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ANALYSIS OF 4-(4-HYDROXYPHENYL)BUTAN-2-ONE IN DIETARY SUPPLEMENTS

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Abstract in English

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Title of thesis: ANALYSIS OF 4-(4-HYDROXYPHENYL)BUTAN-2-ONE IN DIETARY SUPPLEMENTS

Raspberry ketone, (4-(4-hydroxyphenyl)butan-2-one), a key flavouring substance of raspberries, has been used in food supplements to support weight loss for several years. Following diploma thesis determines the present quantities of raspberry ketone in the *Raspberry Keto 400 Liquid* food supplement available on the Italian market. The presences of synephrine and quercetine in that product are also reported. Firstly, a raspberry ketone molecule was identified in the product by preliminary TLC and confirmed by NMR analysis and mass data. To establish fingerprint profile of the product HPTLC technique was used. To obtain quantitative analysis of raspberry ketone quantities, HPLC-DAD method was performed. The chromatographic separation was performed on a reversed phase column ODS-2 with gradient elution with acetonitrile-water. In this condition, linearity, inter- and intra-day precision and accuracy were within acceptable values. The developed method showed satisfactory precision and accuracy with overall intra- and inter-day variations below 1.2%, and the overall recovery of 101%. The proposed approach was successfully applied as a powerful tool for the evaluation of RK content in *Raspberry Keto 400 Liquid.*

Preliminary phytochemical screening of the extract confirmed the presence of RK and showed absence of both, synephrine and quercetin in the product. The quantitative analysis showed unusual RK content, compared with commonly occurring RK levels from natural sources. The quantities of RK reported in this analysis are not in accordance with the natural sources, there must be RK added as

substance probably of synthetic origin. The label of the product, promoting product as totally natural, was found as misleading.

Abstract in Czech

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Název diplomové práce: ANALÝZA 4-(4-HYDROXYFENYL)BUTAN-2-ONU V DOPLŇCÍCH STRAVY

Malinový keton, neboli (4-(4-hydroxyfenyl)butan-2-on), který je klíčovou látkou malinového aroma, je již několik let používán v doplňcích stravy na podporu hubnutí. Následující diplomová práce stanovuje množství malinového ketonu v doplňku stravy *400 Liquid Raspberry Keto,* který je dostupný na italském trhu. Rovněž byla monitorována přítomnost synefrinu a kvercetinu v tomto produktu. Nejdříve byla molekula malinového ketonu v produktu identifikována předběžným TLC, což bylo potom potvrzeno i analýzou NMR a získáním MS dat. K získání „fingerprinting" analýzy vzorku bylo provedeno HPTLC. Kvantitativní zhodnocení obsahu malinového ketonu ve vzorku bylo provedeno metodou HPLC-DAD. K separaci byla použita chromatografická kolona s reverzní fází ODS-2, za použití gradientu směsi acetonitril-voda. Za těchto podmínek stanovená linearita, inter- a intra-denní přesnost byly v přijatelných hodnotách. Vyvinutá metoda ukázala uspokojivou preciznost a přesnost s celkovou intra- a inter- denní odchylkou pod 1.2%, přičemž celková výtěžnost byla 101%. Uvedený postup byl zhodnocen jako účinný nástroj pro hodnocení obsahu malinového ketonu v produktu *Raspberry Keto 400 Liquid*.

Předběžný fytochemický screening potvrdil přítomnost malinového ketonu a vyvrátil přítomnost synefrinu a quercetinu v analyzovaném vzorku. Kvantitativní analýza ukázala neobvykle vysoký obsah malinového ketonu, ve srovnání s běžně se vyskytujícími koncentracemi ketonu v přírodních zdrojích. Zjištěné množství není v

souladu s původem z přírodních zdrojů, a nasvědčuje dodatečnému přidání malinového ketonu do produktu, pravděpodobně syntetického původu. Etiketa produktu propagující produkt jako zcela přírodní byla shledána jako zavádějící.

Table of Contents

List of Figures

List of Tables

Abbreviations

ACS	American Chemical Society
CAS	Chemical Abstracts Service
$CDCl_3$	Deuterated chloroform
CFR	Code of Federal Regulations
Co.	Company
DR	Derivative Reagent
DCM	Dichloromethane
EFSA	European Food Safety Authority
EtOAc	Ethyl acetate
EU	European Union
FDA	U.S. Food and Drug Administration
FEMA	Flavor and Extract Manufacturers Association
FS	Food Supplement
FSA	Food Standards Agency
GC	Gas Chromatography
GC-MS	Gas Chromatography - Mass Spectrometry
GRAS	Generally Recognized as Safe
HPTLC	High Performance Thin Layer Chromatography
ICH	International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid Chromatography
LC-MS	Liquid Chromatography - Mass Spectrometry
MP	Mobile Phase
NMR	Nuclear Magnetic Resonance
NPR	Natural Product Reagent A
PTFE	Polytetrafluorethylene
QSAR	Quantitative structure–activity relationship
RK	Raspberry Ketone

RPE	Analytical grade reagents
SST	System Suitability Test
WHO	World Health Organisation

1 Introduction

According to the World Health Organisation (WHO) definition, overweight and obesity are abnormal or excessive fat accumulations that may impair health. Obesity is causing many risks, ranging from an increased premature death to several debilitating complaints that have lowering effect on life quality. Obesity is also an important risk factor for non-communicable diseases such as diabetes mellitus type II, cardiovascular diseases and cancer, usually associated also with various psychosocial problems. Obesity is considered a public health problem with an epidemic phenomenon, which nowadays causes more deaths worldwide than underweight. The simplest solution of achieving and maintaining weight loss should be changing life-style with focusing to increase levels of physical activity and improving the quality of the food input. However, in practice controlling overweight and obesity through the healthy diets and physical activities can be for many people difficult to achieve [1,2].

When lifestyle changes alone are not sufficient in reducing weight, there are also more radical solutions as gastric surgery or drug therapy. Currently few drugs are approved for weight loss purposes in the European Union. All of them are prescription-only drugs, except orlistat which can be in lower dosage purchased also over-the-counter. Necessity of prescription and higher price of weigh loss medications, supported with free availability of nonprescription supplements have an increasing effect on the weight loss food supplements consumption [1,3].

There are many various dietary supplements products on the market, promoting different health claims, sometimes even without proper content information on the label. At the same time, many studies reporting the low quality of the supplements can be found, especially concerning non-preciously defined compositions.

The possibility to determine exact content of the marketed supplement and define its particular properties is crucial characteristic to evaluate product safety. Various analysing procedures can be used, but in the field of supplements, usually high performance liquid chromatography (HPLC) and gas chromatography (CS) techniques are the methods of choice. Separation techniques are often used in the

combination with spectral analysis, as for example GC with mass spectrometry (GC–MS) or LC with mass spectrometry (LC–MS). Anyway, the concrete technique selection depends on the property to be measured, the type of product to be analyzed, and the reason for carrying out the analysis [4,5].

2 Aims of the project

As a matter of fact, many food supplement products containing unauthorized ingredients could be found on the market. Other problematic issue in the field of dietary supplements is that several herbal food supplements (botanicals) are under accusation that content of active substance is not just as natural as declared. There could be purposely added synthetic compound to increase activity and performance of the marketed product. Therefore, analytic content control of suspicious marketed food supplements is necessary to avoid potential side effects. Analysis of botanicals is a difficult task, considering the composition of a multi-ingredient product, where several natural substances are used. Thus, it is the topic of this diploma thesis to further evaluate the food supplement product, marketed as completely natural, composed of raspberry ketone (RK) as main active substance [2,5,6].

Exposure of RK in common diet from fruits and flavourings has been estimated to be ranged from 1.8 to 3.8 mg/day for an adult (EFSA 2011 and Crispim 2010). Currently, the daily doses of RK recommended by food supplement suppliers are well above that range. On the internet recommended doses ranging up to 1400 mg/day can be found. In fact, that is about 368 times higher dosage than the highest estimated exposure from diet. Additional safety and toxicology analysis are needed to ensure that such concentrations of RK are having health benefits. Without any further research on the effects of consuming such a high concentrations of RK, consumers should be wary of unsubstantiated claims and mindful of potential harmful effects to the health [7,8, 9,10].

This work reports analysis performed with *Raspberry Keto 400 Liquid* product available on the Italian pharmaceutical market. The main object is to determine the present quantities of RK in that product. Detection of synephrine and quercetin presences was also reported, because presence of these constituents is claimed on the product label. Furthermore, synephrine and quercetin occurrences are expected in complex botanical matrices from natural sources.

Firstly, the preliminary TLC analysis was made to detect RK presence in the product.

Further, HPTLC analysis was carried out, to obtain HPTLC fingerprinting of the sample extract. A fingerprint of product was obtained, avoiding the necessary identification of single constituents which could be present. Analysed product was then separated by CC to isolate RK fraction. Structure of RK was confirmed by NMR and mass spectra analysis. Finally, quantitative evaluation of the analysed sample was made by HPLC.

3 Theoretical part

3.1 RK in food supplements

European Food Safety Authority (EFSA) defines food supplements as concentrated sources of nutrients with nutritional or physiological effects whose purpose is to supplement the ordinary diet. Products marketed as food supplements, supply nutrients, micronutrients and other physiologically active substances in predetermined amounts. A lot of those products are based just on plant extracts or plant constituents. Hence, as the popularity of natural sources is increasing, overweight or obese subjects are tempted to buy weight loss food supplements that are freely available in drug and health food stores, groceries and on the Internet. Especially in a competitive Internet market patients can be confronted with claims such as easy and quick weight loss, 100% naturally, and totally safe [6 ,11].

