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Comprehensive HRMS chemical characterization of pomegranate-based antioxidant drinks via a newly developed suspect and target screening workflow

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Abstract: Antioxidants play significant role in human health, protecting against a variety of diseases. Therefore, the development of products with antioxidant activity is becoming increasingly prominent in human lifestyle. New antioxidant drinks containing different percentages of pomegranate, black berries, red grapes, and aronia have been designed, developed, and manufactured by a local industry. The comprehensive characterization of the drinks' constituents has been deemed necessary to evaluate their bioactivity. Thus, LC-qTOFMS has been selected, due to its sensitivity and structure identification capability. The data dependent and independent acquisition modes have been utilized. The data have been treated according to a novel, newly designed workflow based on MS-DIAL and mzmine for suspect, as well as target screening. The classical MS-DIAL workflow has been modified to perform suspect and target screening in automatic way. Furthermore, a novel methodology based on compiled bioactivity driven suspect list was developed and expanded with combinatorial enumeration to include metabolism products of the highlighted metabolites. Compounds belonging to ontologies with possible antioxidant capacity have been identified, such as flavonoids, amino acids, and fatty acids, that could be beneficial to humans' health, revealing the importance of the produced drinks as well as the efficacy of the new in-house developed workflow.

Keywords:
Antioxidant drinks, novel workflows, HRMS, pomegranate, suspect screening methodology

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1. Introduction

Pomegranate (*Punica granatum L.*), classified as a berry, is a member of Pinaceae family and has been cultivated in the Mediterranean region (Turkey, Egypt, Tunisia, Spain), as well as in India and Iran [1]. Pomegranate has been highly valued, due to its nutritional and medicinal properties, as well as its biological and free radical scavenging activity, which are attributed to the antioxidant phytochemicals derived from various parts of the plant (peel, seed, leaf, and flower) [2-4]. Pomegranate juice is a rich source of polyphenols, fructose, carbohydrates, glucose and organic acids (i.e., ascorbic acid, citric acid, fumaric acid, and malic acid), while it contains several amino acids, including proline, methionine, and valine. Additionally, the presence of tannins and flavonoids, as the main type of polyphenols, indicates the pomegranate's pharmacological potential, due to their antioxidant activity [1]. Ellagic acid, a metabolized form of ellagitannin is a powerful antioxidant, and it has an extensive applicability in plastic surgery, preserving the viability of skin flaps.

Furthermore, anthocyanins (water-soluble pigments), flavan-3-ols, and flavanols are some of the flavonoids found in pomegranate related to plausible health benefits. Catechins, which can be found in both juice and the peel of pomegranate, are vital to the biosynthesis of anthocyanins and have antioxidant and anti-inflammatory properties. It should be noted that all the flavonoids appeared in pomegranate have antioxidant capacity and contribute to the indirect suppression of inflammatory indicators, such as tumor necrosis factor-alpha (TNFα) [1]. It has been demonstrated that pomegranate fruits can be utilized for the treatment of human prostate cancer inhibiting cell development and inducing apoptosis.

Aronia melanocarpa, commonly referred as "aronia," is a *Rosaceae* family plant that is native to eastern North America and has lately also been grown in Europe [5]. Aronia extracts demonstrated anti-oxidant, anti-diabetic, and anti-inflammatory activity [6]. *Morus nigra,* which belongs to the *Moraceae* family, is also known as black mulberry [7], and is cultivated in India and China, as well in the Mediterranean region in Greece and Turkey [8]. A number of biological activities, including antidiabetic, antioxidant, anti-inflammatory, and anti-hyperlipidemic, have recently been linked to mulberry fruit [9]. *Vitis vinifera L.* (red grapes), a member of *Vitaceae* family [10], has traditionally been located from South Caucasus toward Mediterranean basin [11], whereas more recently new countries (United States, Australia, China) have been involved in the cultivation of the species [12]. Grapes have known to possess a wide array of biological activities such as antioxidant, antimicrobial, anti-inflammatory, and anti-cancer properties [13].

Pomegranate's beneficial effect on human health resulted in an increasing growth in market share. Therefore, the global pomegranate market is expected to worth 248.4 million USD in 2022 and 338.6 million USD by 2028, with a CAGR of 5.3% during the review period [14]. In the term of this study, pomegranate-based juices were created with the addition of secondary ingredients; black berries, red grapes, and aronia. The selection of these ingredients, that could provide added value to the final drink, was based on their market availability, contribution to the antioxidant capacity of the juice, and effect on the flavor and inner flavor of the juice. Therefore, there is an increasing trend to introduce in the market these pomegranate-based drinks.

Regarding the evaluation of the drinks' value and the determination of its antioxidant capacity, two trends appear in the literature. The first approach is related to the application of conventional techniques (i.e., DPPH, TEAC assay, Folin-Ciocalteu method) and the second one is linked to the determination of the antioxidant content of juice in terms of molecular species. The first approach is prone to various limitations, due to the nature of the matrix with the most common being the interference from the background. To begin with, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is a spectrometric method for determining antioxidants in solid or liquid matrices [15] via measuring substances' ability to act as free radical scavengers or hydrogen donors. This method can be employed specifically for the estimation of the overall antioxidant capacity and the free radical scavenging activity of fruit and vegetable juices [16]. Furthermore, Trolox Equivalent Antioxidant Capacity Assay (TEAC) measures spectrophotometrically the reduction of the radical cation ABTS+ (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) by antioxidant compounds [17], and it is commonly used in scientific research for analyzing foods' and beverages' antioxidant capacity [18]. Another frequently employed method for the determination of the antioxidant capacity is the spectrophotometric method using the Folin-Ciocalteu reagent [19].

