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## Bioavailability of catechins from ready-to-drink tea

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## UNIVERSITÀ DEGLI STUDI DI TORINO

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1 **Bioavailability of catechins from ready-to-drink tea.**

2

3 **Running title:** Catechins from ready-to-drink tea.

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11 recruitment, collection and interpretation of the clinical data, and subjects' management; DDR was  
12 responsible of conception and design of the study, DDR & LC were the primary authors of the  
13 manuscript, and all other authors provided input and approved the final version of the manuscript;  
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20

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24 **Abstract**

25 **Objective:** As consumption of teas may be associated with potential health benefits due to its  
26 content in polyphenols and as in Western countries the consumption of tea is equally divided  
27 between the hot and the ready-to-drink (RTD) cold version of this typical beverage, aim of this  
28 work is studying absorption and metabolism of flavan-3-ols in human volunteers after the ingestion  
29 of a commercial RTD tea.

30 **Research Methods & Procedures:** A feeding study was carried out on 20 healthy human  
31 volunteers and urine samples were collected for 24 hours after tea ingestion. Flavan-3-ols derived  
32 molecules were identified and quantified in urine samples by HPLC with tandem mass  
33 spectrometric detection.

34 **Results:** Eight relevant metabolites were identified in urine, all modified flavan-3-ols with the  
35 exception of unmetabolised gallic acid. The urinary excretion of flavan-3-ols was equal to the 7.2%  
36 of the intake with tea. Gallic acid, which was abundant in the RTD tea used in this study, reached a  
37 4.5% of the drunk amount.

38 **Conclusions:** The bioavailability values observed are in agreement with previous reports even  
39 though the dosage of polyphenols ingested in this study is remarkably lower. Moreover, the use of a  
40 group of twenty volunteers, more than the average number of subjects used for usual human acute  
41 feeding studies involving polyphenols, gives additional credibility to the results. After drinking the  
42 ready-to-drink ice tea used in this study, the internal compartments are exposed to non-marginal  
43 doses of flavanols and flavanol metabolites up to 24 hours.

44

45

46 **Keywords:** Tea, flavan-3-ols, catechins, polyphenols, bioavailability

47

## 48 **Introduction**

49           Tea is one of the world's most popular beverages second only to water, prepared by water  
50 infusion of dried leaves from *Camellia sinensis*. Consumption of teas may be associated with  
51 potential health benefits. Teas are a rich source of polyphenols, with the phenolic content dependant  
52 upon the degree of fermentation. Green tea is unfermented and the dried leaves are a rich source of  
53 flavan-3-ols (catechins) [1]. During preparation of black tea, leaves are crushed allowing  
54 fermentation by polyphenol oxidase. Oxidation followed by polymerisation diminishes catechins  
55 levels while theaflavins, procyanidins and other compounds of higher mass are formed.

56           The major phenolics found in teas are catechins (flavan-3-ols) and flavonols. There are three  
57 predominant families of catechins in teas, the free catechins, including galliccatechins and gallate  
58 esters, the theaflavins and finally the thearubigens. The main flavonols are conjugates of quercetin  
59 and kaempferol [1]. The conjugation varies from mono- to di- and tri- glycosides. In addition to  
60 these two families, flavones and phenolic acids are also reported in teas, in particular the quinic  
61 esters of gallic, coumaric and caffeic acid.

62           Polyphenols in general and catechins in particular are reported to exert various biological  
63 activities. A significant body of evidence relating to the protective effects of tea polyphenols against  
64 cardiovascular disease, stroke and cancer incidence has been amassed [2]. The proposed  
65 mechanisms for the protective effects of tea against coronary heart disease include inhibition of  
66 oxidation of LDL, known to be involved in the development of atherosclerosis, anti-  
67 hypercholesterolemic activity and inhibition of platelet aggregation [3]. Consumption of tea  
68 polyphenols has been linked with prevention of a variety of cancers using animal models, including  
69 prevention of cancers of the skin, digestive tract, liver, bladder and prostate [4]. Inhibition of  
70 tumorigenesis observed in these models may be applicable to human systems, however  
71 epidemiological evidence is again inconclusive. Moreover, protective effects of teas against cancers  
72 are more commonly observed in studies carried out in Asian countries where predominantly green  
73 tea is consumed [3].

