An ultrasensitive and selective ‘turn off’ fluorescent sensor with simple operation for the determination of trace copper (II) ions in water and various beverage samples


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An ultrasensitive and selective ‘turn off’ fluorescent sensor with simple operation for the determination of trace copper (II) ions in water and various beverage samples

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ABSTRACT
Considering vital roles of trace copper ions for the health of humans, it is critical to sensitively detect it in foodstuffs. Herein, an ultrasensitive and cost-effective coumarin based fluorescent strategy with a “turn–off” signal was developed for specific detection of trace Cu²⁺ ions. Synthesis of 6,7-dihydroxy-3-(3-methylphenyl)coumarin (TMAC) probe was performed and characterized. The developed fluorescent probe presented excellent selectivity. The limit of detection of the sensor can reach as low as 5.13 nm in ACN/H₂O (v/v=95/5) buffered by 5 μM HEPES buffer at pH = 7.0, and it is below the World Health Organization (WHO) guideline for drinking water (31.5 μM). The probable stoichiometry of TMAC–Cu²⁺ complex (1:1) was determined by Job’s plot method and confirmed by FT–IR and ESI–MS. We further successfully applied TMAC for Cu²⁺ detection in water and various beverage samples with good recovery (90.24-109.75 %).
Introduction

Copper is one of the vital trace elements in the human body. It plays critical roles in many biological processes, such as producing red blood cells, acting as neurotransmitters for physiological functions and as a cofactor of several enzymes (1–3). The human body receives the copper element either through daily diet or through some mechanisms exist to maintain the copper balance in their body (4, 5). Considering the copper ions are found widely in the environment and may ultimately accumulate, via food chains, in the human body. Short-term or long-term, overexposure to an abnormal amount of copper ions via foodstuffs causes significant harm to human health since many foodstuffs are one of the main ways that copper enters human bodies (5, 6). Nowadays, more and more copper ions are being released into the environment with the growth of the industrialisations (2, 7). The World Health Organisation (WHO) and the European Water Quality Directive (EU-WQD) have reported that the maximum admissible concentration for copper (II) ions in drinking water is 2.0 mg.L\(^{-1}\) (4, 6, 8, 9). Although a normal intake of copper ions below this level is essential for human health, the abnormal copper (10.0 mg.L\(^{-1}\)) concentration in tissues can cause many serious diseases, including cancers, gastrointestinal disturbances, and be quite toxic to the human body. Modification in its cellular homeostasis of copper ions can induce neurodegenerative diseases (1–6, 8–12); therefore, the development of analytical strategies for copper ions sensitively monitoring is considerably important.

Up to now, numerous traditional methods for detecting copper ions have been reported. In this regard, graphite flame atomic absorption spectrometry (AAS) (4, 13), plasmon–resonance Rayleigh scattering spectroscopy (RSS) (14), inductively coupled plasma optical emission spectroscopy (ICP-OES) (15, 16), inductively coupled mass atomic emission spectrometry (ICM-AES) (17), atomic fluorescence spectrometry (AFS) (19) and electrochemical assays (19, 20) are examples of preferred, sensitive and realistic analytical methods that have been employed. However, these methods are complicated and time-consuming, require relatively expensive and sophisticated instrumentations, suffer from serious matrix interferences and high operating costs, and need highly trained personnel (1, 2, 4–6, 8, 9). In recent years, considerable efforts have been made on the development of copper selective fluorescent sensors to overcome these disadvantages of conventional technologies. Fluorescent sensors possess many advantages for trace analyses; such as low cost, high sensitivity and selectivity, fast response time, non-destructive and real-time detection and simplicity for implementation (5, 6, 21–26). Over the past decades, fluorescent sensors have emerged as attractive determination platforms for various anions, cations or biomolecules. Also, they allow several of the above-mentioned attributes for achieving sensitive determination through utilising appropriate chemosensors. In light of this, several literatures based on fluorescent sensors containing such as BODIPY, fluorescein, perylene, anthracene, coumarin and so on for the determination of trace copper ions have been reported in the literatures (27, 28). For example, as in a recent report by Jung and co-workers, a new coumarin-derived Cu(II)-selective fluorescent sensor was produced and employed for the determination of copper (II) ions (29). Additionally, Mani et al. developed a ‘turn-off’ fluorescence sensor for Cu\(^{2+}\) detection with quite a simple operation and presented the applicability of the sensor (30). However, most of the reported copper sensors have disadvantages, especially in terms of long response time, detection limit, sensitivity and selectivity. Undoubtedly, the design of fluorescent chemosensors with low detection limits that can easily monitor the copper ions will expand the utility of these detecting systems. Therefore, the development of sensitive and selective fluorescent sensors with good accuracy and recovery for the trace detection of copper ions is considerably urgent (6, 31–35).