An alternative approach is the use of chromatography-based techniques for the determination of individual analytes that are connected to antioxidant activity. This approach ascertains the selectivity and credibility of the obtained results. High-pressure liquid chromatography with a photodiode array detector (HPLC-PDA) was used for the determination of organic and phenolic acids (11), and anthocyanins in juices (11, 12). In the direction to further enhance the sensitivity and specificity of the acquired results, the recent trend is towards mass spectrometry-based methodologies, i.e. performing LC-

MS/MS methods for the determination of organic acids [20], and HPLC/PDA/MS2 for the quantification of punicalagin, ellagic acids and anthocyanidins [21]. A more comprehensive approach, for the obtain of a more holistic picture of the antioxidant landscape, can be followed through the implementation of High-Resolution (HR) techniques, i.e. conducting analysis of anthocyanins and phenolic compounds via UHPLC-Orbitrap-MS [22, 23], or anthocyanins and other phenolic compounds' analysis (phenolic acids, ellagitannins, and flavonoids) in pomegranate juices via HPLC–DAD–ESI-qTOF-MS [24]. Additionally, HRMS targeted and untargeted analysis in conjunction with chemometrics can be used alongside of bioactive compound determination, as well as a reliable tool for pomegranate adulteration [25]. Additionally, pomegranates' metabolite profiling and implementation of chemometrics was conducted via Nuclear Magnetic Resonance (NMR) spectroscopy [26].

The requirement for developing novel workflows capable of handling the massive amount of data derived from HRMS has emerged. Vendor specific and open access software have been utilized to interpret the acquired data; however, the scientific community has noted the significance of employing and evaluating open-source software due to the variety of algorithms, the files' compatibility between vendors, the codes' transparency, the large community of software developers and the capacity for their modification according to various licensing schemes. Nevertheless, there are still issues using non-targeted MS data derived either from Data Independent Acquisition (DIA-bbCid) or Data dependent (DDA-automs). DDA and DIA modes were employed in conjunction, to maximize the benefits of each mode and ensure the mining of the largest features' number. On the one hand, DDA is the most used strategy for compound elucidation, due to its cleaner and more easily interpretable spectra [27]. On the other hand, since DIA detects and fragments all ions in a sample, it empowers more thorough and repeatable analysis by collecting data within a wide range of known and unknown ions, while the fragmentation spectra are more complicated in interpretation [28].

In the terms of this project, innovative antioxidant pomegranate-based juices have been produced. In this direction, this research serves two supplementary purposes. The first aim is the molecular characterization of the drink *per se* in terms of quantitative and qualitative constitution. The second aim is the development of an advanced mass spectrometry novel workflow for the comprehensive characterization of the drink in terms of compounds' identification that bares antioxidant activity. This has been accomplished via the compilation of an extensive suspect list of antioxidants for the bioactivity characterization, and the assembling of the literature-based suspect list for the usual comprehensive characterization. It is noteworthy, that the aforementioned bioactivity-based characterization is a novel approach aiming towards the fast and efficient exploitation of the chemical domain. A new role for the combined suspect lists as a searchable database has been highlighted, towards the automated suspect screening, and increasing the credibility of the identification results. Therefore, the bioactive-based and literature-based characterization of foods and beverages may pave the way for a more comprehensive identification, while the utilization of open-source software provides an alternative yet efficient tool for the scientific community.

2. Results

2.1. Suspect Screening of the juice employing different workflows- Qualitative results

In total, 29 compounds were identified in all investigated juices, employing the suspect screening methodology reaching to different identification confidence levels depending on the available information. The levels of identification based on the criteria set on the scientific work of Schymanski et al. [29]. Specifically, 17 compounds reached at the level of identification 1, 10 compounds were identified at level of identification 2a, 1 compound reached to level 2b, and 1 compound was identified at level 3.

The identified compounds belonged to several categories with potential beneficial effect on human health. Specifically, 8 organic acids (citric acid, malic acid, gallic acid, gentistic acid, chlorogenic acid, pyroglutamic acid, fumaric acid, quinic acid), 4 fatty acids (linoleic acid, oleic acid, palmitic acid, linolenic acid), 3 amino acids (phenylalanine, leucine, norvaline) and 1 organic acid ester (ethyl gallate) were identified. Additionally, 10 flavonoids and the metabolites thereof (ellagic acid, ellagic acid glucoside, quercetin, rutin, apigenin, 2-Phenylethyl beta-Dglucopyranoside, kaempferol, phlorizin, verbacoside), 2 sugars (Fructose, glucosamine) were also identified.

All the compounds identified are tabulated in Table 1, where the compound name, the molecular formula, the experimental and the predicted retention time, the theoretical and experimental m/z value of the precursor ion, and the ionization mode are provided. Additionally, the 5 most intense MS/MS fragments (if they existed) of the sample and their corresponding ones obtained either from the spectral library or the reference standards are presented. The cosine similarity scores of the investigated spectra for all samples compared, as acquired from MS-DIAL, and the corresponding level of identification are also presented. The samples are coded as 80%, 90% and 100% based on the percentage of pomegranate, which is the basic ingredient.

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Table 1. Compounds identified through suspect screening in the final product.