74 In Western countries, with probably the only exception of the United Kingdom, the  
75 consumption of tea is equally divided between the hot and the Ready-to-drink (RTD) cold version  
76 of this typical beverage [5]. During spring and summer, moreover, this balance falls drastically  
77 towards an increased consumption of RTD teas, easily available and characterised by several  
78 special features beside the cold beverage nature, such as different tastes, vitaminic fortification, and  
79 sweeteners as substitutes of sugar. Among RTD products, however, the quality in terms of  
80 polyphenol content is strongly varied [6], usually depending on the mode of preparation (soluble  
81 extract or proper infusion), on the original tea leaves chosen by each brand and on the technological  
82 treatments used for safety issues. Moreover, little information exists on the bioavailability of tea  
83 catechins in RTD teas.

84 This paper reports on absorption and metabolism of flavan-3-ols in 20 healthy human  
85 volunteers after the ingestion of 500 mL of a commercial RTD tea. The work was performed by  
86 analysing flavan-3-ols and their metabolites in urine samples collected over 4 periods in 24 h after  
87 intake. Catechin metabolites were identified by HPLC-MS/MS and subsequently quantified using  
88 HPLC with MS in the selective ion recording (SIR) mode.

89

## 90 **Materials and methods**

### 91 **Tea and chemicals**

92 Five hundred mL bottles of ready-to-drink tea beverage were supplied by Soremartec Italia  
93 S.r.l. (Alba, CN, Italy). This brand represents the 25% of the Italian market of RTD tea [5]. The  
94 product is made from Sri Lanka tea leaves. The tea is produced by a unique technological system  
95 that reproduces the traditional tea infusion since leaves are dipped into boiling water. The final  
96 product is composed by tea infusion (water, tea), sugar, dextrose, lemon juice, ascorbic acid,  
97 flavour. The tea is produced in Italy (Alba, CN).

98 Pure (-)-epicatechin, (-)-epigallocatechin, (-)-epigallocatechin-3-gallate, (-)-epicatechin-3-  
99 gallate and gallic acid were obtained from Sigma (St. Louis, MO, USA). All the solvents and  
100 reagents were purchased from Carlo Erba reagents (Milano, Italy)

### 101 **Human feedings study**

102 The feeding study was carried out on 20 healthy human volunteers (17 men and 3 women)  
103 selected according to exclusion criteria including diabetes mellitus, cardiovascular events, chronic  
104 liver diseases or nephropathies, cancer, organ failure and taking antioxidant or vitamin integrators.  
105 The volunteers were  $31 \pm 15$ yo (mean  $\pm$  SD) and with an average BMI of  $23 \pm 3$  kg/m<sup>2</sup>. Each  
106 volunteer signed an informed consent and the study protocol was approved by the Ethics Committee  
107 for Human Research of the University of Parma.

108 For two days prior to, and 24 h after the ingestion of tea, the subjects followed a diet almost  
109 deprived of flavonoids and phenolic compounds by avoiding fruit and fruit juices, chocolate, nuts,  
110 vegetables, tea and any kind of herbal tea, coffee, wine and dietary antioxidant supplements. To  
111 check for compliance, the volunteers were asked to fill a 3-day weighed food record during the two  
112 days before the study and the study day. On the day of the study, after an overnight fast, each  
113 subject drank 500 mL of the tea beverage. Urine was collected before the volunteers drank the tea  
114 and 0-4, 4-7, 7-10 and 10-24h after ingestion. The volume of urine collected during each period was

115 measured and aliquots stored at  $-80^{\circ}\text{C}$  prior to analysis of  $0.45\mu\text{m}$  filtered samples by HPLC-  
116 MS/MS without further processing.

### 117 **HPLC- ESI-MS/MS analysis**

118 Flavan-ols and their metabolites in tea and urine were analysed using a Waters 2695  
119 Alliance separation module equipped with a Micromass Quattro Micro Api mass spectrometer fitted  
120 with an electrospray interface (ESI) (Waters, Milford, MA, USA). Separations were performed  
121 using a Waters Atlantis dC18  $3\mu\text{m}$  ( $2,1 \times 150\text{ mm}$ ) reverse phase column (Waters). The mobile  
122 phase, pumped at a flow rate of  $0.17\text{ mL/min}$ , was a 15-min gradient of 5-30 % acetonitrile in 1 %  
123 aqueous formic acid. The tuning of the mass spectrometer was optimised by infusing a standard of  
124 (-)-epicatechin into the source along with the 5% acetonitrile in 1 % aqueous formic acid, the initial  
125 HPLC mobile phase, at a flow rate of  $30\mu\text{L/min}$ . The ESI source worked in negative ionisation  
126 mode. Source temperature was  $120^{\circ}\text{C}$ , desolvation temperature was  $350^{\circ}\text{C}$ , capillary voltage was  
127  $2.8\text{ kV}$ , cone voltage was  $35\text{V}$ . The collision energy for MS/MS identifications was set at  $25\text{ eV}$ .  
128 Following HPLC separation and MS/MS identification, flavan-3-ols and their metabolites were  
129 quantified using HPLC with the MS operating in the selected ion recording (SIR) mode.  
130 Unmetabolised flavan-3-ols were quantified using calibration curves of the appropriate standard  
131 compound while metabolites of epicatechin and epigallocatechin in urine were quantified using  
132 epicatechin and epigallocatechin, respectively.

