PhD Thesis

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Thesis submitted in accordance with the requirements of the Medical University of Lodz for the degree of Doctor of Philosophy in Pharmaceutical Science by:

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Assessment of Potential Impact of Cherry Flavor on Stability of Montelukast Tablets

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I owe you all!

Mariusz Staśkiewicz

To my parents

deceased but alive

to whom almost 20 years ago I made a promise

which this dissertation eventually fulfills

R.I.P.

Table of Contents:

1. Introduction 1
2. Oxidation of sulfides to sulfoxides and sulfoxides to sulfones 7
3. Antioxidant and prooxidant properties of phenolics 9
   3.1. Phenolics 9
   3.2. Flavonoids 12
   3.3. Phenolics as antioxidants 12
      3.3.1. Direct scavenging of ROS 13
      3.3.2. Metal chelating activity 15
   3.4. Prooxidant activity of flavonoids through oxidation by phenoxyl radicals 16
   3.5. Mechanism Preventing Montelukast's Oxidation to Sulfoxide Impurity 18
   3.6. Determination of Total Phenolic Content (TPC) 19
   3.7. Determination of Total Flavonoid Content (TFC) 19
   3.8. Determination of Antioxidative Capacity (AC) 20
   3.9. Conclusions on pro- and antioxidant properties of flavonoids 22
4. Aim of the doctoral thesis 23
5. Materials and Methods 25
   5.1. Related substances by HPLC 25
   5.2. HPLC method for flavonoids (screening analysis) 29
   5.3. HPLC – MS method for identification of flavonoids in Cherry flavor 31
   5.4. Determination of TPC by Folin-Ciocalteu assay 32
   5.5. Determination of TFC by the aluminum chloride colorimetric assay 32
   5.6. Determination of Antioxidative Capacity by modified Brand-Wiliams method 33
   5.7. Water content by Karl Fischer and moisture content by LOD 34
6. Results and discussion 35
   6.1. Analytical assessment of Cherry flavor 35
      6.1.1. Preliminary HPLC screening analysis for flavonoids 35
      6.1.2. HPLC – MS method for identification of flavonoids in Cherry flavor 39
      6.1.3. Determination of TPC, TFC and AC in Cherry flavor 42
   6.2. Physical formulation mixtures 44
      6.2.1. Degradation study using various amounts of Cherry flavor, Quercetin and Ascorbic acid 44
      6.2.2. In-situ degradation during sample preparation for analytical RS testing 48
      6.2.3. MTK2 to Montelukast conversion/degradation induced by antioxidants 50

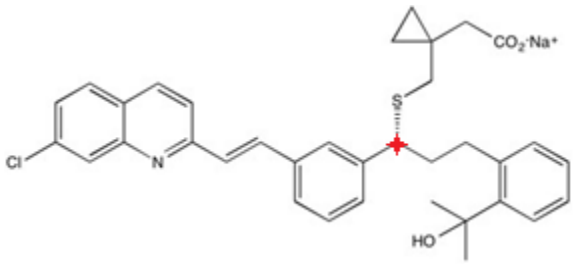
6.2.4. Excipients compatibility 51
6.2.4.1. Excipients compatibility using API and each excipient 51
6.2.4.2. Excipients compatibility using mixtures of API, Cellulose microcrystalline and various amounts of Cherry flavor 53
6.3. Stability assessment of drug substance and registered formulation containing various amounts of Cherry flavor 55
6.3.1. Short term stability assessment applying accelerated stability conditions (40°C/75% RH) – Drug Substance 56
6.3.2. Short term stability assessment applying accelerated stability conditions (40°C/75% RH) – Drug Product 57
6.3.3. Long Term stability assessment applying intermediate stability conditions (30°C/65% RH) – Drug Substance 61
6.3.4. Long Term stability assessment applying normal (Zone II) stability conditions (25°C/60% RH) – Drug Product 64
6.3.5. Water content in investigated formulations 67
7. Final conclusions 68
8. Literature 71
9. List of tables 78
10. List of figures 80
11. List of abbreviations 82
12. Summary of the Thesis 83
13. Streszczenie 84
14. Published Papers 85

1. Introduction

Montelukast Sodium, (Monte (2-[1-[(R)-[3-[2(E)-(7-chloroquinolin-2-yl) vinyl] phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]propyl-sulfanylmethyl]-cyclo-propyl] acetic acid sodium salt)), belongs to leukotriene receptor antagonist family of medications and is used for a number of conditions including asthma, exercise induced bronchospasm, allergic rhinitis, and urticaria [1] [2]. Recently, Montelukast has also been considered as potential drug for new indications such as epilepsy [3] or COVID-19 [4]. This drug is manufactured in the form of tablets, capsules, orally disintegrating tablets (ODT) or chewable tablets. Other pharmaceutical forms are under research and/or development, e.g. aerosolizable modified-release particles [5].

Having one asymmetric center Montelukast can form two stereoisomers of which only one, R, is pharmaceutically active. The structure of Montelukast Sodium, with asymmetric carbon marked with red cross, is presented on Figure 1-1.

Figure 1-1 Structure of Montelukast sodium



Adamed Pharma manufactures three technologically different drug products containing Montelukast (as Sodium) in the form of 5 mg and 10 mg tablets. The drug products are in the form of both uncoated and coated tablets. These formulations exhibit different behavior when subject to stability seasoning at routine, ongoing stability program at 25°C/60% RH.

Formulation details are summarized in Table 1-1 (p. 2), Table 1-2 (p. 2) and Table 1-3 (p. 3).

Note: the actual amounts of all excipients, except for the one under investigation, are not provided to protect intellectual property. This does not affect overall study design, investigation and results. Qualitative composition is not subject to intellectual protection as this information is required by current EU regulations and must be provided on the leaflet.

Table 1-1 Formulation details of Montelukast 10 mg, film-coated tablets

Component	**Quantity (mg/tablet)**	**Function in formulation**
Montelukast Sodium Montelukast free acid	10.38 10.00	Active substance
Cellulose microcrystalline	-	Diluent/binder
Lactose Monohydrate	-	Diluent
Crospovidone	-	Disintegrant
Croscarmellose sodium	-	Colorant
Hydroxypropylcellulose	-	Binder
Magnesium stearate	-	Lubricant
Water, purified	-	Solvent
Total core	200	-
Film coating		
Film-coating system	-	Film coat
Water, purified	-	Solvent
Total film-coated tablet	205.6	-

Table 1-2 Formulation details of Montelukast 5 mg, uncoated tablets (with ethylenediaminetetraacetic acid disodium salt, EDTA)

Component	**Quantity (mg/tablet)**	**Function in formulation**
Montelukast Sodium Montelukast free acid	5.19 5.00	Active substance
Cellulose microcrystalline	-	Diluent
Mannitol	-	Diluent
Crospovidone	-	Disintegrant
Iron oxide red	-	Colorant
Hydroxypropylcellulose	-	Binder
Disodium edetate	-	Chelating Agent
Cherry flavor	2.00	Flavor/Antioxidant
Aspartame	-	Sweetener
Talc		Glidant
Magnesium stearate	-	Lubricant
Water, purified	-	Granulating Fluid
Total	300	-

Table 1-3 Formulation details of Montelukast 5 mg, uncoated tablets (without EDTA, the least stable formulation)

Component	**Quantity (mg/tablet)**	**Function in formulation**
Montelukast Sodium Montelukast free acid	5.19 5.00	Active substance
Mannitol	-	Diluent
Cellulose microcrystalline	-	Diluent
Croscarmellose sodium	-	Disintegrant
Iron oxide red	-	Colorant
Hydroxypropylcellulose	-	Binder
Cherry flavor	0.99	Flavor/Antioxidant
Aspartame	-	Sweetener
Magnesium stearate	-	Lubricant
Water, purified	-	Solvent
Total	300	-

Montelukast formulations are known for their stability issues related to active's oxidation to Sulfoxide impurity. Based on the structure two enantiomers of sulfoxide impurity can be formed, refer to Figure 1-2, and the sum of both is determined due to lack of specificity of Related Substances analytical method (nonchiral separation). Sulfoxide Impurity can be commercially provided by European Medicinal Agency (EMA) as EP Imp C.

As the structure of Monteukast's Sulfoxide impurity is classified as sulfoxide the oxidative number of Sulfur is 0 compared to -2 in case of Montelukast. The oxidative states of some most popular organic compounds are listed in Table 1-4 (p. 4).

Figure 1-2 Structure of Sulfoxide Imp (MTK 2) – two (trans) enantiomers

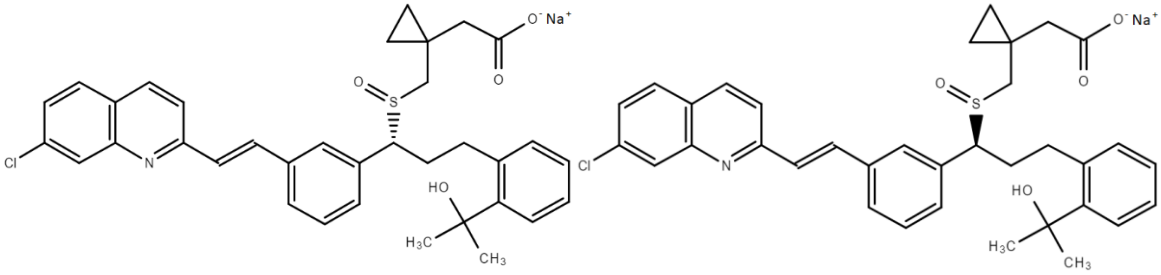


*Table 1-4 Oxidative states of Sulfur in most popular organic compounds including Montelukast API (Sulfide) and its Sulfoxide impurity**

-2	-1	0	+2	+4	+6
H_2S R–S–H thiols R–S–R sulfides R–S(⊕)(R)–R sulfonium ions	R–S–S–R disulfides	S elemental R–S(=O)–R sulfoxides R–S–OH sulfenic acids	R–S(=O)(=O)–R sulfones R–S(=O)–OH sulfinic acids	SO_2 R–S(=O)(=O)–OH sulfonic acids R-O–S(=O)–O-R sulfite esters	SO_3 R-O–S(=O)(=O)–O-R sulfate esters

* Website of the day 06/01/2022
https://chem.libretexts.org/Bookshelves/Organic_Chemistry/Book%3A_Virtual_Textbook_of_OChem_%28Reusch%29_UNDER_CONSTRUCTION/14%3A_Thiols_and_Sulfides

Complex Drug Product (DP) and Dug Substance (DS) stability assessment was carried out by S. H. Tivaria et al. [6]. Montelukast sodium is prone to oxidation reactions owing sensitive moieties in its structure. It is also known to be light sensitive. The study carried out by S. H. Tivaria et al. was aimed to understand the degradation behavior of the drug in different oxidative media containing hydrogen peroxide, AIBN, Fe3+, Fenton's reagent and O_2 environment under normal laboratory light conditions. The degradation behavior of the drug was also evaluated in solid state under ICH recommended accelerated stability condition of 40°C/75% RH to correlate with the degradation products formed in a solid oral formulation. A total of nine degradation products were formed from both the drug substance and the marketed tablet formulation on storage under controlled oxygen environment in normal laboratory light and temperature conditions, Figure 1-3. Trans-Sulfoxide impurity was identified as MTK 2 and cis-Sulfoxide was identified as MTK 1. For structural difference between the two diastereoisomers refer to Figure 1-4 (p. 5).

Figure 1-3 Main oxidation degradants of Montelukast as reported by S. H. Tivaria et al. [6]

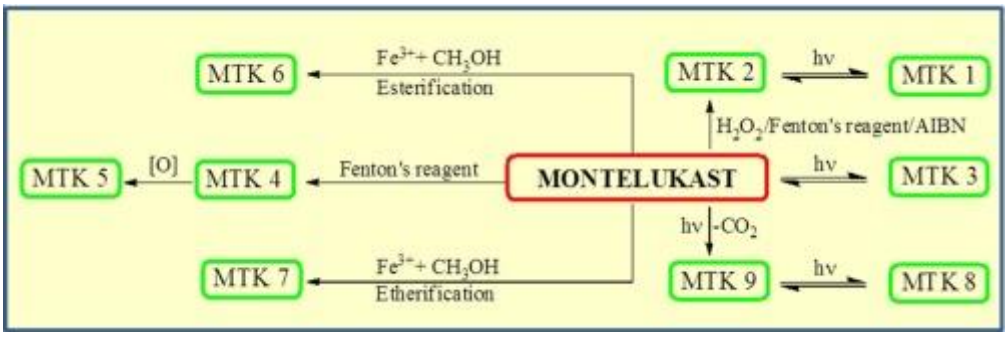
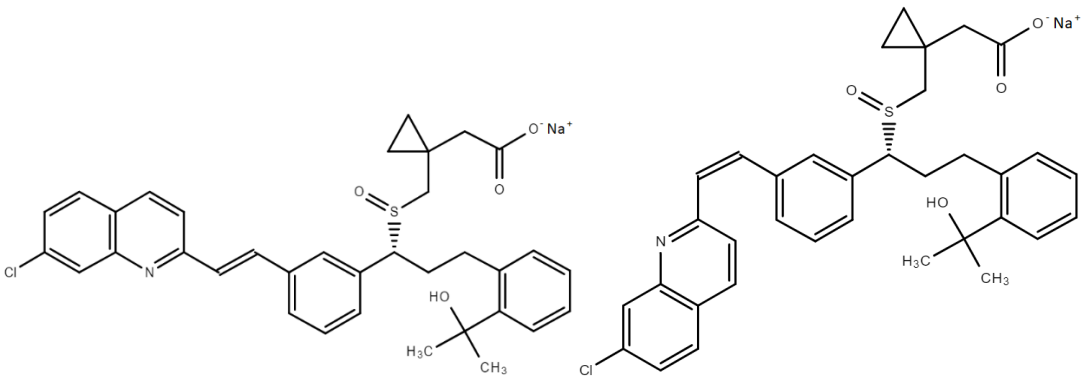


Figure 1-4 Structure of Montelukast Sulfoxide – two diastereoisomers, - trans (MTK2) and – cis (MTK1)



Numerous *in vivo* studies show Sulfoxide impurity is also Montelukast's metabolite ([7], [8], [9]). Sulfoxide impurity is dose-dependent cytotoxic in human peripheral lymphocytes, however, it was found to be non-genotoxic. Leadscope (version 1.7.3) and ToxTree (version 2.6.6.) programs predicted sulfoxide impurity as non-mutagenic, it was also found to be non-mutagenic in Ames MPF Penta I assay (using Ames MPF™ Penta I kit, Microplate Format Mutagenicity Assay; Xenometrix, Switzerland) and can be classified as ordinary impurity according to guidelines [9].

No toxicological data is available on sulfoxide impurity which could potentially support its higher than 0.5% specification limit. However, based on current regulations, human metabolites that can raise a safety concern are those formed at greater than 10 percent of total drug-related exposure at steady state. As sulfoxide impurity is considered minor metabolite with less than 2% conversion ratio [7], specification limit set at 1.5 % could be potentially considered as scientifically justified during regulatory variation process of extending current related substances specification limit.

As expected Adamed's formulations also exhibit different degradation profiles and rates, refer to Table 1-5 (p. 6), showing variable concentrations of investigated Sulfoxide impurity, which is reflected in different Specification limits for Related Substances, refer to Table 1-6 (p. 6).

Table 1-5 Stability (expressed as concentration of Sulfoxide impurity) of three montelukast formulation at 25 /60% RH, 24 months

Formulation	Batch no.	Timepoint (months)						
		0	3	6	9	12	18	24
10 mg	00550413	0.15	0.18	0.23	0.26	0.27	0.32	0.35
	00560413	0.14	0.22	0.23	0.28	0.31	0.37	0.41
5 mg (with EDTA)	41660555	0.33	-*	0.47	-*	0.51	0.49	0.58
	41751487	0.34	-*	-*	-*	0.43	0.43	0.56
5 mg (no EDTA)	**01090413**	**0.20**	**0.41**	**0.47**	**0.60**	**0.63**	**0.71**	**0.79**
	01120413	**0.17**	**0.48**	**0.60**	**0.76**	**0.81**	**0.94**	**1.09**

* Results are not reported as reduced testing was applied (matrix/bracketing)

Table 1-6 Release specification limits for Sulfoxide impurity and Total impurities, Related Substances, for Montelukast 5 mg (with and without EDTA) and Montelukast 10 mg tablets formulations

	5 mg formulation without EDTA, uncoated	5 mg formulation with EDTA, uncoated	10 mg formulation (Coated)
Sulfoxide impurity	≤ 1.2%	≤ 1.0%	≤ 0.5%
Total impurities	≤ 2.0%	≤ 2.0%	≤ 1.0%

As seen in the above table tablet coating significantly decreases the level of Sulfoxide impurity and total impurities which is in line with previous research carried out and published inter alia by Mahmoud M. Al Omari [10].

The most problematic formulation is the one for 5 mg strength, without EDTA. Compared to the formulation with EDTA the amount of Cherry flavor is different: 0.66% w/w for the one including EDTA and 0,33% w/w for the one without EDTA. Comparing the stability data, refer to Table 1-5, and formulation details, refer to Table 1-1 (p. 2), Table 1-2 (p. 2) and Table 1-3 (p. 3), and Specifications, refer to Table 1-6, a hypothesis that amount of Cherry flavor affects stability behavior of Montelukast 5 mg tablets was formed.

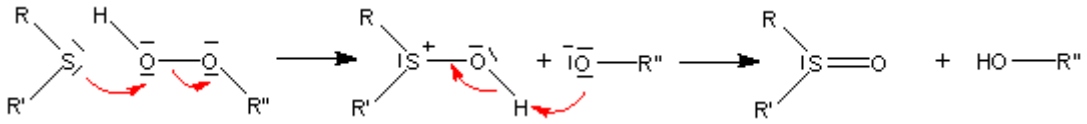
Potential influence of EDTA and/or Iron Oxide Red (Fe_2O_3) and their antioxidative impact on the formulations although also considered has not been subject of this research.

2. Oxidation of sulfides to sulfoxides and sulfoxides to sulfones

The degradation path described in this section is observed in case of all drug substances classified as sulfides including not only Montelukast but also some popular actives as rabeprazole, omeprazole, fluphenazine, promethazine, albendazole, therefore, the potential excipients related issues can be approached applying similar problem-solving techniques.

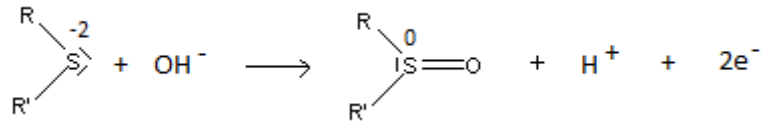
The mechanism by which the Sulfoxide impurity is formed is presented in Figure 2-1.

Figure 2-1 Mechanism of reaction by which Montelukast Sulfoxide impurity is formed



The same reaction can be presented as redox equations as follows:

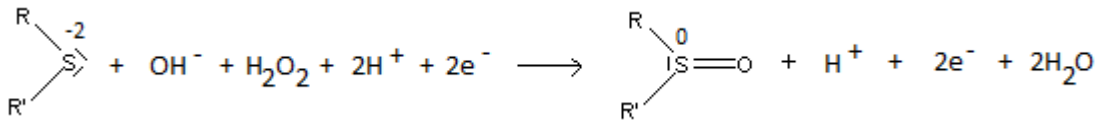
Oxidation half-reaction:

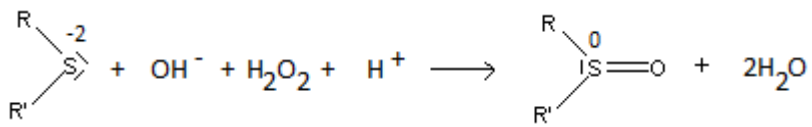


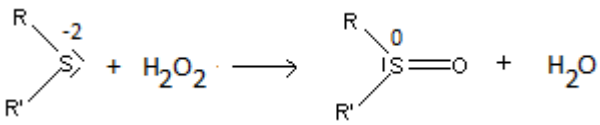
Reduction half-reaction:

$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O$

Summing up both half-reactions result in the following:



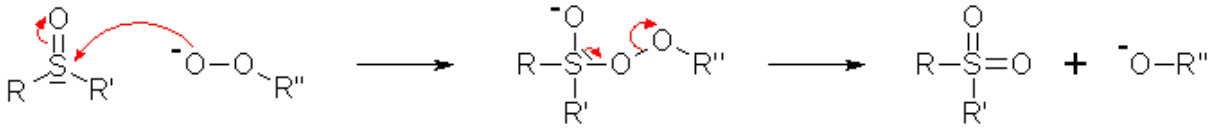




The above reaction is an example of common oxidation of sulfide to sulfoxide type of reaction. In the first step of the reaction, the sulfur atom attacks the terminal oxygen of the peroxide group. Breakage of the peroxide bond results in the formation of an alkoxy anion and a sulfoxide protonated at the oxygen atom. Proton exchange yields the sulfoxide and the alcohol corresponding to the peroxide. If hydrogen peroxide triggers the reaction the product is water which may also trigger other degradation paths and affect physicochemical properties of the tablets (e.g. hardness and dissolution).