Compound Name	Chemical Formula	Exp. t_R (min) (Reference Standard) [a]	Pred. t_R (min)	Application Domain [b]	Exp. m/z [c]	Theor. m/z	ESI Mode	MS/MS Explained Fragments [d]	Reference MS/MS Spectra [e]	Total score			Level of Identification/ Database Reference [i]
										80% [f]	90% [g]	100% [h]	
Citric acid	$C_6H_8O_7$	1.2 (1.2)	1.1	Box 1	191.0213	191.0197	-ESI	57.0353	57.0354	0.76	0.75	0.76	1
								87.0092	87.0089				
								111.0094	111.0088				
								191.0198	191.0199				
Malic acid	$C_4H_6O_5$	1.2 (1.1)	1.0	Box 1	133.0131	133.0143	-ESI	71.01442	71.0139	0.69	0.72	0.65	1
								115.00472	115.0022				
								133.01266	133.0138				
Fructose	$C_6H_{12}O_6$	1.4	1.7	Box 1	179.05754	179.0561	-ESI	89.0224	89.0246	0.66	0.64	0.69	1
								179.0560	179.0558				
Gallic acid	$C_7H_6O_5$	1.6 (1.5)	2.9	Box 2	169.0151	169.0142	-ESI	69.0354	69.0346	0.66	0.66	0.76	1
								97.0271	97.0295				
								125.0245	125.0244				
Gentisic acid	$C_7H_6O_4$	2.2 (2.4)	3.0	Box 1	153.0199	153.0193	-ESI	108.0191	108.0217	0.70	0.70	0.76	1
								109.0289	109.0295				
Chlorogenic acid	$C_{16}H_{18}O_9$	2.9 (2.9)	3.5	Box 1	353.0896	353.0878	-ESI	161.0264	161.0233	0.74	0.76	0.76	1
								173.0479	173.0447				
								191.0557	191.0555				
								192.0564	192.0589				
Fumaric Acid	$C_4H_4O_4$	1.3 (1.2)	1.9	Box 1	115.0035	115.0037	-ESI	71.0136	71.0136	0.68	0.71	0.74	2a MzCloud no 1274
								72.9928	72.9925				
								115.0035	115.004				
Quinic acid	$C_7H_{12}O_6$	1.3 (1.3)	1.2	Box 1		191.0561	-ESI	85.0291	85.0299	0.62	0.64	0.69	1
								191.0564	191.0558				
Phenylalanine	$C_9H_{11}NO_2$	3.0	4.2	Box 2	164.0726	164.0717	-ESI	72.0091	72.0099	0.63	0.66	0.61	1
								147.0447	147.0448				
								164.0728	164.0712				

Compound Name	Chemical Formula	Exp. t_R (min) (Reference Standard) [a]	Pred. t_R (min)	Application Domain [b]	Exp. m/z [c]	Theor. m/z	ESI Mode	MS/MS Explained Fragments [d]	Reference MS/MS Spectra [e]	Total score			Level of Identification/ Database Reference [i]
										80% [f]	90% [g]	100% [h]	
Leucine	$C_6H_{13}NO_2$	2.8	1.8	Box 1	132.1020	132.1019	+ESI	58.0654 69.0697 86.0964 87.0989 132.1019	58.0634 69.0686 86.0956 87.0987 132.1013	0.69	0.68	0.69	1
Norvaline	$C_5H_{11}NO_2$	4.5	1.2	Box 3	118.0646	118.0863	+ESI	65.03932 117.0584 118.0658	65.036 117.057 118.062	0.71	0.73	0.72	2a MonA ID: FiehnHILIC002191
Quercetin	$C_{15}H_{10}O_7$	7.3 (7.3) [a]	7.0	Box 1	301.0361	301.0354	-ESI	151.0042 169.0170 179.0004	151.0037 169.0135 178.9996	0.68	0.61	0.70	1
Rutin	$C_{27}H_{30}O_{16}$	5.7 (5.7) [a]	6.2	Box 1		609.1461	-ESI	300.028 301.0312	300.0256 301.0366	0.62	0.63	0.68	1
Apigenin	$C_{15}H_{10}O_5$	8.3 (7.9) [a]	7.6	Box 1	269.0460	269.0455	-ESI	117.0359 151.0077 269.0463	117.0341 151.0029 269.0459	0.61	0.66	0.61	1
Kaempferol	$C_{15}H_{10}O_6$	7.6 (7.8) [a]	7.2	Box 1	285.042	285.0405	-ESI	133.0305 151.0030 175.0386 285.0421	133.0297 151.0039 175.0388 285.0400	0.76	0.78	0.71	2a GNPS ID: VF-NPL-QEHF014174
Verbascoside	$C_{29}H_{36}O_{15}$	5.0 (4.8) [a]	8.4	Box 4	623.1993	623.1981	-ESI	161.0319 162.0263	161.0244 162.0278	0.62	0.69	0.68	1
Phloridzin	$C_{21}H_{24}O_{10}$	5.9 (5.8) [a]	8.2	Box 2	435.1291	435.1297	-ESI	167.0362	167.0340	0.66	0.67	0.75	1

Compound Name	Chemical Formula	Exp. t_R (min) (Reference Standard) [a]	Pred. t_R (min)	Application Domain [b]	Exp. m/z [c]	Theor. m/z	ESI Mode	MS/MS Explained Fragments [d]	Reference MS/MS Spectra [e]	Total score			Level of Identification/ Database Reference [i]
										80% [f]	90% [g]	100% [h]	
Ethyl gallate	$C_9H_{10}O_5$	4.9 (5.0)	5.0	Box 1	197.0473	197.0455	-ESI	123.0061 140.0102 168.0066 169.0149 197.0460	123.0086 140.0118 168.0074 169.0146 197.0460	0.62	0.63	0.62	3 FoodB ID:FDB012004
Linoleic acid	$C_{18}H_{32}O_2$	13.5 (13.5) [a]	12.9	Box 1	279.2336	279.2330	-ESI	279.2327 280.2345	279.2328 280.2333	0.61	0.60	0.70	1
Oleic acid	$C_{18}H_{34}O_2$	14.0 (14.0) [a]	13.3	Box 1	281.2486	281.2486	-ESI	281.2484 282.2526	281.2468 282.2508	0.69	069	0.74	1
Palmitic acid	$C_{16}H_{32}O_2$	13.8 (13.8) [a]	13.0	Box 1	255.2334	255.2330	-ESI	255.2329 256.2366 257.2349	255.2327 256.2364 257.2395	0.84	0.83	0.86	1
Linolenic acid	$C_{18}H_{30}O_2$	13.0	12.4	Box 1	277.2170	277.2173	-ESI	277.2198	277.2180	0.91	0.92	0.95	2a MoNA ID: MetaboBASE0976
Ellagic acid	$C_{14}H_6O_8$	4.6	4.7	Box 1	300.9993	300.9990	-ESI	201.0183 229.0143 283.9960 299.9924 300.9992	201.0200 229.0144 283.9950 299.9900 300.9994	0.77	0.74	0.80	2a MoNA ID:FiehnHILIC001170
Glucosamine	$C_6H_{13}NO_5$	1.8	1.4	Box1	162.0764	162.0760	+ESI	60.0450 72.0450 84.0450 85.0290 162.0760	60.0443 72.0435 84.0445 85.0284 162.0744	0.94	0.97	0.93	2a GNPS ID: CCM-SLIB00005464276
2-Phenylethyl beta-D-glucopyranoside	$C_{14}H_{20}O_6$	6.5	6.2	Box 1	302.1616	302.1600	+ESI	81.0337 85.0287 97.0289 105.0707 127.0340	81.0330 85.0270 97.0280 105.0710 127.0400	0.89	0.92	0.94	2a GNPS ID: CCMSLIB00000854907

