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A COMPREHENSIVE STUDY OF THE PRESENCE OF SOME FOOD ADDITIVES IN NON-ALCOHOLIC BEVERAGES IN REPUBLIC OF MACEDONIA FROM THE PERIOD 2008- 2012

Vesna Kostik[1*]

[1]Institute of Public Health, 50 Divizija No. 6, 1000 Skopje, Republic of Macedonia

[*]e-mail: vesna2mk@yahoo.com

Abstract

Food additives are substances added to food to preserve flavour or enhance its taste and appearance. The most abundant additives used in production of refreshing non-alcoholic beverages (soft drinks) are: potassium sorbate, sodium benzoate caffeine, some artificial food colourings, artificial sweeteners etc. Different medical studies have shown that the usage of additives have various impact on human's health.

In the current study, the presence of: preservatives (potassium sorbate and sodium benzoate), caffeine, quinine chloride, synthetic food colorants, artificial sweeteners (saccharin, acesulfame potassium, sodium cyclamate and aspartame), citric acid, phosphoric acid and ascorbic acid were characterized by high performance liquid chromatography and ion chromatography. For that purpose, 872 samples of soft drinks (carbonated mineralised and water based flavoured drinks) which were imported (751) and produced (121) in Republic of Macedonia, from the period from 2008 to 2012 were analyzed.

The obtained results showed different distribution of food additives in the imported soft drinks and soft drinks from the domestic producers. The most prominent additives in the imported soft drinks were found to be preservatives potassium sorbate and sodium benzoate, which were present in 80% of tested samples. Synthetic colorants were present in 8% of tested the samples, caffeine in 7%, artificial sweeteners in 2%, quinine chloride in 1% and citric, ascorbic and phosphoric acid in 2% of tested soft drinks. In domestic brands, preservatives were found in 54% of tested samples, artificial sweeteners in 34%, and synthetic colorants in 5%. The concentrations of caffeine, quinine chloride, citric acid, phosphoric and ascorbic acid in domestic brands of soft drinks were found to be similar to those found in imported samples. The results have been discussed with respect to the related regulations.

Soft drinks take an important part in the total daily intake of food additives. Therefore, the constant monitoring of their presence in non alcoholic beverages is needed to ensure compliance with food safety regulations as well as for calculating risk assessment.

***Key words**: Additives, Soft drinks, Preservatives, Food colorants, Artificial sweeteners, High performance liquid chromatography, Ion chromatography.*

1. Introduction

Food additives are used in the modern food industry for maintaining food quality as well as promoting food safety. Additives are used in foods to replace the taste lost in processing, enhance their texture or appearance, prolong shelf life, stop food from decaying and facilitate the preparation of processing. They are also used to enhance the flavour, to give an extra taste to food products and to make foods more appealing [1].

The use of food additives in foodstuffs is regulated by European legislation [2] and only authorised additives may be used in food specified in the legislation. Authorisation in this context means that the additive is included in a "positive" list as laid down in the legislation. The legislation also contains the name of the authorised additive, the Maximum Permitted Levels (MPLs) and the foods in which they are permitted to be used. Each food additive is also identified by a number and classified by the functions it performs [2]. Prior to their authorisation, food additives are evaluated for their safety by the European Food Safety Authority (EFSA). If an additive is deemed acceptable for food use, an Acceptable Daily Intake (ADI) is normally set. In addition to a thorough safety evaluation and a demonstrated technical need of purpose, all additives used in foods must be declared in the list of ingredients in accordance with Council Directive 2000/13/EC [3].

The question of food additive safety is one that has received widespread attention in recent years. While thousands of studies, conducted throughout the world, attest to the overall safety of additives, some studies showed that the usage of certain additives may have genotoxic or mutagenic effects in human's and could have possible influence on children's behavior [4, 5].

The legislation [3] also requires that food additives must be kept under continuous surveillance and must be re-evaluated whenever necessary in the light of changing conditions of use and new scientific information. Surveillance programmes carried out in many of the European member states involve the monitoring of foodstuffs to determine the usage levels of colours, sweeteners and other additives, to ensure that the MPL is not exceeded and also to identify unauthorised use in foodstuffs. It is also possible to estimate the intake of a particular food additive by combing usage levels with food data and to compare this with the ADI that has been established for the additive.