In acidic or neutral conditions, the oxidation follows the same mechanism as that described for the oxidation of sulfide to sulfoxide leading to further oxidation of sulfoxide to sulfone. In basic solution, however, a nucleophilic attack of the peroxide anion takes place at the sulfur atom, refer to Figure 2-2.

Figure 2-2 Mechanism of reaction by which Montelukast Sulfone impurity is formed



As this oxidation is slower than the oxidation of sulfides the concentration Montelukast Sulfone impurity is often omitted in the drug product specification.

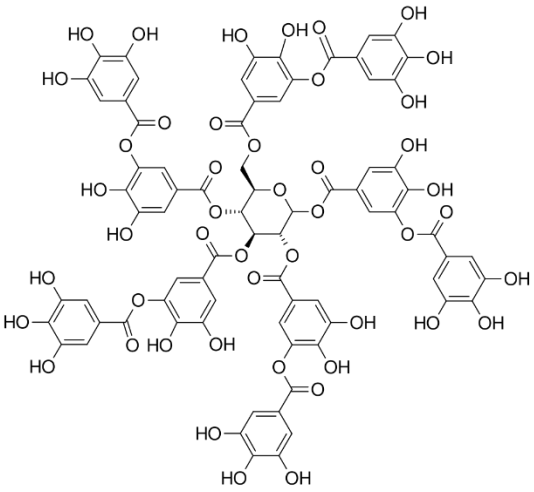
In the light of the above potential analytical issues should be also discussed. If samples are prepared in either basic, neutral or acidic solution in-situ formation of Sulfoxide and conversion of Sulfoxide to Sulfone impurity may take place, especially in the presence of reagents or matrix containing residual peroxides. As long as the concentration of Sulfone formed is kept below the identification threshold (specification limit for the unknown impurity) the situation does not require further action. In case the level exceeds the limit the usage of better quality reagents or even analytical method redevelopment should be considered.

3. Antioxidant and prooxidant properties of phenolics

3.1. Phenolics

In organic chemistry, phenols, sometimes called phenolics, are a class of chemical compounds consisting of one or more hydroxyl groups (- OH) bonded directly to an aromatic hydrocarbon group. The simplest is phenol, C_6H_5OH. Phenolic compounds are classified as simple phenols or polyphenols based on the number of phenol units in the molecule. Polyphenol may form complex molecules, a good example of such a group of compounds is Tannic acid which is a mixture of polygalloyl glucoses or polygalloyl quinic acid esters with the number of galloyl moieties per molecule ranging from 2 up to 12 depending on the plant source used to extract the tannic acid. Example of molecule containing 10 galloyl moieties is presented on Figure 3-1.

Figure 3-1 Tannic acid containing 10 galloyl groups

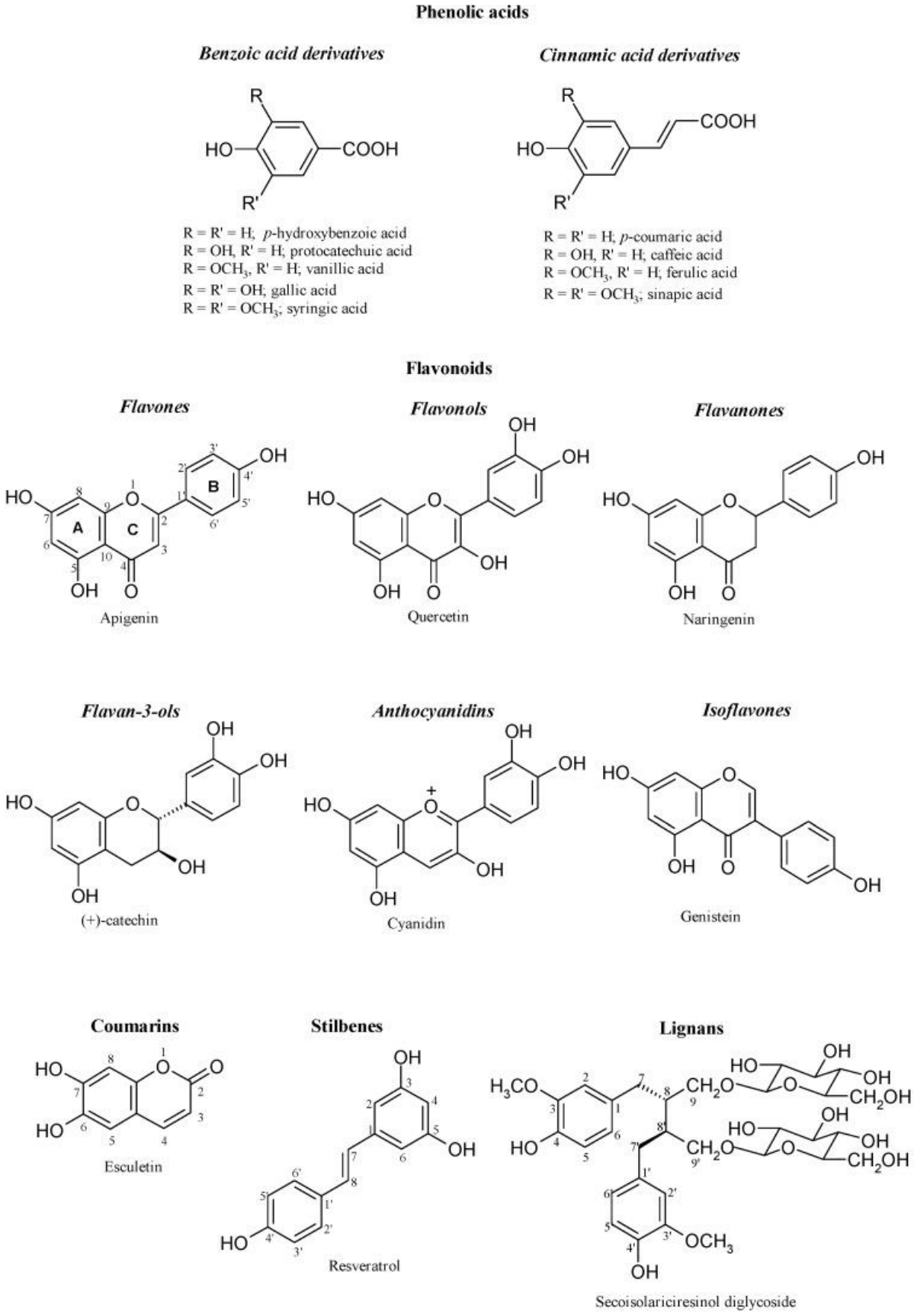


In general, phenolics are able to act as antioxidants in a number of various ways [11] [12] [13]. Phenolic hydroxyl groups are good hydrogen donors: hydrogen-donating antioxidants can react with reactive oxygen and reactive nitrogen species in a termination reaction, which breaks the cycle of generation of new radicals. Following interaction with the initial reactive species, a radical form of the antioxidant is produced, having a much greater chemical stability than the initial radical. The interaction of the hydroxyl groups of phenolics with the π-electrons of the

benzene ring gives the molecules special properties, most notably the ability to generate free radicals where the radical is stabilized by delocalization. The formation of these relatively long-lived radicals is able to modify radical-mediated oxidation processes. The antioxidant capacity of phenolic compounds is also attributed to their ability to chelate metal ions involved in the production of free radicals. However, phenolics can act as pro-oxidants by chelating metals in a manner that maintains or increases their catalytic activity or by reducing metals, thus increasing their ability to form free radicals. Phenolic structures often have the potential to strongly interact with proteins, due to their hydrophobic benzenoid rings and hydrogen-bonding potential of the phenolic hydroxyl groups. This gives phenolics the ability to act as antioxidants also by virtue of their capacity to inhibit some enzymes involved in radical generation, such as various cytochrome P450 isoforms, lipoxygenases, cyclooxygenase and xanthine oxidase.

Main classes of phenolics and flavonoids discussed in Section 3.2 are shown in Figure 3-2 (p. 11) [14].

Figure 3-2 Main classes of phenolics [14].

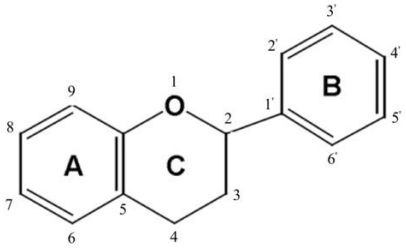


3.2. Flavonoids

Flavonoids (the term is derived from the Latin word “flavus”, meaning yellow) belong to larger class of compounds known as phenolics, i.e. compounds built of at least one phenol unit. Based on their chemical structure, phenolic compounds can be divided into different subgroups, such as phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids. These are colored compounds due to red, blue, and purple anthocyanin pigments of plant tissues [15].

All Flavonoids share the same basic chemical structure of a common three-ring moiety (A-, C- and B-rings) with 15 carbon atoms (C6-C3-C6) (Figure 3-3). The substitution of a functional group of the heterocyclic ring (C-ring) with a methyl, hydroxyl, glycan, acetyl or other group, along with the C-ring oxidation state determines the classification of various subclasses of flavonoids.

Figure 3-3 Chemical core structure of Flavonoids



Over 5000 naturally occurring flavonoids have been characterized from various plants. They have been classified according to their chemical structure, and are subdivided into subgroups [16].

3.3. Phenolics as antioxidants

The antioxidant capacities of many phenolics including flavonoids are much stronger than those of Ascorbic acid [11] and they can prevent injury caused by free radicals, both in vivo and in vitro, by the following mechanisms [12]:

(1) direct scavenging of Reactive Oxygen Species (ROS) [17],

(2) activation of antioxidant enzymes [13],

(3) metal chelating activity [18],

(4) reduction of α-tocopheryl radicals [19], [20],

(5) inhibition of oxidases [20] , [21],

(6) mitigation of oxidative stress caused by nitric oxide [22],

(7) increase in uric acid levels [23],

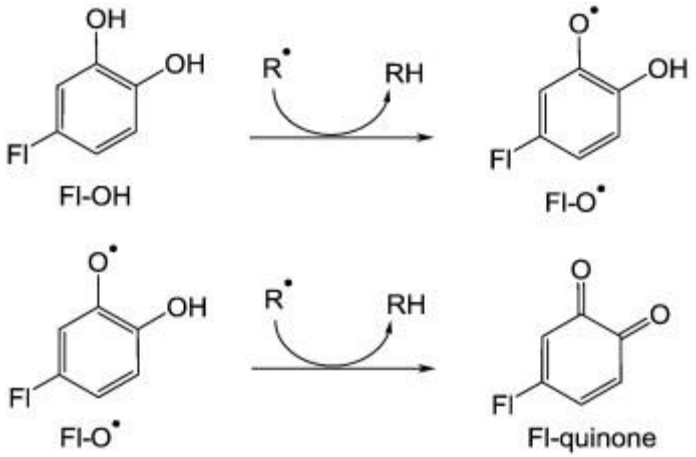
(8) increase in antioxidant properties of low molecular antioxidants [24].

As not all the mechanisms are relevant from the perspective of interaction being subject of this thesis, only (1) and (3), i.e. *in vitro* related, will be furtherly discussed.

3.3.1. Direct scavenging of ROS

Phenolics, including flavonoids are able to scavenge free radicals directly by hydrogen atom donation. Radicals are made inactive according to the following equation, where R**.** is a free radical and Fl-O**.** is a flavonoid phenoxyl radical, Figure 3-4.

Figure 3-4 Scavenging of reactive oxygen species (R) by flavonoid. The free radical Fl-O may react with a second radical, acquiring a stable quinone structure [25]



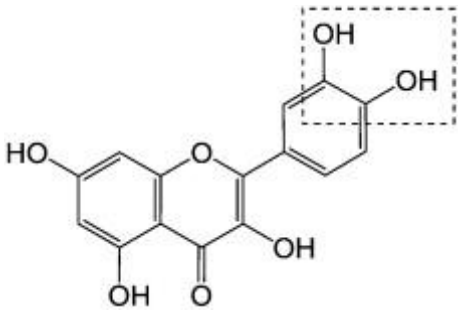
The *in vitro* phenolics antioxidant activity depends on the arrangement of functional groups on its core structure. Both the configuration and total number of hydroxyl groups substantially influence the mechanism of the antioxidant activity [20]. The B ring hydroxyl configuration is the most significant determinant of ROS scavenging [26], whereas substitution of the rings A and C has little impact on superoxide anion radical scavenging rate constants [27], [28]. The *in*

vitro antioxidant activity could be increased by polymerization of flavonoid monomers, e.g. proanthocyanidins (also known as condensed tannins), the polymers of catechins, are excellent *in vitro* antioxidants due to the high number of hydroxyl groups in their molecules. The antioxidant capacity of proanthocyanidins depends on their oligomer chain length and the type of ROS with which they react [29]. The glycosylation of flavonoids reduces their *in vitro* antioxidant activity when compared to the corresponding aglycons [30], [31], [32], [33], [34]. Comparison of Trolox equivalent antioxidant capacity (TEAC) values of quercetin (4.42 mM) and rutin (2.02 mM), quercetin-3-*O*-rutinoside, shows that glycosylation of the 3-OH group has strongly suppressive effect on the antioxidant activity [30]. Similar results were observed also for other pairs of flavonoid aglycon and glycoside (e.g. hesperetin–hesperidin, luteolin–luteolin 4′-glucoside; luteolin–luteolin 7-glucoside; baicalein–baicalin; and quercetin–quercitrin) [30], [31]. Quercetin glycosylation also significantly reduced its superoxide scavenging ability [35], hypochlorite scavenging activity [36] and power to reduce Fe(III) to Fe(II) (determined by Ferric Antioxidant Power (FRAP) assay) [37].

The main structural features of flavonoids required for efficient radical scavenging could be summarized as follows [17], [38]:

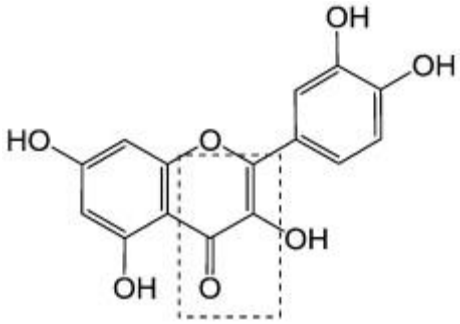
a) an *ortho*-dihydroxy (catechol) structure in the B ring, for electron delocalization, Figure 3-5:

Figure 3-5 An ortho-dihydroxy (catechol) structure in the B ring [39]



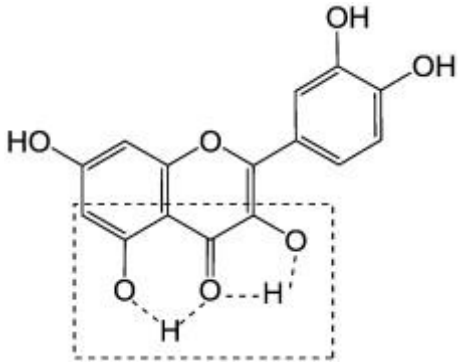
b) 2,3-double bond in conjugation with a 4-oxo function in the C ring provides electron delocalization from the B ring, Figure 3-6 (p. 15):

Figure 3-6 2,3-Double bond in conjugation with a 4-oxo function in the C ring [39]



c) hydroxyl groups at positions 3 and 5 provide hydrogen bonding to the oxo group, Figure 3-7:

Figure 3-7 Hydroxyl groups at positions 3 and 5 [39]

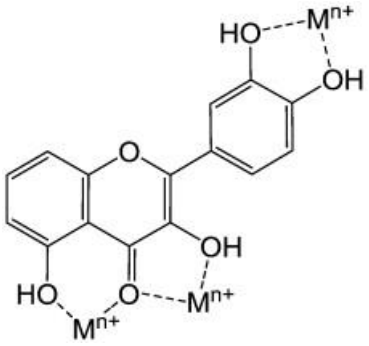


According to the previously stated criteria, flavonols quercetin and myricetin should be the most effective radical scavengers in the aqueous phase, which has been confirmed experimentally [30].

3.3.2. Metal chelating activity

Some flavonoids are known to chelate iron and copper by which they remove a causal factor for the development of free radicals. Quercetin was able to prevent oxidative injury induced in the erythrocyte membrane by a number of oxidizing agents (e.g. acrolein and phenylhydrazine), which cause release of iron in its free, redox active form [18]. Pietta [25] proposed that the binding sites for trace metals in the molecule of flavonoids are the catechol moiety in the ring B, the 3-hydroxyl and 4-oxo groups in the heterocyclic ring C, and the 4-oxo and 5-hydroxyl groups between the C and A rings (Figure 3-8 p. 16).

Figure 3-8 Binding sites for trace metals [38]



The catechol moiety in the B ring has been shown to be important for Cu^{2+}- chelate formation and thus being the major contributory site of the metal chelation [40]. Quercetin, in particular, is known for its iron-chelating and iron-stabilizing properties. Morin and quercetin were shown to form complexes with Cd(II) and exhibit strong antioxidant activity in the *in vitro* studies. Their sulfonic water-soluble derivatives exert low toxicity and therefore could be potential antidotes in cadmium intoxication [41], [42], [43].

3.4. Prooxidant activity of flavonoids through oxidation by phenoxyl radicals

Due to unexpected results of experiments with some known antioxidants, refer to Section 6.2.1, pro-oxidant properties of flavonoids (compounds constituting Cherry flavor responsible for its redox properties) are also discussed.

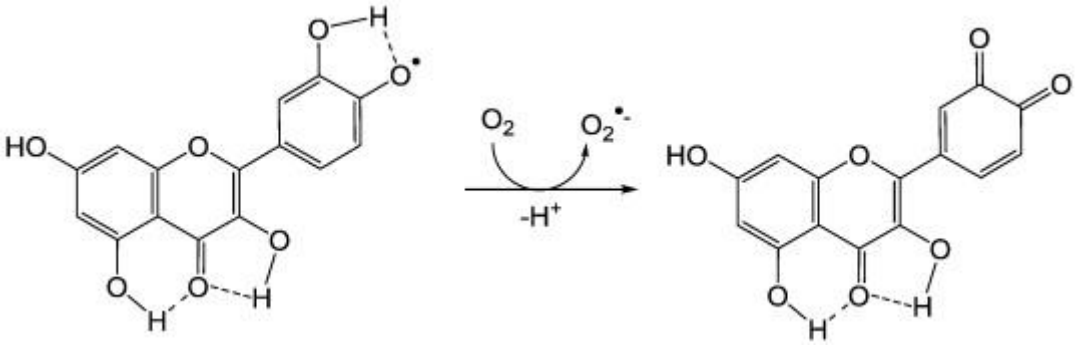
As well as many of so-called antioxidants, also flavonoids can act, under certain circumstances, as prooxidants and, hence, promote the oxidation of other compounds. For the purposes of this study only in vitro prooxidant properties of flavonoids will be discussed.