Raspberry ketone is the essential flavour constituent of raspberries. Since 1965, when RK got GRAS safety status from FEMA (Flavor and Extract Manufacturers Association) [12], it has been commonly used as a flavour and aroma ingredient in food industry, perfumery and cosmetics. It is important to note that the FDA did not confirmed FEMA's GRAS classification. As stated in 21 CFR 172.515 regulation [13], FDA designated RK as an adjuvant which can be used only in the minimal quantities required for intended effect. This flavouring agent purpose is stated also in EU Flavouring Regulation 1334/2000/EC [7, 8,14].

In recent years this molecule grabbed people's attention, when was published the information about possibility of its "miraculous" slimming effect. Anyway, RK for other use, than as a flavouring agent, was not legislatively allowed. That was one of the reasons why in March 2014 Food Standards Agency (FSA) classified RK as a novel food (except of its extract prepared using water or 20% EtOH, 1:4 EtOH to water). In accordance to EU legislation, an ingredient is labelled as a novel food when there is no evidence of its consumption to a significant degree in EU before the 15th May 1997. Novel foods may only be legally sold in the EU countries if they have been authorised for following aspects: a) *does not present a risk to the consumer*; b) *does not mislead the consumer*; and c) *is not nutritionally disadvantageous compared with other foods that it might replace in the diet* [15, 16]. RK molecule has not been evaluated and did not get approval to be sold in EU countries before authorisation. Even though RK apart from some specified extracts is considered novel food, it is still marketed on the Internet market in food supplements. This is challenging fact for the food authorities to ensure that food supplements containing unauthorised constituents are neither sold in groceries nor on online where anyone can distribute these supplements around whole world despite all regulations [2, 6,7].

However, a health claim application at EFSA for *Rubus idaeus* L. extract, named BERI-08, was not approved due to lack of scientific evidences. Later, EFSA reinforced this statement, considering this extract potentially not safe [17].

3.1.1 Botanical overview

Raspberry ketone term defines a phenolic compound with the IUPAC name 4-(*p*-hydroxyphenyl)butan-2-one, that is a key component responsible for natural raspberry fruit aroma. It can be found in several sources. Although predominant occurrence in raspberries (*Rubus idaeus* L.), RK has been detected in huge diversity of species. As other natural sources could be mentioned baby kiwi (*Actinidia arguta*), cranberry (*Vaccinium oxycoccus L.*), yew (*Taxus baccata* L.), pine needles (*Pinus densiflora*), orchid flower lip (*Bulbophyllum apertum*), traditional Chinese

medicine herb *Rheum officinale*, sea-buckthorn (*Hyppophae rhamnoides*) or freshly brewed coffee (*Coffea arabica* L.) [8,18,19].

Although many botanicals contain 4-(*p*-hydroxyphenyl)butan-2-one, considering very low RK amounts reached, the only practically used sources of this volatile compound are raspberry cultivars. RK concentrations in the raspberry fruits vary greatly. The data reported in literature for the most common variety of raspberry, *Rubus idaeus* L., are listed in Table 1 below. As resulting from RK occurrence level in fruits, about kilograms of raspberries would be needed to isolate just 1 gram of RK. Obviously, this fact makes natural 4-(*p*-hydroxyphenyl)butan-2-one (US$3000/kg), much more expensive than synthetic one (US$58/kg) [8,20].

Table 1: Raspberry ketone concentration range in *Rubus idaeus* L., given in µg /100g of fruits

Gallois et al., 1982 [21]	20-370
Borejsza-Wysocki et al.,1992 [14]	10-70
Maquin et al., 1981 [14]	0.9-17.4

3.1.2 Chemical structure

The molecular formula of 4-(*p*-hydroxyphenyl)butan-2-one is $C_{10}H_{12}O_2$ with molecular weight of 164.2 g/mol. Its structure was firstly described by Schinz and Seidel in 1957 [14].

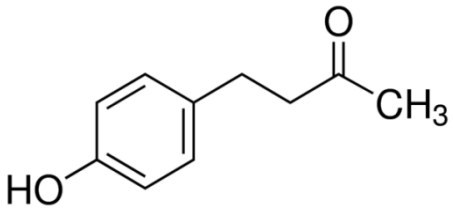

Figure 1: Structure of raspberry ketone
(Drawn in JSDraw V3.2.3)

There is slight chemical similarity in the structure of RK to two other compounds, synephrine and capsaicin [22].

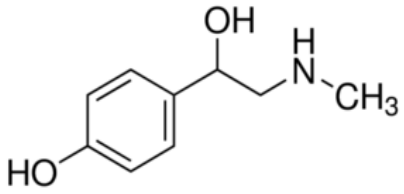

Figure 2: Structure of synephrine
(Drawn in JSDraw V3.2.3)

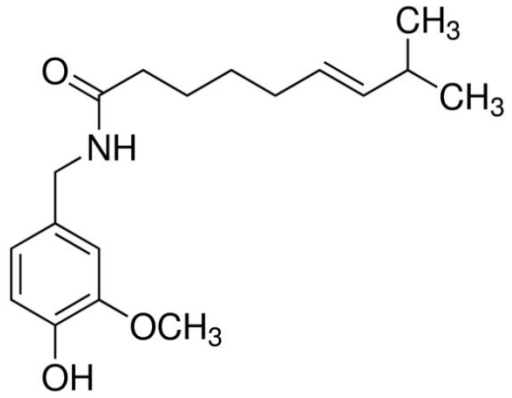

Figure 3: Structure of capsaicin
(Drawn in JSDraw V3.2.3)

In these three compounds the main sequence is maintained in hydroxyphenyl. Other similarity feature is a presence of bonded oxygen, in synephrine boned in alcoholic hydroxyl group, in RK and capsaicin in carbonyl groups. Both, synephrine and capsaicine have been used in food supplements due to their weight-loss effect. For example, anti-obese activity of RK has been estimated 3 times higher than capsaicin [22,23].

A sympathomimetic synephrine, can stimulate lipolysis via beta-adrenergic receptors. It is structurally similar to other sympathomimetic ephedrine. Ephedrine, known as amphetamine-like compound, was banned for its central nervous system stimulatory activity and cardiovascular toxicity [24].

Capsaicin is also a sympathomimetic, which naturally occurs in cayenne pepper, has reported increasing effects on lipid metabolism. In recent years, a few reported studies accusing capsaicin for cardiovascular toxicity as well [25, 26, 27].

Due to the structural similarities which usually elicit similar biological activity, RK has been investigated for its influence in weight loss and potential toxicity [22].

3.1.3 Mechanism of action RK

Despite reported effects of RK in weight loss, the underlying mechanism of RK action has not been thoroughly understood.

In the study of Morimoto et al. (2005) performed with mice fat cells, RK increased lipolysis in white adipocytes with dose-response effect and inhibited intestinal absorption of dietary fat.

The study of Park (2010) investigated higher levels of adiponectin stimulated by RK. Adiponectin is an adipocytokine secreted by adipose tissue, which regulates lipid and glucose metabolism. Therefore, increased level of adiponectin induces lipolysis. On the other hand, decreased level of adiponectin was found to be associated with obesity. Further, Park reported increased fatty acid oxidation by RK treatment [22, 28].

However, considering toxicological studies, where weight loss regards to potentially harmful effect, weight reduction seen in laboratory animals should not be interpreted as a slimming or anti-obesity activity [7].

3.1.4 Toxicological data on RK

3.1.4.1 Toxicological studies *in vivo*

In a literature review, only one published study reviewing RK toxicological activity in laboratory animals was found (Gaunt et al., 1970). No Observed Adverse Effect Level (NOAEL) was estimated in that study for 100 mg/kg of body weight per day, which was based on evaluation of relative weights of liver and kidneys in male rats. No studies reporting genotoxicity, carcinogenicity or reproductive/development toxicity performed with RK were identified in the literature search [7].

3.1.4.2 Toxicological studies *in silico* and *in vitro*

A few studies evaluating RK toxicity based on structure-activity relationship were found. Because of above mentioned structural similarity or RK with cardiotoxic compounds, a hazard for potential cardiotoxicity was identified. Because of hydroxyl group presence on a free benzene ring in the RK structure, some approaches contribute RK ability to interact with the androgen receptor. In a study of Bredsdorff

et al. (2015), performed based on QSAR models *in silico*, RK was evaluated within the structural similarity with 54 models. From these 54 models possible cardiotoxicity was related with 1 model, reproductive toxicity with 2 models and developmental toxicity with 2 models too [7].

Estrogenic activity was studied also *in vitro* on a yeast assay and human breast cancer cells. Compared to 17-β-estradiol, the estrogenic activity of RK was found 30.000.000 times lower [7]. In another study (Ogawa et a. 2010), androgenic antagonistic activity was evaluated with human breast cancer cell line. Results showed its IC_{50} value (concentration of producing 50% inhibition) of 252 μM, which means that RK is very weak androgenic antagonist if at all [23].

In conclusion, toxicological RK evaluations are mostly negative. However, considering the lack of available data, RK cannot be excluded for potential adverse effects on reproduction, development or cardiotoxicity [7].

3.2 Analytical methods in food supplements analysis

The possibility of proper analytical control of food supplement is a major concern of regulatory authorities, which are developing standardization guidelines in order to facilitate the quality control [4].