Compound Name	Chemical Formula	Exp. t_R (min) (Reference Standard) [a]	Pred. t_R (min)	Application Domain [b]	Exp. m/z [c]	Theor. m/z	ESI Mode	MS/MS Explained Fragments [d]	Reference MS/MS Spectra [e]	Total score			Level of Identification/ Database Reference [f]
										80% [f]	90% [g]	100% [h]	
Pyroglutamic acid	$C_5H_7NO_3$	2.4	2.1	Box1	130.0511	130.0507	+ESI	84.0455 85.0483 129.0190 130.0508	84.0460 85.0450 129.0220 130.0510	0.94	0.93	0.91	2a MassBank ID: PR311148
4-Hydroxyquinoline	C_9H_7NO	4.5	6.1	Box 2	146.0606	146.0600	+ESI	77.0389 91.0541 101.0395 146.0598	77.03700 91.0560 101.0440 146.0610	0.93	0.97	0.99	2a RIKEN PLaSMA ID: RIKENPlaSMA000824
Dihydrozeatin	$C_{10}H_{15}N_5O$	5.8	4.31	Box 2	222.1351	222.1349	+ESI	69.0699 136.0615 148.0626 204.1227 222.1347	69.0710 136.0620 148.0620 204.1250 222.1349	0.91	0.88	0.92	2a MoNA ID: FiehnHILIC000308
Ellagic acid glucoside	$C_{20}H_{16}O_{13}$	3.7	4.5	Box 1	463.0514	463.0518	-ESI	300.9976					2b diagnostic ion Using Smilib

[a] retention time of the reference standard, [b] Development and Prediction of Retention Time Indices on-line available at http://rti.chem.uoa.gr [c] experimental *m/z* value with error ± 0.005 Da; $[M + H]^+$ for +ESI and $[M - H]^-$ for -ESI (*with the exception of glucosamine, which *m/z* value corresponds to $[M-H_2O+H]^+$, and 2-phenylethyl beta-D-glucopyranoside which m/z corresponds to $[M+NH_4]^+$), [d] top-five most intense explained peaks (if they existed), [e] MS/ MS fragments of the spectra reference standard or mass spectral library, [f] 80% pomegranate, [g] 90% pomegranate, [h] 100% pomegranate, [i] for the level of identification 2a the database entry is referred.

2.2. Quantitative analysis

The compounds, for which their analytical standards were available in the laboratory, were quantified using calibration curves of reference standards. Specifically, the concentrations of 5 organic acids (abscisic acid, chlorogenic acid, citric acid, gallic acid, quinic acid), 2 flavonoids (quercetin, galangin), one flavonoid glucoside (verbascoside), and phenol glucoside (phlorizin) were determined. The compounds catechin, gentistic acid, epicatechin, genistein, p-coumaric, and pinobanksin were identified through target screening;however their concentrations were below their limit of quantification (LOQ) defined as 0.5 mg/kg. The abovementioned concentrations with their corresponding standard deviation (SD) for the three investigated samples are presented. Additionally, the corresponding calibration equations in the form of $y= (a\pm S_a) x+(b\pm S_b)$, as well as their correlation coefficients are tabulated in Table 2.

Table 2. Quantification results.

Analyte	Concentration (mg/kg) ± SD (n=3)	Concentration (mg/kg) ± SD (n=3)	Concentration (mg/kg) ± SD (n=3)	Equation of the external calibration curve $y= (a\pm S_a) x+(b\pm S_b)$	Correlation coefficient R^2
	80% [a]	90% [b]	100% [c]		
Abscisic acid	0.28 ±0.02	<LOQ	<LOQ	y = (29407± 1108) x +(13932 ±5655)	0.994
Chlorogenic acid	4.07±0.33	1.35±0.08	0.52±0.05	y = (299437 ± 17948) x +(236799 ± 91609)	0.98
Citric acid	203±18.9	204±21.2	199±18.4	y = (114212 ± 3871) x -(27802±19759)	0.991
Galangin	1.41±0.86	0.65±0.05	<LOQ	y = (179124 ± 11301) x -(9729±57678)	0.98
Gallic acid	5.72±0.41	4.57±0.41	5.11±0.47	y = (46623 ± 3535) x +(57556±18043)	0.98
Phloridzin	1.01±0.09	0.65±0.05	0.65±0.06	y = (304319 ± 25920) x +(287534±132294)	0.97
Quinic acid	0.75±0.09	0.38 ±0.04	<LOQ	y = (121371 ± 1970) x +(431844 ± 10055)	0.993
Verbascoside	0.87±0.12	0.51±0.05	<LOQ	y = (58119 ± 1053) x -(14120±5376)	0.996
Quercetin	13.1 ±0.45	11.6 ±0.56	11±0.48	y = (71193 ± 4859) x + (51014 ± 24802)	0.992

[a] 80% pomegranate, [b] 90% pomegranate, [c] 100% pomegranate

3. Discussion

3.1. Development of a novel workflow

The necessity for the development of a novel workflow that combines targeted and suspect screening with the existing DDA and DIA fragmentation methodologies emerged lately. In the current data treatment software landscape, only MS-DIAL has the appropriate algorithms to perform both DDA and DIA (MS2Dec, CorrDec) analysis. On the other hand, MS-DIAL is not designed to perform target screening, whereas MZmine [30] is capable of performing target screening based on MS1 spectra and annotation based only on DDA fragmentation. Therefore, novel workflows that combined these two pieces of software together were designed to overcome these issues.

