

1 **Final version published in: *J. Food Sci.* 2018, 83, 2257-2264. DOI:**  
2 **10.1111/1750-3841.14273**

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4 **Identification and Quantification of Thujone In a Case of Poisoning Due to Repeated**  
5 **Ingestion of an Infusion of *Artemisia vulgaris* L.**

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30 Short title: (Thujone in *Artemisia vulgaris* poisoning)

31

## 32 **Abstract**

33 Plants of the *Artemisia* genus are used worldwide as ingredients of botanical preparations.  
34 This paper describes the case of a 49-year-old man admitted to the emergency room at a  
35 Zurich hospital in a manic state after the ingestion of 1 L of an infusion of *Artemisia vulgaris*.  
36 Two monoterpenic ketones,  $\alpha$ - and  $\beta$ -thujone, are present in various concentrations in  
37 *Artemisia spp*, but adverse effects have previously been associated only with essential oil  
38 from *Artemisia absinthium* and attributed to the inhibition of gamma-aminobutyric acid  
39 receptors, with consequent excitation and convulsions.

40 The aim of this work was to examine and quantify the possible presence of thujone in the  
41 patient's serum and urine. A High Performance Liquid Chromatography (HPLC) method with  
42 isocratic separation and fluorescence detection (FLD) was set up and validated. Serum  
43 thujone concentrations were found to be  $27.7 \pm 3.48$   $\mu\text{g/mL}$  at day 0 and  $24.1 \pm 0.15$   $\mu\text{g/mL}$  on  
44 day 1. Results were confirmed by a gas chromatography with flame ionization detection (FID).  
45 Poisoning due to thujone was thus confirmed, suggesting four possible scenarios: 1) an  
46 unusually high concentration of thujone in the *Artemisia vulgaris* ingested; 2) chronic  
47 exposure as the cause of the poisoning; 3) low metabolic efficiency of the patient; 4)  
48 contamination or adulteration of the plant material with other *Artemisia* species, e.g. *Artemisia*  
49 *absinthium*.

## 50 **Practical application**

51 These results could aid research in the field of adverse effects of botanicals, lead to better  
52 understanding and management of similar cases of poisoning, and promote more informed  
53 use of natural products.

54

55 Word count: 3705

56 **Keywords:** thujone poisoning, *Artemisia spp.*, adulteration, biomarkers, HPLC-FLD, gas-  
57 liquid chromatography, flame ionization detector

58

## 59 **Introduction**

60 *Artemisia vulgaris* L. (mugwort) is a weed in the family of *Asteraceae*, widely distributed in  
61 Europe, Asia and North America. Its traditional use is mainly based on infusions with  
62 supposed antihypertensive, antispasmodic, anti-inflammatory and anthelmintic properties  
63 (Miller, 2000). Other applications have been suggested in the field of gynecology  
64 (dysmenorrhea and problems during labor) (Chevallier, 1996; Lee et al. 1998). *Artemisia*  
65 *vulgaris* was used as a flavor in beers before hops, and infusions of the leaves and flowering  
66 tops have been prescribed for digestive problems (Barney and Di Tommaso, 2003; Miller,  
67 2000). The genus *Artemisia* is highly variable in morphology and phytochemical composition  
68 – approximately 60 different compounds have been identified in it (Abad, Bedoja, Apaza, and  
69 Bermejo, 2012). Thujone is a monoterpene ketone naturally present in two stereoisomeric  
70 forms: (-)-3-isothujone (CAS 546-80-5) or  $\alpha$ -thujone and (+)-3-isothujone (CAS 471-15-8) or  $\beta$ -  
71 thujone (O'Neil, 2013). Figure 1 shows their chemical structures.