Analysis of food supplements is challenging issue, considering the content of various compounds presented in those products. Especially in botanical food supplements (botanicals), where are complexes of biological matrices. Some compound in the analysed product can contain reactive functional groups that interfere with the compound of our interest, influence its solubility, stability and resorption [29].

So far, in the quality control of dietary supplements two analytic approaches are possible. The first one is restricted to the single chemical entities or specific class of compounds in the analysed product. The second approach, sometimes called "fingerprint analysis", is avoiding necessary identification of single constituents and considers global analysis of whole mixture. Fingerprint analysis received attention especially in the field of multi-ingredient natural supplements [4, 5].

3.3 Chromatographic methods

Chromatographic methods are separation techniques based on sample components separation, which are carried out by the mobile phase (liquid or gas) and resolved by sorption-desorption steps on the stationary phase (solid or liquid). Molecules of different analytes have various affinity to stationary phase, which appears that analyte with weaker interactions to stationary phase is less retained.

According to the physical state of mobile phase, chromatography can be classified as gas chromatography, using inert gas as mobile phase, or liquid chromatography, using liquid mobile phase. There are planar liquid chromatographic techniques, utilizing a flat (planar) stationary phase and an open system, as opposed to column chromatography methods using vertical columns [30 , 37].

3.3.1 Planar liquid chromatography

Thin layer chromatography (TLC) and its last planar chromatography evolution, high performance TLC (HPTLC), are easy tools whose major application area is currently control of quality, purity, stability and identity in the analysis of multi-compositional natural products. Both methods have many advantages, as for example short time lasting procedures and the possibility of multicomponent sample analysis, under the same chromatographic conditions, respectively. They are also environmentally friendly, because of the low consumption of mobile phase and its short running time. In addition, it minimizes costs of the analysis [4, 5].

After the sample and standard application on the stationary plate (usually concern silica gel) is the plate developed in the chamber saturated by mobile phase. TLC/HPTLC differs from other chromatography methods in the fact that in addition to stationary and mobile phases, another gas phase is present, which can influence the result of the separation. Developed plate can be detected by fluorescence in UV light or by specific reaction, for compounds that are not naturally coloured. Specific reagents applied by dipping or spraying enable their selective detection. In the obtained chromatogram of HPTLC are several separated spots in a track, each corresponding to a single constituent of analysed product. The total track record,

called fingerprint is useful approach to obtain a proper identification of a plant material and determine biological changes in the analysed product [30, 37].

Qualitative identification can be achieved by comparison of developed spots by retention factors with standards. Retention factor is a value which is the ratio of the migration distance of the compound of interest to the mobile phase front. Furthermore, spot colour of analysed compound should be coherent with standard spot. Quantitative analysis can be done by converting series of peaks by densitometry [29,31].

3.3.2 High performance liquid chromatography

Recently, the most widely used column chromatography techniques is liquid chromatography using high pressure called high performance liquid chromatography (HPLC). HPLC is regarded as standard method for quantitative determination of components in dietary supplements and botanicals, as well like in products of pharmaceutical and food industry.

HPLC is a separation technique, which allows both, qualitative and quantitative determination of components in analysed product. Analysis by HPLC is fast and very sensitive, even only a little amount of analysed sample is necessary. Another advantage of HPLC is its possibility of fully automatic process, which allows sample application in various modes [4, 31].

The stationary phase in the column is usually silica gel modified by bonding various radicals. According to the used stationary phase, various separations techniques can be done. As for example, reversed phase chromatography, which is one of the most used ones. In contrary to normal phase chromatography, where silica gel surface is covered with polar silanol groups, reversed phase technique is using non-polar radicals bonded to the silica gel hydroxyls (usually hydrophobic octyldecylsilane C18 or C8 carbon chain). In the chiral chromatography, chiral stationary phase is used. This technique is designed to separate enantiomeric mixtures, which is allowed by specific interactions of stationary phase with one enantiomeric form. Ion-exchange chromatography is designed to separate ionic and ionizable compounds. If negatively charged molecules or anions are analysed, cationic or positively charged

stationary phase is used. Conversely, in cationic exchange chromatography of positively charged molecules, negative solid support is used. This technique is used mostly for proteins purification.

HPLC analysis done with constant composition of the mobile phase during the separation process is called an isocratic elution. On the other hand, separation when mobile phase composition is changing is described as gradient elution. Usually, the initial mobile phase is very weak (usually water) and the % of organic solvent is increased in time as follows to the specific gradient programme. Benefits of gradients include sharper peaks and according to the increasing MP strange also reduced build-up of contamination. Gradient elution is used when variety of components are present in the analysed sample.

Essential qualitative characteristic of performed HPLC is retention time. Retention time (t_R) is the time between injection and the appearance of the peak maximum. Quantitative evaluation is based on the peak area, which is related to the amount of eluted substance. Peak area is usually measured by an integrator or system software [31,32].

The schematic of an HPLC instrument includes mobile phase reservoir, pump, sample injector, column and the detector. Pump carries mobile phase solvents through the column into the detector. Automatic sample injectors whose have replaced manual injectors are enabling to inject liquid sample to the column within the volume range of 100 nL and 100 μL with high reproducibility. Columns containing stationary phase are made from cylinders of stainless steel or polymers. They are available in various dimensions, with different length and internal diameter (id). Column parameters affect sensitivity and efficiency of the separation, and determine analyte volume that can be loaded onto the column. For example, smaller id columns have higher sensitivity compared to larger id columns, but with reduced loading capacity. Further, there is a detector connected to the computer with appropriate data evaluation units. Most detectors currently used in HPLC also can be used in the analysis of food supplements analysis [31,32,33].

The detection of HPLC is usually made by spectrophotometry, when the measured characteristic is absorbance of electromagnetic radiation (light) by eluted components. Radiation can be measured in the UV spectra or visible light (VIS). UV detectors can have working wavelength fixed during the analysis, absorption spectra can be scanned in the peak maximum or there are diode array detectors (DAD), which can scan the absorption spectra in the several wavelengths modes simultaneously [31, 32].

Recently popular tool in the detection of constituents is HPLC coupling with mass spectroscopy (MS). This combination of HPLC-MS allows structural identification of chemical constituents present even in very low concentrations.

3.3.3 Gas chromatography

Another type of column chromatography, which can be used for the analysis of complex mixtures, such as plant extracts from botanicals, is gas chromatography (GC). Botanicals can be analysed by GC because of the facts, that plant constituents are usually thermostable and can be volatilized at temperatures less than 265-300°C [4, 31]. In the GC procedure is volatilized sample transported by the inert carrier gas through the stationary phase of the column. Common detectors are flame ionization (FID) for compounds containing carbon, electron capture (ECD) for halogenated compounds, and flame photometric (FPD) for compounds with sulphur or phosphorous, and finally nitrogen-phosphorous (NPD) for compounds containing nitrogen or phosphorous. The location of the analyte spot on the gas chromatogram is described by retention time, which is similar to HPLC [37].

3.4 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) is spectroscopic technique routinely used for structure determination of organic compounds. The method principle is that atomic nuclei of the analysed sample have magnetic properties, due them interact with external magnetic field.

Nucleus is spinning positively charged particle, which can have its own spin. Spin is, loosely speaking, angular moment form of a particle. Particles with non zero nuclei

spin are those with odd number of protons (e.g. ^{1}H, ^{14}N) or odd number of neutrons (e.g. ^{13}C). They behave like magnetic dipoles and interacts with magnets, in other words, they can be analysed by NMR spectroscopy.

Analysed sample is placed into a strong magnetic field, which turn spins from random orientation to organised, in the respect to external magnetic field. Spins can be oriented in lower energy stage (spin-up), which is parallel with applied magnetic field or anti-parallel (spin-down). The existence of two various energy levels for a single proton provides the fundamental key for NMR spectroscopy. When a proton in the more stable parallel state is irradiated with electromagnetic radiation at the resonant frequency, it absorbs energy and excites to the more energetic spin-down stage. Further, excited nuclei return to the lower energy stage by relaxation process called spin-lattice relaxation. Both can be observed, the absorption of the energy or the subsequent emission of energy during de-excitation [4, 34].

NMR spectrometer construction contains a strong magnet, radiofrequency transmitter amplifier and detector, connected to computer. Nowadays usually the Fourier transformation is done, when the transmitter is turned on for a few microseconds to excite all nuclei simultaneously by the pulse of radiation. Its response is sampled as a function of time and the NMR spectrum is then calculated by software. Since the spectrometer is controlled by software, the analysis can be easily repeated and the average signal can be calculated.

The NMR spectrum, a plot of absorbed energy against applied radio frequency, provides information about the frequency at the peak response and about the peak properties. Frequency is indicative of the proton characteristics. The peak area is corresponding to the quantity of protons in the sample and the peak width indicates the rate at which protons transfer energy when excited [35].

For a molecular structure determination is particularly important to know resonant frequencies in NMR spectra, because it is related with proton energy. Proton energy is affected by applied magnetic field strength, which is fixed, and proton surrounding in the nucleus. The electron density surrounding the nucleus causes its shielding and therefore is resulting in decreased absorption of the nucleus. Differences in absorbed

energy refer as chemical shifts seen in NMR spectra. The chemical shift of a nucleus is expressed by comparing with resonance frequency of the reference compound (often tetramethysilane, TMS). The quantity of chemical shift (delta, δ) is reported in parts per million (ppm). Shifts in ^{13}C NMR spectra (δ=1-220 ppm) are much greater than shifts in ^{1}H NMR (δ=0-10 ppm) relative to TMS. Zero point on the chemical shift scale is at the right with numbers increasing to the left. A higher chemical shift value indicates a nucleus with lower electron density around [34, 35].