The DDA and DIA approaches, producing fragmentation of the molecular species, differ essentially to the precursor selection. Thus, in DDA, the precursor ion is selected, followed by fragmentation, whereas in DIA, no precursor ion is selected, instead, all ions are fragmented. Therefore, in a formal way only DDA produces MS/MS spectra, while DIA generates MS/MS like fragmentation but in MS1 spectra. Therefore, for the rest of the

manuscript the term "fragmentation derived spectra" will be used to describe the fragmentation pattern derived from either DDA (MS/MS) or DIA (high collision energy).

3.1.2 Compilation of suspect lists

In the direction of deeply mining all the potential information according to their antioxidant contribution, a novel idea was conceived called bioactivity driven interrogation. Therefore, focusing on a desirable property of the final product, substances of a specified activity were highlighted, bypassing the fuzzy information concerning the whole metabolite landscape. A new list that filtered the specific biological activity of the chemical space, i.e., emphasizing in the current case to the antioxidant capacity (the antioxidant active compounds) has been assembled. The compiled list has been entitled as "Bioactivity Driven Suspect List (BDSL)".

Taking into consideration the metabolism of the most abundant antioxidant compounds of the BDSL, another list was compiled through **combinatorial enumeration** in order to predict products from potential metabolic patways, such as the glucolysation and methylation. This assembled list, called Virtual Metabolite Suspect List **(VMSL),** was generated using Smilib v2.0 [31] [32], utilizing the scaffolds, linkers and building blocks according to the software. The scaffolds are the already identified Natural Products (NPs) of the BDSL, while as building blocks have been used one or two glucose units and/or a methyl group. Additionally, to retain the parent compounds, hydrogen has been also selected as a building block. It should be noted that the metabolites of the compounds thereof could potentially have enhanced biological activity compared to their initial non-metabolized counterparts (i.e., quercetin may have similar antioxidant capacity with quercetin glucosides or even enhanced, due to the latters' different hydrophilicity). Alongside with the aforementioned suspect lists, a literature-based list (LBL) has also been compiled.

3.1.3 Mzmine-based workflow (MS1 driven)

Mzmine has a targeted feature detection module, which can interrogate the MS1 experiment using the information from a suspect list and construct the respective Extracted Ion Chromatograms (EICs), as the feature list. These chromatographic peaks were prioritized based on the area under curve. It has to be reminded that the antioxidants need to be in high quantities in order to exert their role. Thus, the most abundant antioxidants (MAA) were selected and used as input to construct a searchable database. This list will be utilized as a part of the MS-DIAL workflow. Therefore, these two lists also function in a confirmatory way as the simultaneous presence of a metabolite enhances the confidence., Thus, 6 common compounds were identified from both lists (VMSL and LBL), i.e., quercetin, kaempferol, apigenin, gentisic acid, gallic acid and chlorogenic acid.

3.1.4 MS-DIAL based workflow (fragmentation driven)

MS-DIAL deems to be a valuable solution, in order to exploit the DIA results, as well as DDA spectra. The compounds are annotated offline by comparing the DDA or DIA deconvoluted spectra to the corresponding ones from the samples. MS-DIAL searches against already assembled local libraries (i.e., the general list ESI(+/-)-MS/MS assembled from authentic standards, which is provided from the software's download page). A novel idea has been conceived about the replacement of the abovementioned library from a narrowed version, encompassing only specific compounds of interest (i.e., target/suspect version). In contrast to the untargeted mode, for which MS-DIAL has originally been used, this novel approach allows the software to function in the target/suspect mode. Therefore, this flexibility allows the construction of custom-made libraries, providing the capacity to narrow down the number of plausible candidates.

MSP files (editable with a simple text editor i.e., notepad, work pad etc.) consist of entries that include the candidates' names, the molecular formula, the exact mass, the theoretical retention time, and the MS/MS fragments. Such files are publicly available from various sources such as the GNPS, MS-DIAL etc. webpages. These MSP files were adjusted to focus on the analytes of interest, thus serving as a database, which is essentially a suspect list. This approach offers the additional advantage of a more complete view for spectra comparison. Thus, the fragments included in this database correspond to experimental spectra and not biased/curated fragments as it happens commonly for the compilation of suspect lists. These files are compatible with the MS-DIAL software, which offers the potential for process both DDA and DIA data. Two lists have been generated (LBL, VMSL) and imported to MS-DIAL. The overall chemical space was searched with the aid of these two suspect lists, aiming to find the bioactive content in terms of antioxidants, and secondly to chemically characterize in a comprehensive way the final drinks.

3.2. Comparative analysis of antioxidant juices

As the main aim of this endeavor was the development of a drink with enhanced antioxidant activity, various combinations of raw materials in different percentages (i.e., 3.3% ,6.6% from other juices) have been used. For clarification purposes, it should be reminded that the secondary ingredients used; were juices of aronia, black berry and red grapes. The contribution of the quantity for the selected antioxidants has been studied. Generally, 3 patterns have been observed based either to the targeted results or the corresponding peak areas (used for the substances, which their reference standards were not available). An increasing trend of the investigated antioxidants when the percentage of other juices was lowered (i.e., quercetin), an opposite effect (i.e., ethyl gallate), or no effect (i.e., fructose). Thus, in the case of ethyl gallate, the drinks containing higher percentage of the secondary ingredients, also contain higher amounts of this substance comparing to the pure pomegranate drink. On the other hand, the amount of quercetin in juices (containing 80 and 90% pomegranate) is lower. Finally, the same amount of fructose has been determined in all three analyzed juices. This is depicted in the figure 1. No discrepancies for these patterns were noticed, which validates the results of the analysis.