Prooxidant activity is thought to be directly proportional to the total number of hydroxyl groups in a flavonoid molecule [44]. Series of mono- and dihydroxy flavonoids demonstrated no detectable prooxidant activity, while multiple hydroxyl groups, especially in the B-ring, significantly increased production of hydroxyl radicals in Fenton reaction [20], [45]. The latter compounds include baicalein containing a pyrogallol structure in the A-ring, which has also been reported to promote hydrogen peroxide production [20], [46] from which highly reactive hydroxyl radicals may be generated via Fenton reaction [20], [47]. There is also evidence that the 2,3-double bond and 4-oxo arrangement of flavones may promote the formation of ROS induced by divalent copper in the presence of oxygen [35].

Flavonoids prooxidant properties, at least in vivo, seem to be concentration-dependent [48], however, as in vivo behavior is not being investigated in this thesis no further discussion is provided and invitro mechanism through oxidation by flavonoid phenoxyl radicals will only be discussed.

According to the "classical" definition, antioxidant is a molecule: (1) that could donate electrons or hydrogen atoms, (2) yields an antioxidant-derived radical that (3) is efficiently quenched by other electron or hydrogen sources to prevent cellular damage, and (4) whose properties are spatially and temporally correlated with oxidative stress events [49], [50], [51].
The end products of ROS scavenging by flavonoids are flavonoid phenoxyl radicals (Fl-O$\bullet$) with a lifetime of 200 μs [52]. They are highly reactive and subjected to further oxidation, yielding, among other possible products, the more stable flavonoid quinones (Figure 3-4 p.13). Flavonoid quinones are still reactive but they can be stabilized by conjugation with nucleophiles, such as GSH, cysteine or nucleic acids [51], [53], [54], [55]. This reaction is responsible for one of the prooxidant effects of flavonoids [29], [56]. The prooxidant properties of the flavonoids apigenin, naringenin, and naringin have been described by showing that their phenoxyl radicals rapidly oxidize NADH, resulting in extensive oxygen uptake and $O_2\bullet^-$ formation [57], [58], [59]. Another reaction, which may be responsible for undesired prooxidant properties of flavonoids, could be the interaction of Fl-O$\bullet$ with oxygen in the presence of high levels of transition metals (Figure 3-9), generating quinones and $O_2\bullet^-$ [29], [28].

Figure 3-9 Prooxidant activity of flavonoids [28]



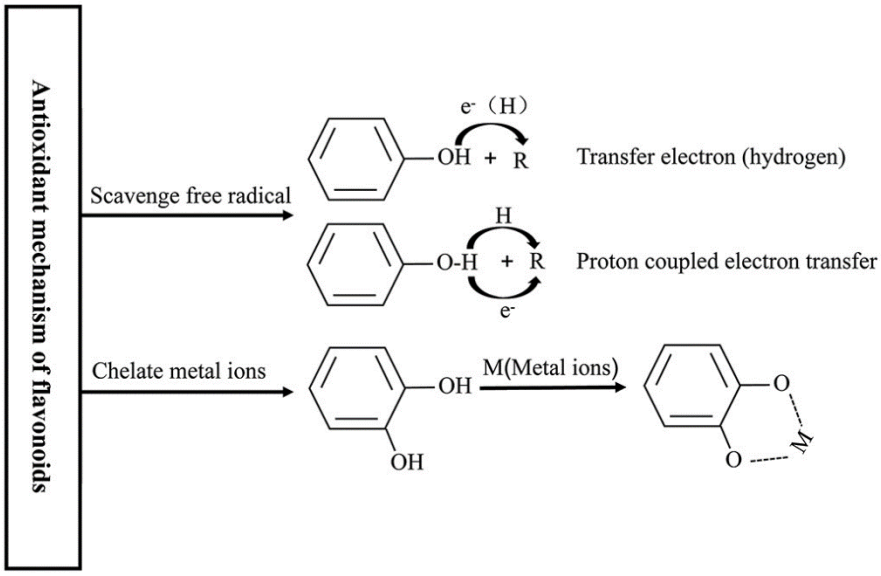
The source of phenoxyl radicals could be an autoxidation as well. Canada et al. [60] found that the rate of autoxidation for both quercetin and myricetin was highly pH-dependent with no autoxidation detected for quercetin at physiological pH. The rate of quercetin autoxidation was substantially increased both by an addition of iron and by an addition of iron followed by SOD.

The addition of iron increased the rate of autoxidation of myricetin as well. On the other hand, neither kaempferol, a monohydroxylated flavonol, nor rutin, a glycosylated quercetin, showed any ability to autoxidize. Autoxidation of quercetin accompanied by rapid accumulation of H_2O_2 was observed also in the presence of copper ions at neutral pH [61].

3.5. Mechanism Preventing Montelukast's Oxidation to Sulfoxide Impurity

Two in-vitro mechanisms of action describing antioxidative properties of polyphenolic compounds are presented on Figure 3-10 [62]: (a) the phenolic hydroxyl groups of polyphenolic compounds act as hydrogen donors to directly react with radicals and reduce the activities of $\cdot O_2$ -, H_2O_2, $\cdot OH$, $ROO\cdot$, $^1O_2$, and other active radicals; (b) polyphenolic compounds chelate metal ions that induce free radical production, thus reducing the generation of free radicals. Both mechanisms are described in Sections 3.3.1 and 3.3.2, respectively.

Figure 3-10 In-vitro mechanisms of action describing antioxidative properties of polyphenolic compounds



As the major excipient by which Montelulast's oxidation takes place is cellulose, due to residual peroxides it is contaminated with, scavenging free radicals by flavonoids is predominant mechanism.

3.6. Determination of Total Phenolic Content (TPC)

Antioxidative properties of Cherry flavor were assessed by commonly applied Folin–Ciocalteu method which is described in several pharmacopoeias including European Pharmacopoeia starting from 6th Edition (2007). The reaction forms a blue chromophore constituted by a phosphotungstic/phosphomolybdenum complex, where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds. However, this reagent rapidly decomposes in alkaline solutions, which makes it necessary to use an enormous excess of the reagent to obtain a complete reaction. This excess can result in precipitates and high turbidity, making spectrophotometric analysis impossible. To solve this problem, Folin and Ciocalteu included lithium salts in the reagent, which prevented the turbidity. The reaction generally provides accurate and specific data for several groups of phenolic compounds, because many compounds change color differently due to differences in unit mass and reaction kinetics.

The resulting solution after reaction was measured at 550 nm and compared with a standard curve generated with gallic acid standard solutions. The details of method used are provided in Section 5.4.

3.7. Determination of Total Flavonoid Content (TFC)

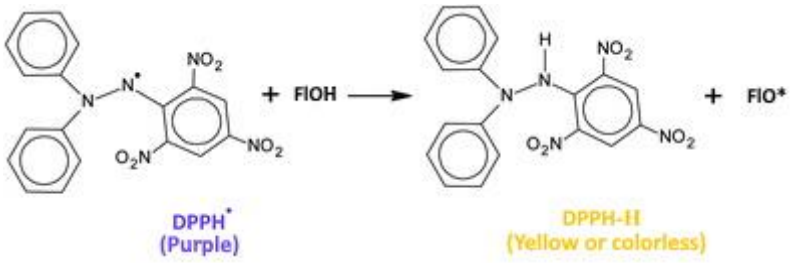
Determination of TFC was carried out employing aluminum chloride colorimetric assay method [64]. In this procedure complexation reaction is carried out in the presence of $NaNO_2$ in alkaline medium. The method is based on the nitration of any aromatic ring bearing a catechol group with its three or four positions unsubstituted or not sterically blocked. After addition of Al(III), a yellow solution of complex is formed, which then turned immediately to red after addition of NaOH, and the value of absorbance is measured at 510 nm. The details of method used are provided in Section 5.5.

3.8. Determination of Antioxidative Capacity (AC)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH), radical discovered by Goldschmidt and Renn in 1922, is widely known agent used to evaluate the properties of potential antioxidants for scavenging free radicals. This radical is colored and stable, exhibiting two properties that have made it one of the most popular radicals in a wide range of studies. For the purposes of this study UV-Vis method of quantitative assay was applied, however, other detection techniques are also available including Raman, FT-IR, ATR-IR and HPLC [63] [64].

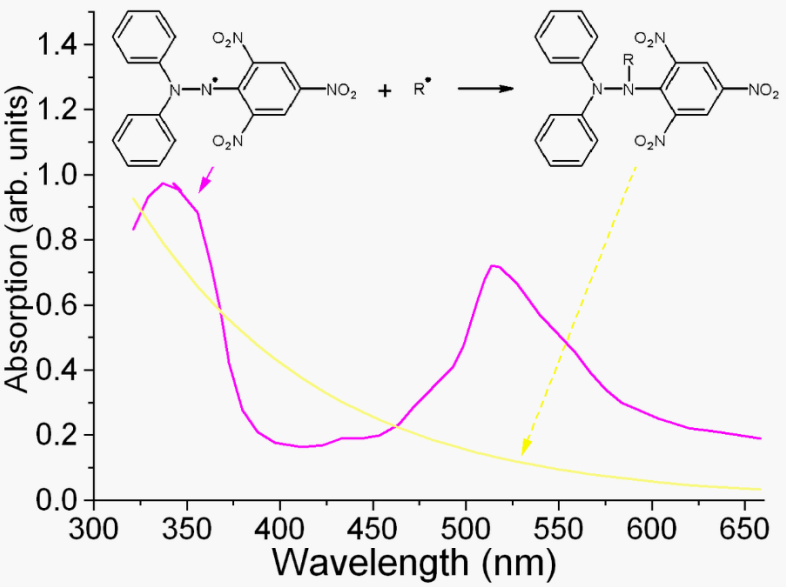
The method employed in this thesis is based on the spectrophotometric measurement of the DPPH concentration change resulting from the reaction with an antioxidant. The absorbance of DPPH measured at λ_{Max} = 517 nm decreases when the radical is reduced by antioxidants, refer to Figure 3-11.

Figure 3-11 Reduction of DPPH to DPPH-H*



The overlay UV-Vis spectra of both DPPH and DPPH-H are presented in Figure 3-12 (p. 22)

Figure 3-12 Change in absorption spectrum (from magenta to yellow) upon reaction of DPPH with a radical [65]



Because of a strong absorption band located at about 517 nm, the DPPH radical has a deep violet color in solution, and, depending on the solvent used, it becomes colorless or pale yellow when neutralized. This property allows visual monitoring of the reaction.

Several techniques have been developed for this assay using different conditions such as different reaction times, solvents, pH and different compounds used as antioxidant standards. The influence of these parameters have been investigated by K. Pyrzynska and A. Pekal [66]

In this thesis modified Brand-Wiliams method for determination of antioxidative capacity was used [67].

As Antioxidative Capacity parameter is investigated as potential test by which Cherry flavor is to be assessed during routine pharmaceutical Quality Control process of raw material the method has been modified to be suitable for the above purposes. As exact qualitative and quantitative composition of the excipient is unknown and may significantly vary from one supplier to another supplier the method is not based on molar considerations and weigh-to-weight (w/w) approach has been applied, refer to Section 5.6 for more details.

There are many methods of calculating and reporting the final results of AC testing employing DPPH assay [68]. For substrates of known structure and molar mass, working in terms of molar units completely obscures the interpretation of the data on a molecular basis; it is more appropriate to apply the relative molar mass for DPPH [69]. Use of only a single mass-in-

volume concentration does not help to elucidate the structural basis of antioxidant activity, since it provides only two points on the titration curve [70]. Working in terms of numbers of free radicals necessitates the use of the Avogadro number to bring the values on to a mole basis [71]. However, in case of complex mixtures such as plant extracts, the results can be expressed as DPPH equivalents per gram of material or as per the relative absorbance read after the reaction or absorbance drop.

3.9. Conclusions on pro- and antioxidant properties of flavonoids

Flavonoids cannot only be considered purely as antioxidants, since under certain reaction conditions they can also display prooxidant activity. This unexpected behavior could explain, in part, the observed toxicity of some flavonoids *in vivo* [72]. It seems that prooxidant or antioxidant properties of a particular flavonoid depend most of all on its concentration.

The extent to which are flavonoids able to act as anti- or prooxidants *in vivo* is still poorly understood and this topic clearly requires further studies.

4. Aim of the doctoral thesis

The aim of the work was to investigate and modify, if required, the least stable formulation of Montelukast tablets (5 mg, chewable tablets, formulation without EDTA) manufactured by Adamed Pharma to decrease the degradation rate and final concentration of Sulfoxide impurity measured at the end of product's shelf-life.

Following formulation analysis three excipients were chosen as potentially exhibiting antioxidative properties:

- EDTA, which is well known for its chelating properties of metal ions (potential antioxidant)
- Iron Oxide Red, Fe_2O_3, containing iron in its +3 oxidation state (potential alternative to Monelukast electron donor)
- Cherry flavor

For the purposes of this research Cherry flavor was chosen as potential stabilizing agent. Cherries contain anthocyanins which are examples of phenolic compounds, and other phenolic compounds including polyphenolics and flavonoids. These compounds are known for their anti-oxidative properties acting as electron donors and, as consequence, inhibiting oxidation of coexisting in the matrix molecules.

The work was split into the following research trials:

- Analytical screening of Cherry flavor for the presence of flavonoids (HPLC with UV detection)
- LC-MS analysis for the purposes of verification of flavonoid's presence in Cherry flavor
- Determination of: Total Phenolic Content, Total Flavonoid Content and Antioxidative Capacity of Cherry flavor by UV-Vis method
- Stress study in the stability chamber, 40°C/75% RH, mixture of placebo and API seasoned for one month at 40°C/75% RH containing 3 different antioxidants (Cherry flavor, Quercetin and Ascorbic acid)
- Investigation of potential in-situ degradation to Sulfoxide impurity during sample preparation for analytical RS testing

- Excipients compatibility study using mixtures of API, Cellulose microcrystalline and various amounts of Cherry flavor

- Compression of 5 mg tablets (1) without Cherry flavor, (2) containing Cherry flavor as per the investigated formulation and (3) containing 200% Cherry flavor (relative to the original formulation). These tablets were subject to seasoning at accelerated conditions (40°C/75% RH) for 6 month and at normal conditions (25°C/60% RH) for 24 months (full shelf-life time) as per the stability plan detailed in Table 4-1.

Table 4-1 Stability plan for Montelukast tablets, three compression trials

	Initial	**2 days**	**1 month**	**3 months**	**6 months**	**12 months**	**18 months**	**24 months**
40°C/75% RH	✓	✓	✓	✓	✓			
25°C/60% RH				✓	✓	✓	✓	✓

- Testing carried out on the three above trial batches and Cherry flavor for water content by Karl Fischer and moisture content by LOD to assess potential effect of residual H_2O on Montelukast's stability results.

Apart from Cherry flavor investigated by means of technological trials and laboratory analyses other excipients with potential antioxidant properties were discussed. This discussion lays a background for further research to optimize Montelukast formulations exhibiting lower concentration of Sulfoxide impurity formed during product's shelf-life.

5. Materials and Methods

The following analytical methods and equipment were used for the purposes of this research. The method for Related Substances is an in-house Adamed Pharma's method. Flavonoid's presence was confirmed by screening testing employing modified HPLC method developed and published by t. Seal [73].

5.1. Related substances by HPLC

Note: the below described method is validated according to current ICH Q2 requirements, however, due to intellectual property rights full validation is not included as part of this thesis.

Preparation of mobile phase

Buffer: Weigh accurately 3.9 g of Sodium dihydrogen phosphate dihydrate into a beaker containing 1000 mL of Milli-Q-grade water and mix to dissolve. Adjust the pH to 3.7 ± 0.05 with orthophosphoric acid. Filter through 0.45 micron or finer porosity membrane filter and degas.

Mobile phase: A Buffer and Acetonitrile (lot 1058/11/19, 1237/11/19, 0801/04/20, 1076/11/20 purchased from The Porch Company) in the ratio of 80:20

Mobile phase: B Buffer and Acetonitrile in the ratio of 20:80

Diluent: Acetonitrile and Water (MiliQ water supplied by Merck KGaA, Milli-Q IQ 7000, no. F9AA5806D F8PA348899) in the ratio of 60:40

Preparation of solutions

Prepare the standard and sample solutions in an amber colored volumetric flask and inject fresh preparations only.

Note: Check the water content of Montelukast sodium working standard at the time of analysis. Water content should be within the drug substance specifications.

System suitability solution: Prepare a solution having known concentration of about 0.5 mg/ml Montelukast for peak identification (EP Standard) in diluent.

For example: Weigh accurately 5.0 mg of Montelukast for peak identification (EP Standard) into a 10 ml, clean and dry volumetric flask, dissolve in and dilute to volume with diluent.

Standard solution (0.2%): Prepare a solution having known concentration of about 0.001 mg/ml as Montelukast in diluent.

For example: Weigh accurately 52 mg of Montelukast sodium working standard into a 100 ml clean, dry volumetric flask, add 70 ml of diluent and sonicate to dissolve. Make up to volume with diluent and mix. Dilute 5 mL of this solution to 50 mL with diluent. Further dilute 2 ml of this solution to 100 ml with diluent.

Alternative standard by using Montelukast dicyclohexylamine standard: Weigh accurately 32.7 mg of Montelukast dicyclohexylamine standard into a 50 ml clean, dry volumetric flask, add 35 ml of diluent and sonicate to dissolve. Make up to volume with diluent and mix. Dilute 5 ml of this solution to 50 mL with diluent. Further dilute 2 ml of this solution to 100 ml with diluent.

Sample solution: Take 20 tablets into a clean, dry mortar and crush the tablets to fine powder. Weigh accurately powder equivalent to 50 mg of Montelukast (3000 mg) into a 100 ml clean, dry volumetric flask, add 70 ml of diluent and sonicate for 15 minutes, shake for 5 minutes and make up to volume with diluent. Filter through 0.45 μm membrane filter.

Placebo solution: Transfer 2950 mg of placebo into a 100 ml clean, dry volumetric flask, add 70 ml of diluent and sonicate for 15 minutes, shake for 5 minutes and make up to volume with diluent. Filter through 0.45 μm membrane filter.

Note: Placebo solution shall be injected for discarding the peaks due to placebo in sample solution chromatogram. Examine the placebo solution chromatogram for any extraneous peaks and discard corresponding peaks observed in the chromatogram of sample solution. If placebo solution not injected, discard the peaks below 3 minutes and peak about 14.9 minutes in sample solution chromatogram which corresponds to placebo solution.

Chromatographic conditions

Column: Hypersil BDS-C18, 100 x 4.6 mm ID, 3µm or equivalent

Flow rate: 1.0 ml/min

Detection: UV, 225 nm

Injection Volume: 10 µl

Data acquisition time: 70 min

Pump mode: Gradient

Gradient program:

Table 5-1 Gradient applied during HPLC analysis

	Time	Flow	% MP A	% MP B	Gradient type
1	0	1.00	53.0	47.0	-
2	35	1.00	5.0	95.0	Linear
3	58	1.00	5.0	95.0	Linear
4	62	1.00	53.0	47.0	Linear
5	70	1.00	53.0	47.0	Linear

Evaluation of system suitability

- Separately inject 10 µl of the system suitability solution, into the chromatograph, record the chromatogram and evaluate the system suitability
- The resolution between the peaks corresponding to the Montelukast and Keto impurity (EP Impurity-F) should not be less than 1.5. Theoretical plates of peaks corresponding to Montelukast should not be less than 4000. Asymmetry should not be more than 1.5 for the same peak
- Separately inject 10 µl of the standard solution, six times into the chromatograph, record the chromatograms and measure the peak areas corresponding to the Montelukast
- % RSD for peak areas of six injections from standard solution should not be more than 5.0%
- Inject 10 µl of diluent and placebo solution into the chromatograph and record the chromatograms

- Inject 10 µl of sample solutions into the liquid chromatograph and record the chromatogram.

Examine the diluent, placebo solution chromatograms for any extraneous peaks and disregard corresponding peaks observed in the chromatogram of sample solution.

Disregard any peak with an area less than 0.05% in the chromatogram of sample solution.

Retention time of Montelukast is about 22 minutes.