133

## 134 **Results**

### 135 **Analysis of tea**

136 The relevant flavan-3-ol content of 500 mL of RTD tea was  $22.9 \pm 1.7\mu\text{mol}$  (mean  $\pm$  SD) (-)-  
137 epigallocatechin;  $25.7 \pm 3.1\mu\text{mol}$  (-)-epigallocatechin-3-gallate;  $14.1 \pm 1.0\mu\text{mol}$  (-)-epicatechin;  
138  $24.8 \pm 2.7\mu\text{mol}$  (-)-epicatechin-3-gallate; making a total of  $87.4 \pm 8.4\mu\text{mol}$  of total flavan-3-ols.  
139 The content of gallic acid was equal to  $59.4 \pm 4.7\mu\text{mol}$ . A sample chromatogram is reported in  
140 figure 1.



141 **Identification of flavan-3-ols and their metabolites in urine.**

142 Urine samples collected at different time points after the ingestion of 500 mL of tea were  
143 analysed by HPLC-MS/MS. Eight relevant compounds were identified in urine, all modified flavan-  
144 3-ols with the exception of unmetabolised gallic acid. No metabolites of either epicatechin-3-gallate  
145 or epigallocatechin-3-gallate were detected in urine. The MS/MS criterion of identification is  
146 mainly based on previously reported mass analyses [7] and is reported in table 1 together with the  
147 number of detected peaks for each metabolite. Methyl-epigallocatechin was identified based only  
148 on molecular ion ( $m/z = 319$ ) and retention time.

149 **Quantitative analysis of urine**

150 After the initial qualitative analysis, urine samples were analysed by MS in the selected ion  
151 recording (SIR) mode and typical HPLC-SIR traces obtained and used to quantify metabolites are  
152 reported in figure 2. The excretion of tea-related components in urine are reported in figure 3. The  
153 main excreted flavan-3-ol metabolites were epigallocatechin-O-glucuronide and methyl-  
154 epicatechin-sulphate, whereas the sulphate metabolite of epigallocatechin was almost negligible  
155 along all the 24 hours (not shown in figure). It is interesting to note that, while most of the  
156 catabolites reach their peak excretion within the first 4 hours, the methylated compounds show their  
157 maximum excretion after 10h (figure 3).

158 Bioavailability was calculated as a ratio between the total excretion and the total intake of  
159 flavan-3-ols and gallic acid. However, to try to distinguish between absorption rate of epicatechin  
160 and epigallocatechin (being the metabolites derived from these two molecules), the assumption that  
161 EGC-catabolites derived from both EGC and EGCG (after degalloylation) and EC-catabolites  
162 derived from both EC and ECG (after degalloylation), was made. It is in fact impossible to discern  
163 between EC as a product of ECG and EGC as a product of EGCG degalloylation, reaction that has  
164 been reported to occur also in the mouth [8]. Following this assumption, the bioavailability (table 3)  
165 of EC-components (namely EC and ECG) was equal to approximately 7.1%, similarly to the  
166 bioavailability of EGC-related compounds (namely EGC and EGCG), which excretion accounted

167 for almost the 7.3% of the total intake. This leads to a total flavanols excretion equal to the 7.2% of  
168 the intake with tea. Excretion of gallic acid, which was abundant in the RTD tea used in this study,  
169 reached a 4.5% of the amount ingested.