Since the consumption of soft drinks and non alcoholic beverages is a widespread habit all around the world, such foodstuff products are considered to be of great importance economically, as well as socially. According to United States Department of Agriculture (USDA) the per capita soft drink consumption in the United States of America (USA) has increased almost 500% over the past 50 years [6]. Carbonated soda is the soft drink most frequently consumed, especially among the children and teenagers.

Soft drinks can contain many additives including artificial colorants, artificial sweeteners, preservatives, stimulants (caffeine), flavor components, etc. In order to assure the quality of soft drinks as well as their safety for human consumption, the presence of food additives in soft drinks in the Republic of Macedonia is regulated by legislation [7 - 9], which is in compliance with EU legislation [2]. According to the legislation, the periodical checks of soft drinks on the presence of additives are mandatory.

Considering the remarks mentioned above, the main objective of this research study was determination of the content of several food additives in different types of non alcoholic beverages available on the Macedonian market. The current surveillance study was carried out in order to establish the compliance of the products with the existing legislation on MPLs of these additives.

2. Materials and Methods

2.1 Materials

A total of 872 samples were collected at retail level by officers of the Macedonian Food and Veterinary Agency over the period March 2008 to December 2012, for analysis of artificial food colours, artificial sweeteners, food preservatives, caffeine, quinine chloride, citric acid, phosphoric acid and ascorbic acid. The samples mainly comprised carbonated mineralised, and water based non-alcoholic flavoured drinks, which were imported (751 samples) and produced (121 samples) in the Republic of Macedonia.

2.2 Methods

2.2.1 Method for determination of artificial food colorants

2.2.1.1 Sample preparation

The samples were filtered in vacuum on a micro filtration membrane (pore size of 0.45 μm) and were placed on an ultrasonic bath for 15 min. in order to degas. The pH sample was adjusted at 6.50 with a sodium hydroxide (NaOH) solution (10%).

2.2.1.2 Chemicals

Tartazine (95% purity), Sunset Yellow (95.0% purity), Amaranth (98.0% purity), and Erythrosine B (95.0% purity) were obtained from Sigma - Aldrich (Germany); Carmoisine (98.0% purity), Allura red (98.0% purity) Quinoline Yellow (95.0% purity) Ponceau 4R (98.0% purity), Brillliant Black BN (96.0% purity) and Brilliant Blue FCF (95.0% purity) were obtained from Fluka (Germany).

Potassium dihydrogen phosphate (KH_2PO4), and disodium hydrogen phosphate (Na_2HPO4) were purchased from Merck (Darmstadt Germany); NaOH and all solvents were Chromasolv purity from Merck (Darmstadt, Germany). Water was purified using a Milli-Q Ultrapure water purification system (Millipore, Bedford, MA, USA).

All aqueous solutions were prepared using ultra pure water. Mixed stock standard solution (1000 mg/L) was prepared by weighing 0.1000 g of each color to 100 mL and its preservation at 2 - 8 ^{0}C temperature and is kept for 6 months. Mixed working calibration standards were prepared by diluting stock solution taking the appropriate volumes after its equilibration to room temperature.

Mobile phase A consisted of 1.4196 g Na_2HPO4 and 0.1200 g KH_2PO4 diluted to 1000 mL with ultra pure water.

Mobile phase B was prepared as a mixture of acetonitrile and methanol (1 + 3).

2.2.1.3 Chromatographic conditions

The separation and determination of Tartazine, Sunset Yellow, Quinoline Yellow, Amaranth, Ponceau 4R, Carmoisine, Allura red, Erythrosine B, Brilliant Black BN, and Brilliant Blue FCF, were performed using a high performance liquid chromatography (HPLC) system Agilent Series 1100 equipped with diode array detec-

tion (DAD), auto-sampler and column oven. The colorants were separated on a Hypersil C8 column, 250 x 4.6 mm with 5 µm particle size (Agilent). Gradient elution was used for colorants separation. The initial conditions (95% A) were maintained for 2 min., then a linear gradient was applied (5 - 55% mobile phase B) over 16 min. and to 95% mobile phase A in 20 min. Conditioning of the column with 95% mobile phase A was carried out for 20 min. The flow rate was set up at 1.0 mL/min and the injection volume was 10 µL. The oven temperature was 40 ^{0}C. DAD was used to monitor the colorants between 350 and 800 nm.

2.2.2 Method for determination of artificial sweeteners, preservatives and ascorbic acid

2.2.2.1 Sample preparation

The samples of soft drinks were degassed for 15 min. in an ultrasonic bath before dilution with water. 10 mL of the sample was diluted to 100 mL with ultra pure water. Aliquot of the sample was filtered through a 0.45 µm membrane filter into an autosampler prior to HPLC analysis.