Note: To calculate the % area of sulfoxide, area of main peak of sulfoxide at RRT~0.47 and the area of corresponding diasteromer of it at RRT~0.46 should sum up and calculate as total sulfoxide area percentage

No.	Name of impurity	RRT (approx.)
1	Diastereomer of Sulfoxide	0.46
2	Sulfoxide	0.47
3	Montelukast Sodium	1.0

Calculation:

Calculation by using Montelukast sodium standard

$$\%\ Known\ impurity = \frac{A_k\ \cdot\ W_S \cdot 5 \cdot 2 \cdot 100 \cdot 586{,}2 \cdot\ Aw \cdot (100 - W) \cdot P}{A_S \cdot 100 \cdot 50 \cdot 100 \cdot W_t \cdot 608{,}2 \cdot L_c \cdot 100}$$

$$\%\ Unknown\ impurity = \frac{A_u\ \cdot\ W_S \cdot 5 \cdot 2 \cdot 100 \cdot 586{,}2 \cdot\ Aw \cdot (100 - W) \cdot P}{A_S \cdot 100 \cdot 50 \cdot 100 \cdot W_t \cdot 608{,}2 \cdot L_c \cdot 100}$$

Calculation by using Montelukast dicyclohexylamine standard

$$\%\ Known\ impurity\ = \frac{A_k\ \cdot\ W_m \cdot 5 \cdot 2 \cdot 100 \cdot 586{,}2 \cdot\ Aw \cdot P_m}{A_S \cdot 50 \cdot 50 \cdot 100 \cdot W_t \cdot 767{,}5 \cdot L_c}$$

$$\%\ Unknown\ impurity = \frac{A_u\ \cdot\ W_m \cdot 5 \cdot 2 \cdot 100 \cdot 586{,}2 \cdot\ Aw \cdot P_m}{A_S \cdot 50 \cdot 50 \cdot 100 \cdot W_t \cdot 767{,}5 \cdot L_c}$$

Total impurity = Total known impurities + Total unknown impurities.

A_k - Area of peak corresponding to known individual impurity in sample solution chromatograms.

A_u - Area of peak corresponding to unknown impurity in sample solution chromatograms.

A_s - Average area of peak corresponding to Montelukast in standard solution chromatograms.

W_s - Weight in mg of Montelukast sodium working standard

W_t - Weight in mg of sample taken.

P - % of assay of Montelukast sodium working standard (Anhydrous basis)

W - Water content of Montelukast working standard

A_w - Average weight of Montelukast chewable tablets

L_c - Label claim of Montelukast chewable tablets in mg

W_m - Weight in mg of Montelukast dicyclohexylamine standard.

P_m - % of assay of Montelukast dicyclohexylamine standard (As is basis)

586.2 and 608.2 are the molecular weights of Montelukast and Montelukast sodium respectively.

586.2 and 767.5 are the molecular weights of Montelukast and Montelukast dicyclohexylamine respectively.

5.2. HPLC method for flavonoids (screening analysis)

It is assumed that the impact of Cherry flavor on the overall formulation's stability is caused by the content of antioxidants. To confirm the presence of flavonoids modified HPLC method developed and published by t. Seal was used [73]. The results were not quantitated as the purpose was to screen the material only. As the precise and accurate content of flavonoids in Cherry flavor is not subject of this investigation such approach is justified.

HPLC equipment

HPLC analyses were performed with Agilent Infinity II 1260 liquid chromatograph with binary pump, diode array detector (1260 DAD HS), vial sampler and Empower 3 system manager as data processor. The separation was achieved by a reversed-phase Hypersil BDS C18 column (5 m particle size, i.d. 4.6 x 250 mm).

Preparation of standard solutions

Ascorbic acid

The stock solution of concentration 1 mg/ml was prepared by dissolving 5 mg of Ascorbic acid in 2.5 ml HPLC-grade methanol followed by sonication for 5 minutes and the resulting volume was made up to 5 ml with the solvent for the Mobile phase (acetonitrile and 1% aq. acetic acid 1: 9).

Rutin

The stock solution of concentration 0.8 mg/ml was prepared by dissolving 4 mg of Rutin in 2.5 ml HPLC-grade methanol followed by sonication for 5 minutes and the resulting volume was made up to 5 ml with the solvent for the Mobile phase (acetonitrile and 1% aq. acetic acid 1: 9).

Quercitin

The stock solution of concentration 1 mg/ml was prepared by dissolving 10 mg of Quercitin in 5 ml HPLC-grade methanol followed by sonication for 5 minutes and the resulting volume was made up to 10 ml with the solvent for the Mobile phase (acetonitrile and 1% aq. acetic acid 1: 9). Than 1 ml of solution was transferred into 50 ml volumetric flask – (solution with concentration of 0,02 mg/ml was obtained).

The standards solutions were filtered through 0.45 μm PVDF-syringe filter and the mobile phase was degassed before the injection of the solutions.

Mixture of Ascorbic acid, Rutin and Quercitin

1 ml of each stock standard solution was transferred into 5 ml volumetric flask and mixed. Concentration of Ascorbic acid and Rutin in this solution is 0.33mg/ml and concentration of Quercitin is 0.0066mg/ml.

Preparation of Sample solutions

The sample solutions of concentration of 1mg/ml were prepared by dissolving 10 mg of Cherry flavor sample in 5 ml HPLC-grade methanol followed by sonication for 15 minutes and the resulting volume was made up to 10 ml with the solvent for the Mobile phase (acetonitrile and 1% aq. acetic acid 1: 9).

Sample solutions were filtered through 0.45 μm PVDF-syringe filter and the mobile phase was degassed before the injection of the solutions.

Chromatographic analysis of phenolic compounds and ascorbic acid

The mobile phase contains 1% aq. Acetic acid solution (Solvent A) and Acetonitrile (Solvent B), the flow rate was adjusted to 0.7 ml/min, the column was thermostatically controlled at 28C and the injection volume was kept at 20 μl. A gradient elution was performed by varying the proportion of solvent B to solvent A. The gradient elution was changed from 10 % to 40% B in a linear fashion for duration of 28 min, from 40 to 60 % B in 39 min, from 60 to 90 % B in 50 min. The mobile phase composition back to initial condition (solvent B: solvent A: 10: 90) in 55 min and allowed to run for another 10 min, before the injection of another sample. Total analysis time per sample was 65 min. HPLC chromatograms were detected using a photo diode array UV detector at three different wavelengths (272, 280 and 310 nm) according to absorption maxima of analysed compounds. Each compound was identified by its retention time and by spiking with standards under the same conditions. The quantification of the sample was done by the measurement of the integrated peak area and the content was calculated using the calibration curve by plotting peak area against concentration of the respective standard sample. The data were reported with convergence limit in triplicate.

5.3. HPLC – MS method for identification of flavonoids in Cherry flavor

Same HPLC method (and reagents, however, at mass spectroscopy grade) to separate mixture of flavonoids was used as the one described in Section 5.2 except for the detector type.

The analysis was performed using an Ultimate 3000 series HPLC system coupled with an Amazon SL (Bruker, Bremen, Germany) ion trap mass spectrometer.

The results are discussed in Section 6.1.2.

5.4. Determination of TPC by Folin-Ciocalteu assay

The total phenolics content was determined by using the Folin-Ciocalteu assay [9].

Standard curve

1ml of standard solution of gallic acid (20, 40, 40, 60, 80 and 100 µg/ml in water) was added to a 25 ml volumetric flask, containing 9 ml of distilled water. A reagent blank was prepared using distilled water. One milliliter of Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of 7% Na_2CO_3 solution was added to the mixture. The solutions were made up to volume. Standard solutions were incubated at ambient temperature for 90 min.

Sample solution

5 ml of sample solution (1000µg/ml in water) were added to a 25 ml volumetric flask, containing 4 ml of distilled water. One milliliter of Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of 7% Na_2CO_3 solution was added to the mixture. The solutions were made up to volume. Sample solutions were incubated at ambient temperature for 90 min.

Test

After 90 min of incubation the absorbance against the blank solution was determined at 550 nm with an UV/Vis spectrophotometer. Total phenolics content was expressed as mg gallic acid equivalents (GAE). Solutions were dark blue colored - the higher the concentration of phenols, the more intense the color.

The results are summarized in Section 6.1.2.

5.5. Determination of TFC by the aluminum chloride colorimetric assay

Total flavonoid content was performed by the aluminum chloride colorimetric assay [74].

Standard curve

1 ml of standard solutions of quercetin (20, 40, 60, 80 and 100 µg/ml in Methanol) was added to a 10 ml volumetric flask containing 4 ml of distilled water. To the flask, 0.30 ml of 5% $NaNO_2$ was added and after 5 min, 0.3 ml of 10% $AlCl_3$ was added. After 5 min, 2 ml of 1M NaOH was added and the solution was made up to volume with distilled water.

Sample solution

5 ml of sample solutions (1000 µg/ml in Methanol) was added to a 10 ml volumetric flask. To the flask, 0.30 ml of 5% $NaNO_2$ was added and after 5 min, 0.3 ml of 10% $AlCl_3$ was added. After 5 min, 2 ml of 1M NaOH was added and the solution was made up to volume with distilled water.

The absorbance of standards and sample was read using spectrophotometer set at 510 nm.

Test

Solutions were yellow colored - the higher the concentration of flavonoids, the more intense the color. All standard curve solutions were yellow, from light to dark, however sample solution was completely transparent. It means that either Cherry flavor does not have flavonoids at all or the concentration in sample solution was too low in order to detect flavonoids.

The results for total flavonoid content was below detection level which was in line with LC-MS results provided in Section 6.1.2.

5.6. Determination of Antioxidative Capacity by modified Brand-Wiliams method

The method was carried out as per modified Brand-Wiliams method [67] described below.

Solutions preparation:

Standard solution: 0.06 mM (around 0.0237 mg/ml) solution of DPPH in methanol was prepared.

Sample solution: Three different concentration of Cherry flavor were prepared - 1 mg/ml, 10

mg/ml and 100 mg/ml. As visual examination confirmed solubility issue in methanol three different diluents were used - water, methanol, and dimethyl sulfoxide (DMSO).

Procedure:

7.8 ml of standard solution and 0.2 ml of sample solution were mixed and incubated overnight. As blank sample 7.8 ml of standard solution and 0.2 ml of diluent incubated overnight was used. Since Cherry flavor is not fully soluble in methanol nor in water on the next day sample solutions were filtered through RC syringe filter with 0.45 μm pore size. Cherry flavor dissolved completely in DMSO, however, to avoid adding another variable the filtering step was also applied. Following sample preparation and filtering the absorbance of blank solution and test solutions was read at 515 nm.

The results are expressed as the percentage decrease of absorbance measured at 515 nm.

5.7. Water content by Karl Fischer and moisture content by LOD

Twelve months stability samples of three trial batches kept in normal conditions (25C/60%RH) were tested for water content by Karl Fischer and Loss on Drying methods. LOD was carried out as per Ph. Eur. 2.2.32. Automated KF titration was carried out using KF Titrant 5 (Aquastar, Merck, BN HX99074610) and solvent (Aquastar, Supelco, BN HX90433115).

Samples for KF titration were prepared by weighing out accurately approximately 850 mg of ground tablets. Cherry flavor used for 100% and 200% samples was also tested by weighing out approximately 250 mg. Samples were prepared in duplicate.

For the LOD purposes approximately 850mg of powdered tablets was used, the sample was dried to the constant weight. The samples were prepared in duplicate, Cherry flavor was not tested for LOD due to insufficient amount of sample (KF was determined as described above).

The results are discussed in Section 6.3.5.

6. Results and discussion

6.1. Analytical assessment of Cherry flavor

To verify potential impact of Cherry flavor on stability of Montelukast tablets two factors need to be investigated:

1. Properties of Cherry flavor that may affect chemical stability of formulations
2. Cherry flavor should exhibit the above properties, regardless of knowing or not knowing the mechanism or root cause of the action, in particular formulation being investigated.

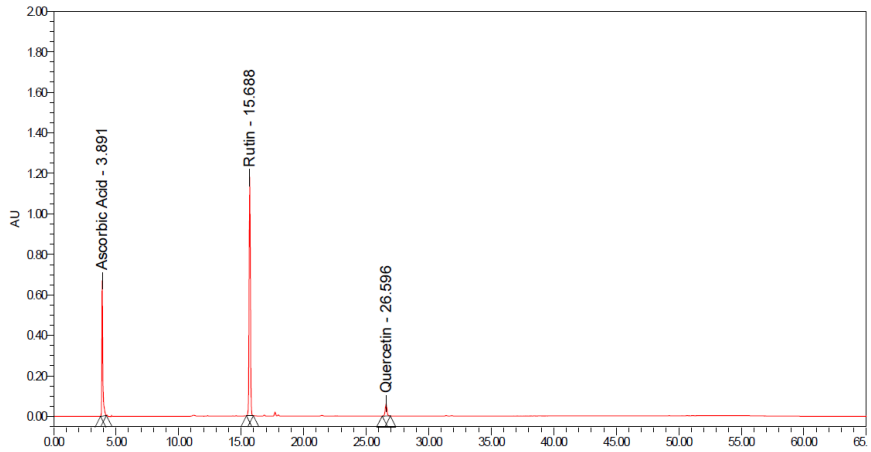
The first factor in not a must, however, if such properties identified and confirmed, solid scientific background built at this stage would facilitate further work and analyses on given formulations. Therefore, Section 6 of this thesis is focused on analytical trials by which postulated antioxidative properties of Cherry flavor are confirmed. It must be remembered that all the conclusions are applicable for the Cherry flavor used by Adamed Pharma, not all the Cherry flavors available on the market. The composition of each of them may and will vary from manufacturer to manufacturer, some of them may exhibit similar properties, some may exhibit them at different level/concentration, and some may exhibit no such properties. Therefore, the decision of choosing supplier or changing current supplier should be supported by suitable analytical trials or solid risk analysis, respectively.

6.1.1. Preliminary HPLC screening analysis for flavonoids

Two batches of Cherry flavor (all available in Adamed Pharma) were tested as per modified analytical method suggested by T. Seal [73], refer to Section 5.2. Quercetin, Ascorbic acid and Rutin, antioxidants listed in the original method and available at Adamed Pharma, were injected as nonquantitative standards to position the peaks and enable further identification by Relative Retention Times.

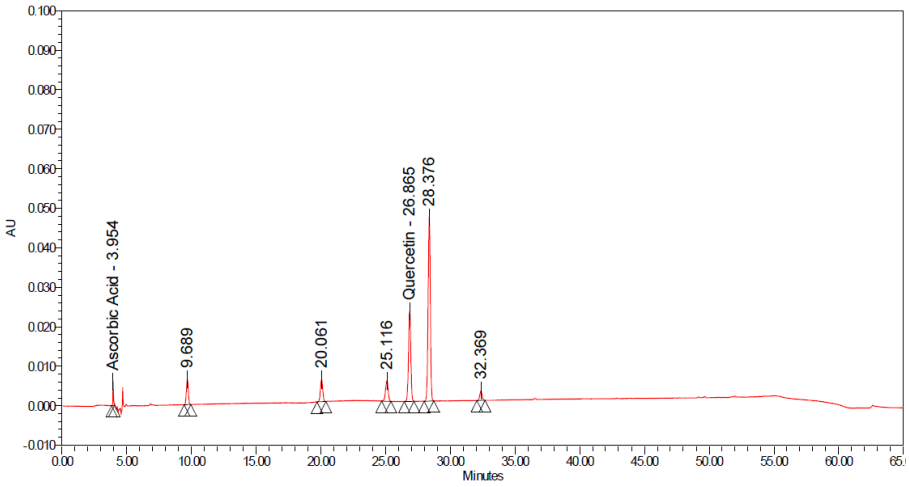
The chromatogram of solution containing mixture of Ascorbic Acid, Rutin and Quercetin is presented in Figure 6-1 (p. 36).

Figure 6-1 Chromatogram of standard solution containing Ascorbic Acid, Rutin and Quercetin analyzed as per the method described in Section 5.2



Example chromatogram of Cherry flavor solution is presented in Figure 6-2.

Figure 6-2 Chromatogram of Cherry flavor, BN 41726644 (sample solution), analyzed as per the method described in Section 5.2



Seven peaks were recorded out of which two were identified based on RRTs. The results of screening HPLC analysis are summarized in Table 6-1 Potential flavonoids identified in Cherry flavor employing analytical method as per Section 5.2, all peaks found in the Cherry flavor sample are in bold.

Table 6-1 Potential flavonoids identified in Cherry flavor employing analytical method as per Section 5.2

Peak identified	**Original method by T. Seal** [73]		**Modified method for the screening purposes as per Section 5.2.**	
	RT	**RRT** Quercetin as Reference	**RT**	**RRT** Quercetin as Reference
Ascorbic acid	4.02	0.14	4.00	0.15
Gallic acid	6.01	0.21	ND	N/A
Unknown 1	ND	N/A	9.70	0.36
Catechin	11.28	0.39	ND	N/A
Methyl gallate	12.60	0.43	ND	N/A
Caffeic acid	13.96	0.48	ND	N/A
Syringic acid	14.33	0.49	ND	N/A
Rutin	16.80	0.58	15.69	0.58
P-coumaric acid	18.37	0.63	ND	N/A
Sinapic acid	19.29	0.66	ND	N/A
Ferrulic acid	19.77	0.68	ND	N/A
Unknown 2	ND	N/A	20.10	0.75
Unknown 3	ND	N/A	25.10	0.93
Quercetin	29.02	1.00	26.87	1.00
Unknown 4	ND	N/A	28.40	1.06
Apigenin	33.16	1.14	ND	N/A
Kaempferol	34.23	1.18	ND	N/A
Unknown 5	ND	N/A	32.40	1.21

As can be noticed Rutin was not found in Chery flavor but its RRT calculated against Quercetin for both methods confirm the RRTs using original and modified methods are comparable, refer to Table 6-2 (p. 38).

Table 6-2 Comparison of RRTs for the original and modified HPLC method (Section 5.2)

Peak identified	**Original method by T. Seal** [73]		**Modified method for the screening purposes as per Section 5.2.**	
	RT	**RRT** Quercetin as Reference	**RT**	**RRT** Quercetin as Reference
Ascorbic acid	4.02	**0.14**	4.00	**0.15**
Rutin	16.80	**0.58**	15.69	**0.58**
Quercetin	29.02	1.00	26.87	1.00

The two batches of Cherry flavor tested were compared in terms of qualitative and quantitative composition by relative approach. The areas corresponding to each identified peak, corrected by the actual weights used to normalize raw data, are tabulated in Table 6-3.

Table 6-3 Comparison of peak areas found in two batches of Cherry flavor tested as per modified method described in Section 5.2.

Peak identified	**~ RRT** Quercetin as Reference	**Peak area**	
		BN 12054770	**BN 1726644**
Ascorbic acid	0.15	22256	20188
Unknown 1	0.36	62091	66087
Unknown 2	0.75	60949	69365
Unknown 3	0.93	64593	73250
Quercetin	1.00	245367	281109
Unknown 4	1.06	483396	551027
Unknown 5	1.21	29070	31439

No quantitative (absolute) calculations were performed as absolute values are not significant from the perspective of the purpose of this research. There is no significant difference between the two batches in terms of both qualitative and quantitative compositions. Oxidative capacity of Cherry flavor will be determined employing modified Brand-Wiliams at al. method with the aid of synthetic radical DPPH (2,2-Diphenyl-1-picrylhydrazyl) and using UV-Vis spectrometry [67].

6.1.2. HPLC – MS method for identification of flavonoids in Cherry flavor

HPLC-MS technique was employed to verify HPLC-UV screening analysis results described in Section 6.1.1. Both batches of Cherry flavor were tested, however, as the chromatograms are very similar only those for BN 12054770 are presented. The following chromatograms were recorded.