72 Thujone occurs in different quantities in *Artemisia* species. Its toxicological potential was  
73 emphasized by EMA (European Medicines Agency) in its monograph on both *Artemisia*  
74 *absinthium* L. herba (EMA, 2009a) and *Salvia officinalis* L. folium: “Thujone is reported to be  
75 neurotoxic and chemotypes with low content of thujone should be preferred. The intake of  
76 thujone should not exceed 3.0 mg/day” (EMA, 2009b). The content of  $\alpha$ - and  $\beta$ -thujone in  
77 *Artemisia vulgaris* L. is normally below the levels found in *Artemisia absinthium* L., but the  
78 concentration is variable (Pelkonen, Abass, and Wiesner, 2013). The essential oil from the  
79 herbal stem and flowers of *Artemisia vulgaris* L. contain approximately 56.3%  $\alpha$ -thujone and  
80 7.5%  $\beta$ -thujone (EFSA, 2012) but, because of their low solubility in water, it is difficult to  
81 predict the quantity extracted by traditional infusion.

82  $\alpha$ -thujone and  $\beta$ -thujone are both responsible for neurotoxic effects,  $\alpha$ -thujone being 3-4  
83 times as potent as  $\beta$ -thujone (Höld, Sirisoma, and Casida, 2001). The neurotoxicity is due to  
84 the rapid action of thujone in modulating the GABA-gated chloride channels and accounts for  
85 the epileptiform convulsions that are usually present in cases of acute poisoning (EMA, 2012).  
86 Convulsions are normally preceded by other less specific symptoms, such as vasodilation  
87 leading to hypotension, tachycardia and respiratory problems (IPCS, 1981).

88 Some case reports of severe intoxication due to the consumption of herbal preparations  
89 containing thujone have been published. Blindness, hallucinations and epileptiform  
90 convulsions sometimes progressing to unconsciousness are the most usual clinical patterns  
91 described (Burkhard, Brukhardt, Haenggeli, and Landis, 1999; Holstege, Baylor, and  
92 Rusyniak, 2002; Lachenmeier, Walch, Padosch, and Kröner, 2006; Strang, Arnold, and  
93 Peters, 1999). Acute poisoning due to thujone is most frequently associated with the  
94 consumption of *Artemisia absinthium* L. and alcoholic beverages containing its essential oil.  
95 Like other famous artists, Vincent Van Gogh suffered from absinthism, which differs from  
96 alcoholism in presenting episodes of delirium and epilepsy (Arnold, 1988; Holstege et al.  
97 2002).

98 The acute oral toxicity (LD<sub>50</sub>) of thujone in laboratory animals (mouse, rat, guinea pig) has  
99 been reported at doses between 192 and 500 mg/kg body weight (EMA, 2012; SCF, 2002). In  
100 a study performed in rats, where thujone was administered by gavage on five days a week for  
101 13 weeks, a NOEL (no-observed effect level) of 12.5 mg/kg bw for convulsion was  
102 established in males (Surber, 1962). In a similar study, where thujone was administered by  
103 gavage 6 times/week for 14 weeks, the NOEL for convulsive effect was 10 mg/kg bw in males  
104 and 5 mg/kg bw in females (Margaria, 1963). In 2-year studies performed by the National  
105 Toxicology Program (NTP, 2011) a NOEL for mortality of 12.5 mg/kg bw was identified in rats  
106 (although clonic seizures were observed at this level - the lowest administered), while in mice  
107 a NOEL for both mortality and seizure was established at 12 mg/kg bw.

108 Few data on the pharmacokinetic and toxicokinetic of thujone in humans are available. Max  
109 (1990) reported that a dose of 2-4 mg of thujone (0.03-0.06 mg/kg body weight), consumed  
110 with an alcoholic drink, did not induce the acute effects described in the scientific literature.