The compounds quinic acid, kaempferol, quercetin, chlorogenic acid, rutin, verbacoside were found higher in the juice supplemented with 6.66 % of each secondary raw material. Their elevated quantity is connected to the enhanced antioxidant activity.

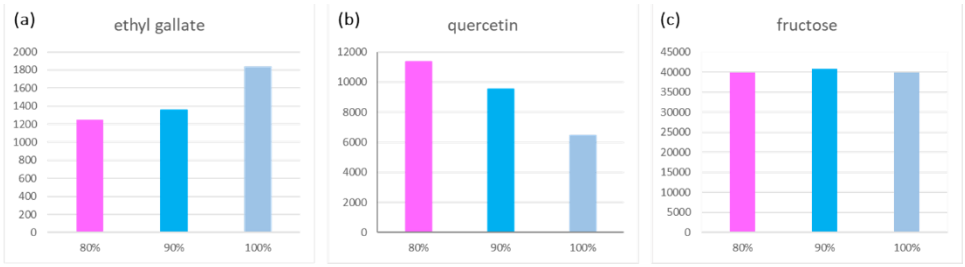

Figure 1: Quantity of (a) ethyl gallate, (b) quercetin (c) fructose for the designed juices with 80%, 90%, 100% of pomegranate.

3.3. Beneficial role of the identified compound in humans' health

One prevalent criterion for the selection and the final percent contribution of each ingredient to the produced drink is their antioxidant capacity in terms of the existence and content of bioactive substances. Hence, the presence of the antioxidants should be adequately high, as their activity is not excreted by the interaction of the substance with a pharmacological target/receptor. The obtained results revealed the presence of the antioxidants compounds belonging to several categories (organic acids, fatty acids, amino acids,

flavonoids and metabolites, etc.). The beneficial effect of the most important identified compounds is briefly discussed.

Ellagic acid is a well-known antioxidant that has been shown to be effective in preventing neurodegeneration by repairing mitochondrial damage and scavenging free radicals [33]. Quercetin is a powerful antioxidant known for its capacity to prevent tissue damage [34]. Kaempferol has anticarcinogenic, antioxidant and anti-inflammatory [35], as well as antibacterial, antifungal, and antiprotozoal activities [36].

Fumaric acid has anti-inflammatory, neuroprotective, chemo preventive activities [37], and acts against multiple sclerosis (MS) [38]. Chlorogenic acid has antioxidant antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, antiviral, anti-microbial, anti-hypertension activity [39]. Citric acid is a secondary antioxidant [40], yielding synergistically to enhance primary antioxidants' activity [41], while its chelating and acidulating properties (25) are well- known [42].

4. Materials and Methods

4.1. Methodology of preparation Pomegranate based drink

A thorough literature review was conducted for 16 potential raw materials that might serve as additional ingredients in the pomegranate-based juice to enhance its nutritional value. The raw materials investigated were *Prunus cerasifera, Vaccinium vitis-idaea L, Prunus cerasus, Aronia melanocarpa, Citrus, Ribes rubrum, Vitis vinifera L, Hippophae, Actinidia deliciosa, Opuntia ficus-Indica, Ficus carica, Rubus occidentalis, Morus alba, Morus nigra.*

The *Morus nigra* (black berries), *Aronia melanocarpa* (aronia), and *Vitis vinifera L* (red grapes) were chosen based on their market availability, antioxidant contribution, and effect on the flavor and the lingering of the final product.

Then, two mixtures of juices were created, only differing in the proportion of their ingredients. Specifically, the % percentage of pomegranate, black berries, aronia, red grapes were (90, 3.33, 3.33, 3.33, v/v) and (80, 6.66, 6.66, 6.66, v/v), respectively. Their flavor (sweetness, sour taste, acidity), lingering flavor (sour taste, acidity) as well as color, fragrance, and texture have been assessed. The second mentioned juice had a higher overall score in the majority of the investigated categories.

The manufacturing process of the final product was initialized with the defrosting of the raw components (juices of pomegranate, red grape, black berry and aronia) until they reached at room temperature. Afterwards, the transfer of the juices to tanks and their combination through stirring followed. Next, a pasteurization step in a tube heat exchanger at 83 ° C was performed. Next, hot filling took place, and the bottles were sealed using an automatic sealing machine. The juices' temperature has been decreased in a cooling tunnel. The bottles were kept at a temperature of 20 °C, protected from light.

4.2. Reagents and Materials

All standards and reagents used were of analytical grade (<95%), unless differently stated explicitly. Methanol (MeOH, LC–MS grade) was purchased from Merck (Darmstadt, Germany), while formic acid 99% and acetic acid were acquired from Fluka (Buchs, Switzerland). Ammonium acetate and ammonium formate were obtained from Fisher Scientific (Geel, Belgium). The ultrapure water (H_2O) was provided by a Milli-Q device (Millipore Direct-Q UV, Bedford, MA, USA). Regenerated cellulose syringe filters (RC filters, pore size 0.2 μm, diameter 15 mm) were acquired from Macherey-Nagel (Düren, Germany). Citric acid, chlorogenic acid, gallic acid, malic acid, quercetin, apigenin, phloridzin, ethyl gallate, L-phenylananine, leucine, fatty acid methylesters, D (-) fructose, catechin, epicatechin, pinobanksin and p-coumaric acid were obtained from Sigma Aldrich (Stenheim, Germany. Verbascoside was purchased from HWI pharma services (Rülzheim, Germany), while quinic acid, genistein, gentistic acid, and galangin were acquired from

Supelco (Stenheim, Germany). Apigenin was purchased from Alfa Aesar (Karlsruhe, Germany).