Figure 6-3. UV vs. MS chromatograms of Cherry flavor, BN 12054770

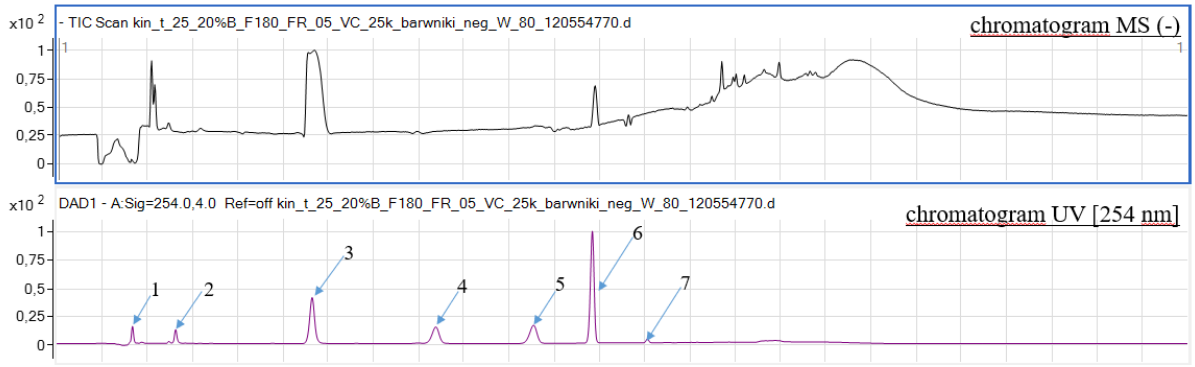
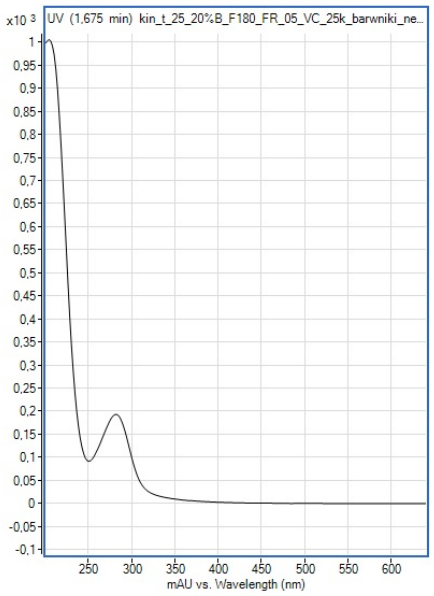


Figure 6-4 Separate UV/MS peaks chromatograms of Cherry flavor, BN 12054770, peaks 1-3

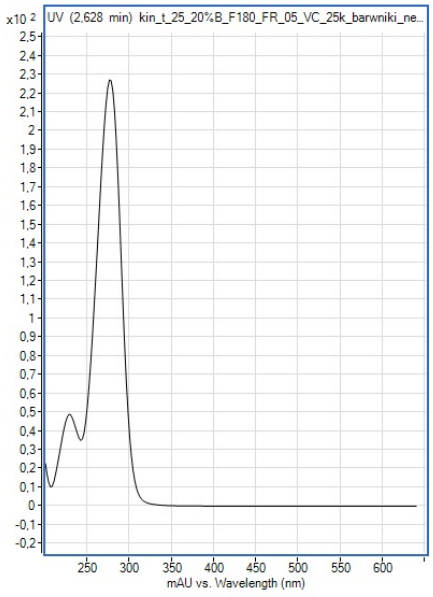
Peak 1

Elution at dead time



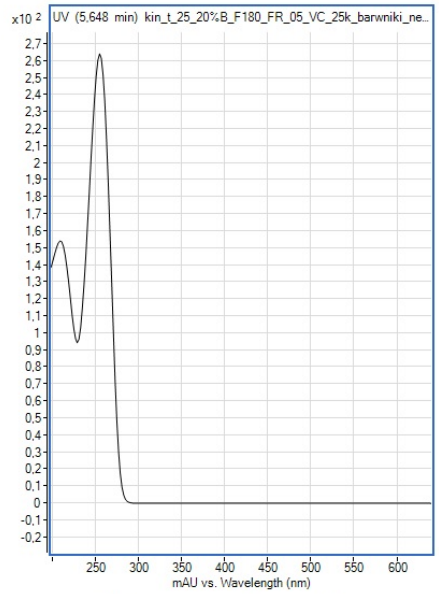
UV spectrum for peak 1

Peak 2

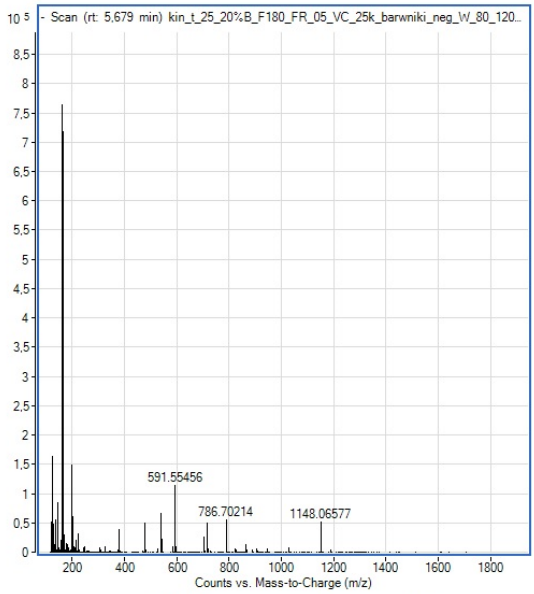


UV spectrum for peak 2

Peak 3



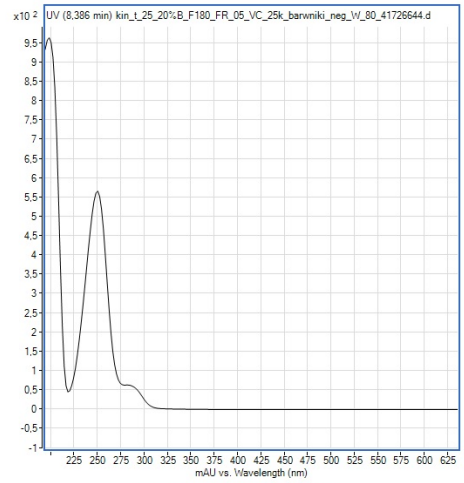
UV spectrum for peak 3



MS (-) spectrum form peak 3

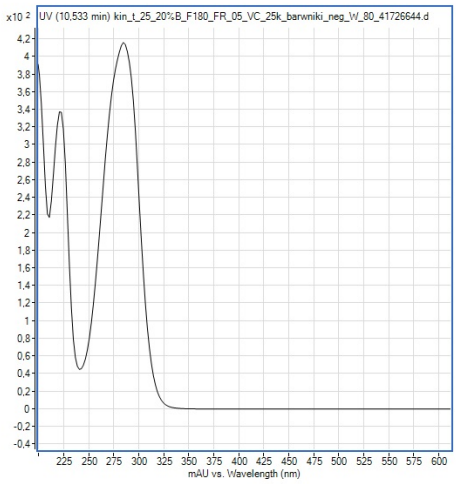
Figure 6-5 Separate UV/MS peaks chromatograms of Cherry flavor, BN 12054770, peaks 4-7

Peak 4



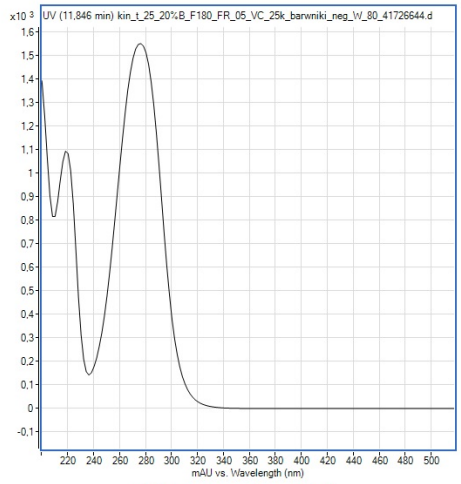
UV spectrum for peak 4

Peak 5

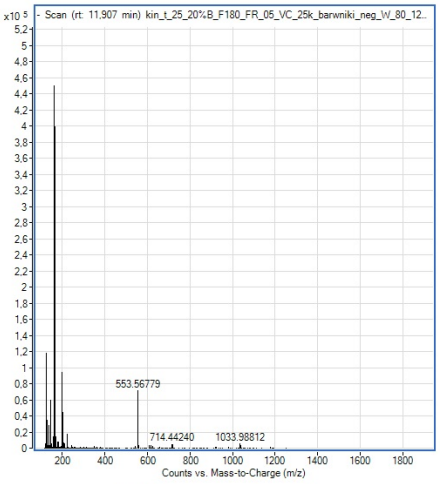


UV spectrum for peak 5

Peak 6

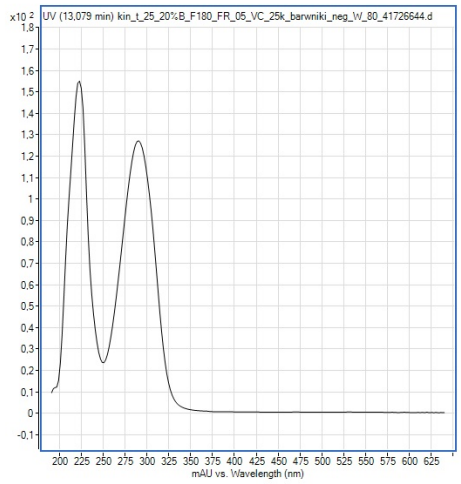


UV spectrum for peak 6



MS (-) spectrum for peak 6

Peak 7



UV spectrum for peak 7

Conclusions:

During the testing the following technical problems were encountered which did not allow to identify the components of the sample mixture:

- Lack of ionization for some of the components (Peaks 1, 2, 4, 5 and 7)
- Lack of spectra standards in the databases for which no ionization was observed
- Low molecular masses of searched molecules resulted in multiple peaks which are seen by the equipment as noise
- Based on the above testing there is no evidence that flavonoids are presented in the samples which is in line with the results obtained in Section 6.1.3.

6.1.3. Determination of TPC, TFC and AC in Cherry flavor

All the tests were carried out as per Sections 5.4, 5.5 and 5.6, the results of TPC and TFC are summarized in Table 6-4. The absorbance against blank was determined at 550 nm and 510 nm with UV-Vis spectrophotometer for phenolics and flavonoids, respectively. For determination of Antioxidant Capacity, the absorbance was measured at 517 nm. Total Phenolic Content was expressed as mg Gallic acid Equivalents (GAE) and % w/w and Total Flavonoid content was expressed as mg Quercetin Equivalents (QE).

Table 6-4 Results of TPC and TFC in Cherry flavor, BN and 1726666

Cherry flavor BN	**TPC**		**TFC**
	mg GAE/100g	**% w/w**	**mg QE/100g**
12054770*	62.12	0.06	Below LOD
41726644	92.47	0.09	Below LOD

* used to prepare artificial mixtures for the purpose of this research

Based on previous trials it was confirmed that Cherry flavor contains phenols which are likely responsible for its antioxidative properties. Knowing the above TPC (Total Phenolic Content) has been determined, refer to Section 5.4 for further details. As the flavor is of plant origin hypothesis was formed that the antioxidative properties are triggered by flavonoids, however, when TFC (Total Flavonoid Content) was carried out the result of test was negative, flavonoids were not found in the tested samples. To unequivocally confirm the antioxidative potential of Cherry flavor the excipient was screening tested for Antioxidative Capacity.

The antioxidative potential was determined by the DPPH· assay [67]. α, α-diphenyl-β-picrylhydrazyl (DPPH) is a dark-colored crystalline powder composed of stable free-radical molecules. The solution of DPPH is deep violet colored and express maximum UV absorption at around 515 nm in methanol. When the solution is exposed to oxidants DPPH is reduced and the solution loses its violet color when mixed with a substance that donates a hydrogen atom. That property allows for determination of antioxidant potential of a tested compound.

Results and conclusions:

All results of test solutions were compared to blank solution. The results confirmed previous observation and initial hypothesis that Cherry flavor exhibits antioxidative properties.

Table 6-5 Results of screening testing of Antioxidative Capacity expressed as relative absorbance in Cherry flavor, BN 12054770, in three different diluents

Ratio ChF/DPPH (w/w)	**Absorbance expressed as percentage of initial (Blank)**		
	MeOH	**Water**	**DMSO**
0 (reference)	100.0	100.0	100.0
1	98.4	99.1	96.5
11	96.2	94.7	94.5
108	91.0	76.2	74.4

- Methanol does not dissolve all the ingredients responsible for antioxidative properties of Cherry flavor. There was sediment at the bottom of the test tube used for sample preparation and overall relative absorbance at 108 ratios dropped down to 91% only
- Using water did not improve visual solubility as the sediment was still present, however, the relative absorbance dropped down to 76%
- DMSO completely dissolved the powder, however, there was no significant drop in relative absorbance compared to water. The above indicates that the residues dissolved in DMSO does not exhibit antioxidative properties
- Water is recommended as solvent for routine AC testing. The recommended ratio of Chf/DPPH is approximately 100. The specification and limits for the parameter should be stated based on analysis of at least 10 batches to confirm batch-to-batch reproducibility/variability.

6.2. Physical formulation mixtures

Antioxidative properties of Cherry flavor used by Adamed Pharma have been proven in Section 6 by screening analysis by HPLC and by determining antioxidant capacity by UV-Vis method.

In this Section the flavor and other common antioxidants are checked if they exhibit antioxidative properties in simulated, physical mixtures of Montelukast and placebo.

6.2.1. Degradation study using various amounts of Cherry flavor, Quercetin and Ascorbic acid

Antioxidizing properties of various antioxidizing excipients has been investigated including Quercetin, Cherry flavor and Ascorbic acid. Mixtures of all placebo ingredients as per original formulation was prepared, without Cherry flavor (base matrix). The following mixtures were prepared, initially tested and put for 30 days into stability chamber at 40°C/75% RH (Accelerated conditions).

- Mixture 1: Base matrix only
- Mixture 2: Base matric + Cherry flavor in amount as per the formulation
- Mixture 3: Base matric + Cherry flavor in amount 5 times as per the formulation
- Mixture 4: Base matric + Quercetin (amount corresponding to 5 x the amount of Cherry flavor in the formulation)
- Mixture 5: Base matric + Ascorbic acid (amount corresponding to 5 x the amount of Cherry flavor in the formulation)

Validated analytical method for related substances, details given is Section 5.1, was used to test the samples. The results were calculated and reported applying on peak's normalization, unknown impurities are color coded to facilitate data analysis.

Table 6-6 Stability of Base matrix, 40°C/75% RH, initials vs. 1 month, as percentage of normalized areas

	Relative RT	Initial	After 30 Days
Sulfoxide	**0.47**	**0.40**	**0.34**
MOK	1.00	99.11	99.24
Keto	N/D	N/D	N/D
Styren	1.56	0.06	0.07
Unknown 1	0.46	0.05	0.04
Unknown 2	0.79	0.18	0.15
Unknown 3	1.75	0.04	N/D
Unknown 4	1.86	0.09	0.05
Unknown 5	2.00	0.06	0.04
Unknown 6	2.16	ND	0.06
Sum (Unknown impurities)		**0.43**	**0.35**

Table 6-7 Stability of Base matrix plus Cherry flavor as per formulation, 40°C/75% RH, initials vs. 1 month, as percentage of normalized areas

	Relative RT	Initial	After 30 Days
Sulfoxide	**0.47**	**0.35**	**0.29**
MOK	1.00	99.14	99.39
Keto	N/D	N/D	N/D
Styren	1.55	0.07	0.07
Unknown 1	0.46	0.04	N/D
Unknown 2	0.79	0.18	0.18
Unknown 3	1.72	0.06	N/D
Unknown 4	1.85	0.11	0.06
Unknown 5	1.99	0.06	N/D
Sum (Unknown impurities)		**0.45**	**0.25**

Table 6-8 Stability of Base matrix plus 5 times amount of Cherry flavor compared to the original amount as per formulation, 40°C/75% RH, initials vs. 1 month, as percentage of normalized areas

	Relative RT	Initial	After 30 Days
Sulfoxide	**0.47**	**0.35**	**0.70**
MOK	1.00	99.06	98.72
Keto	N/D	N/D	N/D
Styren	1.57	0.07	0.07
Unknown 1	0.45	0.04	0.08
Unknown 2	0.79	0.18	0.12
Unknown 8	0.97	ND	0.05
Unknown 3	1.75	0.06	0.07
Unknown 7	1.82	0.05	0.08
Unknown 4	1.87	0.11	N/D
Unknown 5	2.01	0.07	0.04
Unknown 6	2.16	N/D	0.06
Sum (Unknown impurities)		**0.52**	**0.50**

Table 6-9 Stability of Base matrix plus Quercetin, 40°C/75% RH, initials vs. 1 month, as percentage of normalized areas

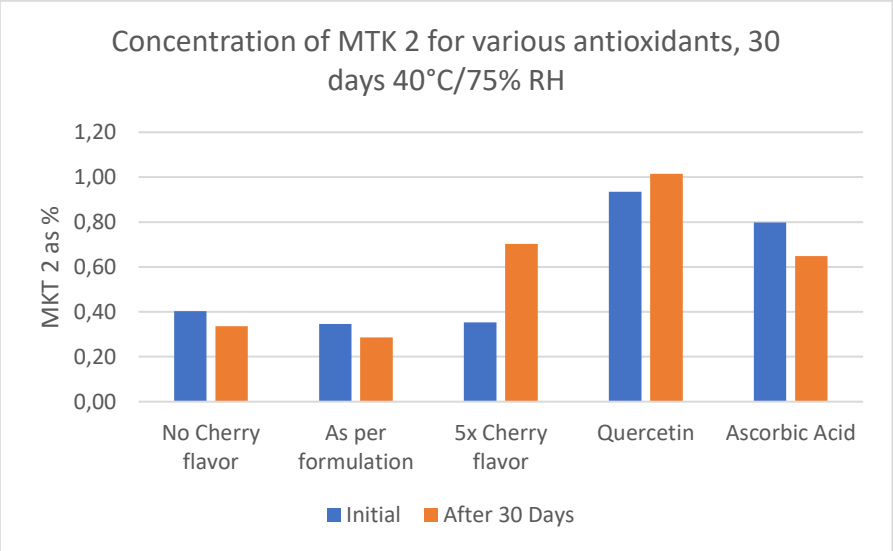
	Relative RT	Initial	After 30 Days
Sulfoxide	**0.46**	**0.93**	**1.01**
MOK	1.00	98.74	98.72
Keto	N/D	N/D	N/D
Styren	1.57	0.07	0.07
Unknown 1	0.45	0.10	0.11
Unknown 2	0.79	0.09	0.08
Unknown 4	1.87	0.06	N/D
Sum (Unknown impurities)		**0.32**	**0.26**

Table 6-10 Stability of Base matrix plus Ascorbic Acid, 40°C/75% RH, initials vs. 1 month, as percentage of normalized areas

	Relative RT	Initial	After 30 Days
Sulfoxide	**0.46**	**0.80**	**0.65**
MOK	1.00	98.73	98.84
Keto	N/D	N/D	N/D
Styren	1.58	0.06	0.07
Unknown 1	0.44	0.12	0.09
Unknown 2	0.79	0.16	0.11
Unknown 8	0.97	0.08	0.07
Unknown 4	1.88	0.05	0.07
Unknown 5	1.98	N/D	0.05
Unknown 6	2.17	N/D	0.05
Sum (Unknown impurities)		**0.41**	**0.44**

The relative concentrations of Sulfoxide impurity, main oxidative impurity investigated in this thesis, for various physical mixtures containing same base formulation and one antioxidative agent are summarized in Figure 6-6.

Figure 6-6 The impact of various antioxidants and Cherry flavor on formation of MTK 2 in investigated Montelukast formulation.



Some of the reported data exhibit opposite trend than expected. None of the antioxidant used significantly slows down the rate Sulfoxide impurity is formed, although some of them are well known for having antioxidizing properties (Ascorbic acid and Quercetin). It is reported that most antioxidants can act as prooxidants under certain conditions [12], and such a behavior could justify gathered results. High concentration of antioxidant compared the concentration of

substance being protected from oxidation, may lead to opposite effect and may actually increase the oxidative degradation.

Also, in case of Quercetin and Ascorbic acid the results are unexpected for another reason: the concentration of Sulfoxide Impurity is high initially and does significantly change after 1 month at accelerated stability conditions. The above may indicate that observed degradation to Sulfoxide impurity does not take place in powder mixture but is triggered during sample preparation, i.e. takes place in the solution. The above hypothesis can be verified by preparing a sample without antioxidant and spiking it with various amount of investigated agents (Cherry flavor, Quercetine and Ascorbic acid). If such a mechanism was confirmed sample preparation should be modified by choosing diluent in which Montelukast sodium passes into solution whilst antioxidation agents keep undissolved. Such approach would not eliminate presence of Sulfoxide Impurity during seasoning, but would decrease its concentration by a certain, constant amount, at each timepoint.