111 Hinkelbein (2004) confirmed that a dose of 3.5 mg of thujone, corresponding to 0.05 mg/kg  
112 bw, should be safe.

113 In 2012, EMA published a statement on the use of herbs containing thujone with the  
114 evaluation of acute and chronic toxicity of thujone in humans (EMA, 2012). The conclusions  
115 were that animal studies can be considered significant in calculating the human sensitivity to  
116 thujone, even though a direct extrapolation of the dose responsible for acute poisoning is  
117 uncertain. According to the available data, daily doses of 1.5-3.85 mg would not produce  
118 neurological disorders, while doses of 15 mg could affect attention and mood. In agreement,  
119 Dettling, Grass, Schuff, Skopp, Strohbeck-Kuehner, and Haffner (2004) showed that the  
120 intake of 17-20 mg in a person of 70 kg bw (0.24-0.28 mg/kg bw day) could be responsible for  
121 mild effects, such as problems in driving or operating machinery. On the basis of these results,  
122 EMA indicated 3 mg as the maximum safe daily dose for humans (EMA, 2009a).

123

124 The study reported in this paper describes a case of poisoning, which occurred in a 49-year-  
125 old man presenting a manic state after the ingestion of 1 L of *Artemisia vulgaris* infusion. The  
126 specific clinical symptoms suggested poisoning by thujone and this was confirmed by its  
127 presence in the patient's serum and urine. Two analytical approaches were used: 1) a newly  
128 developed and validated HPLC-FLD method, and 2) a published method based on gas  
129 chromatography coupled with a flame ionization detector (FID).

130

131

### 132 **Case report**

133 A 49-year-old man was admitted to the University Hospital of Zurich in a manic state.  
134 According to the patient, he was habitually drank 1 L/day of an infusion prepared from  
135 *Artemisia vulgaris*. There were no details on how the infusion was prepared, but he had  
136 continued the practice for about three years.

137 Blood samples were taken on the day of admission (day 0) and on the following day  
138 (day 1), while urine was sampled only on day 1. Approval from an ethics committee or  
139 institutional review board was not necessary for the analyses performed, since they had been

140 requested to establish the source of the poisoning. The patient gave Informed consent for  
141 publication of this case.

142

## 143 **Materials and methods**

### 144 ***Purified standards***

145 The purified standard of  $\alpha,\beta$ -thujone (80% purity; 70%  $\alpha$ -thujone and 10%  $\beta$ -thujone) was  
146 from Sigma Aldrich (Steinheim, Germany). This mixture was the product with the highest  
147 quality available in the short time necessitated by the patient's condition and its standard of  
148 purity was considered sufficient for our purposes.

### 149 ***Reagents***

150 The reagents (LC grade) were: acetone (Farmitalia Carlo Erba, Milano, Italy); L-ascorbic acid  
151 (Sigma-Aldrich, Steinheim, Germany); acetonitrile, methanol and water (VWR International,  
152 Fontenay-sous-Bois, France).

153

## 154 **HPLC-FLD METHOD**

### 155 ***Preparation of standard solutions and calibration curve***

156 The reference standard solution contained 0.724 g/mL of  $\alpha$  and  $\beta$ -thujone (taking into  
157 consideration purity of 80.0% and density 0.925 g/mL). An aliquot of 10  $\mu$ L of the standard  
158 solution was diluted in 10 mL of methanol to a final concentration of 0.724 mg/mL. Standard  
159 solutions were added to a healthy volunteer's serum ("control" sample) to concentrations of  
160 14.5, 29.0, 57.9 and 72.4  $\mu$ g/mL of thujone.

### 161 ***Preparation of biological samples***

162 Aliquots of 700  $\mu$ L of the patient's and control serum (the latter without and with the addition  
163 of purified thujone) were added to 20  $\mu$ L of an aqueous solution of L-ascorbic acid (1% w/v),  
164 used as a preservative; 700  $\mu$ L of acetone was added to each and the resulting solutions  
165 were thoroughly vortexed and centrifuged at 2500 r.c.f. for 5 minutes (Hermle Labortechnik,  
166 Wehingen, Germany). The supernatants were filtered through a 0.45  $\mu$ m syringe filter (VWR  
167 International, Fontenay-sous-Bois, France) and injected into the HPLC.

168 The urine sample was filtered as such on a 0.45  $\mu\text{m}$  filter and injected into the  
169 chromatographic equipment.