170

## 171 **Discussion**

172 The consumption of ready-to-drink tea, generally called iced tea, is increasing in the western  
173 countries and is often overcoming the intake of traditional hot tea [5]. Considering the evidence that  
174 tea is one of the most significant sources of polyphenols in the human diet and that tea consumption  
175 [2] and polyphenol intake in general [9] are strongly related to reduced risk for several chronic  
176 diseases, the aim of our investigation was to assess the actual exposure of human volunteers to the  
177 most representative and bioavailable tea polyphenols (namely, flavan-3-ols) after acute consumption  
178 of a ready-to-drink tea prepared by infusion of tea leaves. The tea used in this study was marketed  
179 as black, but contained a relevant amount of flavan-3-ols which are known to be highly bioavailable  
180 [10] and linked to most of the health benefits demonstrated for this beverage and its non-fermented  
181 counterpart, green tea.

182 Before conclusions can be drawn on the potential *in-vivo* effects of tea polyphenols on  
183 human health, a more complete understanding of the mechanisms of absorption, bioavailability and  
184 biotransformations is necessary. Recent studies indicate that following tea consumption catechins  
185 are metabolised and circulate as sulphated, methylated or glucuronidated derivatives [11-13].  
186 Absorption is believed to occur through the small intestine as bacterial degradation within the colon  
187 is hypothesised to break down the flavonoids into smaller phenolic acids [14].

188 The ready-to-drink tea used in this study is a source of flavan-3-ol monomers, contained at a  
189 level of  $87 \pm 8 \mu\text{mol}/500 \text{ mL}$ . Based on this data, the present work represents the feeding and  
190 bioavailability study with the lowest dosage of this class of components present in the literature.  
191 However, urine excreted over a 24 h period after ingestion of the tea contained several flavan-3-ol  
192 metabolites of EC and EGC. In fact, neither EGCG or ECG were detectable in urine (as metabolites

193 or aglycones), in agreement with previous studies [15]. This is probably due to biliary excretion  
194 which these galloylated catechins undergo, investigated and clarified in rats after intravenous  
195 administration of (-)-[4-<sup>3</sup>H] EGCG [16].

196 In total,  $6.3 \pm 3.1$   $\mu$ mol of metabolites were excreted, corresponding to 7.2 % of the  
197 ingested dose of tea flavan-3-ols. Moreover, considering that epicatechin metabolites derive not just  
198 from epicatechin but also from its galloylated form, and making the same argument for  
199 epigallocatechin, this demonstrates that the bioavailability of these two subclasses does not differ  
200 (table 2). Previous works have reported higher bioavailability for epicatechin [17], but excluding  
201 the galloylated forms as potential source of EC and EGC is at least questionable, in particular given  
202 the evidence that human saliva contains an esterase that can convert epigallocatechin-3-gallate to  
203 epigallocatechin [8].

204 By observing the excretion profiles peculiar behaviours for different catabolites emerge.  
205 Most of the metabolites show their peak excretion during the first 4 hours of urine collection, while  
206 two of them (namely methyl-epigallocatechin and its sulphated version) reach their peak excretion  
207 later in time, during the 7 to 10 hour period.

208 A limitation of the study is the absence of colonic metabolites of polyphenols from the  
209 analysis, as there is a growing appreciation that a significant portion of ingested dietary flavonoids  
210 and related compounds are not absorbed in the small intestine but pass to the large intestine where  
211 they are degraded by the colonic microflora to simple phenolic acids which can be absorbed into the  
212 circulatory system. Flavan-3-ols can interact with the human colon microbiota, giving rise to 5-  
213 (3',4',5'-trihydroxyphenyl)- $\gamma$ -valerolactone, 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactone and 5-  
214 (3',5'-dihydroxyphenyl)- $\gamma$ -valerolactone [18] all of which also appear as sulphated and  
215 glucuronidated derivatives. These ring fission metabolites are found at high concentrations in urine  
216 and plasma of human volunteers after drinking green tea. However, due to the low amount of  
217 catechin ingested and to the absence of commercially available pure standards, they were not  
218 investigated in this study. A second study with RTD green tea is presently ongoing.

219 **Conclusion**

220 After drinking the ready-to-drink ice tea used in this study, the internal compartments are  
221 exposed to non-marginal doses of flavanols and flavanol metabolites up to 24 hours. Whether the  
222 excretion in urine continues beyond such period can only be hypothesised considering literature  
223 data on ring fission metabolites generated in the colon. The bioavailability values observed in this  
224 study are in agreement with previous reports [17] even though the dosage of polyphenols ingested  
225 in this study is remarkably lower. Moreover, the use of a group of twenty volunteers, more than the  
226 average number of subjects used for usual human acute feeding studies involving polyphenols,  
227 gives additional credibility to our results.