2.2.2.2 Chemicals

Potassium sorbate (99.0 % purity), sorbic acid (99.0% purity), sodium benzoate (99.5% purity), benzoic acid (99.0% purity), L - ascorbic acid (99.0% purity), caffeine (99.0 % purity), acesulfame K (98.0 % purity), aspartame (98.0% purity), sodium saccharin (98.0 %), and sodium cyclamate (98.0% purity) were purchased from Sigma – Aldrich (Germany). HPLC-grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Water was purified using a Milli-Q Ultrapure water purification system (Millipore, Bedford, MA, USA). Potassium dihydrogen phosphate (KH_2PO4), phosphoric acid, sulphuric acid, heptane, sodium hypochlorite, sodium carbonate, and sodium sulphate were obtained from Merck (Darmstadt, Germany).

Stock solutions were prepared separately by dissolving reference compounds in ultra pure water (1000 mg/L).

Working standard solutions of aspartame, acesulfame K, sodium saccharin, caffeine, L - ascorbic acid, sorbic acid, and benzoic acid were prepared with mixing and diluting aliquots of stock solutions with ultra pure water. Sodium cyclamate in 30 mL stock solution was derivatised into cyclohexylsulphamic acid because of its poor absorbance.

2.2.2.3 Procedure for derivatization of sodium cyclamate

To determine sodium cyclamate, 30 mL stock solutions were derivatized into cyclohexylsulphamic acid, as follows: the liquid sample, 1 mL of sulphuric acid, 10 mL of heptane, and 2.5 mL of sodium hypoclorite solution were added into a separating funnel. After separating the phases, the aqueous phase was discarded and heptane layer washed with 25 mL of sodium carbonate solution (50 g/L). The lower phase was discarded and heptane phase dried with 1 g of sodium sulphate and filtered through a fluted filter paper and then through 0.45 µm membrane filter into vials.

2.2.2.4 Determination of sodium cyclamate

The determination of sodium cyclamate was performed using an HPLC system Agilent Series 1100 equipped with diode array detection (DAD), and auto-sampler. Cyclohexylsulphamic acid was separated on a C18 column, 150 x 4.6 mm with 5 µm particle size (Supelco, USA). Isocratic elution was used for the separation. Mobile phase consisted of 85% methanol and 15% ultra pure water. The flow rate was 1.5 mL/min and the injection volume was 10 µL DAD was used to monitor the cyclohexylsulphamic acid at 314 nm.

2.2.2.5 Determination of aspartame, acesulfame K, sodium saccharin, caffeine, L - ascorbic acid, potassium sorbate, sorbic acid, sodium benzoate and benzoic acid

The separation and determination of aspartame, acesulfame K, sodium saccharin, caffeine, L - ascorbic acid, potassium sorbate, sorbic acid, sodium benzoate and benzoic acid was performed using a HPLC system Agilent Series 1100 equipped with diode array detectior (DAD), auto-sampler and column oven. The analytes were separated on a ProntoSil 120-3 C18, 100 x 2.0 mm with 5 µm particle size (Wicom, Germany). Gradient elution was used for the separation. Mobile phase A was phosphate buffer pH = 3, and mobile phase B was acetonitrile. The initial conditions (100% A) were maintained for 1.5 min., then a linear gradient was applied (0 - 45% mobile phase B) in 5 min., and held 45% mobile phase B for 15 min. Conditioning of the column with 100% mobile phase A was carried out for 20 min. The flow rate was set up at 0.4 mL/min. and the injection volume was 5µL. The oven temperature was 40 ^{0}C. DAD was used to monitor the analytes at 220 nm. Standards were analysed on a separate chromatogram and quantification was based on a comparison between the peak areas of the sample and of the reference standards.

2.2.3 Method for determination of phosphoric acid and citric acid

2.2.3.1 Sample preparation

The samples were filtered in vacuum on a micro filtration membrane (pore size of 0.45 µm) and were placed on an ultrasonic bath for 15 min. in order to degas.

2.2.3.2 Chemicals

Phosphoric acid (90% w/v), and citric acid (99.9% purity) were obtained from Sigma Aldrich (Germany). 20 mM potassium hydroxide (KOH) solution was obtained from Dionex (USA).

Stock solutions were prepared separately by dissolving reference compounds in ultra pure water (1000 mg/L). Working standard solutions of phosphoric acid and citric acid were prepared with mixing and diluting aliquots of stock solutions with ultra pure water.