Following the above analysis additional research was carried out which is described in the following Sections 6.2.2 and 6.2.4.

6.2.2. In-situ degradation during sample preparation for analytical RS testing

Stability of Montelukast samples have been already investigated as part of numerous analytical validations and scientific trials [10] and the following, general conclusions can be drawn:

- The sample solutions are stable in basic solutions
- Acidic and H_2O_2 solutions exhibit high API degradation to Sulfoxide impurity

As each drug product formulation is different either in terms of qualitative or quantitative composition or in terms of drug substance and excipients suppliers, the investigated formulation has been checked if MTK2 impurity is generated during sample preparation or sample storage.

Three stock samples, each dedicated to one and only one agent investigated, i.e. one for Cherry flavor, one for Quercetine and one For Ascorbic acid, containing base formulation (all ingredients without Cherry flavor) were prepared as per analytical method. 5 ml from each sample was taken, filtered and a portion injected onto an HPLC system. To 20 ml of this sample 0.5 mg of the reagent the sample was dedicated to was added. Sample was sonicated and left

for 1h, filtered and injected onto HPLC system along with reference sample. To 10 ml of sample containing 0.5 mg of the reagent the sample was dedicated to another 1mg of agent was added. Sample was sonicated and left for 1h, filtered and injected onto HPLC system along with reference sample and sample containing 0.5 mg of agent. 12 months samples (25/50%RH, no Cherry flavor formulation was used) were used for the purposes of this trials, the results were calculated and reported by peak normalization (% area).

Table 6-11 Content of Sulfoxide Impurity as normalised % area in base formulation plus Cherry flavor, Quercetine and Ascorbic acid.

	Analysis 1	**Analysis 2**		**Analysis 3**		
	Reference (No ChF tablets)	**Reference (No ChF tablets)**	**+ 0.5 mg Reagent**	**Reference (No ChF tablets)**	**+ 0.5 mg Reagent**	**+ 1 mg Reagent**
Cherry flavor	0.71	0.70	0.70	0.72	0.71	0.71
Quercetine			0.67		0.70	0.71
Ascorbic Acid			0.85		0.91	1.10

Conclusions:

The experiment was carried out to investigate the results generated in Section 6.2.1 and summarized in Table 6-6 Stability of Base matrix, 40°C/75% RH, initials vs. 1 month, as percentage of normalized areas (p. 45). Based on the above results there is no evidence of in situ degradation to Sulpoxide impurity related to the amount of Cherry flavor or Quercetine. Analysis 3 also proves that for both above mentioned antioxidants the samples are stable during the course of analysis. Slight increase was observed in case of Ascorbic Acid.

The results reported in Table 6-6 may be explained by significantly higher concentrations of antioxidants used for forced degradation trials described in Section 6.2.1. The amount of Quercetin and Ascorbic Acid was 5 times higher than the amount of Cherry flavor in the original formulation. Samples for the experiment carried out in this section were prepared as follows (the HPLC method was carried out as per Section 5.1):

Reference: 750 mg of powdered tablets (no ChF tablets), corresponding to 12.5 mg API was transferred to a 25 ml volumetric flask. 15 ml diluent was added and the sample was sonicated for 15 mins, shaken for 5 mins, made up to volume with the diluent and filtered through 0.45 μm RC syringe filter (c=0.5 mg/ml)

Sample: 750 mg of powdered tablets (no ChF tablets), corresponding to 12.5 mg API was transferred to a 25 ml volumetric flask. 0.5 mg of Cherry flavor, Quercetin and Ascorbic acid, each in separate flask, was added. 15 ml diluent was added and the sample was sonicated for 15 mins, shaken for 5 mins, made up to volume with the diluent and filtered through 0.45 μm RC syringe filter (c=0.5 mg/ml). Same procedure was carried out for 1.0 mg samples.

Based on the above 0.5 mg of antioxidant investigated added to 750 mg of ground tablets corresponded to approximately 0.07% w/w compared to 0.3% w/w of Cherry flavor in investigated formulation. Therefore, for the purposes of study carried out in Section 6.2.1 approximately 1.5% of each Cherry flavor, Quercetin and Ascorbic acid was used and such high concentration could lead, and very likely led based on the results presented in Table 6-6, to different sample behavior. Such high amounts were justified for the purposes of Degradation study described in Section 6.2.1, however, for the trials carried out on *No ChF* formulation the amounts were reduced down to reasonable levels.

To confirm there is no risk of undesirable reversed conversion of MTK2 back to Montelukast (or MTK2 decomposition to any other impurity) verification trial as per Section 6.2.3 was carried out. Reversed reaction or further decomposition could potentially lead to false Sulfoxide Impurity level during testing, i.e. the tablets would contain the above impurity generated during either manufacture or shelf-life, however, during sample preparation the impurity would be converted back to the active substance.

6.2.3. MTK2 to Montelukast conversion/degradation induced by antioxidants

The purpose of the trial designed as below was to prove the antioxidants cannot trigger decomposition of MTK2 impurity (back to active substance or other impurity).

Amount of ground tablets enough to prepare sample as per Section 5.1 was stressed by 5 ml of 1% H_2O_2, 1h. To stop the degradation the solution was heated to approximately 50°C, 1h, to enhance full H_2O_2 decomposition. The above self-decomposition process is described by redox half-reactions:

Oxidation: $H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$

Reduction: $H_2O_2 + 2H^+ + 2e^- \rightarrow 2\ H_2O$

and the balanced equation:

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

The sample was split into four aliquots: reference and Sample 1-3 containing only one investigated reagent (1.0 mg of each Cherry flavor, Quercetine and Ascorbic Acid was added to each Sample1-3). All four samples were tested as per Section 5.1 and results are tabulated below.

Table 6-12 Influence of investigated antioxidants on concentration of MTK2 impurity.

	Reference (Oxidized sample)	**Reference + 1.0 mg Reagent (Samples 1-3)**
Cherry flavor	5.6%	5.7%
Quercetine		5.6%
Ascorbic Acid		7.8%

Conclusions:

Use of any of the antioxidants did not lead to decrease of Sulfoxide impurity concentration compared to the reference sample. In case of Cherry flavor and Quercetine the level of Sulfoxide impurity did not significantly change, in case of Ascorbic Acid the concentration increased from 5.6% up to 7.8% which is in line with the results generated in Section 6.2.2.

Same conclusions can be withdrawn based on the results generated in Section 6.2.2. which was designed to verify in-situ formation of Sulfoxide Impurity during the process of sample preparation.

6.2.4. Excipients compatibility

6.2.4.1. Excipients compatibility using API and each excipient

Compatibility of Montelukast (as Sodium) with all excipients of investigated formulation has been checked in accelerated conditions (40°C, 75% RH). Physical mixtures of the drug substance and each excipient were prepared, analyzed and results are summarized in Table 6-13 (p. 52).

Table 6-13 Combinations of Montelukast Sodium with excipients in various mass ratios

Excipient	API: Excipient ratio (w/w)	API : Excipient ratio per tablet	Sulfoxide Imp Content (% w/w)	
			Start	1 month
Cellulose microcrystalline	**1:20**	**1 : 12**	**0.04**	**0.59**
	1:40		**0.04**	**0.45**
Hydroxypropylcellulose	1:3	1 : 0.17	0.24	0.35
	1:6		0.46	0.66
Aspartame	1:1	1 : 0.24	0.04	0.21
Red Iron Oxide	1:1	Not significant	0.03	0.07
Crosscarmellose sodium	1:20	1 : 1.84	0.04	0.16
Cherry flavor	1:1	1 : 0.003	0.04	0.07
Magnesium stearate	1:1	Not significant	0.04	0.07
Mannitol	1:40	1 : 42	0.03	0.08
	1:60		0.03	0.22

Four excipients were picked as potential agents inducing Montelukast's oxidation by which Sulfoxide Impurity is formed: Cellulose microcrystalline, Hydroxypropylcellulose, Aspartame and Mannitol. Hydroxypropylcellulose and Aspartame were rejected for further analyses as tiny amount of each is used and 1:3 mixture shows degradation of 0.35% and 1:1 mixture shows degradation of 0.21% only, respectively. Mannitol was rejected due to insignificant degradation at amount present in the formulation, ~ 0,08%.

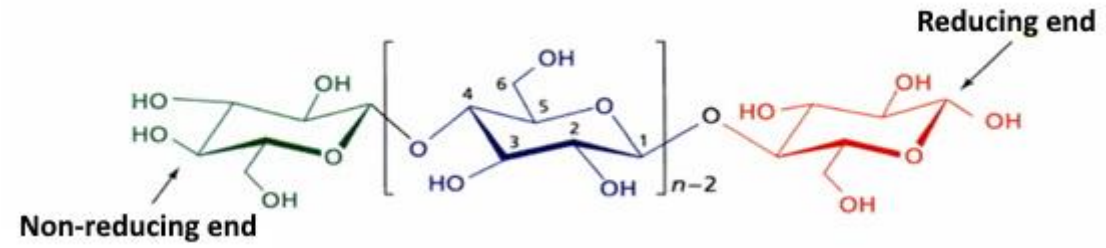
Potential redox reaction in which cellulose microcrystalline acts as a reducing sugar, which cellulose is not considered, needs deeper investigation.

A reducing sugar is any sugar that is capable of acting as a reducing agent because it has a free aldehyde group or a free ketone group. [75, p. 626] All monosaccharides are reducing sugars, along with some disaccharides, some oligosaccharides, and some polysaccharides. The monosaccharides can be divided into two groups: the aldoses, which have an aldehyde group, and the ketoses, which contain in their structure a ketone group. It has to be noted that ketoses must first tautomerize to aldoses before they can act as reducing sugars, as it is the aldehyde functional group that allows the sugar to act as a reducing agent. The cyclic hemiacetal forms of aldoses can open to reveal an aldehyde, also certain ketoses can undergo tautomerization to become aldoses.

Disaccharides consist of two monosaccharides and may be either reducing or nonreducing. Even a reducing disaccharide will only have one reducing end, as disaccharides are held together by glycosidic bonds, which consist of at least one anomeric carbon. With one anomeric carbon unable to convert to the open-chain form, only the free anomeric carbon is available to reduce another compound, and it is called the reducing end of the disaccharide. Similarly, most polysaccharides have only one reducing end.

Cellulose, as 1,4 - linked glucans, has one reducing end containing un unsubstituted hemiacetal (red), and one non-reducing (green) end containing an additional hydroxyl group at C4, refer to Figure 6-7 [76, p. 22].

Figure 6-7 Molecular structure of cellulose showing the numbering of the carbon atoms, the reducing end in red with hemiacetal, and non-reducing end in green with a free hydroxyl at C4 [76, p. 22]



6.2.4.2. *Excipients compatibility using mixtures of API, Cellulose microcrystalline and various amounts of Cherry flavor*

The analysis carried out in the previous section indicates that Cellulose microcrystalline may be the main ingredient responsible for the oxidation of Montelukast API to Sulfoxide impurity. To verify antioxidative impact of Cherry flavor on such a mixture containing API and Cellulose microcrystalline physical mixtures as per Table 6-14 were prepared, tested initially, left for one month at accelerated stability conditions (40°C/75% RH, glass bottle with open lid to facilitate humidity penetration) and retested. The effect of cellulose aging and its oxidative capacity increase or reduction with time was also assessed by testing mixture of API and fresh cellulose (1:12 w/w) as reference and API with cellulose kept at 40°C/75%RH for 1 month. The results expressed as % Area are summarized in tables Table 6-14 The impact of Cherry flavor on oxidative properties of Microcrystalline cellulose (p. 54) and Table 6-15 (p. 54).

Table 6-14 The impact of Cherry flavor on oxidative properties of Microcrystalline cellulose

		API: Cellulose microcrystalline: Cherry flavor (w/w/w)				
		1:12:0 (Reference)	1:12:0.1	1:12:0.2	1:12:0.3	1:12:0.5
Initial	MTK2 as % Area	0.10	0.10	0.09	0.12	0.09
	Montelukast as % Area	99.63	99.58	99.51	99.37	99.27
1 month at 40°C/75% RH	MTK2 as % Area	2.16	1.23	1.26	1.12	1.16
	Montelukast as % Area	97.52	98.47	98.43	98.57	98.52

Table 6-15 The impact of aged cellulose on formation of MTK2 impurity

	API + fresh cellulose	API + cellulose kept for 1 month at 40°C/75%RH
MTK2 as % Area	0.10	0.12
Montelukast as % Area	99.64	99.45

Sample preparation:

Note: the HPLC method was carried out as indicated in Section 5.1.

API : cellulose : ChF (1:12:0.1 1:12:0.2 1:12:0.3 1:12:0.5) mixture – 50 mg of Montelukast sodium accurately weighed was added to a weighing boat along with 600 mg of microcrystalline cellulose and Cherry flavor in amount of 5 mg, 10 mg, 15 mg and 25 mg. Each sample was well mixed and tested at T0. Second set of samples was prepared, placed in an open lid securitainer in a stability chamber and tested after 1 month. Prior to HPLC analysis all powder was quantitatively transferred to a 100 ml volumetric flask, 50 ml of diluent was added and the content was sonicated for 15 mins followed by 5 mins shaking. The solution was made up to volume with solvent, filtered through RC 0.45 μm filter and forwarded for the analysis.

API + cellulose – 50 mg of Montelukast sodium accurately weighed was added to a weighing boat along with 600 mg of microcrystalline cellulose. The sample was well mixed and tested at T0. Second sample was prepared, placed in an open lid securitainer in a stability chamber and tested after 1 month. Prior to HPLC analysis all powder was quantitatively transferred to a 100 ml volumetric flask, 50 ml of diluent was added and the content was sonicated for 15 mins

followed by 5 mins shaking. The solution was made up to volume with solvent, filtered through RC 0.45 μm filter and forwarded for the analysis.

Reagents and standards:

Montelukast sodium, interanl standard, B/N WMKT0009

Cellulose microcrystaline 101 (VIVAPUR 101), B/N 11913767

Cherry flavor B/N 12054770, supplied by Firmenich Switzland

Conclusions:

Slight amount of Cherry flavor significantly decreases the rate MTK2 impurity is formed. When 0.1% w/w was added the rate was decreased by 43%, when the amount was increased to 0.5% additional 3% were observed (46% reduction in total). Based on the above it is concluded that both quality of Cherry flavor and Cellulose may have significant impact on formation of Sulfoxide impurity. The quality of Cherry flavor could be potentially verified by Total Phenolic Content or Antioxidative capacity tested as per Sections 3.6 and 3.8, respectively.

The quality of Cellulose could be assessed by testing of residual peroxides which triggers oxidation and MTK2 formation.

6.3. Stability assessment of drug substance and registered formulation containing various amounts of Cherry flavor

Antioxidative properties of Cherry flavor used by Adamed Pharma have been proven in Section 6. In Section 6.2 the flavor and other common antioxidants were checked if they exhibit antioxidative properties in simulated, physical mixtures of API and placebo. This section focuses on the formulation being investigated, i.e. most problematic 5 mg tablets without EDTA.

Based on previous investigations and findings granulate as per the formulation, but without Cherry flavor has been prepared. A portion of tablets has been compressed from this granulate, then Cherry flavor in amount as per the formulation has been added. A portion of tablets has been compressed again from resulting granulate, then second portion of Cherry flavor in total amount corresponding to 200% as per the formulation has been added. The third set of tablets has been compressed. Refer to the formulation details given in Table 6-16 (p. 56).

Table 6-16 Formulation of laboratory scale batches of Montelukast (as Sodium) tablets with various amounts of Cherry flavor

Component	Quantity (mg/tablet)		
	No ChF	100% ChF	200% ChF
Montelukast Sodium Montelukast free acid	5.19 5.00	5.19 5.00	5.19 5.00
Mannitol	-	-	-
Cellulose microcrystalline	-	-	-
Croscarmellose sodium	-	-	-
Iron oxide red	-	-	-
Hydroxypropylcellulose	-	-	-
Cherry flavor	0	0.99	1.98
Aspartame	-	-	-
Magnesium stearate	-	-	-
Water, purified	-	-	-
Total	299	300	301

The tablets from three formulations were tested for Related Substances immediately after compression, after 2 days of exposure to room temperature/humidity and as per the stability program summarized in Table 4-1 Stability plan for Montelukast tablets, three compression trials (p. 24).

6.3.1. Short term stability assessment applying accelerated stability conditions (40°C/75% RH) – Drug Substance

Stability data for the active pharmaceutical ingredient supplied by Morepen, stored at accelerated conditions for 6 months, are summarized in Table 6-17 (p. 57). Three investigated formulations, i.e. without Cherry flavor, with 100% and 200% Cherry flavor were manufactured using the above API source.

API samples were kept in double polythene bag (inner transparent outer black) and then in aluminum bag. Silica bags were placed between both of polythene bag and also between outer polythene bags and aluminum bag under nitrogen atmosphere and sealed. The packed samples were kept in small HDPE containers and labeled suitably.

The data generated by the manufacturer shows the molecule is stable and Sulfoxide impurity does not generate above the LOQ level.

Table 6-17 Summary of 6 months stability of Montelukast API supplied by Morepen, BN MK14-1011/I, accelerated conditions (40 ℃/75%RH)

Impurities	40°C/75% RH*				
	Initial analysis	**1 months**	**2 months**	**3 months**	**6 months**
Sulfoxide Imp	Below LOQ **	Below LOQ	Below LOQ	Below LOQ	Below LOQ
Sum	0.14	0.13	0.14	0.14	0.15

* the results are published by curtesy of Morepen, Montelukast's supplier.

** LOQ = 0.5%

6.3.2. Short term stability assessment applying accelerated stability conditions (40°C/75% RH) – Drug Product

The results of Sulfoxide imp content for tablets kept at accelerated conditions are summarized in Table 6-18, the overall results including all impurities quantitated are summarized in Table 6-21 (p. 59).

The plot showing stability trends for all three formulations is shown as Figure 6-8 Graphical presentation of accelerated stability study on Montelukast 5 mg (no EDTA) formulation (p. 60).

Table 6-18 Summary of 6 months stability of Montelukast 5 mg tablets (without EDTA), accelerated conditions, with various amounts of Cherry flavor (ChF)

	Sulfoxide Imp as %		
Timepoint	**No ChF**	**100% ChF**	**200% ChF**
Initial	0.42	0.42	0.42
1 month	0.61	0.57	0.51
3 months	1.67	1.67	2.00
6 months	2.46	2.44	2.17
Total increase	**2.04**	**2.02**	**1.75**

The comparison of routine stability results of two commercial batches (packed in PVDC blisters) and stability of the three investigated formulation (kept in stability chambers in sealed aluminum bags), expressed as concentration of Sulfoxide impurity and Total impurities, are summarized in Table 6-19 (p. 58) and Table 6-20 (p. 58), respectively.

Table 6-19 Stability of investigated formulations vs. commercial batches, 40°C 75%RH, Sulfoxide impurity

Formulation	Batch No/ID	Timepoint (months)			
		0	1*	3	6
5 mg (no EDTA)	01090413	0.20		1.04	1.44
	01120413	0.17		1.01	1.38
Investigated formulations	No flavor	0.42	0.67	1.67	2.46
	100%	0.42	0.57	1.67	2.44
	200%	0.42	0.51	2.00	2.17

* timepoint not covered by routine, accelerated stability plan

Table 6-20 Stability of investigated formulations vs. commercial batches, 40°C 75%RH, Total Impurities

Formulation	Batch No/ID	Timepoint (months)			
		0	1*	3	6
5 mg (no EDTA)	01090413	0.34		1.36	1.93
	01120413	0.30		1.31	2.06
Investigated formulations	No flavor	0.55	0.72	1.98	3.26
	100%	0.55	0.69	2.01	3.33
	200%	055	0.63	2.62	3.09

* timepoint not covered by routine, accelerated stability plan

Investigated formulations show higher initial concentration of Sulfoxide impurity which may be caused by the fact the samples were tested approximately 6 weeks after the manufacture while commercial batches were tested within two weeks. The rate Sulfoxide impurity is formed is also significantly higher for the investigated batches compared to the commercial ones. The above can be explained by the primary packaging material which was different in both cases. Commercial batches were seasoned in stability chambers in PVDC blisters which contained very limited amount of air. Investigated batches were kept in sealed Aluminum foil and each bag contained significant amount of trapped air and moisture.