If two or more protons (or indeed carbons) are located in an equivalent chemical environment, then they are resulting in the same chemical shift which appears in spectra like one signal. In fact, most of organic compounds contain more than one hydrogen atom, so interactions between them must be considered. Non-equivalent nuclei which are close to each other in the distance of maximally three bond lengths, they have an influence on each other's magnetic fields. Power of interaction is expressed by *J* constant, which is measured in Hertz (Hz). This effect is called spin-spin coupling. The coupling splits the peaks in the spectrum into a group of peaks called a multiplet. According to general rule (n+1) is a signal of hydrogen atom, which is surrounded by n equivalent hydrogens splitted into (n+1) lines. Multiplet is specifically called according to the number of lines in the peak doublet, triplet, quartet, quintet etc. Coupling is often seen in ^{1}H spectra, but rarely with ^{13}C, because C^{13} makes only 1.1% of naturally occurring carbon [34].

Particularly, NMR allows simultaneous determination of natural components and low-molecular-weight additives in the food supplements. Finally, a more useful approach for structure determination is the combination of NMR spectra with other techniques, as for example mass spectroscopy (MS). In MS are ionized atoms and molecules separated according to their mass to charge ratio (*m/z*). Therefore, it provides information of mass, atomic composition and structure through fragmentation process [4].

4 Experimental part

4.1 Analysed product description

Analyses were performed with food supplement *Raspberry Keto 400 Liquid* product, made by Dietalinea® (General Dietetics Pharma Co. from Genoa, Italy), packed in 500 mL bottle. The marketed product reports in the label content of several juices or extracts as active substances. There is extract of raspberry fruits (*Rubus idaeus* L. *fructus*): 400 mg (containing 260 mg of RK), extract of bitter orange (*Citrus aurantium* L. sub. *amara fructus immaturus*) 160 mg containing 9.6 mg of an alcaloid synephrine; and an extract of onion (*Allium cepa* L.) 40 mg accounted for 18 mg of flavonoid quercetin. There are also small amounts of proteins (<0.2 g) and lipids (<0.2 g). Carbohydrates present 4.7 g and the rest is of inorganic origin, represented by 40 μg of chrome (no oxidation reported). The recommended use for weight loss is 20 mL/ day for at least 25 days treatment.

4.2 Solvents, chemicals and standards

4.2.1 Chemicals

Chemicals were purchased from Sigma-Aldrich or Carlo Erba Reagenti, both from Milan, Italy.

Acetonitrile (HPLC grade)

Ammonium hydroxide (ACS grade, NH_4OH)

p-Anisaldehyde 98%

Chloroform (ACS grade, $CHCl_3$ containing 0.5-1.0% EtOH as stabilizer)

Dichloromethane (HPLC grade, CH_2Cl_2 containing 40-150 ppm amylene)

Ethanol 95%, denatured with methanol (EtOH)

Ethyl acetate (EtOAc, puriss. p.a.)

Formic acid ≥98 % (puriss. p.a.)

Glacial acetic acid ≥99.8% (AcOH, puriss. p.a.)

n-Hexane ≥99% (puriss. p.a.)

Methanol (MeOH, HPLC grade)

Sodium sulphate (Na_2SO_4) granulated drying agent (puriss. p.a.)

Sulfuric acid 95-97% (puriss. p.a.)

Toluene ≥99.7% (puriss. p.a.)

Ultrapure Milli-Q water

4.2.2 Standards

All reference standards were obtained from Sigma-Aldrich Co., (Milan, Italy)

RK standard (99.0%), CAS Number 5471-51-2

Synephrine standard (≥95.0%), CAS Number 94-07-5

Quercetin 4′-glucoside standard (≥97.0%), CAS Number 20229-56-5

4.2.2.1 Preparation of standards solutions

Raspberry ketone solution: Stock solution of RK was prepared by dissolving 5.0 mg of RK standard in 5.00 mL of MeOH in a volumetric flask.

Quercetine solution: Stock solution of quercetin was prepared by dissolving 5.0 mg of quercetin 4′-glucoside standard in 5.0 mL of MeOH in a volumetric flask.

Synephrine solution: Stock solution of synephrine was prepared by dissolving 5.0 mg of its standard in 5.0 mL of MeOH in a volumetric flask.

Aqueous and chloroform phases of synephrine were prepared in a separatory funnel. Synephrine standard was transferred to the funnel. Secondly chloroform and distilled water were added to the funnel (1:1). Extraction was accomplished by shaking the funnel for 3 minutes. After equilibration both phases were separated and secondly used for HPTLC determination.

4.2.3 Chromatographic mobile phases (MP)

MP_1: Ethyl acetate: Toluene *v/v* (8:2) – was used for preliminary TLC, CC, TLC of fractions after CC and HPTLC

MP_2: Methanol: Ethyl acetate: Dichloromethane: Ammonium hydroxide *v/v/v/v* (2:2:1:0.05) – was used for HPTLC determination of synephrine

MP_3: Ethyl acetate: Dichloromethane: Acetic Acid: Formic acid: purified water *v/v/v/v/v* (100:25:10:10:11) – was used for HPTLC determination of quercetin

MP_{HPLC}: purified water (channel A), acetonitrile (channel B); Linear gradients of B into A were eluted with constant flow rate of 1 mL/min as follows: 0–15 min: 70% A with 30% B; 15–35 min: 20% A and 80% B, 35-40 min: 100% B.

4.2.4 Detection reagens

Anisaldehyde-sulfuric reagent: was prepared from 1.5 mL *p*-anisaldehyde, 2.5 mL H_2SO_4 and 1 mL AcOH in 37 mL EtOH

Natural Product Reagent A: 1 g diphenylborinic acid aminoethylester in 200 mL of ethyl acetate, purchased from Sigma-Aldrich Co., (Italy)

4.2.5 Stationary phases

TLC and HPTLC: glass-backed silica gel layers, 2 μm thin with pores size 60 Å, covered with F254 fluorescent indicators, bought from Fluka Chemicals (Milan, Italy).

CC: silica gel for column chromatography, high-purity grade, pore size 60 Å, particle size 70-230 mesh, obtained from Sigma-Aldrich (Italy)

HPLC: C18 carbon chain bonded silica (octadecyl, ODS-2) reversed-phase column (250 mm × 4.6 mm i.d., 5 μm particle size), obtained from Merck KGaA (Darmstadt, Germany)

4.3 Laboratory instruments and equipment

4.3.1 Laboratory instruments

HR-120 Analytical Balance (A&D Instruments, Japan)

Laboratory water purification system MilliQ (Millipore SpA, USA)

Disposable syringe filters:

- Minisart SRP4, PTFE membrane, diameter 4 mm, pore size 0.45 μm, Sartorius Stedim Biotech GmbH (Germany)
- Chromafil 20/25, PET membrane, diameter 25 mm, pore size 0.20 μm, Macherey-Nagel GmbH (Germany)

Commonly used lab instruments

4.3.2 Equipment

NMR spectral analysis of ^{1}H and ^{13}C NMR were performed with Varian (nowadays Agilent Technologies, USA) Mercury 300 MHz instrument, using $CDCl_3$ as internal standard; the chemical shift was expressed in ppm from TMS.

To perform mass spectra was used spectrometer Q-TOF MICRO (Micromass, now Waters, United Kingdom) operating at 70 eV ionizing voltage. This spectrometer enables also electrospray ionisation (ESI-MS), with mobile phase solution: MeOH /H_2O + formic acid 0.2 %. This ion source transfer neutral compounds to negative or positive charged ions.

The HPTLC system (CAMAG, Muttenz, Switzerland) consists of an automatic sample applicator Linomat 5 using 100 µL syringes. Samples are carried out under a nitrogen pressure to the glass plates 20 cm x 10 cm (Merck, Darmstadt, Germany) with ready-to-use glass-backed silica gel layers as stationary phase. Elution occurs within an Automatic Developing Chamber (ADC 2, CAMAG-Muttenz in Switzerland), containing of twin trough chamber 20 x 10 cm. The documentation and interpretation of the results was done by the visualizer (CAMAG-Muttenz, Switzerland), an electronic device that allows you to capture images of developed plates at different wavelengths.

HPLC-DAD analysis was carried out on Agilent Technologies 1100 liquid chromatography system (Palo Alto, USA), equipped with an automatic sampler, a binary solvent pump and a column temperature controller. The system was coupled with a diode array detector (DAD) from Alltech Associates, USA. Quantitative data were captured and processed by Agilent Chemstation for LC 3D systems software (Santa Clara, USA).

4.4 Mobile phases preparation

4.4.1 Mobile phase for TLC

We have tried number of solvents systems as a mobile phase, but the satisfactory resolution was obtained after saturation of developing chamber for 20 minutes at room temperature with solvent combination of ethyl acetate: toluene (8:2) *v/v*.

The same solvents mixture was used as a mobile phase for preliminary TLC, column chromatography, TLC analysis of obtained fractions and finally also for HPTLC analysis.

4.4.2 Mobile phase for HPTLC of synephrine and quercetine

Mobil phase for HPTLC determination of synephrine was made by mixing MeOH, ethyl acetate, dichloromethane and ammonium hydroxide in the ratio 2:2:1:0.05 (*v/v/v/v*).