#### 170 ***Chromatographic conditions***

171 For the identification and quantification of thujone in the patient's serum, a specific HPLC  
172 method combined with fluorimetric detection was developed. This method proved incapable  
173 of separating the two isomers, but this was achieved by gas chromatography, see below. The  
174 equipment consisted of an Intelligent PU-880 pump (Jasco, Tokyo, Japan), a fluorescence  
175 detector FP-1520 (Jasco, Tokyo, Japan), a sample injection valve Rheodyne 7725 with 20  $\mu\text{L}$   
176 loop (Cotati, California, USA) and a column LiChrospher<sup>®</sup> 100, RP-8, 250 x 4 mm, particle  
177 size 5  $\mu\text{m}$  (Merck KGaA, Darmstadt, Germany) heated at 30  $^{\circ}\text{C}$ . ChromNAV software (Jasco,  
178 Tokyo, Japan) was used for data integration. The analysis was performed by isocratic elution  
179 at a flow rate of 1 mL/min with a mobile phase containing acetonitrile:water 55:45 (v/v). The  
180 fluorescence detector was set at 220/290 nm ( $\lambda_{\text{ex}}/\lambda_{\text{em}}$ ).

#### 181 ***Method validation***

182 The HPLC method was validated according to internationally recognized guidelines for  
183 analytical methods (FDA, 2013; Peters, Drummer, and Musshoff, 2007; Shabir, 2003). The  
184 following parameters were calculated: system suitability test (SST), linearity, sensitivity as  
185 LOD (Limit of Detection) and LOQ (Limit of Quantitation), selectivity, accuracy and precision.

#### 186 **GC-FID**

##### 187 ***Chromatographic conditions***

188 Gas-chromatographic analyses were performed according to Dybowski and Dawidowicz  
189 (2016) with some modifications. The equipment included a DANI 8610 gas-chromatograph  
190 (DANI Instruments, Cologno Monzese, Italy) with a flame ionization detector (DANI 86/10,  
191 DANI Instruments, Cologno Monzese, Italy). For chromatographic separation of  $\alpha$ - and  $\beta$ -  
192 thujone and an internal standard, naphthalene, a Supelco SLB<sup>®</sup>-5ms fused silica capillary  
193 column (length 30 m, i.d. 0.25 mm,  $d_f$  0.25  $\mu\text{m}$ ) was used (Sigma-Aldrich, Steinheim,  
194 Germany). Helium was used as a carrier. The injector temperature was set at 290  $^{\circ}\text{C}$ . The  
195 oven temperature program employed was: 1 min at 50  $^{\circ}\text{C}$  followed by an increase of 6  $^{\circ}\text{C}/\text{min}$   
196 up to 110  $^{\circ}\text{C}$  and then at a rate of 20  $^{\circ}\text{C}/\text{min}$  up to 280  $^{\circ}\text{C}$ .

##### 197 ***Preparation of standard solutions and calibration curve***

198 A calibration curve was prepared by injecting solutions of purified  $\alpha$ - and  $\beta$ -thujone in the  
199 presence of naphthalene as an internal standard (ISTD) (Sigma-Aldrich, Steinheim,  
200 Germany). Standard solutions were prepared by diluting the commercial standard in  
201 dichloromethane. The final concentrations were: for  $\alpha$ -thujone 109, 52, 42, 18, 1  $\mu\text{g/mL}$ ; and  
202 for  $\beta$ -thujone 20, 10, 8.3, 0.2  $\mu\text{g/mL}$ . Internal standard concentration was 25  $\mu\text{g/mL}$  in all the  
203 samples. Two microliters of the sample were injected into the GC at least three times.