Stock solutions of the reference standards (1000 mg L^{-1}) were prepared in MeOH (LC-MS grade) and stored at −20 °C in amber glass vials. A solution of 50 mg L^{-1} was prepared by appropriate dilution of the individual stock standard solutions. Following that, dilutions with a mixture of MeOH: H_2O (80:20, v/v) were carried out in order to prepare working solutions with concentrations of 0.5, 1, 2.5, 5,10 mg L^{-1}.

4.3. Sample Pre-Treatment for HRMS Analysis

In an eppendorf tube, 200 mg of the drink were weighed and the addition of 200 μL MeOH: H_2O (80:20, v/v) was followed. The mixture was vortexed vigorously and filtered through RC syringe filters. The extracts were transferred to 2-mL autosampler glass vials and injected into the UPLC-QToF-MS system in both ionization modes.

4.4. Instrumentation

4.4.1. UPLC-QToF-MS Instrumentation

The chemical analysis of the pomegranate-based juice was carried out using Ultra-High-Pressure Liquid Chromatography-Quadruple Time of Flight Mass Spectrometry (UPLC-QToF-MS) employed with an HPG-3400 pump (Dionex Ultimate 3000 RSLC, Thermo Fisher Scientific, Dreieich, Germany) coupled to a time-of-flight mass analyzer (Hybrid Quadrupole time of Flight Matic Bruker Daltonics, Bremen, Germany). The chromatographic column utilized was an Acclaim RSLC 120 C18 column (2.2 μm, 2.1 × 100 mm, Thermo Fisher Scientific, Dreieich, Germany), equipped with a pre-column (Van guard Acquity UPLC BEH C18 (1.7 μm, 2.1 × 5 mm, Waters, Ireland) and its temperature (30 °C) was maintained constant during the analysis. In the positive ionization mode, the mobile phases consisted of (A) aq. 5 mm ammonium formate: MeOH (90:10, *v/v*) acidified with 0.01% formic acid and (B) 5 mM ammonium formate in MeOH acidified with 0.01% formic acid. In the negative ionization mode, the mobile phases were (a) aq. 10 mM ammonium acetate: MeOH (90:10, *v/v*) and (B) 10 mM ammonium acetate in MeOH. The same gradient elution program was used in both ionization modes. The gradient program is described in detail in a previous work of our group [43]. The values selected for the MS parameters were capillary voltage of 3500 V, nebulizer gas pressure of 2 bar (N_2), drying gas flow rate of 8 L min^{-1}, and capillary temperature of 200 °C. The sodium formate calibrant, which was prepared in H2O: isopropanol (50:50, v/v), was injected at the beginning of each run to calibrate the Q-ToF system on a daily basis.

4.5. Mass Spectrometry Data Analysis

4.5.1. Identification Confidence

The feature annotation was performed according to the Schymanski et al. scheme, considering the five levels of confidence in identifying a plausible candidate [29]. At level 5, the only confirmed information is the exact mass of interest, while there is no information concerning its molecular mass. At level of identification 4, the candidate's molecular formula is confirmed [44], whereas at the next level (level 3), the tentatively identification via evaluation of candidates' MS/MS fragment is realized utilizing *in silico* fragmentation tools (MetFrag [45] or CFM-ID [46]). Additionally, at this identification level, prioritization methods, such as retention time prediction [47] and ionization efficiency estimation [48] can be used to enhance the identification confidence. In the cases, in which diagnostic ion exist, the plausible candidate can reach at level identification 2b. At level identification 2a, potential candidates can reach, when the corresponding MS/MS spectra are available at spectral libraries and their similarity score is higher than 0.7. At level of identification 1, the candidates' reference standards are available and their MS/MS spectra, the retention time being in accordance.

4.5.2 Data processing and identification workflows

4.5.2.1 Workflows for the compilation of suspect lists.

4.5.2.1.1 Bioactivity driven suspect list.

A suspect list compiled of 734 antioxidant substances was retrieved using Orange statistical language (version 3.33.0) through the text mining module using PubChem data. The molecular formula and the exact mass, alongside with the compound name, were deposited in a csv file, which in turn was uploaded to MZmine 2.53. The raw data were calibrated and converted to mzxml files using Data Analysis software (Bruker Daltonics, Bremen, Germany) to be compatible with MZmine. The most abundant substances i.e., those with the highest chromatographic peak areas were selected for the evaluation of the drink's antioxidant capacity. The mass spectral databases used for the assembling of the suspect list were: MoNa [49], MassBank-Europe [50], METLIN, Human Metabolome Database (HMDB) [51], and the Global Natural Products Social Molecular Networking (GNPS) [52]. The features were annotated through the comparison of their MS/MS spectra with the corresponding ones from the spectral libraries or the reference standards in the cases that they were available in the laboratory. Due to the lack of entries concerning the metabolites derived from the enumeration process as well as their MS/MS spectra from the aforementioned libraries, their fragmentation was estimated based solely on the characteristic diagnostic ions (i.e., for ellagic glucoside, the fragment of the aglucone part and the corresponding fragment of the sugar moiety). These pieces of information were also added to the VMSL list.

4.5.2.1.2. Comprehensive literature based suspect list.

An exhaustive literature-based, text-mining-defined suspect list was created using the Orange statistical language. This list encompasses the compounds retrieved from PubChem that were specified for pomegranate, black berries, red grapes, and aronia. In the same direction, a literature- based suspect list [2, 20-26, 53-55] was assembled with the traditional way and merged with the one obtained from the text mining procedure. This list has been used for the suspect/target screening protocol and as a supporting tool to enhance the confidence of the acquired results from the BDSL. Furthermore, these two workflows act synergistically to provide a holistic picture of the plant's chemical composition. The workflows employed for the compilation of the suspect lists is illustrated to figure 2.