Herein, we aimed to develop an ultrasensitive and cost-effective coumarin-based fluorescent sensor for the detection of trace level of copper (II) ions in water and various beverage samples with a simple operation. A coumarin–based probe (6,7-dihydroxy-3-(3-methylphenyl) coumarin (TMAC)) was synthesised according to literature (36–38) and successfully characterised. The developed turn-off probe presented excellent selectivity of copper ions with good recovery and had no obvious response on other metal ions. We further applied TMAC for the copper (II) ion detection in real drinking samples.

Experimental

General information

All the materials used in the synthesis, purification of the compounds and spectroscopic studies were obtained from commercial suppliers. Distilled water was obtained by Millipore Ultra-Pure Water Purification System. Various metal ion solutions were performed with the corresponding perchlorate salts. \(^{1}\)H and \(^{13}\)C spectra were recorded on a Bruker DPX 400 MHz spectrometer with DMSO-\(d_6\) and CDCl\(_3\) as solvent. The FT-IR spectra of compounds were measured using on Perkin Elmer FT-IR spectrum 100. Fluorescence spectra were recorded with Agilent Cary Eclipse spectrometer using quartz cells of
1.0 cm path length. Mass spectra were obtained on a Bruker Daltonics Microflex Mass Spectrometer equipped with an ESI source. A PerkinElmer model PinAAcle™ 900T atomic absorption spectrometer (PerkinElmer, Inc., Norwalk, CT, USA) combined with flame/longitudinal Zeeman furnace system was used for the determination of copper ions. The wavelength, lamp current and slit width parameters for copper were adjusted as 324.75 nm, 30 mA and 0.7 nm, respectively. 

The acetylene–air flame was used and flow rates were adjusted as 2.0 and 17.0 L min⁻¹, respectively. The data were recorded by Syngistix™ for AA Software. A Mettler Toledo benchtop pH metre was used for pH adjustments. To mix the water and beverage samples, a Vortex-Mixer VM-10 orbital motion was used. A Hettich Universal 320R model benchtop centrifuge (Andreas Hettich GmbH & Co, Germany) was used for centrifuging.

**Synthesis of probe**

Synthesis and purification of compounds 2-(2,4,5-trimethoxyphenyl)-1-(3-(methylphenyl))acrylonitrile (TMAN) and 6,7-dihydroxy-3-(3-methylphenyl)coumarin (TMAC) were done based on the literature (36–38).