#### 204 **Sample preparation**

205 Sample preparation was modified from the original method, since the biological fluids most  
206 usually received by emergency rooms are serum (not plasma) and urine. Dichloromethane  
207 (200  $\mu\text{L}$ ), containing 25  $\mu\text{g/mL}$  of ISTD, was added to 200  $\mu\text{L}$  serum and the two phases were  
208 subjected to vortex mixing for one minute. The resultant emulsion was centrifuged at 2500  
209 r.c.f. for 5 minutes. The upper aqueous phase was then separated and extracted again with  
210 200  $\mu\text{L}$  dichloromethane containing ISTD. The organic layers were combined and analyzed.

#### 211 **RESULTS**

212 The patient arrived at the emergency room in a manic state. He reported a habit of drinking  
213 (daily and in large quantity) an infusion of *Artemisia vulgaris*. The clinical symptoms  
214 presented by the patient were compatible with an exposure to thujone, a neurotoxic molecule  
215 contained in variable quantity in *Artemisia* spp, but normally hardly present in *Artemisia*  
216 *vulgaris* (Abad et al. 2012).

217 To test the hypothesis that thujone was responsible for the observed poisoning, thujone was  
218 quantified in the patient's serum and urine by HPLC-FLD and by gas chromatography coupled  
219 with FID.

#### 220 **HPLC-FD METHOD**

##### 221 **System suitability test (SST)**

222 In this test the following parameters were calculated: retention factor (K), separation factor  
223 between two neighboring peaks ( $\alpha$ ), peak tailing factor and column efficiency (number of  
224 theoretical plates). The analysis was performed five times. **Table 1** shows the results of the  
225 SST obtained from a control serum spiked with standard thujone at a concentration of 54.3

226  $\mu\text{g/mL}$ . The chromatographic system proved to be efficient and suitable for the quantification  
227 of thujone, with retention factor ( $K$ )  $\geq 2$ , separation factor ( $\alpha$ )  $> 1$ , and symmetry factor ( $SF$ )  $\leq$   
228 2. However, the method does not separate the two isomers of thujone, unlike gas  
229 chromatography, see below.

### 230 **Linearity**

231 Standard solutions (0.724 mg/mL) were added to a “blank” serum to the final concentration of  
232 14.5, 29.0, 58.0 and 72.4  $\mu\text{g/mL}$  and were prepared as previously described; each solution  
233 was analyzed at least three times.

234 A linear regression was obtained by plotting the areas of analyte peaks vs. the nominal  
235 concentrations. The method was linear between 14.5 and 72.4  $\mu\text{g/mL}$ , with a correlation  
236 coefficient ( $R^2$ ) of  $>0.99$ . The linear regression equation was  $y = 253\ 137x - 189\ 570$ , where  $y$   
237 refers to the peak area and  $x$  refers to concentration.

### 238 **Sensitivity (LOD and LOQ)**

239 The limit of detection (LOD) and the limit of quantitation (LOQ) were determined by spiking  
240 control serum with decreasing concentrations of thujone standard solutions: LOD was  
241 established at a signal-to-noise ratio of 3, while LOQ at a signal-to-noise ratio of 10.

242 Furthermore, the precision at LOQ was evaluated with five independent injections.

243 The LOD and LOQ calculated in spiked serum calibration samples were 1.36  $\mu\text{g/mL}$  and 4.53  
244  $\mu\text{g/mL}$ , respectively. The precision at LOQ was 19.4%, below the acceptable value of 20%.

245 All values reported above were suitable for an accurate determination of thujone in serum  
246 samples.

### 247 **Selectivity**

248 The possible interaction between analytes and endogenous matrix (serum) compounds was  
249 investigated by adding standard thujone to a control serum at the final concentration of 54.3  
250  $\mu\text{g/mL}$ . Selectivity requires that the peak area of compounds eluting together with the analyte  
251 of interest is less than 20% of the peak area of sample at LOQ. As shown in **Figure 2**,  
252 thujone is clearly identified, since no significant peak was present at its retention time. The  
253 two isomers are not separated by HPLC; they are shown to have slightly different retention  
254 times in gas chromatography, as shown below.