2.2.3.3 Chromatographic conditions

A Dionex reagent-free ion chromatography (IC) system equipped with an electrolytic eluent generator, EluGen EGC II KOH cartridge, continuously regenerated anion trap column (CR-ATC), dual-piston pump with vacuum degas, six-port injection valve fitted with a 1.25 µL sample loop, heated conductivity cell and column heater were used in this work. An IonPac Fast Anion III analytical column (3 x 250 mm) with its respective guard column, Fast Anion III guard column (3 x 250 mm), was used for all separations. Phosphoric acid and citric acid were detected by suppressed conductivity with an ASRS ULTRA II suppressor (2 mm) operated at 70 mA in the recycle mode. Chromeleon 6.6 chromatography management software was used for system control and data processing.

2.2.4 Method for determination of quinine

2.2.4.1 Sample preparation

The samples were filtered in vacuum on a micro filtration membrane (pore size of 0.45 µm) and were placed on an ultrasonic bath for 15 min. in order to degas.

2.2.4.2 Chemicals

Quinine monohydrochloride dihydrate (90.0% purity) was obtained from Sigma - Aldrich (Germany). HPLC grade methanol (CH_3OH), acetonitrile (CH_3CN) and ammonium acetate (CH_3COONH_4) were obtained from Merck (Darmstadt, Germany). Stock solution of quinine monohydrochloride was prepared in ultra pure water (1000 mg/L). Working standards were prepared with mixing and diluting aliquots of stock standard solution.

2.2.4.3 Chromatographic conditions

The determination of quinine hydrochloride was performed using an HPLC system Agilent Series 1100 equipped with fluorescence detectior (FI), and auto-sampler. Quinine hydrochloride was separated on a C18 column, 250 x 4.0 mm with 5 µm particle size (Supelco, USA) at ambient temperature. Isocratic elution was used for the separation. The mobile phase consisted of CH_3OH–CH_3CN–CH_3COONH_4 0.1 M, (45 : 15 : 40% v/v/v) and was delivered at a flow rate of 1.0 mL/min. Fluorescence detection was performed at 325 nm (excitation) and 375 nm (emission). For the quantitative determination of quinine, salicylic acid was used as internal standard (0.5 ng/µL).

3. Results and Discussion

3.1 A study of artificial colorants

The presence of artificial food colorants: Tartrazine (E102), Sunset Yellow FCF (E110), Carmoisine (E122), Amaranth (E123), Ponceau 4R (E124), Allura Red AC (E129), Brilliant Black BN (E151), Brilliant Blue FCF (E133), Quinoline Yellow (E104), and Erythrosine B (E127) was analyzed in the samples of non-alcoholic beverages. The obtained results are shown in Table 1 and Table 2.

Table 1. The results of the presence of artificial food colorants in imported soft drinks

Food colorant	Number of tested samples	Number of positive samples	Concentration range (mg/L)	Maximum permitted level - MPL (mg/L)	Number of samples with colour content above MPL (mg/L)	Number of samples with colour detected but not labelled
Tartazine (E102)	550	283	< 1.0 – 68.3	100	-	3
Sunset Yellow FCF (E 110)	560	260	< 1.0 – 70.1	50	8	2
Quinoline Yellow (E 104)	505	242	< 2.0 – 65.4	100	-	2
Carmoisine (E 122)	210	43	< 1.0 – 70.5	50	5	1
Amaranth (E123)	185	55	< 1.0 - 56.8	100	-	3
Ponceau 4R (E 124)	158	65	< 1.0 – 69.8	50	3	2
Allura red (E 129)	149	48	< 1.0 – 35.5	100	-	-
Erythrosine B (E 127)	92	58	< 0.9 – 23.4	100	-	-
Brilliant Black BN (E 151)	87	42	< 0.8 - 25.4	50	-	-
Brilliant Blue FCF (E 133)	21	10	< 1.0 - 30.1	100	-	-

Table 2. The results of the presence of artificial food colorants in domestic soft drinks