Mobil phase for HPTLC determination of quercetin was prepared mainly from ethyl acetate, then with dichloromethane, acetic acid, formic acid and purified water in ratio 100:25:10:10:11 (*v/v/v/v/v*).

4.4.3 Mobile phase for HPLC-DAD

The mobile phase consisted of water (channel A) and acetonitrile (channel B), fluxed in 1 mL injection volume as to the followed gradient programme with a constant flow rate of 1 mL/min. The mobile phase was prepared by a series of linear gradients of B into A. The elution system was starting with 0% B in A, followed in 0–15 minutes with 30% B and 70% A; in 15–35 minutes with 80% B and 20% A and in 35-40 minutes 100% B. The temperature was stable at 35°C; and the working pressure was 30-36 atm.

4.5 Samples preparation

4.5.1 Sample for preliminary TLC

50.0 mL of *Raspberry Keto 400 Liquid* product was transferred to the separating funnel. Stepwise totally 100.0 mL of EtOAc and 100.0 mL of distilled water were added to the funnel, always followed by shaking the funnel. Extraction of the sample was accomplished by shaking for 3 minutes. After equilibration was the aqueous phase removed and organic one was used for TLC.

4.5.2 Samples for CC separation and fractions analysis

After a preliminary TLC of the supernatant, organic phase of *Raspberry Keto 400 Liquid* was mixed with MeOH in the equal volume (1:1). Then the solvent was evaporated and the solid residue was suspended in water and partitioned with ethyl acetate in the separating funnel. The ethyl acetate phase was applied to the silica gel column.

4.5.3 Samples for HPTLC analysis

Dried extracts of selected fractions: 3-8-12-14-24-29-42 were weighted, dissolved in MeOH (6 mg/ mL) and finally filtered before use.

4.5.4 Sample for HPLC-DAD

Bottle of *Raspberry Keto 400 Liquid* product was shaken according to the recommendation of its producer written on the label. 2.5 mL of the product were taken as sample and diluted with 10 mL of ultrapure Milli-Q water in separating funnel. Additional 15 mL of dichloromethane (CH_2Cl_2) were added to the funnel and was shaken for 2 minutes. This process was repeated four times followed by equilibration, to extract the organic phase. The supernatant was collected and dehydrated using Na_2SO_4 into a 100 mL flask. Solvents were completely removed using a rotary evaporator under reduced pressure at 60 mbar and T<30°C. Then the extract was prepared for the analysis by suspending in 5 mL of acetonitrile. This procedure was performed both, with normal samples and with fortified ones (with 1.6 and 4.0 mg/mL of analyte).

4.6 Methods of validation

The developed method was validated in accordance of the local "in-house" guidelines for validation of analytical procedures at the Department of Environmental Biology of Sapienza University in Rome.

4.6.1 Validation of HPTLC method

In accordance to perform validity assay of HPTLC developed method, stability of the samples and repeatability of the method were determined.

4.6.1.1 Stability

Stability of the prepared samples was evaluated before and after application to chromatographic plates. Sample solutions were prepared and stored at room temperature for 3 days and then applied on the same HPTLC plate. After performing the chromatographic run, the chromatogram was evaluated for additional band. Similarly, band stability was checked by keeping the resolved peaks and inspecting at intervals of 12, 24 and 49 hours.

4.6.1.2 Repeatability

Repeatability was determined by running a minimum of three analyses and then retention factors deviations were calculated. Repeatability was expressed as relative standard deviation (RSD %), also known as coefficient of variation.

4.6.2 Validation and system suitability test of HPLC method

Validation studies were assessed by using the fundamental parameters, including accuracy, precision, repeatability, linearity, selectivity and system suitability test (SST).

4.6.2.1 System suitability test

System suitability determines the capability of the chromatographic system as a whole to be reliable to use for the intended purpose. Suitability test considers that all

used equipment, electronics, analytical operations and samples can be evaluated. The particular analysis can be done only if the system successfully passes SST [36].
SST is in our case performed by evaluating Retention time (t_R) parameter.

4.6.2.2 Linearity

Linearity of the method is its ability to obtain test results which are wholly proportional to the concentration of analyte in the sample [37].
Linearity was studied for various amounts of RK standard (0.1, 0.5, 1.0, 1.5, 2.0 mg/ mL) diluted in acetonitrile. Each concentration was prepared in triplicate. Those various concentrations were plotted versus the corresponding peak areas to determinate the calibration plot.

4.6.2.3 Precision and repeatability

The precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the exact conditions. Precision may be determined by repeatability, intermediate precision or reproducibility.
Precision determination is expressed as relative standard deviation (RSD%), also known as coefficient of variation, which was reported for each type of investigated for repeatability and intermediate precision measurements.
Repeatability, also known as intra-day precision, expresses the precision under the same operating conditions over a short period of time. The repeatability was evaluated by assaying three samples of the same analyte in the same concentration during the same day.
Intermediate precision, called also as inter-day precision, describes a precision over different conditions within the laboratory: analysis made different days, by different analysts or with different equipment. Inter-day precision was studied by comparing the results on three different days.
Reproducibility expresses the precision between different laboratories. Reproducibility was not evaluated in this thesis [36].

4.6.2.4 Accuracy

According to the International Conference on Harmonization guidelines for validation of analytical procedures (ICH), the definition, accuracy is the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Accuracy was described as recovery (R), calculated by the following formula:

$$R\ (\%) = {}^{c_i}/_{c_o} \cdot 100$$

where: c_o - inserted concentration

c_i - concentration to be determined

Recovery study was carried out by spiking the sample at two fortification levels (1.6 and 4 mg/mL) and the average recovery was calculated [36, 37].

4.6.2.5 Limits of Detection and Quantitation

The limit of detection (LOD) of an analytical procedure is characterized by the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

The limit of quantitation (LOQ) of an analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy [37].

LOD and LOQ, under the present chromatographic conditions, were determined at a signal-to-noise ratio (S/N). Standard solution was prepared by sequential dilutions and injected at lower concentrations. LOD was taken as the concentration of analyte at which signal to noise ratio (S/N) was 3. LOQ was taken as the concentration of analyte at which S/N was 10.

4.7 Methods of results evaluation

For the quantification of the sample an equation of the calibration curve was used, constructed with external standard method (solution of following concentrations 0.1, 0.5, 1.0, 1.5, 2.0 mg/mL of RK standard diluted in acetonitrile).

5 Results and discussion

5.1 Development of analysis and chromatographic conditions

5.1.1 RK identification by TLC

A preliminary screening of analysed product was carried by TLC to check the RK presence. Previously prepared RK standard solution in 1.0 mg/mL concentration and ethyl acetate phase of the sample solution were applied to the pre-coated silica gel plate in the same volume. Chromatography was carried out in ascending order in a standard chromatographic chamber saturated with mobile phase for 30 minutes before analysis.

The detection of developed plates was made by inspection under UV light at 254 nm and 366 nm wavelength.

5.1.2 CC separation and analysis of fractions

Column chromatography was carried out over silica gel. Silica gel system, deactivated by containing 10% to 12% water was prepared beforehand. Silica gel system covered with sample was eluted with an EtOAc /Toluen mixture (8:2, *v/v*).

TLC analysis was performed with each obtained fraction ($MP_{1,}$ DR: anisaldehyde, T=120°C). As it was detected by TLC, the highest concentrations of RK were found in the fractions 4-6. These close fractions 4-6 were combined to produce one, which was further purified by silica gel and eluted with *n*-hexane-ethyl acetate to isolate RK. Structure of RK was confirmed by NMR and mass spectra analysis.

5.1.3 Determination of RK by HPTLC

Glass plates with silica gel were previously prewashed with MeOH and dried for 3 minutes at 100 °C. Secondly, filtered solutions were automatically applied to the pre-coated silica gel by nitrogen flow. Following solutions were injected by syringes: 3-8-12-14-24-29-42 CC fractions, 1 mg/ mL concentrated RK standard and referential *Raspberry Keto 400 Liquid* extract, application data summary is in the Table 4 below (Page 47).

Saturation of ADC 2 developing chamber was made for 20 minutes at room temperature with mobile phase MP_1. Then the HPTLC plate was developed in linear ascending order to a distance of 70 mm.

To render plates easier detectable were derivatized by using anisaldehyde spraying. Plates were thoroughly dried on plate heater for 5 minutes at 120°C. Finally, images of the HPTLC plates were gained with CAMAG visualizer before and after derivatization. Detection was performed under reflectance and transmission of white light (WRT), UV 245 nm and UV 366 nm wavelengths modes, controlled by winCATS software.

5.1.4 Determination of synephrine and quercetine by HPTLC

A further phytochemical analysis by HPTLC was performed to detect presence of the other two components reported on the label of *Raspberry Keto 400 Liquid*, concretely to check presence of synephrine and quercetine.

HPTLC conditions in both cases were used the same as with previous determination of RK, with the CAMAG system.

Regarding to synephrine analysis, on the pre-coated silica gel have been applied following samples: fractions after CC, Reference RK 400 liquid, methanol solution of synephrine standard (1 mg/ mL concentrated), chloroform and aqueous phases of synephrine standard. As a mobile phase was mixture MP_2 described above. Then the HPTLC plate was developed and secondly inspected under UV 245 nm, without any derivatization.

Regarding to the analysis of quercetin we performed HPTLC with these samples: methanolic solutions of CC fractions 12 and 14 in comparison with methanol quercetin solution, application data resume is in the below. MP_3 was used as the mobile phase. As a detection reagent was used NPR. Developed plates were detected using UV detection at 254 nm and 366 nm wavelengths.