255 ***Accuracy and precision***

256 Accuracy and precision were determined by spiking control serum with two quantities of  
257 purified thujone, at final concentrations of 18.1 and 54.3 µg/mL. Accuracy describes the  
258 closeness of a measurement to the true value and is calculated as the percentage ratio  
259 between the experimentally measured values and the nominal ones. Precision was  
260 determined by calculating the variation coefficient (RSD%) of the peak areas of five replicates  
261 injected in the same day. The method was precise and accurate, since RSD% was below  
262 15% (5.1-5.7%) and the calculated accuracy was always within ±15% (106.8 to 108.5) of the  
263 nominal concentration.

264 ***Recovery***

265 To evaluate the recovery in biological fluids, control serum was spiked with a standard  
266 solution at the final concentration of 25 µg/mL. Recovery was 94.2±10.3%.

267

268 ***Quantification of thujone in patient's serum***

269 The chromatograms of the patient's serum at day 0 and day 1 are illustrated in **Figure 3**.  
270 Using the regression line prepared in serum, thujone concentrations were determined and  
271 corresponded to 27.7±3.5 and 24.1±0.15 µg/mL (mean ± SD), at day 0 and 1, respectively.  
272 The identification of thujone was confirmed by spiking the serum with a solution of purified  
273 standard (**Figure 4**).

274 ***Quantification of thujone in patient's urine***

275 The method was similarly validated for the analysis of urine, by using a linear regression  
276 obtained by spiking control urine with known quantities of purified standard. **Figure 5** shows  
277 the chromatograms of a control urine without and with addition of a standard solution of  
278 thujone at a final concentration of 72.4 µg/mL.

279 No interfering peaks were present at the retention time of thujone in the control sample. No  
280 peak at the retention time of thujone was detectable (<LOD) in the patient's urine (not shown).

281 **GC-FID METHOD**

282 To confirm unambiguously the presence of thujone in the patient's biological fluids, the  
283 method of Dybowski and Dawidowicz (2016) was also applied. An example of the gas-  
284 chromatographic separation of the two stereoisomers of thujone and the internal standard  
285 naphthalene is illustrated in **Figure 6**.

286 A calibration curve was obtained plotting the ratio between the area of the analyte and that of  
287 the internal standard (ISTD, naphthalene) vs. the respective concentrations. Within the  
288 concentrations used, the linearity was highly satisfactory, having  $R^2 > 0.999$  for both thujone  
289 isomers.

290 The limit of detection (LOD) was calculated at a signal-to-noise ratio of 3 and LOQ at a signal-  
291 to-noise ratio of 10. The latter was obtained by comparing the area of thujone signals and that  
292 of six peaks (average) of baseline noise from four injections of extracts (dichloromethane) of  
293 control serum samples spiked with a standard solution of thujone. LOD and LOQ were 0.11  
294  $\mu\text{g/mL}$  and 0.37  $\mu\text{g/mL}$ , respectively, for  $\alpha$ -thujone, and 0.10  $\mu\text{g/mL}$  and 0.34  $\mu\text{g/mL}$ ,  
295 respectively, for  $\beta$ -thujone.

296 To evaluate the recovery in biological fluids, the control serum was spiked with standard  
297 thujone to a concentration of 25  $\mu\text{g/mL}$  ( $\alpha = 23.0 \mu\text{g/mL}$ ,  $\beta = 4.2 \mu\text{g/mL}$ ) and injected. The  
298 results showed an average value of  $20.5 \pm 1.1 \mu\text{g/mL}$  ( $\alpha = 15.5 \pm 0.9 \mu\text{g/mL}$ ,  $\beta = 2.8 \pm 0.16$   
299  $\mu\text{g/mL}$ ). The recovery was acceptable, being close to 82% for both total thujone and  
300 separated stereoisomers.

301 **Figure 7** shows the gas-chromatogram of the patient's serum at day 0, when the total thujone  
302 concentration was:  $22.3 \pm 1.3 \mu\text{g/mL}$  ( $\alpha = 18.9 \pm 1.0 \mu\text{g/mL}$ ,  $\beta = 3.4 \pm 0.2 \mu\text{g/mL}$ ). In agreement  
303 with the HPLC method, no thujone could be detected in urine ( $< \text{LOD}$ ) (not shown).