**Synthesis of 2-(2,4,5-trimethoxyphenyl)-1-(3-(methylphenyl))acrylonitrile (TMAN)**

2,4,5-trimethoxybenzaldehyde (TMA) (5.4 mmol) in ethanol (50 mL) was mixed with 3-methylphenylacetonitrile (1) (5.4 mmol) and the temperature of the reaction mixture was promoted to 75°C. Sodium hydroxide solution (25%) was added dropwise until observing the blurry of the mixture. After the termination, the reaction mixture was subjected to precipitation in ice-water. Then, filtration, washing with ethyl acetate/n-hexane mixture and the obtained part was dried under vacuum. The residue was precipitated via ethyl acetate/n-hexane mixture and the obtained part was subjected to column chromatography using chloroform/hexane to obtain pure desired product TMAC. Yield: 80% (0.28 g). Fawn coloured solid. Anal. Calc. for C₆H₇NO₃: C, 66.82; H, 5.79; N, 4.53. Found: C, 66.80; H, 5.76; N, 4.53. FT-IR (KBr, cm⁻¹): 3505, 3181 ν(OH), 2934, 2832 (Ar-Aliphatic), ν: 2199 (C≡N), 1613, 1578, 1512 and 1468 (C = C). ¹H-NMR (400 MHz, CDCl₃): δ 2.44 (3H, s, H(18)), 3.98 (3H, s, H(19)), 3.97 (3H, s, H(15)), 3.91 (3H, s, H(16)), 6.55 (1H, s, H(7)), 7.2 (1H, d, H(16)), 7.35 (1H, t, H(17)), 7.48–7.50 (2H, m, H(14) and H(15)), 7.96 (2H, m, H(3) and H(19)). ¹³C-NMR (CDCl₃) δ 21.56 C(18); 102.66 C(2); 112 C(6); 112.88 C(5); 122.77 C(10); 125.93 C(13); 128.48 C(17); 128.98 C(15); 129.26 C(16); 135.75 C(12); 137.65 C(14); 141.48 C(9); 143.50 C(11); 148.50 C(4); 150.89 C(3); 160.80 C(11).

**General procedure for fluorescence measurements**

Unless otherwise stated, all fluorescence experiments were performed in ACN/HEPES buffer solution (95:5, v/v, 5 μM, pH 7.0) as a vehicle in 3 mL quartz cuvettes. A stock solution of a probe (TMAC) (10 mM) was prepared in HPLC grade acetonitrile (ACN) and then the stock solution was diluted 5 μM in ACN/HEPES solution.

![Scheme 1. Synthetic scheme of TMAN and TMAC.](image-url)
(v/v = 95/5) for fluorescence experiments. Aliquots of 10 mM perchlorate salts (M(ClO₄)ₓH₂O) were then added into the probe solutions. All fluorescence measurements were performed at room temperature. The excitation wavelength was 364 nm and the slit width was 10 nm.

**Determination of the detection limit**

The limit of detection (LOD) for Cu²⁺ was evaluated based on fluorescence titration experiment. For the calculation of the standard deviation of blank, the fluorescence intensity of (TMAC) in the absence of Cu²⁺ at 481 nm was measured by three times. The detection limit of (TMAC) for Cu²⁺ was obtaining utilising the following equation (39, 40).

\[
\text{Detection limit} = \frac{3 \sigma}{k}
\]

where \(\sigma\) is the standard deviation of blank solution measurements; \(k\) is the slope of the calibration curve between the fluorescence intensity of (TMAC) versus Cu²⁺ concentration.

**Selectivity studies**

In order to evaluate the selectivity performance of TMAC, the interference studies were performed in presence of 10-fold excess of metal cations. The fluorescence measurements were investigated in the presence of a series of metal ions (Na⁺, Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺, Mn²⁺, Fe²⁺, Fe³⁺, Co²⁺, Zn²⁺, Cd²⁺, Hg²⁺, Al³⁺ and Pb²⁺) and Cu²⁺ by excitation at 364 nm.

**Preparation of water and beverage samples**

The tap and dam water samples were collected from our laboratory and Fisandun dam, respectively. Beverage samples were purchased from local supermarkets in Karaman city, Turkey. Prior to analysis, tea and coffee samples were placed into a beaker and 50 mL of distilled boiled water was added and waited for 15 min. Also, fizzy drink samples were placed in an ultrasonic bath for 15 min to degas carbonate present in the samples. Afterwards, all samples were first centrifuged at 10,000 for 10 min and then they were filtered through a 0.45 µm pore size membrane to get rid of suspended species or contamination. In addition, the standard addition method was utilised to perform the accuracy of the method and so two different concentrations (0.1 and 0.2 µM) of copper (II) ions were used.