5.1.5 Determination of RK by HPLC-DAD

In order to performed quantitative analysis by HPLC-DAD method of raspberry ketone content in *Raspberry Keto 400 Liquid* we have been collaborating with the University of Camerino.

Liquid separation was carried out with ODS-2 reversed-phase column (250 mm × 4.6 mm i.d., 5 μm particle size) from Merck KGaA (Darmstadt, Germany). The column temperature was maintained at 35°C. Separations were affected by a series of linear gradients of B into A with a constant flow rate of 1 mL/min. The elution system was starting with 0% B in A as follows to the gradient programme, 0–15 min: 30% B and 70% A; in 15–35 minutes with 80% B and 20% A and in 35-40 minutes 100% B. The sample volume of single injection was 1 μL. To perform quantitative analysis, the eluent was monitored using UV detection at 279 nm wavelength.

5.2 Validation of developed HPTLC

5.2.1 Stability

Solutions of the sample extracts were found to be stable for at least one month stored at 4°C in the refrigerator. Stability after application on the HPTLC plates was established for at least 3 days.

5.2.2 Repeatability

Repeatability was determined by running a minimum of three analyses. Retention factors values for observed compounds (RK, quercetin, synephrine) varied ± 0.02%. Differences were assumed as effects of small changes in the mobile phase composition and mobile phase volume, which were reduced by direct comparison. As matter of fact, the results were critically dependent on pre-washing HPTLC plates with MeOH.

5.3 Validation of developed of HPLC-DAD method

5.3.1 System suitability test

The system suitability test was performed on fundamental parameters including: the retention time, method efficiency and peak symmetry.

RK was eluted in less than 8 minutes.

5.3.2 Linearity

Linearity of the method was investigated by analysing five different concentration samples in triplicate. Calibration equation was found by plotting the peak area (y) versus the RK concentration (x) expressed in µg/mL: y = 565.09x, with a regression coefficient R^2 = 0.9999.

5.3.3 Precision and repeatability

Precision was determined by evaluating the RSD values for repeatability and intermediate precision. These intra- and inter-day measurements were performed three times each. The variability of the results was low with RSD values below 1.2% in both cases.

5.3.4 Accuracy

The accuracy which was attained as the average recovery obtained by spiking sample at two fortification levels (1.6 and 4 mg/mL, respectively) was 101%.

5.3.5 Detection and Quantitation Limits

The detection limit (LOD) and quantification limit (LOQ) were determined based on signal-to-noise ratio (S/N) of about 3 and 10; they were 0.6 mg/L and 2.L mg/l, respectively with defined precision and accuracy under the presented experimental conditions.

5.4 Results

To achieve the objectives set at the beginning of this thesis, it was necessary to combine the information from all obtained analysis.

Preliminary TLC was made to prove presence of RK, which was evidencing that compound in reference to the standard sample. The RK's spot can be seen at Rf=0.82 (chromatogram records are listed below, Figure 4).

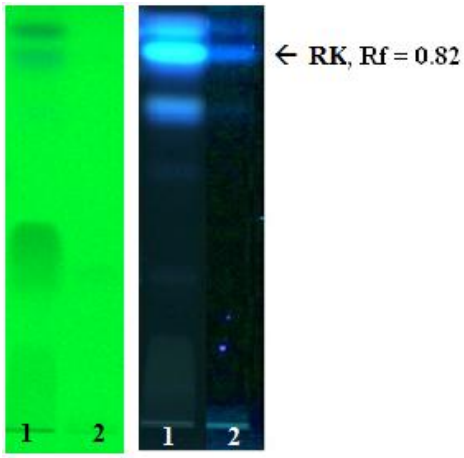

Figure 4: TLC profiles of tested sample (1) and RK standard (2)
Detection: UV 336 nm (left) and 254 nm (right)
MP_1: Toluene:Ethyl acetate 2:8 (*v/v*)

As can be seen in Figure 4, there were more fluorescent spots visible, one close to the RK's one. Because of less complex result by preliminary TLC, product was separated by column chromatography. Collected fractions were all analysed by TLC. One of obtained chromatogram is shown below in Figure 5.

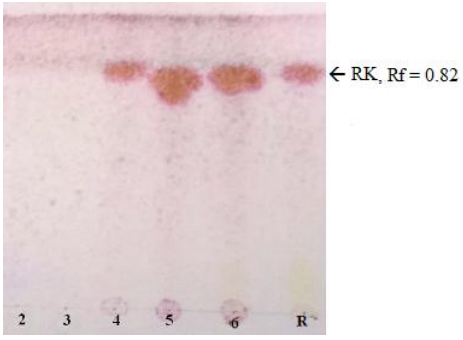

Figure 5: TLC profiles of fractions (2-6) after CC compared with RK standard (R)
Detection: white light
MP_1: Toluene: Ethyl acetate 2:8 (*v/v*)
Derivatization: Anisaldehyde

As it was detected by TLC, the highest concentrations of RK were found in the fractions 4-6, these close fractions were combined and purified to isolate RK. Isolated RK, was treated by NMR spectroscopy.

Obtained NMR spectrum of ^{1}H (Figure 6) is shown and described (Table 2) below. The hydrogen of hydroxyl protic group is not seen in the spectrum, because of its huge shielding by boned oxygen.

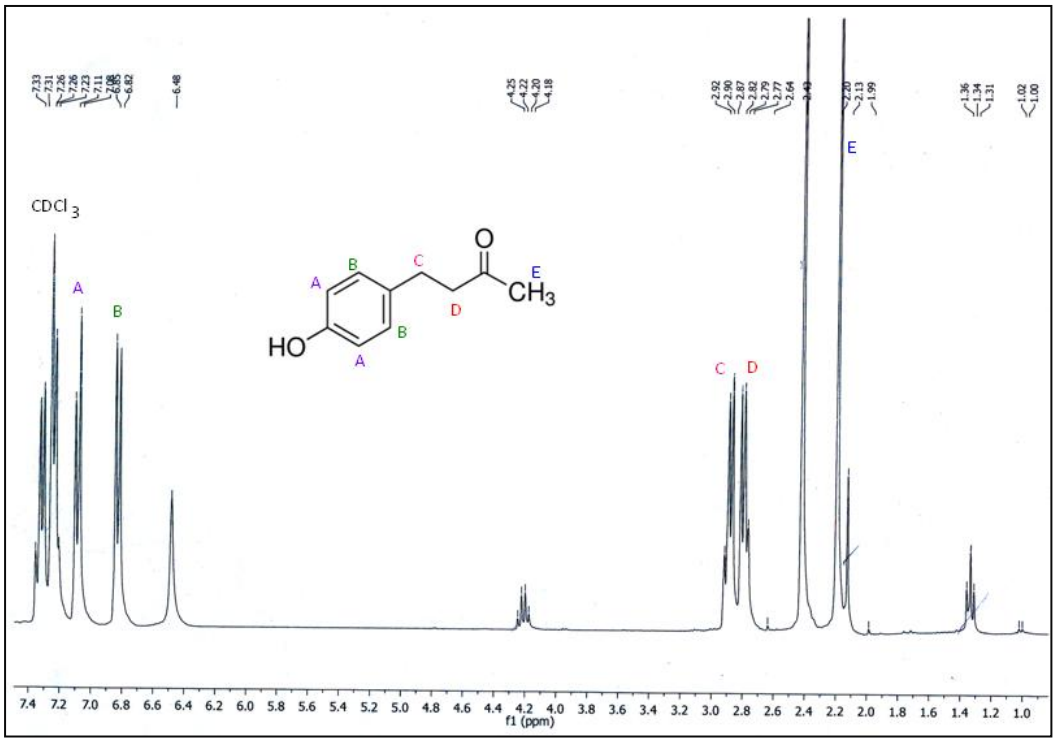

Figure 6: 300 MHz ^{1}H-NMR spectrum of the extract from 4-6 CC fractions
Standard: TMS
Solvent: $CDCl_3$

Table 2: Description of 300 MHz ^{1}H-NMR spectrum of the extract from 4-6 CC fractions

δ (25°C)/ ppm	splitting (coupling constant)	integration	assign
2.13	singlet	3H	E
2.77	triplet (J = 7.5 Hz)	2H	D
2.82	triplet (J = 7.5 Hz)	2H	C
6.82	doublet (J = 8.5 Hz)	2H	B
7.08	doublet (J = 8.5 Hz)	2H	A

Similarly also ^{13}C NMR spectrum was obtained with the extract from 4-6 CC fractions (Figure 7, Table 3).

