a 5 mm x 10 cm chromatographic column, introduce a piece of fat-free cotton and 0.5 g of silica gel treated as follows. Heat chromatographic silica gel in oven 150° for at least 4 h. Allow to cool, and add dropwise a quantity of water corresponding to 1.5% of the weight of silica gel used. Shake vigorously until agglomerates have disappeared, and continue shaking by mechanical means for 2 h. Condition the column with 1.5 ml of hexane. [NOTE – Prepacked columns containing about 0.50 g of suitable silica gel may also be used, provided they have been previously validated.] Concentrate *Solution B* almost to dryness, with the aid of a stream of helium or oxygen-free nitrogen, and dilute with toluene to a suitable volume (200 µl to 1 ml, according to the volume injected in the preparation of *Solution B*). Quantitatively transfer this solution to the column, and proceed with the chromatography, using 1.8 ml of toluene as the mobile phase. Collect the eluate (*Solution C*).

4.2.2.2.6. *Quantitative analysis*

4.2.2.2.6.1. *Quantitative analysis of organophosphorus insecticides*

Test Solution – Concentrate *Solution B* almost to dryness, with the aid of a stream of helium, dilute with toluene to 100 µl, and mix.

Standard Solution – Prepare at least three solutions in toluene containing each of the pesticides of interest and carbophenothion at concentrations suitable for plotting a calibration curve.

Chromatographic System-The gas chromatograph is equipped with an alkali flame-ionization detector or flame photometric detector and a 0.32 mm x 30 m fused silica column coated with a 0.25 µm layer of phase G1. Hydrogen is used as the carrier gas. Other gases, such as helium or nitrogen, may also be used. The injection port temperature is maintained at 250°, and the detector is maintained at 275°. The column temperature is maintained at 80° for 1 min, then increased to 150° at a rate of 30°/min, maintained at 150° for 3 min, then increased to 280° at a rate of 4°/min, and maintained at this temperature for 1 min. Use carbophenothion as the internal standard. [NOTE – If necessary, use a second internal standard to identify any possible interference with the peak corresponding to carbophenothion.] inject the chosen volume of each solution, record the chromatograms, and measure the peak responses. Calculate the content of each pesticide from the peak areas and the concentrations of the solution.

4.2.2.2.6.2. Quantitative analysis organochlorine and pyrethroid insecticides

Test solution – Concentrate Solution C almost to dryness, with the aid of a stream of helium or oxygen-free nitrogen, dilute with toluene to 500 μl, and mix.

Standard Solution – Prepare at least three solutions in toluene containing each of the pesticides of interest and carbophenothion at concentrations suitable for plotting a calibration curve.

Chromatographic System – The gas chromatograph is equipped with an electron-capture detector, a device allowing direct on-column cold injection, and a 0.32 mm x 30 m fused silica column coated with a 0.25 μm layer of phase G1. Hydrogen is used as the carrier gas. Other gases, such as helium or nitrogen, may also be used. The injection port temperature is maintained at 275°, and the detector is maintained at 300°. The column temperature is maintained at 80° for 1 min, then increased to 150° at a rate of 30°/min, maintained at 150° for 3 min, then increased to 280° at a rate of 4°/min, and maintained at this temperature for 1 min. Use carbophenothion as the internal standard. [NOTE – If necessary, use a second internal standard to identify any possible interference with the peak corresponding to carbophenothion.] Inject the chosen volume of each solution, record the chromatograms, and measure the peak responses. Calculate the content of each pesticide from the peak areas and the concentrations of the solutions.

Acknowledgements

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