**The procedures of Cu²⁺ determination by the fluorescence spectroscopy**

5 µM ligand solution was prepared in ACN/HEPES (95/5) and 3 mL probe solution was added to a cuvette to evaluate detection of Cu²⁺. Afterwards, 15 µL various water and beverage samples were added into a probe solution and mixed to obtain a homogenous mixture. Then, the fluorescence intensities of mixtures were measured by the help of the fluorescence spectroscopy utilising \(\lambda_{ex} = 364\text{ nm}, \lambda_{em} = 481\text{ nm} \) and slit widths = 10 nm, 10 nm.

**The procedures of Cu²⁺ determination by the atomic absorption spectrometry**

Before the atomic absorption spectrometry (AAS) analysis, the sample preparation procedure was applied according to the reported method by Mendil et al. (2015) (41) with some modifications. The water and beverage samples were acidified with 1% with nitric acid and filtered through a 0.45 µm pore size membrane filter. Then, the pH values of the solutions were adjusted to 9 by ammonia/ammonium chloride buffer solution. After 10 min, the sample solution was centrifuged at 10,000 rpm for 10 min. The upper layer was removed, and then the precipitate remained in the tube was dissolved with 0.5 mL of concentrated HNO₃. At the final stage, the sample was diluted to 2.5 mL with ultrapure water. Copper (II) ions in the sample solutions were determined by AAS and the measurements were performed by three replicates. The obtained results were reported as the mean value ± standard deviation.

To construct the calibration curves, standard copper solutions were diluted from the stock standard by dilution with nitric acid to the appropriate concentrations (10–1000 µg.L⁻¹). Various amounts of Cu²⁺ standard solution (10 and 20 µg.L⁻¹) were also spiked to these samples and measured by AAS in triplicate.

**Results and discussion**

**Synthesis and structural characteristics of TMAC**

6,7-Dihydroxy-3-(3-methylphenyl)coumarin (TMAC) was synthesised by using pyridinium hydrochloride and TMAN with supporting material silica gel under microwave and non-solvent conditions. Elemental analysis, $^1$H, $^{13}$C-NMR, 2D HETCOR, MALDI-TOF MS and FT-IR spectroscopy have been used to characterise the structure of compounds. General presentation of all the compounds is shown in Scheme S1. When analysing the NMR and FT-IR spectra of TMAC and TMAN, clear evidences do exist so as to prove the formation TMAC. The presence of -OH
stretching vibration at 3181 and 3505 cm\(^{-1}\) for TMAC proves conversion of methoxy to the hydroxy group and also the existence of \(-\text{C} = \text{O}\) stretching vibrations at 1669 cm\(^{-1}\) is a proof for the characteristic of coumarin compound (Figure S4). Besides these information, the methoxy protons of TMAN were not observed at \(^1\text{H} \text{NMR}\) spectrum of TMAC and the \(-\text{OH}\) protons (7 and 8 number protons in the Scheme S1) of TMAC have been observed at 9.41 and 10.19 ppm that was shown in Figure S2 and Figure S5. Besides, the characterisation of the compounds was supported with ESI-MS and the peaks at 268.7 m/z, 291 m/z and 307.2 m/z in the ESI-MS spectra of TMAC were corresponding to the TMAC, (TMAC,+ Na\(^+\)) and (TMAC,+ K\(^+\)) in Figure S7, respectively.