Food colorant	Number of tested samples	Number of positive samples	Concentration range (mg/L)	Maximum permitted level - MPL (mg/L)	Number of samples with colour content above MPL (mg/L)	Number of samples with colour detected but not labelled
Tartazine (E102)	52	48	< 1.0 - 43.3	100	-	3
Sunset Yellow FCF (E110)	105	105	< 1.0 – 80.3	50	3	2
Quinoline Yellow (E 104)	43	40	< 2.0 – 55.4	100	-	
Carmoisine (ER 122)	18	15	< 1.0 – 61.8	50	6	1
Amaranth (E123)	15	12	< 1.0 - 66.8	100	-	1
Ponceau 4R (E 124)	23	18	< 1.0 – 75.3	50	5	1
Allura red (E129)	22	18	< 1.0 – 15.5	100	-	-
Erythrosine B (E 127)	18	10	< 0.9 – 13.4	100	-	-
Brilliant Black BN (E151)	19	12	< 0.8 – 28.3	50	-	-
Brilliant Blue FCF (E133)	10	6	< 1.0 - 15.1	100	-	-

According to the obtained results (Table 1 and Table 2), the most abundant artificial food colorants in tested samples (imported and domestic), were Tartazine (E102), Sunset Yellow FCF (E110), and Quinoline Yellow (E104). Tartazine was found in 51.4% of tested imported soft drinks (283 samples) and in 92.3% of tested domestic brands (48 samples). Tartazine is sulfonaptophenyl azo dye, often used in food industry. Tartrazine is used to color drinks, sweets, jams, cereals, snack foods, canned fish, and packaged soups [10]. Tartrazine is an azobenzene artificial yellow. As a nitrous derivative it is reduced in the organism to an aromatic amine which is highly sensitizing main metabolite identified as sulfanylic acid [11]. Tartrazine has been implicated as the food additive most often responsible for allergic reactions, having thus been targeted by the scientific community [12]. ADI for Tartrazine is set to 7.5 mg/kg body weight [10]. Some countries such as Sweden, Switzerland and Norway have withdrawn Tartrazine on the grounds of its anaphylactic potential [11].

In order to enhance the yellow color of the food product, Tartazine is used in combination with Sunset Yellow FCF and Quinoline Yellow.

Sunset Yellow was found in 46.43% of tested imported samples (260 samples), and in 100% of tested domestic brands (105 samples). Eight of the imported and three of domestic tested samples exceeded the establish MPL of 50 mg/L [8, 13]. Sunset Yellow belongs to the chemical class of azo dyes with naphthalene structure. In the study conducted on albino rats, Hashem *et al*. [14] revealed that oral administration of Sunset Yellow at doses equalling 10 times ADI could impair hepatic function. Sunset yellow is currently banned in Norway and Finland. European Food Safety Authority (EFSA) in 2008 specified that food and drinks containing any of six artificial colorants that may be linked to hyperactive behaviour in children, including Sunset Yellow, will have to carry warnings. In 2009 EFSA decided to lower the ADI for Sunset Yellow from 2.5 mg/kg to 1.0 mg/kg bodyweight per day [5]. The Republic of Macedonia harmonized the legislation for labelling food colorants [8]. This included recommendation that each food product containing any of the following colorants: Tartazine, Sunset Yellow, Quinoline Yellow, Ponceau 4R, Carmoisine and Allura red, should carry warnings that their consumption may be linked to hyperactive behaviour in children.

Quinoline Yellow was found in 47.9% of tested imported samples (242) and in 93.0% of tested domestic brands (40). No one sample, exceeded established MPL of 100 mg/L [8, 13]. Quinoline Yellow belongs to quino-phthalone chemical class of compounds. Repeated or prolonged exposure to the substance can produce target organs damage [10].

Amaranth (E123), Ponceau 4R (E124) and Carmoisine (E122) belong to the same chemical class of naphtalenesulphonic azo dyes. The presence of Amaranth was found in 29.7% of imported tested samples (55 samples), and in 80% of tested domestic brands (12 samples). No one sample, exceeded established MPL of 100 mg/L. Ponceau 4R was tested in 158 of imported samples and in 23 of local brands. The presence of Ponceau 4R was found in 41.1% of imported and in 78.3% of local brands. Three of the imported and five of the local brands exceeded the establish MPL of 50 mg/L [8, 13]. Carmoisine was found in 20.5% of

imported tested samples (43), and in 83.3% of local brands [15]. Five imported samples, and six domestic samples, exceeded MPL value of 50 mg/L. The study of Bateman *et al*. [15], suggested that consumption of certain mixtures of artificial food colours including Sunset Yellow, Tartazine, Quinoline Yellow, Carmoisine, Amaranth and Ponceau 4R, together with preservative sodium benzoate (E211) is associated with increases in hyperactive behaviour in children.