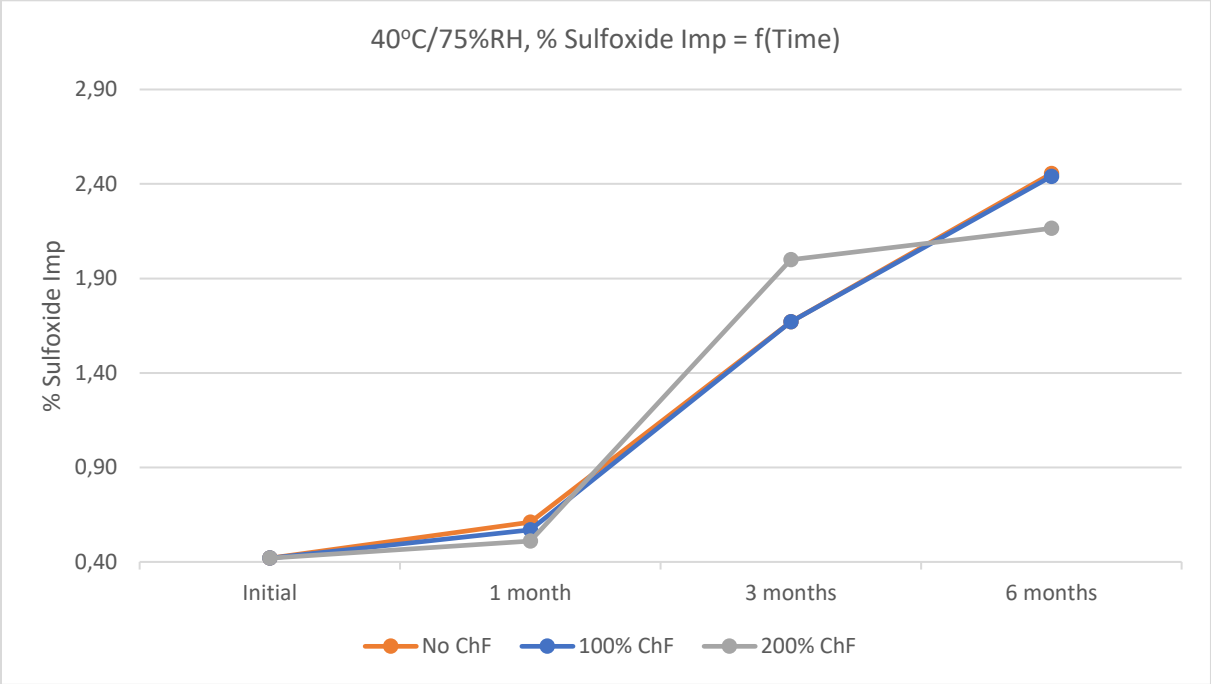
It should also be noticed that in case of both commercial batches the results are very similar in each timepoint. When investigation batches are compared the results for “No flavor” batch and “100%” batch are similar and the results for “200%” batch are significantly different, higher at three months and lower in six months.

Table 6-21 Detailed summary of 6 months stability of Montelukast 5 mg tablets (without EDTA), accelerated conditions, with various amounts of Cherry flavor

Impurities	Relative Retention Time	40°C/75% RH (Start of Stability Program 17/01/2020)													
		Initial Analysis			Tablets initially exposed to light/humidity for 2 days and retested			1 month			3 months			6 months*		
		No ChF	100% ChF	200% ChF	No ChF	100% ChF	200% ChF	No ChF	100% ChF	200% ChF	No ChF	100% ChF	200% ChF	No ChF	100% ChF	200% ChF
Sulfoxide Imp	**0.44**	**0.42**	**0.42**	**0.42**	**0.43**	**0.43**	**0.44**	**0.61**	**0.57**	**0.51**	**1.67**	**1.67**	**2.00**	**2.46**	**2.44**	**2.17**
Unkn Imp	0.24	ND	ND	ND	ND	ND	ND	ND	ND	ND	< 0.05	< 0.05	< 0.05	0.06	0.06	0.06
Unkn Imp	0.47	ND	ND	ND	ND	ND	ND	ND	ND	ND	< 0.05	< 0.05	0.07	< 0.05	< 0.05	< 0.05
Unkn Imp	0.63	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.06	0.07	0.08	0.08
Unkn Imp	0.68	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Unkn Imp	0.70	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Unkn Imp	0.79	ND	ND	ND	ND	ND	ND	ND	ND	ND	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Unkn Imp	0.88	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.05	0.06	0.06	0.08	0.09	0.13	0.16	0.17	0.17
Unkn Imp	0.96	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.09	0.11	0.16	0.22	0.24	0.24
Keto Imp	1.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Unkn Imp	1.17	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.05	0.05	0.06
Unkn Imp	1.25	ND	ND	ND	ND	ND	ND	ND	ND	ND	< 0.05	< 0.05	< 0.05	0.11	0.11	0.10
Unkn Imp	1.42	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	< 0.05	< 0.05	< 0.05	0.07
Styren Imp	1.60	0.12	0.12	0.12	0.12	0.12	0.12	0.06	0.06	0.06	0.14	0.14	0.14	0.13	0.12	0.11
Unkn Imp	1.79	ND	ND	ND	0.07	0.06	0.08	ND	ND	ND	< 0.05	< 0.05	0.06	< 0.05	0.05	0.06
Unkn Imp	1.90	ND	ND	ND	0.08	0.05	0.08	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sum		**0.55**	**0.55**	**0.55**	**0.71**	**0.66**	**0.71**	**0.72**	**0.69**	**0.63**	**1.98**	**2.01**	**2.62**	**3.26**	**3.33**	**3.09**

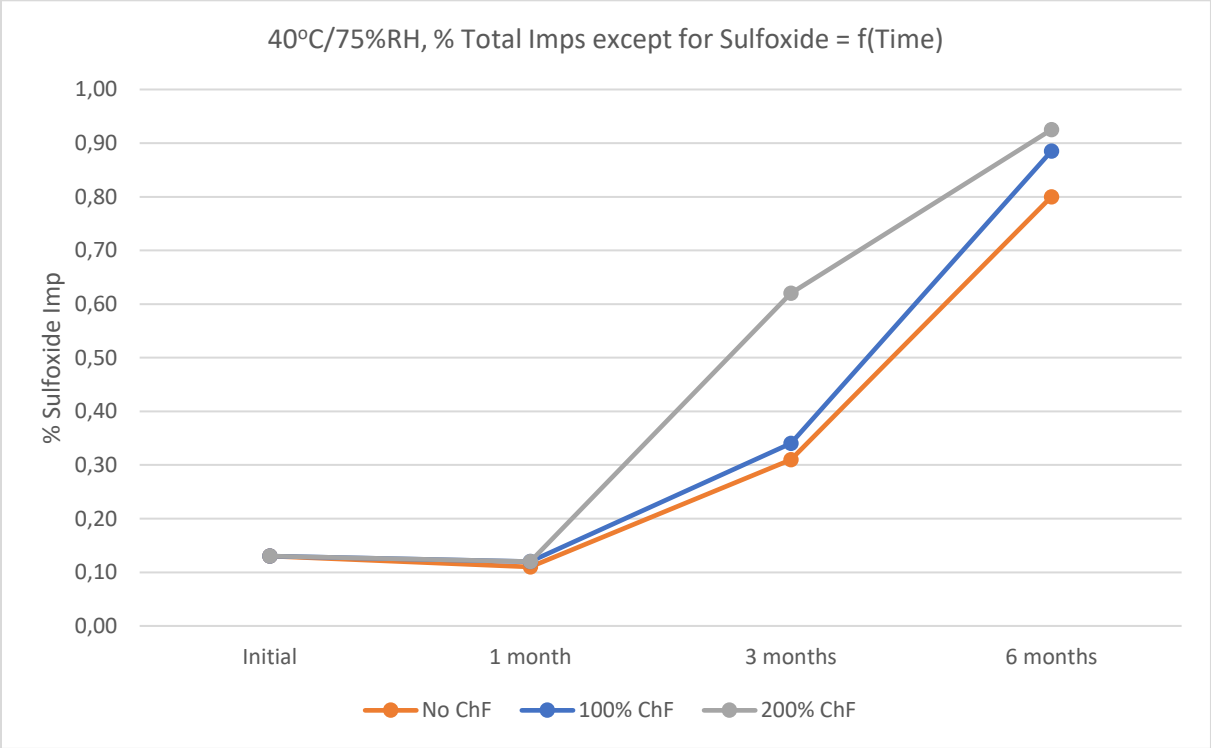
* as unexpected trend appeared the samples were re-analyzed to eliminate analytical error. Both sets of results were similar, average values are reported.

Figure 6-8 Graphical presentation of accelerated stability study on Montelukast 5 mg (no EDTA) formulation



Potential impact of Cherry flavor on Total impurities (without Sulfoxide) was also assessed for all three investigated formulations and summarized in Figure 6-9.

Figure 6-9 Impact of Cherry flavor on the formation of impurities other than Sulfoxide at 40 ℃/75%RH



At three months the relative difference between 200% sample and two other samples is approximately 50%, however, this is mainly caused by the presence of three unknown impurities at the quantity above the reporting threshold (RRT=0.47, RRT=0.63 and RRT=1.79), which are also present in two other samples but below the 0.05% and therefore not reported at all. When six months timepoint is analyzed the Total amounts of impurities other than Sulfoxide ranges from 0.80% for the sample without Charry flavor, through 0.89% for the sample containing the flavor as per the current formulation (100%), up to 0.93% for the sample containing 200% of Cherry flavor. Although the increase is observed its influence on the quality of the product is negligible.

Based on the information collected during the accelerated stability study the following conclusions regarding concentration of Sulfoxide impurity in investigated Montelukast tablets can be drawn:

- There is no significant difference between the three investigated formulations up to and including 1 month timepoint
- There is no significant difference between samples without sherry flavor and samples as per the original formulation (100%)
- 200% sample shows significant increase of degradation at 3 months (2.00% compared to 1.67%) and significant decrease at 6 months (2.17% compared to 2.44% seen in the original formulation)

The last observation requires further analysis of trends observed during stability study curried out at normal conditions.

6.3.3. Long Term stability assessment applying intermediate stability conditions (30°C/65% RH) – Drug Substance

Similarly to Section 6.3.1 stability data regarding Sulfoxide impurity available for the drug substance, intermediate conditions, was tabulated in Table 6-17 (p. 57) and presented for the reference purposes. Stability at normal conditions (25^0C/60%RH) are not reported as the manufacturer carried out the stability program at more strict conditions during the full length of product's shelf-life.

API samples were kept the same way they were kept for the purposes of accelerated program, i.e. in double polythene bag (inner transparent outer black) and then in aluminum bag. Silica bags were placed between both of polythene bag and also between outer polythene bags and aluminum bag under nitrogen atmosphere and sealed. The packed samples were kept in small HDPE containers and labeled suitably.

The data generated by the manufacturer shows the molecule is stable and Sulfoxide impurity does not generate above the LOQ level. In conjunction with accelerated stability data summarized in Section 6.3.1 the data indicates that any Sulfoxide impurity generated during drug product's storage is generated due to either formulation incompatibility and/or inappropriate primary packaging material choice.

Table 6-22 Summary of 36 months stability of Montelukast API supplied by Morepen, BN MK14-1011/I, intermediate conditions (30℃/65%RH)

Impurities	30°C/65% RH*							
	Initial analysis	**3 months**	**6 months**	**9 months**	**12 months**	**18 months**	**24 months**	**36 months**
Sulfoxide Imp	Below LOQ **	Below LOQ	Below LOQ	Below LOQ	Below LOQ	Below LOQ **	Below LOQ	Below LOQ
Sum	0.14	0.15	0.15	0.15	0.15	0.15	0.16	0.23

* the results are published by curtesy of Morepen, Montelukast's supplier.

** LOQ = 0.5%

6.3.4. Long Term stability assessment applying normal (Zone II) stability conditions (25°C/60% RH) – Drug Product

The results of Sulfoxide imp content for tablets kept at normal conditions are summarized in Table 6-23, the overall results including all impurities quantitated are summarized in Table 6-25 (p. 65).

The plot showing stability trends for all three formulations is shown as Figure 6-10 Graphical presentation of normal stability study on Montelukast 5 mg (no EDTA) formulation (p. 66)

Table 6-23 Summary of 24 months stability of Montelukast 5 mg tablets (without EDTA), normal conditions, with various amounts of Cherry flavor

	Sulfoxide Imp as %		
Timepoint	**No Cherry flavor**	**100% Cherry flavor**	**200% Cherry flavor**
Initial	0.42	0.42	0.42
3 months	0.48	0.48	0.47
6 months	0.66	0.63	0.73
12 months	0.73	0.77	0.84
18 months	0.84	1.01	1.01
24 months	1.30	1.34	1.67

The comparison of routine stability results of two commercial batches and stability of the three investigated formulation are summarized in Table 6-24. It has to be remembered that routine stability batches are blisters packed whilst investigated formulations are packed in sealed aluminum foil.

Table 6-24 Stability of investigated formulations vs. commercial batches, 25°C 60%RH

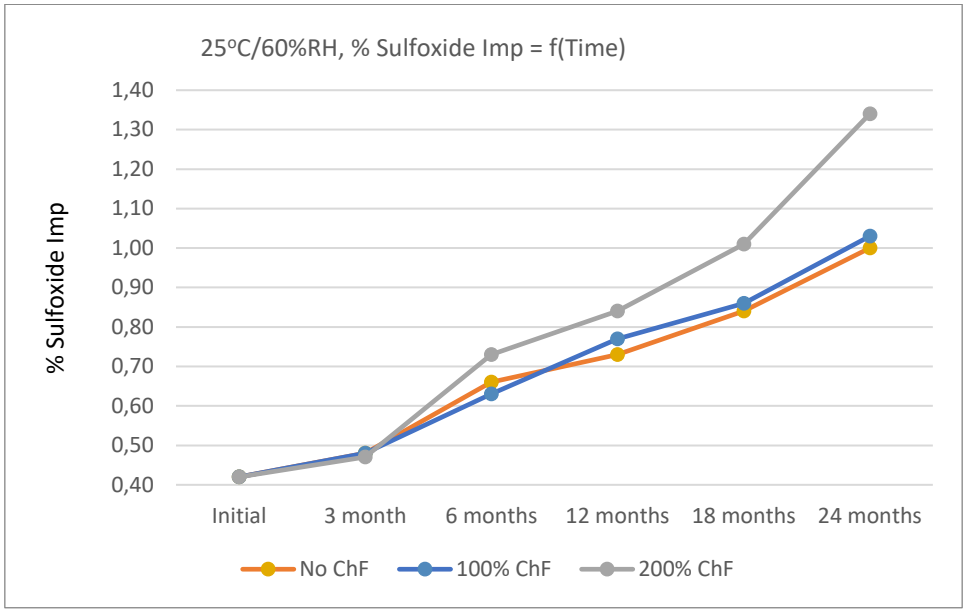
Formulation	**Batch No/ID**	**Timepoint (months)**						
		0	**3**	**6**	**9**	**12**	**18**	**24**
5 mg (no EDTA)	01090413	0.20	0.41	0.47	0.60	0.63	0.71	0.79
	01120413	0.17	0.48	0.60	0.76	0.81	0.94	1.09
Investigated formulations	No flavor	0.42	0.48	0.66		0.73	0.84	1.30
	100%	0.42	0.48	0.63		0.77	0.86	1.34
	200%	0.42	0.47	0.73		0.84	1.01	1.67

For grayed areas the timepoint was not included in the testing program.

Table 6-25 Detailed summary of 24 months stability of Montelukast 5 mg tablets (without EDTA), normal conditions, with various amounts of Cherry flavor

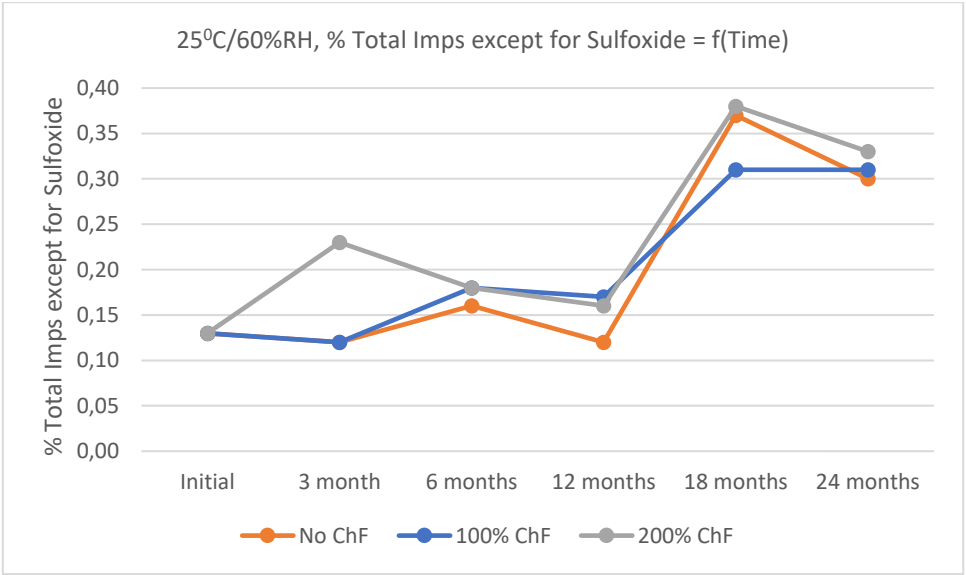
Impurities	RRT	25°C/60% RH (Start of Stability Program: 09/12/2019)																				
		Initial analysis			Tablets initially exposed to light/humidity for 2 days and retested			3 months			6 months			12 months			18 months			24 months		
		No ChF	100% ChF	200% ChF	No ChF	100% ChF	200% ChF	No ChF	100% ChF	200% ChF	No ChF	100% ChF	200% ChF	No ChF	100% ChF	200% ChF	No ChF	100% ChF	200% ChF	No ChF	100% ChF	200% ChF
Sulfoxide Imp	0.44	0.42	0.42	0.42	0.43	0.43	0.44	0.48	0.48	0.47	0.66	0.63	0.73	0.73	0.77	0.84	0.84	0.86	1.01	1.00	1.03	1.34
Keto Imp	1.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	< 0.05	< 0.05	0.05	< 0.05	< 0.05	< 0.05
Unkn Imp	0.63	< .05	<0.05	<0.05	<0.05	<0.05	<0.05	ND	ND	ND	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Unkn Imp	0.68	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0,05	<0.05	0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Unkn Imp	0.70	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.05	<0.05	<0.05	<0.05	<0.05	<0,05	<0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Unkn Imp	0.79	ND	ND	ND	ND	ND	ND	ND	ND	0.05	<0.05	<0.05	<0.05	<0.05	0.05	0.05	0.07	0.07	0.08	< 0.05	< 0.05	< 0.05
Unkn Imp	0.88	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.05	0.06	0.06	0.05	0.05	0.06	<0.05	<0,05	<0.05	0.05	0.06	0.06	0.07	0.07	0.08
Unkn Imp	0.96	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	<0.05	<0.05	<0.05	< 0.05	< 0.05	< 0.05	0.06	0.06	0.08
Unkn Imp	1.25	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	<0.05	<0.05	ND	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Unkn Imp	1.60	0.12	0.12	0.12	0.12	0.12	0.12	0.07	0.06	0.07	0.11	0.12	0.12	0.12	0.12	0.11	0.13	0.11	0.13	0.12	0.13	0.12
Unkn Imp	1.79	ND	ND	ND	0.07	0.06	0.08	ND	ND	ND	<0.05	<0.05	<0.05	ND	ND	ND	0.06	0.06	0.07	0.06	0.05	0.05
Unkn Imp	1.90	ND	ND	ND	0.08	0.05	0.08	ND	ND	ND	<0.05	<0.05	<0.05	ND	ND	ND	ND	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Sum		0.55	0.55	0.55	0.71	0.66	0.71	0.60	0.60	0.70	0.82	0.81	0.91	0.85	0.94	1.00	1.21	1.32	1.39	1.30	1.34	1.67
Sum (except Sulpoxide Imp)		0.13	0.13	0.13	0.28	0.23	0.27	0.12	0.12	0.23	0.16	0.18	0.18	0.12	0.17	0.16	0.37	0.31	0.38	0.30	0.31	0.33

Figure 6-10 Graphical presentation of normal stability study on Montelukast 5 mg (no EDTA) formulation



Similarly to accelerated study carried out at 40°C/75%RH, refer to Figure 6-9 (p. 60), potential impact of Cherry flavor on Total impurities (without Sulfoxide) was also assessed at 25°C/60%RH for all three investigated formulations and summarized in Figure 6-11.