**Fluorescence studies**

The fluorescence response properties of TMAC were investigated by fluorescence spectroscopy in ACN/HEPES buffer (v/v, 95/5, 5 \(\mu\)M, pH = 7.0). In order to investigate the sensitivity of the developed sensor to metal ions, the solutions of metal ions were added into TMAC solution and fluorescence spectrum of each addition was recorded at 481 nm (ex: 364 nm) which is given in Figure 1. As shown in Figure 1, free TMAC displayed strong fluorescence band at 481 nm with a very large Stokes shift more than 100 nm, when excited at 364 nm. The excitation spectrum for the emission at 481 nm (indicates a maximum excitation wavelength around 364 nm, which is used in the further experiments). While the addition of 5.0 fold excess of metal ion solutions (K\(^+\), Al\(^3+\), Ba\(^2+\), Ca\(^2+\), Cd\(^2+\), K\(^+\), Zn\(^2+\), Hg\(^2+\), Fe\(^3+\), Fe\(^2+\), Cd\(^2+\), Mg\(^2+\), Mn\(^2+\), Pb\(^2+\), Sr\(^2+\), Cu\(^{2+}\)) into the ligand solution did not cause any remarkable influence on the fluorescence intensity of the TMAC, copper (II) was the only metal ion that leads to prominent quenching on the fluorescence intensity of the ligand. This observed quenching may be attributed to strong binding and rapid chelation kinetics of Cu\(^{2+}\) ions with the OH groups on the TMAC (42). The quenching capability of TMAC, stated as I\(_0\)/I, was 38.7 for Cu (II), but for the other metal ions, it was changing in a narrow range, from 1.03 to 2.04 (Figure S9).

To further investigate the sensing ability of the TMAC, the fluorescence titration of TMAC with Cu\(^{2+}\) was conducted, and the change in fluorescence intensity was monitored. With the gradual addition of Cu\(^{2+}\) (0–4 equiv.) to the TMAC solution (5 \(\mu\)M), the fluorescence intensity of TMAC was decreased gradually and saturated with 2.0 equiv. of Cu\(^{2+}\) at 481 nm, almost completely quenched (Figure 2).

**Job’s plot of TMAC with Cu\(^{2+}\)**

The Job’s plot was conducted to estimate the binding stoichiometry between TMAC and Cu\(^{2+}\). The maximum

Figure 1. Fluorescence intensity changes of TMAC (5 \(\mu\)M) in the presence of various cations (5 \(\mu\)M) in ACN/HEPES buffer solution (95:5, v/v, 5 \(\mu\)M, pH 7.0) (ex = 364 nm).
relative fluorescence intensity was around 0.5, which indicated that the stoichiometric ratio was 1:1 as shown in Figure S10. The stoichiometry of TMAC-\(\text{Cu}^{2+}\) was verified by ESI-MS and a peak at 329.39 m/z in the ESI-MS spectrum of TMAC-\(\text{Cu}^{2+}\) complex was corresponding to (TMAC-\(\text{Cu}^{2+}\)) which indicated 1:1 ratio in Figure 3. Also, the structure of the TMAC-\(\text{Cu}^{2+}\) complex was characterised utilising FT-IR spectroscopy. As shown in Figure S8, \(-\text{OH}\) groups of TMAC disappeared after complexation reaction.

The binding constant of TMAC for \(\text{Cu}^{2+}\) was determined by the fluorescence titration based on the Benesi–Hildebrand equation \((43)\) and was found to be \(8.8 \times 10^5 \text{ M}^{-1}\) \((Figure 4(a))\). For analytical applications, the detection limit of the sensor is a significant parameter. Therefore, the fluorescence titration data were used to obtain the detection limit of TMAC for \(\text{Cu}^{2+}\). The concentration of \(\text{Cu}^{2+}\) was plotted against the fluorescence intensity at 481 nm \((Figure 4(b))\). The detection limit of TMAC for \(\text{Cu}^{2+}\) ions was found to be 5.13 nM according to \(S/N = 3\) \((44)\), which was sufficiently low for the detection of \(\text{Cu}^{2+}\) ions in food samples and much lower than the WHO for drinking water \(31.5 \text{ µM}\) \((4, 6, 8, 9)\).

Most of the reported copper sensors have disadvantages, especially in terms of long response time, detection limit, sensitivity and selectivity, but as seen in Table S1, the detection limit (LOD) of TMAC is found to be lower than other researches based on the analysis of \(\text{Cu}^{2+}\). In addition, our sensor is compared to other coumarin-based sensor in literature, it has better behaviours in terms of selectivity and response time. These obtained results showed that TMAC could be employed as a probe for the detection of nanomolar concentrations of \(\text{Cu}^{2+}\) ions.