228  
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230

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280

281 Table 1: MS/MS identification of phenolic compounds and metabolites in human urine collected 0-  
 282 24 h after the ingestion of 500 mL of ready-to-drink tea.

<i>Compound</i>	<i>Molecular ion (ESI-)</i>	<i>MS/MS identification fragments</i>	<i>Number of isomers detected in urine</i>
<b>Gallic acid</b>	169	125	1
<b>Methyl-epigallocatechin</b>	319		2
<b>Epicatechin-O-sulphate</b>	369	289 (EC)	3
<b>Methyl-epicatechin-O-sulphate</b>	383	303 (Methyl-EC)	4
<b>Epigallocatechin-O-sulphate</b>	385	305 (EGC)	2
<b>Methyl-epigallocatechin-O-sulphate</b>	399	319 (Methyl-EGC)	2
<b>Epicatechin-O-glucuronide</b>	465	289 (EC)	1
<b>Epigallocatechin-O-glucuronide</b>	481	305 (EGC)	1
<b>Methyl-epigallocatechin-O-glucuronide</b>	495	319 (Methyl-EGC)	2

283 ESI – Electron Spray Ionisation; EGC – Epigallocatechin; EC - Epicatechin

284

285 Table 2: Quantification of flavan-3-ols and gallic acid in tea, total excretion of metabolites and  
286 gallic acid in urine and bioavailability of these compounds after the ingestion of 500 mL of ready-  
287 to-drink tea by twenty human volunteers. Data expressed as mean values in  $\mu\text{mol} \pm$  standard error  
288 (n = 20) with the exception of bioavailability values, expressed as percentage of ingested  
289 component.

	<i>Tea (500 ml)</i>	<i>Urine</i>	<i>Bioavailability (%)</i>
<b>Gallic acid</b>	59.43 $\pm$ 4.67	2.62 $\pm$ 1.01	4.49 $\pm$ 1.69
<b>EC + ECG</b>	38.90 $\pm$ 3.66	2.77 $\pm$ 1.23	7.13 $\pm$ 3.16
<b>EGC + EGCG</b>	48.53 $\pm$ 4.69	3.54 $\pm$ 2.04	7.30 $\pm$ 4.21
<b>Total flavan-3-ols</b>	87.43 $\pm$ 8.34	6.32 $\pm$ 2.68	7.23 $\pm$ 3.06

291

292



293 Figure captions.

294

295 Fig 1: HPLC-SIR chromatograms of flavan-3-ols in tea. Chromatograms represent gradient reversed  
296 phase HPLC analysis with detection of flavan-3-ols using the selected ion recording mode.

297

298 Fig 2: HPLC-SIR chromatograms of principal flavan-3-ol metabolites in urine. Chromatograms  
299 represent gradient reversed phase HPLC analysis with detection of metabolites using the selected  
300 ion recording mode.