Figure 6-11 Impact of Cherry flavor on the formation of impurities other than Sulfoxide at 25°C/60%RH



6.3.5. Water content in investigated formulations

So far laboratory trials proved there is formulation incompatibility between Montelukast and cellulose, refer to Section 6.2.4.1 Table 6-13 (p. 52). Further investigation on physical mixtures of cellulose, API and Cherry flavor proved the presence of latter one decreases the rate Sulfoxide impurity is formed, refer to Section 6.2.4.2 and Table 6-14 (p. 54). Correlation between the amount of Cherry flavor and concentration of Sulfoxide impurity generated during shelf-life was confirmed during accelerated stability studies (refer to Section 6.3.2). Although there is no significant difference between No Cherry flavor formulation and 100% Cherry flavor formulation, the 200% trial batch shows lower concentration of MTK2 at six months timepoint (1.75% vs. 2.04% obtained for No Cherry flavor batch). As the above results are not yet confirmed by the testing of three investigated formulations at normal conditions (so far 12 month stability samples are tested and the program is scheduled for another 12 months), water content by KF and LOD was checked to identify any potential relation between the concentration of Sulfoxide impurity and the content of water in investigated formulations.

Three batches were tested for H_2O by KF and LOD as per Section 5.7, the results are summarized in Table 6-26. 12 months stability samples (25C/60%RH) were tested, 100% Cherry flavor formulation. Cherry flavor was also tested for KF for information purposes. As the result is relatively low and in trend with ground tablets results no additional analysis was provided.

Table 6-26 KF and LOD results of three investigated formulations and Cherry flavor

	MOK 5 mg tablets batch 12112717 (0 % ChF)	**MOK 5 mg tablets batch 12112717 (100 % ChF)**	**MOK 5 mg tablets batch 12112717 (200 % ChF)**	**Cherry flavor batch 1004672949 (12054770)**
	12 months, 25 ºC, 60 %			----------
Loss on drying	3.1 %	3.1 %	3.2 %	----------
Water content	2.8 %	2.7 %	2.9 %	3.4 %

Conclusions:

Water content and LOD is similar for all batches investigated there.

7. Final conclusions

Formation of Montelukast's Suphoxide impurity during its storage is caused by two major factors: 1) API-cellulose incompatibility as shown in table Table 6-13 (p. 52) 2) sulfoxidation caused be other free radicals formed in the matrix during drug shelf-life.

In case of investigated formulation cellulose microcrystalline was found the excipient mainly responsible for Montelukast's oxidation to Sulfoxide impurity. The molecule itself does not contain any groups of atoms that could potentially lead to Montelukast's oxidation, however, the manufacturing step called bleaching, during which residual peroxides are cross-contaminants, may contribute to cellulose's oxidative properties.

Based on provided long term stability data, refer to Table 6-25 (p. 65), Cherry flavor affects the ratio Sulfoxide impurity is formed. In case current supplier of the flavor is to be changed it should be remembered that deeper analysis should be carried out. The main property expected from the flavor is its odor or taste masking ability, however, other properties hidden behind its composition should always be taken into account. As the excipient is of natural, plant origin it contains ingredients exhibiting oxidative or antioxidative properties. When the molecule exhibits oxidative stability, i.e. the drug product specification does not contain any know degradants being products of APIs oxidation, the qualification of new supplier can be simple and straightforward. However, to minimize the risk of any incompatibility it is recommended to carry out comparable stress study during which current supplier and new supplier are tested following similar stress conditions. The sample containing mixtures of active and flavor should be degraded and qualitative and quantitative analyses should be carried out. Regardless of the above it is recommended to test drug product containing old and new source of the excipient following stress degradation. Simulated laboratory mixtures reflecting the actual formulation details can be used if it is not possible to compress the tablets employing the real manufacturing process. All the above actions are strongly recommended in case the API is prone to oxidation.

Cherry flavor currently used by Adamed Pharma exhibits antioxidative properties and inhibits formation of the main oxidative degradant of Montelukast which is Sulfoxide impurity. The knowledge regarding such a property, once identified and confirmed, can be successfully used to modify current formulation(s) to decrease final concentration of MTK 2 at the end of product

shelf-life. If MTK 2 concentration at the end of shelf-life requires attention and action, the amount of Cherry flavor can be increased to meet specification limit with assumed safety margin. Alternatively, to avoid submission of regulatory variation, another source of Cherry flavor can be considered exhibiting higher antioxidative properties.

As the composition of Cherry flavor significantly affects the quality of medicinal product additional measures should be considered to assess its quality during Quality Control testing and raw material release process. Test such as Total Phenolic Content (TPC), Total Flavonoid Content (TFC) or Antioxidative Capacity (AC) should be considered as routine tests introduced to the excipient's specification. Non-quantitative tests, such as HPLC screening test for the identification of critical components, can also be considered to assess the quality of Cherry flavor, however, the identity of the components should have been confirmed first (e.g. by LC coupled with MS detector). Such a raw material's specification change, which does not require regulatory variation submission, secures the product in case of current supplier of the excipient is changed, or, the supplier changed his contractors or modified manufacturing process, which may result in different final composition and different antioxidative properties of material provided.

The influence of EDTA and Iron Oxide Red on stability of Montelukast tablets has not been subject of research described in this thesis, however, based on the data provided in Table 1-5 (p. 6) both EDTA and Fe_2O_3 seem to be the excipients of potentially significant properties inhibiting the increase of Sulfoxide impurity during product's self-life.

Alternative, regulatory approach to solve similar drug product problem should also be mentioned. In case the company is dealing with quality issues regarding exceeded specification limit for Sulfoxide impurity, and technological investigation does not provide conclusive answers, other than product reformulation approach may also be considered. Stability issues, not seen at the time the product was developed, are often results of some variations introduced, e.g. change of API supplier, change of excipient(s) supplier or slight modifications of manufacturing equipment or process. From regulatory point of view, it is easy to tighten specification limits when the whole manufacturing process is improved and better controlled, however, increasing initially setup limits at the time the dossier was submitted is always problematic and requires detailed and strong scientific justification. Also, decreasing specification limits is Type I variation which requires notification only and can be implemented immediately, opposite to Type II variation applied in case of any attempt to increase the limits, which may take - depending on different countries/authorities - up to 12 months before getting

the approval. In case of drug substances for which problematic, increasing with time over the limit impurity is drug's metabolite the chances of getting final approval are higher, however, the time required for the approval is often unacceptable. Six to twelve months without the product on the market, potential product recalls, will very likely force clients to find other suppliers and the product, if does not die, no doubt will be less profitable. Therefore, from business perspective, it is worth to take the effort and carry out expensive and very often time consuming research to find the root cause – to keep the profit the product generates.

8. Literature

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9. List of tables

Table 1-1 Formulation details of Montelukast 10 mg, film-coated tablets 2
Table 1-2 Formulation details of Montelukast 5 mg, uncoated tablets (with ethylenediaminetetraacetic acid disodium salt, EDTA) 2
Table 1-3 Formulation details of Montelukast 5 mg, uncoated tablets (without EDTA, the least stable formulation) 3
Table 1-4 Oxidative states of Sulfur in most popular organic compounds including Montelukast API (Sulfide) and its Sulfoxide impurity* 4
Table 1-5 Stability (expressed as concentration of Sulfoxide impurity) of three montelukast formulation at 25 /60% RH, 24 months 6
Table 1-6 Release specification limits for Sulfoxide impurity and Total impurities, Related Substances, for Montelukast 5 mg (with and without EDTA) and Montelukast 10 mg tablets formulations 6
Table 4-1 Stability plan for Montelukast tablets, three compression trials 24
Table 5-1 Gradient applied during HPLC analysis 27
Table 6-1 Potential flavonoids identified in Cherry flavor employing analytical method as per Section 5.2 37
Table 6-2 Comparison of RRTs for the original and modified HPLC method (Section 5.2) .. 38
Table 6-3 Comparison of peak areas found in two batches of Cherry flavor tested as per modified method described in Section 5.2. 38
Table 6-4 Results of TPC and TFC in Cherry flavor, BN and 1726666 42
Table 6-5 Results of screening testing of Antioxidative Capacity expressed as relative absorbance in Cherry flavor, BN 12054770, in three different diluents 43
Table 6-6 Stability of Base matrix, 40°C/75% RH, initials vs. 1 month, as percentage of normalized areas 45
Table 6-7 Stability of Base matrix plus Cherry flavor as per formulation, 40°C/75% RH, initials vs. 1 month, as percentage of normalized areas 45
Table 6-8 Stability of Base matrix plus 5 times amount of Cherry flavor compared to the original amount as per formulation, 40°C/75% RH, initials vs. 1 month, as percentage of normalized areas 46
Table 6-9 Stability of Base matrix plus Quercetin, 40°C/75% RH, initials vs. 1 month, as percentage of normalized areas 46
Table 6-10 Stability of Base matrix plus Ascorbic Acid, 40°C/75% RH, initials vs. 1 month, as percentage of normalized areas 47
Table 6-11 Content of Sulfoxide Impurity as normalised % area in base formulation plus Cherry flavor, Quercetine and Ascorbic acid. 49
Table 6-12 Influence of investigated antioxidants on concentration of MTK2 impurity. 51
Table 6-13 Combinations of Montelukast Sodium with excipients in various mass ratios 52
Table 6-14 The impact of Cherry flavor on oxidative properties of Microcrystalline cellulose 54
Table 6-15 The impact of aged cellulose on formation of MTK2 impurity 54
Table 6-16 Formulation of laboratory scale batches of Montelukast (as Sodium) tablets with various amounts of Cherry flavor 56

Table 6-17 Summary of 6 months stability of Montelukast API supplied by Morepen, BN MK14-1011/I, accelerated conditions (40°C/75%RH) 57
Table 6-18 Summary of 6 months stability of Montelukast 5 mg tablets (without EDTA), accelerated conditions, with various amounts of Cherry flavor (ChF) 57
Table 6-19 Stability of investigated formulations vs. commercial batches, 40°C 75%RH, Sulfoxide impurity..... 58
Table 6-20 Stability of investigated formulations vs. commercial batches, 40°C 75%RH, Total Impurities 58
Table 6-21 Detailed summary of 6 months stability of Montelukast 5 mg tablets (without EDTA), accelerated conditions, with various amounts of Cherry flavor..... 59
Table 6-22 Summary of 36 months stability of Montelukast API supplied by Morepen, BN MK14-1011/I, intermediate conditions (30°C/65%RH) 63
Table 6-23 Summary of 24 months stability of Montelukast 5 mg tablets (without EDTA), normal conditions, with various amounts of Cherry flavor 64
Table 6-24 Stability of investigated formulations vs. commercial batches, 25°C 60%RH 64
Table 6-25 Detailed summary of 24 months stability of Montelukast 5 mg tablets (without EDTA), normal conditions, with various amounts of Cherry flavor 65
Table 6-26 KF and LOD results of three investigated formulations and Cherry flavor 67

10. List of figures

Figure 1-1 Structure of Montelukast sodium 1
Figure 1-2 Structure of Sulfoxide Imp (MTK 2) – two (trans) enantiomers..... 3
Figure 1-3 Main oxidation degradants of Montelukast as reported by S. H. Tivaria et al. [6] .. 4
Figure 1-4 Structure of Montelukast Sulfoxide – two diastereoisomers, - trans (MTK2) and – cis (MTK1)..... 5
Figure 3-1 Mechanism of reaction by which Montelukast Sulfoxide impurity is formed..... 7
Figure 3-2 Mechanism of reaction by which Montelukast Sulfone impurity is formed..... 8
Figure 4-1 Tannic acid containing 10 galloyl groups 9
Figure 4-2 Main classes of phenolics [14]. 11
Figure 4-3 Chemical core structure of Flavonoids..... 12
Figure 4-4 Scavenging of reactive oxygen species (R) by flavonoid. The free radical Fl-O may react with a second radical, acquiring a stable quinone structure [25]..... 13
Figure 4-5 An ortho-dihydroxy (catechol) structure in the B ring [39] 14
Figure 4-6 2,3-Double bond in conjugation with a 4-oxo function in the C ring [39]..... 15
Figure 4-7 Hydroxyl groups at positions 3 and 5 [39] 15
Figure 4-8 Binding sites for trace metals [38]..... 16
Figure 4-9 Prooxidant activity of flavonoids [28]..... 17
Figure 4-10 In-vitro mechanisms of action describing antioxidative properties of polyphenolic compounds 18
Figure 4-11 Reduction of DPPH* to DPPH-H..... 20
Figure 4-12 Change in absorption spectrum (from magenta to yellow) upon reaction of DPPH with a radical [65] 21
Figure 6-1 Chromatogram of standard solution containing Ascorbic Acid, Rutin and Quercetin analyzed as per the method described in Section 5.2 36
Figure 6-2 Chromatogram of Cherry flavor, BN 41726644 (sample solution), analyzed as per the method described in Section 5.2..... 36
Figure 6-3. UV vs. MS chromatograms of Cherry flavour, BN 12054770..... 39
Figure 6-4 Separate UV/MS peaks chromatograms of Cherry flavour, BN 12054770, peaks 1-3..... 40
Figure 6-5 Separate UV/MS peaks chromatograms of Cherry flavour, BN 12054770, peaks 4-7..... 41
Figure 6-6 The impact of various antioxidants and Cherry flavor on formation of MTK 2 in investigated Montelukast formulation..... 47
Figure 7-2 Molecular structure of cellulose showing the numbering of the carbon atoms, the reducing end in red with hemiacetal, and non-reducing end in green with a free hydroxyl at C4 53
Figure 6-8 Graphical presentation of accelerated stability study on Montelukast 5 mg (no EDTA) formulation..... 60
Figure 6-9 Impact of Cherry flavor on the formation of impurities other than Sulfoxide at 40°C/75%RH..... 60
Figure 6-10 Graphical presentation of normal stability study on Montelukast 5 mg (no EDTA) formulation..... 66

Figure 6-11 Impact of Cherry flavor on the formation of impurities other than Sulfoxide at 25°C/60%RH..... 66

11. List of abbreviations

The following table describes the significance of various abbreviations and acronyms used throughout the thesis. The page on which each one is defined or first used is also given. Standard, well known and recognizable acronyms that are used in some places are not in this list.

AC - Antioxidative Capacity 20
AIBN - Azobisisobutyronitrile 4
API - Active Pharmaceutical Ingredient, Drug Substance 23
ATR-IR - Attenuated Total Reflectance Infrared Spectroscopy 20
ChF - Cherry flavor 61
DMSO – Dimethyl sulfoxide 43
DP - Drug Product 4
DPPH - 2,2-Diphenyl-1-picrylhydrazyl 38
DS - Drug Substance, Active Pharmaceutical Ingredient, API 4
EDTA – Ethylenediaminetetraacetic acid disodium salts 2
EMA – European Medicines Agency 3
FRAP - Ferric Antioxidant Power 14
FT-IR - Fourier-transform infrared spectroscopy 20
ICH – The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use 25
Imp – Impurity 53
MeOH - Methanol 43
MTK 1 - Sulfoxide impurity of Montelukast (cis) 4
MTK 2 - Sulfoxide impurity of Montelukast (trans) 4
ND - Not Detected i.e. below quantitation limit 63
ROS - Relative Oxygen Species 12
RS - Related Substances 49
RT - Retention Time 45
TEAC - Trolox Equivalent Antioxidant Capacity 14
TFC - Total Flavonoid Content 19
TPC - Total Phenolic Content 19

12. Summary of the Thesis

Montelukast, originally invented by Merck (MSD) and marketed as Singulair, belongs to leukotriene receptor antagonist family of medications and is used for a number of conditions including asthma, exercise induced bronchospasm, allergic rhinitis, and urticaria. Since patent expiry in 2012 many generic versions have been developed and registered around the word, including over 40 drug products in Poland. Common issue with increasing concentration of Sulfoxide impurity, main oxidation degradant, has been identified and addressed. The impact of Cherry flavor, one of the excipients commonly used to mask active substance's taste, has been investigated on the least stable formulation manufactured by Adamed Pharma. Tablets containing different amounts of Cherry flavor have been compressed and put on stability program at accelerated conditions (40°C/75% RH, 6 months) and long-term conditions (at 25°C/60% RH, 24 months). Seasoned tablets were tested for Related substances employing validated analytical method and gathered results indicate that the amount of Cherry flavor have significant impact on analyzed formulation, and may have impact on other formulations with Montelukast (as Sodium) as Active Ingredient.

13. Streszczenie

Montelukast, wynaleziony przez firmę Merck (MSD) i wprowadzony na rynek pod nazwą Singulair, należy do grupy leków będących antagonistami receptora leukotrienowego i stosowany jest min. w leczeniu astmy, powysiłkowego kurczu oskrzeli, alergicznego nieżytu nosa oraz pokrzywki. Od czasu wygaśnięcia ochrony patentowej w 2012 roku na świecie zostało rozwiniętych oraz zarejestrowanych wiele preparatów generycznych, w tym ponad 40 odpowiedników w Polsce.

Wytwórcy produktu leczniczego z Montelukastem doświadczają problemu z zanieczyszczeniem leku degradantem oksydacyjnym będącym sulfotlenkiem substancji aktywnej. Przedmiotem pracy badawczej jest ocena wpływu Aromatu wiśniowego, jednej z najczęściej stosowanych substancji pomocniczych mającej na celu zniwelowanie gorzkiego smaku substancji czynnej, na najmniej stabilną pod kątem zawartości w/w zanieczyszczenia formulację wytwarzaną w Adamed Pharma. Na potrzeby badań wytworzono serie tabletek zawierających różne ilości Aromatu wiśniowego, które poddano starzeniu w komorach stabilnościowych w warunkach przyspieszonych (40°C/75% RH, 6 miesięcy) oraz normalnych (25°C/60% RH, 24 miesiące). Sezonowane tabletki zostały poddane badaniom na czystość stosując wcześniej zwalidowaną metodę analityczną a uzyskane wyniki wskazują, że stężenie Aromatu wiśniowego ma istotny wpływ na stabilność badanej formulacji oraz może mieć wpływ na inne receptury zawierające Montelukast (w postaci soli sodowej) jako substancji aktywnej.

14. Published Papers

Publication as the basis of the dissertation:

1. **Mariusz Staśkiewicz**, Michał Nowicki, Klaudia Majewska, Justyna Nadajczyk, Aleksandra Rogut, Kamila Czarnecka, Paweł Szymański Evaluation of the potential impact of cherry flavour on the stability of montelukast tablets. *Acta Poloniae Pharmaceutica – Drug Research* (2022) accepted for publication APPDR-01208-2022-04; 23 June 2022

IF = 0,555; 100 p. MNiSW

Other scientific achievements:

1. Kamil Zawada, Kamila Czarnecka, Małgorzata Girek, Paweł Kręcisz, František Trejtnar, Jana Mandíková, Jakub Jończyk, Marek Bajda, **Mariusz Staśkiewicz,** Przemysław Wójtowicz, Katarzyna Dziubek, Robert Skibiński, Paweł Szymański New hybrids of tacrine and indomethacin as multifunctional acetylcholinesterase inhibitors. *Chemical Papers.* (2021) 75:249–264 https://doi.org/10.1007/s11696-020-01295-y

IF = 2.146; 40 p. MNiSW