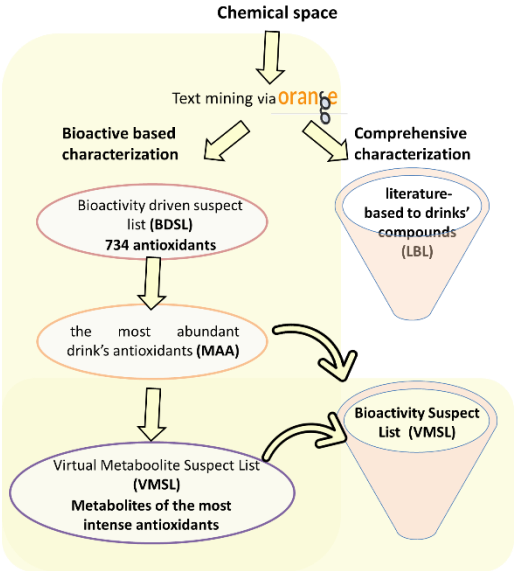

Figure 2. Workflow of suspect lists compilation.

4.5.2.2 Methodology of the development of Workflow MS1 Driven

The raw data were calibrated, converted to mzxml files and uploaded to MZmine 2.53. The list of 734 antioxidants in csv form were imported and the ones with the highest peak area derived from the feature list were used as scaffolds for the compilation of VMSL.

4.5.2.3 Methodology of the development of Workflow MS2 Driven

The calibrated raw data were converted to abf files (ABF converter) [56], and then uploaded to the open-source MS-DIAL software (version 4.92) [57]. Both acquisition modes, DDA and DIA, were examined. DDA was selected for the most abundant compounds, whereas DIA was utilized for the compounds found in lower quantities. The different acquisition modes have been processed separately.

Based on the compounds mentioned in the literature (LBL), an in-house database was created and imported into MS-DIAL (MSP file format). Additionally, the VMSL (MSP file format) was also imported to MS-DIAL. These two MSP files were processed separately to evaluate the antioxidant content, as well as the compounds discovered through the comprehensive drinks' characterization. The online "Retention time prediction tool" (available on http://rti.chem.uoa.gr/) was utilized to predict the theoretical retention time of each compound by uploading the canonical smiles. For compounds with reference standards not available in the laboratory, the corresponding spectra were retrieved from public spectral libraries. A procedure blank was also prepared. The chromatographic peak areas of the procedure blank have to be 5-fold lower than the ones in the sample, in order not to be excluded as false positives.

The entire workflow is depicted in figure 3.

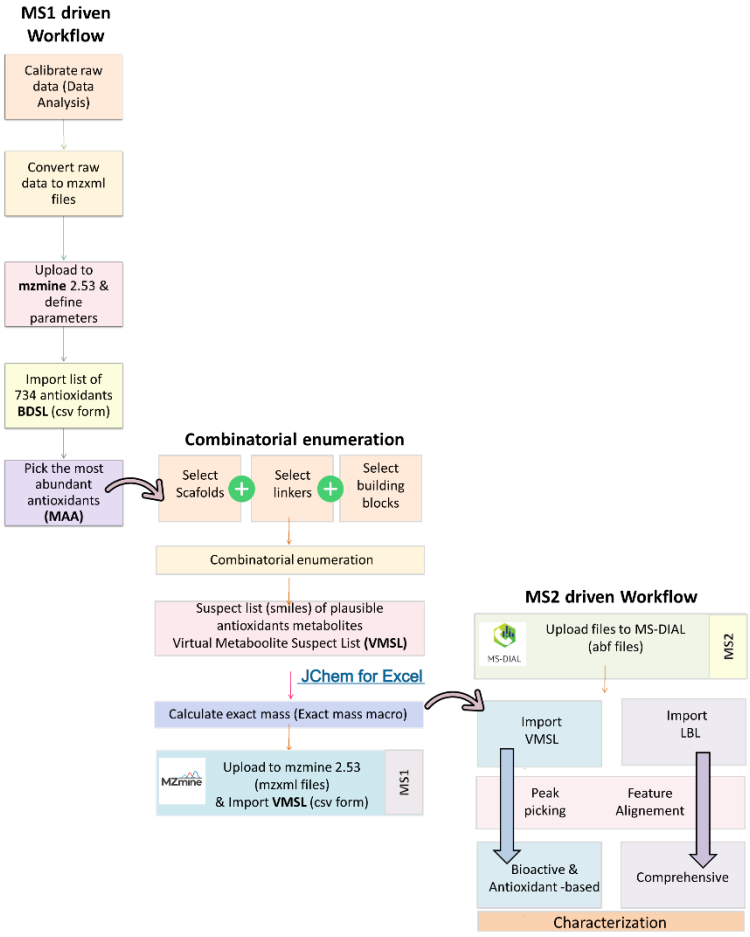

Figure 3. Workflow of the novel suspect screening methodology

4.6. Target screening Methodology

For the determination of the compound's concentration, TASQ 1.4 (Bruker Daltonics, Bremen, Germany) was used. For the compounds with available reference standards, and belonging in the category of bioactive compounds, their quantification was followed. The quantification of the analytes was performed using standard calibration curves and based on a validated method developed in our laboratory [25]. Satisfactory linearity was achieved for all the analytes (R^2 values ranging from 0.97 to 0.996).

5. Conclusions

Pomegranate-based juices with antioxidant capacity has been designed, produced, and characterized employing novel suspect and target screening methodologies through open-source software using UPLC-QToF-MS. A total of 29 compounds, including fatty acids, amino acids, organic acids, flavonoids and their metabolites were identified in the drinks via the developed methodologies in both ionization modes. The significant amount of quercetin, as well as the high concentration of citric acid sparked a lot of interest, due to their plausible positive impact on human health.

In this context, novel suspect, and target screening methodologies for the elucidation of drinks' compounds have been developed to ascertain a faster and less effort-consuming data treatment, ensuring results with enhanced credibility. Bioactivity-/combinatorial- and literature-based lists have been assembled as searchable database in combination to the mass spectrometry analysis using open-source software (MZmine, MS-DIAL).

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