Allura Red (E129) is azo dye with similar chemical structure with Amaranth and Ponceu 4R. The presence of Allura Red was tested in 149 imported and 22 local brands of soft drinks. 32.2% tested imported samples and 81.8% of local brands contained Allura Red, all below the MPL. Allura Red has fewer health risks associated in comparison to other azo dyes [12].

Erythrosine B (E127) is an organoiodine compound, a derivative of fluorone. Erythrosine is primarily used as food colouring. The presence of Erythrosine B was found in 63.0% imported tested samples (58), and in 55.5% domestic tested brands (10), all well bellow established MPL of 100 mg/L. According to the findings of Aziz *et al*., the potential adverse effects of Erythrosine on the spermatogenesis process were found in adult mice [16].

Brilliant Black BN (E151) is a synthetic black diazo dye, used in food decoration and coatings, desserts, sweets, ice cream, red fruit jams, caviar, soft drinks etc. The presence of Brilliant Black BN was tested in 87 imported samples, and 19 domestic brands. The obtained results showed that 48.3% of imported and 63.1% of domestic soft drinks contained Brilliant Black BN, all bellow established MPL of 50 mg/L.

Brilliant Blue FCF (E133) is an azo dye with complex structure used as a colorant in ice cream, canned processed peas, blue raspberry flavored products, non-alcoholic and alcoholic drinks (blue Curaçao liqueur), etc. It can induce allergic reactions in individuals with pre-existing moderate asthma [12]. In our investigations 21 imported and 10 domestic brand were tested on the presence of Brilliant Blue FCF. 47.6% of imported and 60% of domestic bands contained Brilliant Blue FCF in the concentrations well bellow established MPL (100 mg/L).

3.2 A study of preservatives

The preservatives considered in this study are benzoic acid (E210), sodium benzoate (E211), sorbic acid (E200), and potassium sorbate (E202). The obtained results are shown in Table 3 and Table 4.

Table 3. The results of the presence of food preservatives in imported soft drinks

Type of preservative	Number of tested samples	Number of positive samples	Concentration range (mg/L)	Maximum permitted level – MPL (mg/L)	Number of samples with preservative content above MPL (mg/L)	Number of samples with preservative detected but not labelled
Benzoic acid (E211)	85	80	83.4 - 178.5	150	3	3
Sodium benzoate (E210)	289	268	77.5 - 180.4	150	4	5
Sorbic acid (E200)	121	78	180.3 - 250.4	300	-	3
Potassium sorbate (E202)	156	95	175.6 – 310.2	300	2	4

Table 4. The results of the presence of food preservatives in domestic brands of soft drinks

Type of preservative	Number of tested samples	Number of positive samples	Concentration range (mg/L)	Maximum permitted level – MPL (mg/L)	Number of samples with preservative content above MPL (mg/L)	Number of samples with preservative detected but not labelled
Benzoic acid (E211)	55	5	83.4 - 108.5	150	-	-
Sodium benzoate (E210)	99	34	80.3 - 160.2	150	1	2
Sorbic acid (E200)	48	7	140.3 - 145.3	300	-	-
Potassium sorbate (E202)	59	8	155.6 – 140.2	300	-	-

The MPL for benzoates in soft drinks is 150 mg/L (expressed as benzoic acid). The MPL for sorbates is also expressed as the free acid and is 300 mg/L when used singly or 250 mg/L when used in combination with benzoates [7].

According to the obtained data (Table 1 and Table 2), the most prominent preservative in imported soft drinks, as well as, in domestic brands was sodium benzoate (93.3%, and 34.3%, respectively). Sodium benzoate was present over the MPL in 1.5% of the tested imported samples and in 2.94% of domestic brands. In the survey ran between January and May 2008 in the UK, ninety nine percents of the tested samples (250) of fizzy and still soft drinks were within the legal limits and were labelled correctly [17].

Potassium sorbate (E202] is used to prevent mould growth in foods such as cheese, cheese based products, yoghurt, wine, dried meat, pickles, apple cider, soft drinks etc. According to the data obtained in our investigation, potassium sorbate was found in 61% of imported and 13.5% of domestic brands. Ninety four percent of tested imported samples were bellow the MPL set for potassium sorbate and were labelled correctly [7].

The study performed by Mamur *et al*. [4] found out potassium sorbate to be genotoxic to the human peripheral blood lymphocytes *in vitro*.