**Selectivity studies**

Good selectivity for the target ion detection over other potentially relevant ions is important for metal ion sensors. The selectivity studies of TMAC towards the various cations \((2.00 \text{ eq.})\) in the presence of \(\text{Cu}^{2+}\) \((2.00 \text{ eq.)}\) were investigated \((Figure 5)\). The results obviously indicate our probe has good selectivity for \(\text{Cu} (\text{II})\) ion over other cations, only \(\text{Zn}^{2+}\) ion has small interference. This is owing to the similarity in the coordination number of \(\text{Zn}^{2+}\) and \(\text{Cu}^{2+}\) \((45)\). Thus, the sensor TMAC could be confidently utilised as a selective and sensitive turn-off fluorescent probe for the detection of \(\text{Cu}^{2+}\) ions even with being competing other cations present.

**Time-dependence of the sensor**

Due to the fact that it is a significant point for practical applications, time-dependence studies of the sensor were performed and illustrated in Figure S11. After the addition of \(\text{Cu}^{2+}\) to TMAC, the emission intensity of TMAC-\(\text{Cu}^{2+}\) complexes was balanced within 60 s, indicating that a very rapid reaction between TMAC and \(\text{Cu}^{2+}\) was
Figure 3. ESI-MS spectrum of TMAC-Cu\(^{2+}\) complex.

Figure 4. (a) Benesi-Hildebrand plot (em = 481 nm) of 1/(I-I\(_0\)) vs 1/[Cu\(^{2+}\)] based on 1:1 association stoichiometry between TMAC-Cu\(^{2+}\). (b) The plot of emission intensities of TMAC at 481 nm versus with various Cu\(^{2+}\) concentrations.

Scheme 2. The proposed binding mechanism of TMAC and Cu\(^{2+}\) complex in ACN/HEPES buffer solution (95:5, v/v, 5 μM, pH 7.0).
considered, which was very important for the real-time monitoring of Cu$^{2+}$ ion.

Real sample applications

In order to evaluate the efficiency of the fabricated probe to determine Cu$^{2+}$ in real samples, tap and dam water (our laboratory and fisandun dam, respectively) and various beverage samples (a local market in Karaman city, Turkey) were collected and detected using the proposed probe. The analysis revealed that the fabricated probe has a good performance for detection of Cu$^{2+}$ in a series of real drinking samples (Table S2). Each analysis was studied in triplicate. As illustrated in Table S2, the standard deviation in the real analysis was found below 0.1 and RSD results for copper (II) in real drinking samples were between 0.26%-5.46%, demonstrating feasibility and sensitivity of the prepared sensor. Furthermore, two different concentrations (0.1 and 0.2 μM) of copper (II) were added to probe solution to prove the accuracy of obtaining results. As it is shown in Table S2, the recoveries of copper (II) were found in the range of 90.24%-108.95%. Studies on the coumarin-based fluorescent sensor for the determination of Cu$^{2+}$ in drinking samples are limited and most of them are not relevant for the detection of Cu$^{2+}$ in drinking samples. The limit of detection for the prepared TMAC fluorescent probe was found to be less than 5.13 nM and it was lower than the other coumarin-based fluorescent probes in the literature using for analysis of Cu$^{2+}$ in real samples (46, 47). Overall, it is clear that the developed TMAC fluorescent sensor could be utilised as a tool to determine copper (II) in water and various beverage samples with good accuracy and recovery.

The AAS procedure combined with a simple sample preparation procedure was also applied as a second technique (41), for the determination of Cu$^{2+}$ in the water and beverage samples. The results of the non-spiked and spiked samples were given in Table S3. The good agreements between the spiked and found amounts of copper ions were obtained and the recovery values were between 90.28% and 105.68%. Lower relative standard deviation values (0.11%-7.60%) were also obtained from AAS analyses. The accuracy of the proposed TMAC procedure was also assessed. As shown in Table 1, a good agreement between determined values with TMAC probe and the AAS values was obtained. Furthermore, these results were statistically treated using Student’s t-test, where the calculated t values were less than tabulated t values at 2 of freedom and 95% confidence level; therefore, there are no significant differences between the obtained results with TMAC.
and the AAS values (Table 1). Thus, quantitative results of the AAS method confirm the obtained results from TMAC probe for detection of Cu²⁺ in a series of drinking samples.