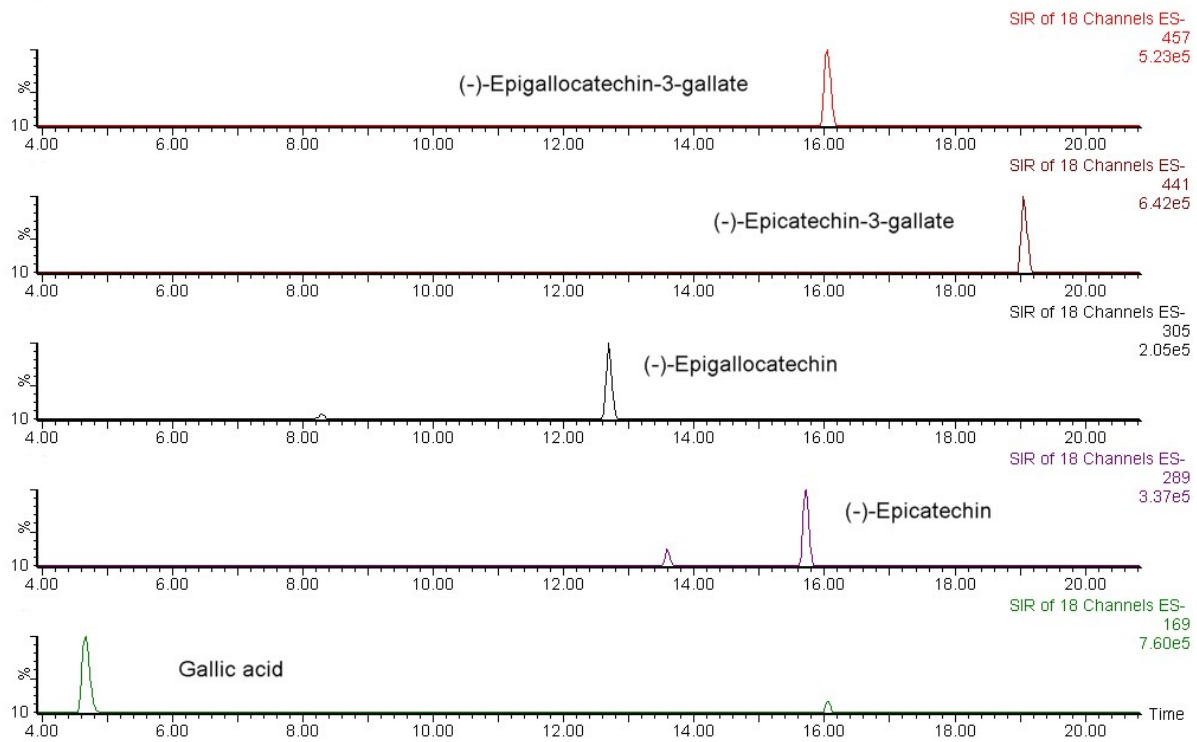
301

302 Fig 3: Excretion profiles of flavan-3-ol metabolites in urine during the 0-4, 4-7, 7-10 and 10-24h  
303 periods in 20 volunteers. Values are expressed as mean  $\pm$  standard error.

304

305

306 Figure 1



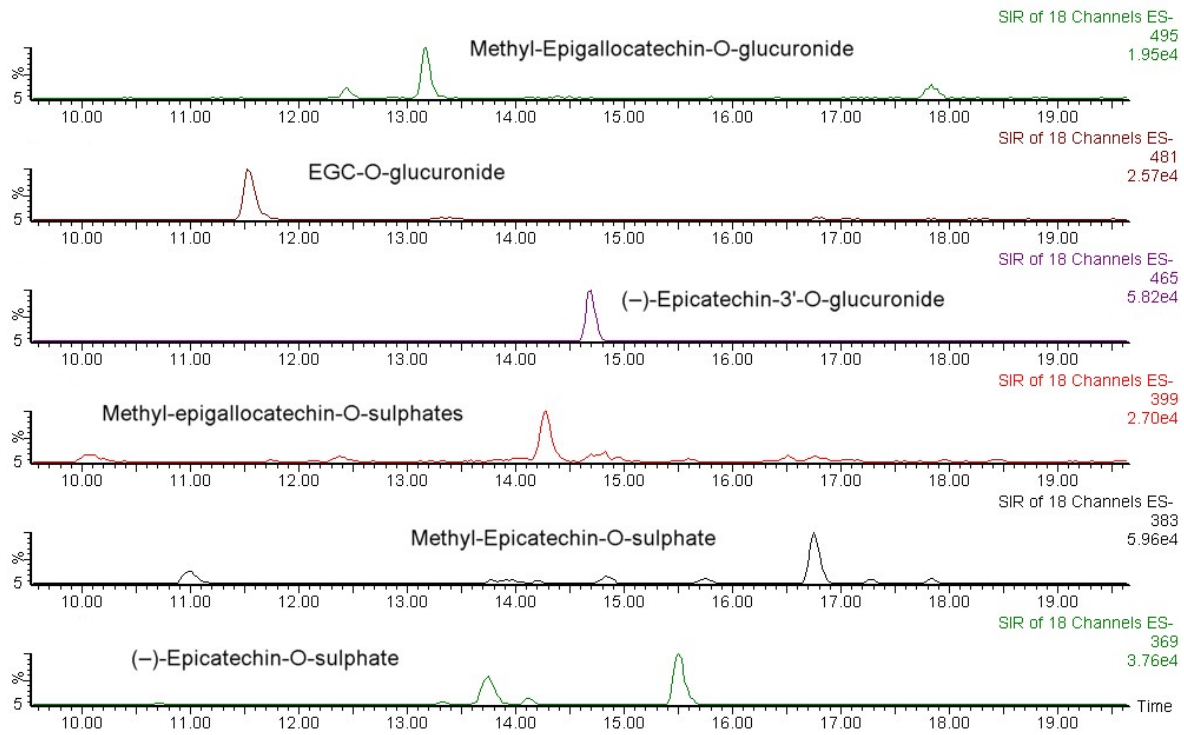
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311 Figure 2



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