3.3 A study of artificial sweeteners

The presence of artificial sweeteners aspartame, acesulfame potassium, sodium saccharin and sodium cyclamate in the samples of sugar reduced soft drinks was analyzed. The obtained results are shown in Table 5 and Table 6.

Artificial sweeteners are often used to control calorie intake and in certain medical conditions such as diabetes and hyperglycemia. The most common are aspartame, sodium cyclamate, acesulfame K, and sodium saccharin, which are marketed in many countries of the world [18]. The usage of the artificial sweeteners in the food industry has provoked strong controversy over theirs possible carcinogenic effects [19]. The Republic of Macedonia has established regulation for MPL of artificial sweeteners in food [7]. According to ours survey for the presence of artificial colorants in soft drinks, aspartame was the most predominant sweetener in imported and domestic brands as well. The results of our findings for the concentrations of artificial sweeteners in soft drinks were similar to those of Serdar and Knežević [18].

Table 5. The results of the presence of food sweeteners in imported soft drinks

Type of sweetener	Number of tested samples	Number of positive samples	Concentration range (mg/L)	Maximum permitted level – MPL (mg/L)	Number of samples with sweetener content above MPL (mg/L)	Number of samples with sweetener detected but not labelled
Aspartame (E951)	125	87	198.3 - 420.4	600	-	-
Acesulfame potassium (E950)	115	95	180.5 - 330.1	350	-	-
Saccharin sodium (E954)	52	43	55.8 - 77.9	80	-	-
Cyclamate sodium (E952)	48	40	230.5 - 270.1	250	1	2

Table 6. The results of the presence of food sweeteners in domestic brands

Type of sweetener	Number of tested samples	Number of positive samples	Concentration range (mg/L)	Maximum permitted level – MPL (mg/L)	Number of samples with sweetener content above MPL (mg/L)	Number of samples with sweetener detected but not labelled
Aspartame (E951)	35	20	178.6 - 395.6	600	-	-
Acesulfame potassium (E950)	28	21	202.4 - 334.6	350	-	-
Saccharin sodium (E954)	26	22	67.8 - 115.3	80	1	3
Cyclamate sodium (E952)	26	23	118.5 - 220.5	250	-	-

3.4 A study of citric acid, phosphoric acid, L – ascorbic acid, caffeine and quinine

The results obtained for the presence of citric acid, phosphoric acid, L – ascorbic acid, caffeine and quinine in imported and domestic brands of soft are presented in Table 7 and Table 8.
According to the obtained results (Table 7 and Table 8), all of the tested samples of imported and domestic brands of soft drinks were within the legal limits and were labelled correctly [7].

Citric cid is a naturally occurring weak organic acid. It is small, very soluble, and easily manufactured. Although it is naturally occurring it is used as an additive to many drinks to enhance flavour and increase stability in soft drinks and syrups. It is also used to prevent colour change by oxidation. The habitual and abusive use of foods containing citric acid can cause serious dissolution effects on human tooth enamel [20]. According to literature sources, ready-to-drink orange juices contain around 16 g/L of total citric acid, juice from actual oranges contains less, around 9 g/L, and soft drinks contain around 1.2 g/L (1200 mg/L) [21]. In our investigation the content of citric acid was found within the range 1220 - 1680 mg/L (imported brands of soft drinks), and from 1345 - 1560 mg/L (domestic brands).

Ascorbic acid (vitamin C) is used extensively in the food industry, not only for its nutritional value but for its many functional contributions to product quality. Acting as an antioxidant, ascorbic acid can improve the colour and palatability of many kinds of food products. When mixed with vitamin C, sodium benzoate forms benzene, a known carcinogen. The rate at which benzene is formed is affected by exposure. In the presence of sucrose, L - ascorbic acid, causes the degradation of artificial food colorants with azo benzene structure [22]. Our investigation showed low concentration of ascorbic acid in imported and domestic soft drinks, within the range 35.8 - 110.0 mg/L.