304

## 305 **DISCUSSION**

306 The case reported in this paper describes a patient who experienced a manic state after the  
307 consumption of an infusion of *Artemisia vulgaris*. The patient reported having consumed  
308 similar infusions for at least three years with no adverse effect. No neurotoxic effect related to  
309 *Artemisia vulgaris* derivatives could be found in the scientific literature, not surprisingly in view  
310 of the small amount of thujone in this species (Abad et al. 2012). Neurotoxic effects have  
311 been reported only for the essential oils of *Artemisia absinthium*, which contain significantly

312 higher amounts of thujone (Lachenmeier et al. 2006). On the other hand, the symptoms  
313 described by the first-aid physicians attending this patient were identical with those  
314 associated with thujone intoxication by Lachenmeier et al. 2006 and Pelkonen et al. 2013).  
315 Although the presence of thujone in essential oils and in alcoholic extracts is well documented,  
316 aqueous extracts have been considered safe because of the low water solubility of this  
317 neurotoxic compound (Capasso, Grandolini, and Izzo, 2006).

318 To confirm the presence of thujone in this patient's serum and urine, two analytical  
319 approaches were used: 1) an HPLC-FLD method, set up and validated for this study; and 2)  
320 the gas-chromatographic method using a flame ionization detector (FID) published by  
321 Dybowski and Dawidowicz (2016).

322 For gas chromatography, the sample preparation was slightly modified from the original  
323 method since the biological fluids most usually obtained in the emergency room are serum  
324 and urine, and values measured in these fluids could be underestimated, because the fraction  
325 bound to plasma proteins would not be taken into account

326 Although slightly different in performances (HPLC gives superior recovery, but shows lower  
327 sensitivity), both methods were considered useful in the identification and quantification of  
328 thujone in the case reported. With both methods, the concentration of total thujone in serum  
329 at day 0 was close to 25 µg/mL confirming the intake of this molecule with the infusion. The  
330 differences in recovery could account for the small difference in serum concentrations  
331 obtained by the two methods (27.7 and 22.3 µg/mL, by HPLC and GC, respectively). With  
332 both methods, thujone was below LOD when measured in urine. Since the raw material  
333 (herbal mixture) and the infusion were not available for further analytical assessments, it is  
334 only possible to hypothesize the following:

- 335 1) An unusually high concentration of thujone in the *Artemisia vulgaris*. Even though  
336 there is no direct correlation between solubility in water and oil, a recent paper  
337 showed that the content of thujone in essential oil can vary significantly (Obistioiu et  
338 al. 2014).
- 339 2) The patient has a low metabolic efficiency, which increases the half-life of 24 hours  
340 determined by Lis-Balchin et al. (2006). The very similar levels of thujone in the  
341 patient's serum at day 0 and day 1 supports this hypothesis.

- 342 3) Thujone poisoning due to the chronic exposure (three years). Chronic toxicity of  
343 thujone has been described in rats and mice, which experienced clonic and tonic  
344 seizures and increased incidence of non-neoplastic lesions in brain, spleen, kidney  
345 and pituitary gland after two years of  $\alpha,\beta$ -thujone intake (NTP, 2011);  
346 4) Possible adulteration/contamination of the *Artemisia vulgaris* with other species, e.g.  
347 *Artemisia absinthium*.