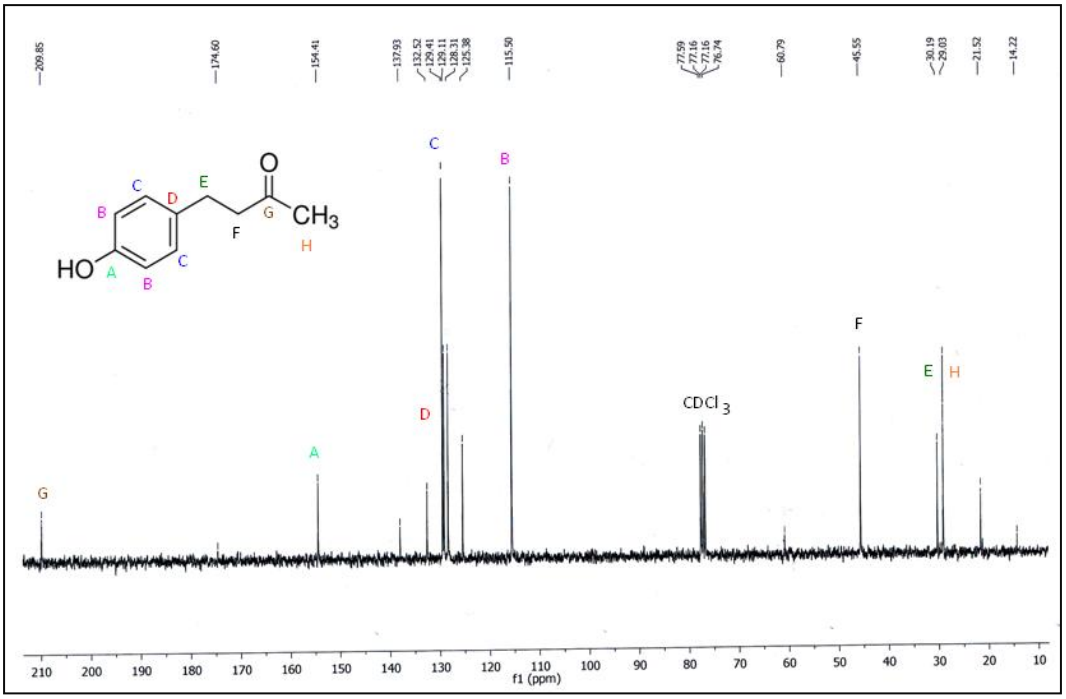

Figure 7: 300 MHz ^{13}C-NMR spectrum of the extract from 4-6 CC fractions
Standard: TMS
Solvent: $CDCl_3$

Table 3: Description of 300 MHz ^{13}C-NMR spectrum of the extract from 4-6 CC fractions

δ (25°C)/ ppm	assign
209.85	G
154.41	A
132.52	D
129.40	C
115.50	B
45.55	F
30.19	E
29.03	H

Obtained NMR spectra (shown in Figure 6 and Figure 7) were compared with literature [19,38]. Data combination of ^{1}H, ^{13}C NMR and MS data proved that this fraction was containing almost pure RK.

There was any amount of synephrine and quercetin detectable by NMR. Unspecified peaks were assumed as rest of solvents and potassium-sorbate, which is reported in the label of *Raspberry Keto 400 Liquid*, added as preservative.

To establish a fingerprint profile of the product, HPTLC was performed under described conditions (application data summary in Table 4), obtained chromatogram is shown below in Figure 8 and Figure 9.

Table 4: Application samples order in HPTLC determination of RK

Track	Application position	Application Volume	Vial of
1	12.0 mm	4.0 µL	RK 400 Liquid product extract
2	21.5 mm	4.0 µL	RK Standard
3	31.0 mm	16.0 µL	Fraction 3
4	40.5 mm	4.0 µL	Fraction 8
5	50.0 mm	4.0 µL	Fraction 12
6	59.5 mm	8.0 µL	Fraction 14
7	69.0 mm	16.0 µL	Fraction 24
8	78.5 mm	16.0 µL	Fraction 29
9	88.0 mm	16.0 µL	Fraction 42

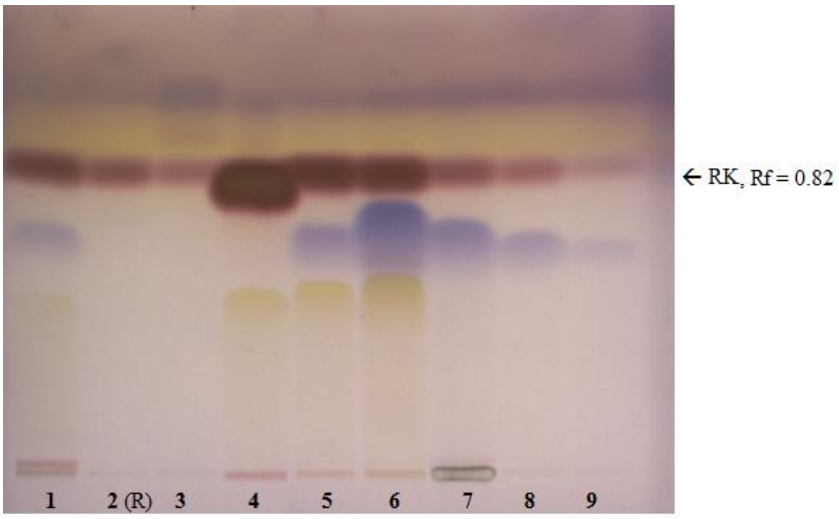

Figure 8: HPTLC plate of analysed product and its CC fractions
MP_1: Toluene: Ethyl acetate 2:8 *(v/v)*
Derivatization: Anisaldehyde reagent
Detection: upper and lower white lamp
Tracks: **1**, Rasberry Keton 400; **2**, RK standard; **3-9**, CC fractions in elution order

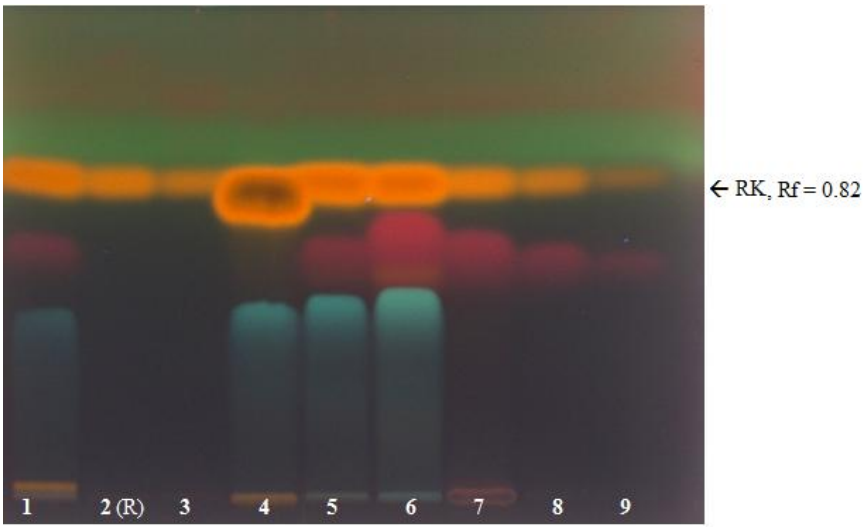

Figure 9: HPTLC plate of analysed product and its CC fractions
MP_1: Toluene: Ethyl acetate 2:8 *(v/v)*
Derivatization: Anisaldehyde reagent
Detection: UV 366 nm
Tracks: **1**, Rasberry Keton 400; **2**, RK standard; **3-9**, CC fractions in elution order

The obtained HPTLC chromatograms proved the presence of RK corresponding to spots at Rf 0.82. Whereas there was RK standard (R) used in concentration 1 mg / mL in track 2 for comparison. The comparison between tracks 1 and 2 showed higher concentration of RK in the sample than in the standard. The fraction 3 in third track was formed almost just with pure RK. Spot with Rf 0.53 was later assigned to potassium sorbate, a constituent reported on the label of *Raspberry Keto 400 Liquid*, added to the product as a preservative.

A further HPTLC analyses were performed to detect presence of the other two components reported on the label of *Raspberry Keto 400 Liquid*, concretely to check presence of synephrine and quercetin. Samples were applied in order shown in Table 5 and Table 6, with obtained results in Figure 10 and 11.

Table 5: Application order of samples in HPTLC determination of synephrine

Track	Application Position	Application Volume	Vial of
1	11.0 mm	4.0 µL	RK 400 Liquid product extract
2	20.5 mm	4.0 µL	Fraction 24
3	30.0 mm	4.0 µL	Synephrine in MeOH
4	39.5 mm	4.0 µL	Synephrine phase in $CHCl_3$
5	49.0 mm	4.0 µL	Synephrine H_2O phase

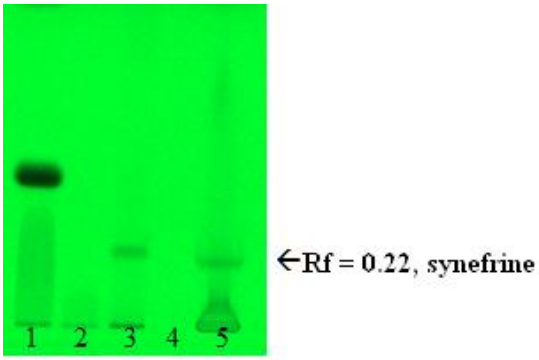

Figure 10: HPTLC determination of synefrine
MP_2: MeOH: EtOAc: DCM: NH_4OH in ratio 2: 2: 1: 0,05 *(v/v/v/v)*
Derivatization: none
Detection: UV 254 nm
Tracks: **1,** Rasberry Keton 400; **2,** Fraction 24; **3**, Synefrine in MeOH; **4,** $CHCl_3$ phase of synefrine; **5,** aqueous phase of synefrine

Table 6: Application order of samples in HPTLC determination of quercetin

Track	Application	Application	Vial of
1	15.0 mm	4.0 µL	Fraction 12
2	25.0 mm	8.0 µL	Fraction 14
3	35.0 mm	1.0 µL	Quercetin

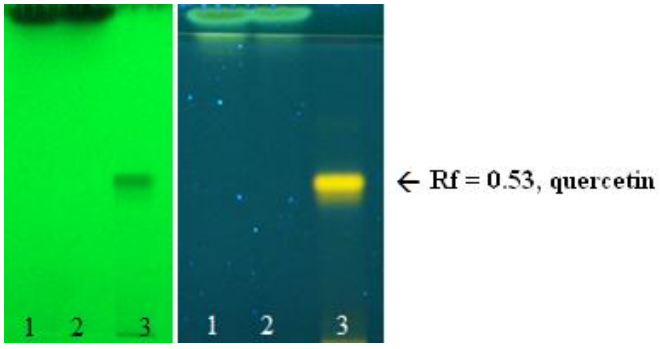

Figure 11: HPTLC determination of quercetin
MP_3: EtOAc: DCM: Acetic Ac.: Formic ac.: water in ratio 100: 25: 10: 10: 11 (*v/v/v/v/v)*
Derivatization: NPR
Detection: UV 254 nm (left) and 366 nm (right)
Tracks: **1,** Fraction 12; **2,** Fraction 14**; 3,** Quercetine standard

There was not any detectable spot evidencing presences of any of those. Obtained results confirmed previous assumption that there is no synephrine and quercetin present in analysed product.