Phosphoric acid is used as a stabilizer and preservative in cola drinks. It also influences taste, making cola drinks tart. The phosphoric acid concentration in cola drinks is monitored to maintain product quality and minimize production costs. Its concentration varies with the beverage formulation, but is usually in the

Table 7. The results of the presence of citric acid, phosphoric acid, L – ascorbic acid, caffeine and quinine in imported soft drinks

Type of additive	Number of tested samples	Number of positive samples	Concentration range (mg/L)	Maximum permitted level – MPL (mg/L)	Number of samples with additive content above MPL (mg/L)	Number of samples with additive detected but not labelled
*Citric acid (E330)	156	128	1220 - 1680	-	-	-
*Ascorbic acid (E300)	143	95	55.4 - 110.0	-	-	-
Phosphoric acid (E338)	98	68	530.4 - 551.2	700	-	-
Caffeine	98	68	140.6 - 149.7	150	-	-
Quinine hydrochloride	48	38	76.5 - 83.5	85	-	-

*MPL is not established (The amount which is needed – Quantum satis)

Table 8. The results of the presence of citric acid, phosphoric acid, L – ascorbic acid, caffeine and quinine in domestic soft drinks

Type of additive	Number of tested samples	Number of positive samples	Concentration range (mg/L)	Maximum permitted level – MPL (mg/L)	Number of samples with additive content above MPL (mg/L)	Number of samples with additive detected but not labelled
*Citric acid (E330)	45	40	1345 - 1560	-	-	-
*Ascorbic acid (E300)	35	30	35.8 - 85.6	-	-	-
Phosphoric acid (E338)	16	16	480.5 - 550.6	700	-	-
Caffeine	16	16	135.6 - 145.3	150	1	-
Quinine hydrochloride	10	10	78.6 - 80.7	85	-	-

*MPL is not established (The amount which is needed – Quantum satis)

range of 500 mg/L ± 100 mg. The consumption of cola is associated with a bone mineral density (BMD) in older woman. In addition to displacing healthier beverages, colas contain caffeine and phosphoric acid, which may adversely affect bone [23].

Caffeine is a xanthine alkaloid, which is added to soft drink because it plays an integral role in the flavour profile. It is valuable for the general public, the medical community, and regulatory agencies to recognize that the high rates of consumption of caffeinated soft drinks more likely reflect the mood-altering and physical dependence-producing effects of caffeine as a central nervous system-active drug than its subtle effects as a flavouring agent [24]. MPL for caffeine in soft drinks is limited to 150 mg/L. In our study, the content of caffeine in all the tested samples didn't exceed MPL [7].

Quinine is an alkaloid which is added as a flavouring agent as a quinine chloride to some types of soft drinks as tonic water, bitter lemon etc. The usage of quinine may cause some unwanted effects as: diarrhoea, nausea, stomach cramps or pain, and in the case of overdose symptoms are: blindness, blurred vision or change in vision, chest pain, dizziness, double vision, fainting, rapid or irregular hearth beat [25]. Therefore, the MPL of quinine in soft drinks is set to 85 mg/L. The content of quinine chloride in all tested samples (imported and domestic), was found to be bellow established MPL [7].

4. Conclusions

- The consumption of non-alcoholic beverages has increased almost 500% over the past 50 years, especially among the children and teenagers [6]. Non-alcoholic beverages can contain different types of additives such as artificial food colorants, artificial sweeteners, food preservatives, flavourings, flavour enhancers, stimulants etc. All of these substances can represent a health hazard. Therefore, the usage of food additives in non-alcoholic beverages is regulated by certain legislation [7]. The aim of our study was to identify and quantitatively analyze some food additives in non-alcoholic beverages, which were imported or produced in the Republic of Macedonia in order to evaluate their safety according to the Official Regulation [7]. For that purpose, a total of 872 samples of non alcoholic beverages (carbonated mineralised, and water based non alcoholic flavoured drinks), were analyze on the presence of artificial food colorants (E102, E104, E110, E122, E123, E124, E127, E129, E131, E151); artificial food sweeteners (E950, E951, E952, E954); food preservatives (E200, E202, E210, E211); antioxidants, acidity regulators (E300, E330, E338); and other additives (quinine and caffeine).

- The results obtained from the study showed that the most prominent additives in the imported soft drinks were found to be preservatives potassium sorbate and sodium benzoate, which were present in 80% of tested samples. Synthetic colorants were present in 44% of tested the samples, caffeine in 69% , artificial sweeteners in 78%, quinine chloride in 74% and citric, ascorbic and phosphoric acid in 73% of tested soft drinks. In domestic brands, preservatives were found in 21.0% of tested samples, artificial sweeteners in 75%, and synthetic colorants in 87.4%. The concentrations of caffeine, quinine chloride, citric acid, phosphoric and ascorbic acid in domestic brands of soft drinks were found to be similar to those found in imported samples.

-Soft drinks take an important part in the total daily intake of food additives. Therefore, the constant monitoring of their presence in non alcoholic beverages is needed to ensure compliance with food safety regulations as well as for calculating risk assessment.

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