348 To our knowledge, this is the first study where thujone was measured in human serum after a  
349 case of poisoning. Other quantifications in human serum have been reported in studies  
350 performed on volunteers (Kröner, Padosch, Lachenmeier, and Madea, 2005; Dybowski and  
351 Dawidowicz 2016).  
352 Kröner et al. (2005) described a pilot study in which two subjects consumed 110 mL of  
353 absinthe containing 3.85 mg thujone within 15 min; thujone was undetectable in their blood  
354 (LOD: 0.34 ng/mL). Dybowski and Dawidowicz (2016) detected thujone in plasma from five  
355 volunteers one hour after the consumption of an alcoholic solution containing approximately  
356 300  $\mu\text{g}$  of  $\alpha,\beta$ -thujone. Plasma values ranged from 22.3 to 37.6 ng/g. Comparing the values  
357 found in our patient's serum ( $\mu\text{g/mL}$ ) with those measured in volunteers' serum (ng/mL), it is  
358 evident that our patient had been exposed to very high doses of thujone, during his long-term  
359 intake (daily for three years) with chronic accumulation of thujone, which is lipophilic.

360

## 361 **CONCLUSION**

362 This paper describes the first case of poisoning due to *Artemisia vulgaris*, in which thujone  
363 was identified as a biomarker of toxicity caused by prolonged excessive exposure. The case  
364 points out some general critical issues related to the consumption of botanicals. The  
365 increased use of botanicals/herbs in recent years is not always associated with suitable  
366 quality and safety control. There is a general belief by consumers that “natural” is always safe,  
367 but adverse effects of botanicals are far from rare.

368 The case also allowed the comparison of two different analytical approaches to measure  
369 thujone in serum and urine. The method developed for this study (HPLC-FLD) proved to be  
370 simple, relatively cheap and sensitive enough to measure small quantities of thujone, as in a  
371 case of poisoning.

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373 **CONFLICTS OF INTEREST**

374 The authors declare no potential conflicts of interest with respect to the authorship and/or  
375 publication of this article.

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377 **Acknowledgements**

378 *This research received funding from the European Community's Seventh Framework*  
379 *Programme (FP7/2007-2013) under grant agreement n° 245199, and has been carried out*  
380 *within the PlantLIBRA project. This paper does not necessarily reflect the Commission's*  
381 *views or future policy in these areas.*

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383 **Author Contributions**

384 Chiara Di Lorenzo designed the study; Francesco Ferretti and Gianfranco Frigerio produced  
385 different parts of the analytical data; Enzo Moro collected test data; Alessandro Ceschi and  
386 Francesca Colombo collected and interpreted the results; Saskia Lude revised the work;  
387 Patrizia Restani drafted and reviewed the work.

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499 **Table 1** - System suitability test for thujone (n = 5)

<b>t<sub>R</sub><sup>a</sup> (min)</b> <b>(mean ± SD)</b>	<b>K<sup>b</sup></b> <b>(mean ± SD)</b>	<b>α<sup>c</sup></b> <b>(mean ± SD)</b>	<b>SF<sup>d</sup></b> <b>(mean ± SD)</b>	<b>N<sup>e</sup></b> <b>(mean ± SD)</b>
18.22 ± 0.05	12.84 ± 2.54	1.29 ± 0.01	0.97 ± 0.02	10316 ± 405

500 LEGEND:

501 <sup>a</sup>t<sub>R</sub> = Retention time; <sup>b</sup>K (Retention factor) = (t<sub>R</sub> - t<sub>0</sub>)/t<sub>0</sub>, where t<sub>R</sub> and t<sub>0</sub> are retention times

502 of thujone and solvent, respectively; <sup>c</sup>α (Separation factor) = (t<sub>R</sub> - t<sub>0</sub>)/(t<sub>R1</sub> - t<sub>0</sub>), where t<sub>R</sub>

503 and t<sub>R1</sub> are retention times of thujone and a neighboring peak, respectively; <sup>d</sup>SF (symmetry

504 factor) = W<sub>0.05</sub>/2f, where W<sub>0.05</sub> is width of the peak at 5% height and f the distance from the

505 peak maximum to the leading edge of the peak, the distance being measured at a point 5% of

506 the peak height from the baseline. <sup>e</sup>N (number of theoretical plates) = 16/(t<sub>R</sub>/W)<sup>2</sup>, where W is

507 the peak width at its base.

508