Further HPLC-DAD method was performed to obtain exact determination of RK content in the analysed product. Obtained chromatograms are showed below (Figure 12 and 13).

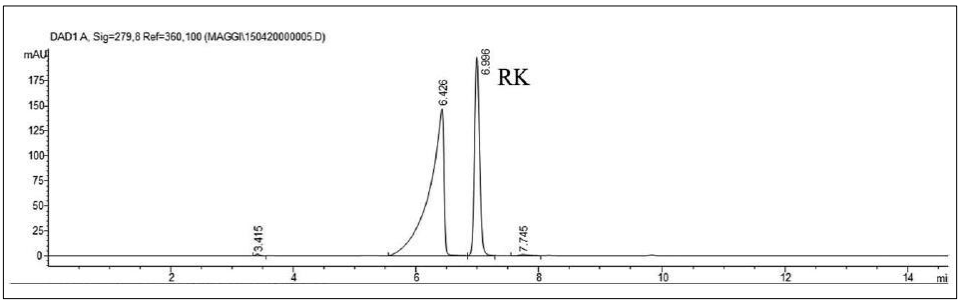

Figure 12: HPLC-DAD chromatogram of the food supplement extract

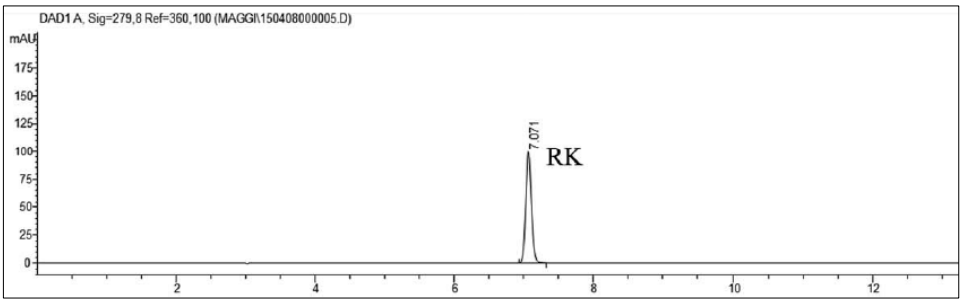

Figure 13: HPLC-DAD chromatogram of the RK standard (1 mg/mL)

Representative chromatograms for RK were found at a retention time of 7.0 min for the standard solution. RK peak in samples of analysed product was observed at a retention time of 6.95 min. Both measured under the under the chromatographic conditions given above. Besides RK peak, additional little peaks were found, presumably also impurity compounds. These peaks did not overlap with the RK peak.

The RK content of the samples was calculated using external standard method with the calibration curve (Figure 14). The average RK concentration suspended in the analysed food supplement was calculated as 4.2 mg/mL.

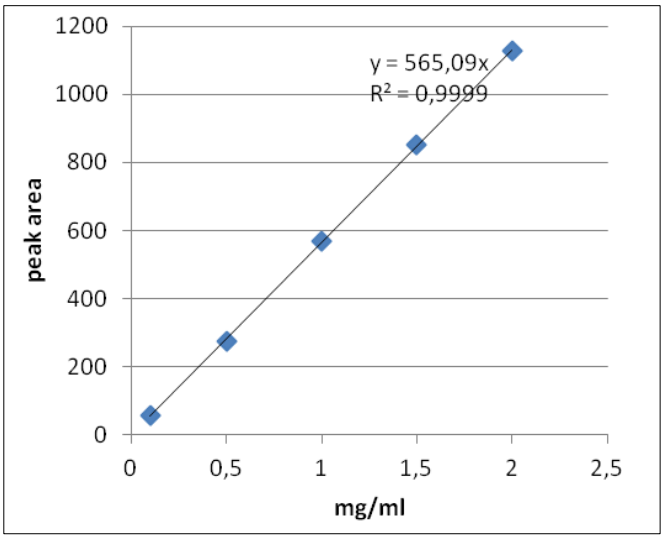

Figure 14: Calibration line constructed by injectining different concentrations of RK standard diluted in acetonitrile (0.1, 0.5, 1.0, 1.5, 2.0 mg/ml)

5.5 Discussion

Firstly, a basic TLC method was used, as an easy tool to check the RK presence in the sample. Preliminary HPTLC was made as more sophisticated tool to prove presence of RK, which was evidencing that compound clearly visible in reference to the standard sample with a spot at RK's Rf (track 2 at ca. 0.82). There were visible more fluorescent spots, one close to the RK's one. Because of this less complex result, the product was separated by column chromatography.

After obtaining a fraction with isolated RK as confirmed by ^{1}H and ^{13}C NMR analysis with MS data, a fingerprint HPTLC of the sample was performed. RK was evidenced in the chromatograms with Rf =0.82. A second compound presented with Rf = ca. 0.53 was assigned as potassium-sorbate, preservative of *Raspberry Keto 400 Liquid* product. Considering the RK standard was used in 1 mg/mL concentration,

comparison of tracks 1 and 2 showed a higher concentration of RK in the sample, than in the standard.

To obtain exact quantitative evaluation of *Raspberry Keto 400 Liquid* product a further HPLC-DAD method was performed. The analysis and the obtained results have confirmed previous assumption, that concentration of RK in product is many times higher than the highest amount occurring present in the literature. The average RK concentration suspended in the analysed food supplement was calculated as 4.2 mg/mL. Considering the maximal potential concentration level of RK in raspberry fruits reached, (370 μg /100g of fruits, reported in Table 1, page 21), that would be necessary to use about 1.1 kg of fruits to get just 1 mL of analysed food supplement. In fact, for one 500 mL marketed bottle we would need approximately 568 kilograms of raspberries. Other natural sources of RK than the *Rubus* family, according to the extremely low RK concentrations contained do not allow assume addition of RK to the product from other natural source [8].

Further, synthetically obtained RK, by the chemical or biotechnological reaction is relative cheap [20]. Moreover, the absence of other natural constituents such as synephrine and quercetin signifies less complex composition than is expected from natural plant origin [5]. Therefore, the unusually high concentration of RK in the analysed food supplement product may be explained.

The safe intake limit for RK in flavourings was assumed to be 0.03 mg/kg of body weight per day [39]. Considering a person of 60 kg body weight, the safe daily intake would be suggested maximally 1.8 mg in a day. Obviously, concentration levels of RK in the analysed product are not compatible with the regulation for flavourings in the European Union (EC 1334 2008) [16]. Especially considering the daily recommended dose of 20 mL per day, the daily intake of RK from the analysed product wound range up to 84 mg/day. In fact, that value is about 47 times higher than the highest safe estimated exposure from common diet [9,10].

5.6 Conclusion

A complex of analytical methods were used to determinate present quantities of 4-(4-hydroxyphenyl)-2-butanone (RK) in the food supplement product and confirm its natural origin, as was claimed on the product label. Detection of synephrine and quercetin presences was also reported, in addition to their claiming presence on the product label too. Furthermore, these two constituents would be expected in matrices of natural origin.

The preliminary planar chromatography methods successively proved RK presence in the analysed product and disproved contents of two other claimed constituents, synephrine and quercetin. The HPLC method proved to be suitable for required measurements with values in desired range. The separation was completed with a reasonable analysis time and acceptable chromatographic parameters.

In conclusion, the amount of RK detected in *Raspberry Keto 400 Liquid* is definitely not compatible with presenting the product as natural and there must be RK added as a substance, probably of synthetic origin. Extraction of RK from raspberry and other fruits is expensive and provides only limited amount of RK, on the other side, it can be easily obtained by chemical synthesis. In any case, of synthetic or natural origin, the risks of 4-(4-hydroxyphenyl)-2-butanone overdose are the same.

The toxicity of RK, although limited, has been recently reported. In December 2014 24-year-old woman died after an overdose of multi-componental *Forza's Rasberry K2* over-the-counter supplement. Which component caused her cardiovascular collapse is not sure, but her death is sad example proving the necessity of adequate quality controls of food supplements [40]. The quantity of every single compound in the food supplements should be known and evaluated for potential harmful effects. Especially considering that food supplements are not assessed as medical drugs, do not need prescription and in their consumption is usually without any medical control. Therefore, possibility of overdose is real [6].

Currently, the data reported in this paper confirm the need of strict legal rules and adequate control mechanisms on marketed food supplements, in order to minimize their possible harmful effects and confirm the complete adherence between labelling and real constitution of the products.

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