### Conclusion

In conclusion, highly sensitive and selective coumarin-based fluorescent sensor for copper (II) detection in ACN/HEPES buffer solution (95:5, v/v, 5 µM, pH 7.0) was developed. The stoichiometry of the complexes between TMAC and Cu²⁺ ions was determined by the Job's method and supported with ESI-MS technique and found to be 1:1 ratio. Moreover, the limit of detection value for Cu²⁺ ion was found to be 5.13 nM according to S/N = 3. Finally, real sample analyses were carried out successfully by using developed sensor. The analysis indicated that the standard deviation in the real analysis was found below 0.1 and RSD ratio for copper (II) in real drinking samples were found in the range 0.26%-5.46%. The facile preparation of TMAC, the detection value of TMAC for Cu²⁺ and the demonstration of its high accuracy and recovery value in real sample analysis warrant its ability to utilise as a tool for detection of copper (II) in water and various beverage sample analysis.

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### Disclosure statement

No potential conflict of interest was reported by the authors.

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**Table 1. The analysis of Cu²⁺ in various water and beverage samples by TMAC probe and AAS.**

| Sample                  | Cu(II) found (µmol L⁻¹)          | Difference of the means | t test statistics (df = 2) | Probability>|t| |
|-------------------------|----------------------------------|-------------------------|---------------------------|----------------|
| Fizzy drink samples     |                                  |                         |                           |                |
| Soda water (unfruitful) | 2.7959 ± 0.0073                  | 0.0367                  | 3.0602                    | 0.0922         |
| Soda water (grape&mulberry–flavoured) | 0.1179 ± 0.0035 0.1083 ± 0.0009 | 0.0093                  | 3.4931                    | 0.0730         |
| Energy drink (mixed fruit-flavoured) | 0.2521 ± 0.0070 0.2487 ± 0.0029 | 0.0034                  | 0.7522                    | 0.5304         |
| Fizzy drink (orange–flavoured) | 0.1646 ± 0.0055 0.1605 ± 0.0004 | 0.0041                  | 1.1963                    | 0.3541         |
| Cola                    | 0.0404 ± 0.0020 0.0395 ± 0.0004 | 0.0009                  | 0.7597                    | 0.5267         |
| Fizzy drink (mixed fruit–flavoured) | 0.0521 ± 0.0006 0.0499 ± 0.0005 | 0.0022                  | 3.0893                    | 0.0907         |
| Tea and coffee samples  |                                  |                         |                           |                |
| Ice tea (nectar fruit–flavoured) | 0.6929 ± 0.0068 0.7009 ± 0.0025 | −0.0080                 | −1.9848                   | 0.1855         |
| Green tea (mint and lemon–flavoured) | 0.4767 ± 0.0064 0.4686 ± 0.0011 | 0.0080                  | 2.3524                    | 0.1429         |
| Coffee                  | 6.3404 ± 0.075 6.2546 ± 0.0091  | 0.0858                  | 1.7678                    | 0.2191         |
| Black tea (bergamot–flavoured) | 0.2931 ± 0.005 0.2907 ± 0.0008 | 0.0023                  | 0.7825                    | 0.5158         |
| Milk samples            |                                  |                         |                           |                |
| Milk                    | 2.8303 ± 0.0701 2.8487 ± 0.0030 | −0.0183                 | −0.4487                   | 0.6975         |
| Milk (strawberry–flavoured) | 1.6960 ± 0.0200 1.6856 ± 0.0028 | 0.0104                  | 1.0507                    | 0.4036         |
| Latte                   | 2.7405 ± 0.112 2.7154 ± 0.0073  | 0.0251                  | 0.3666                    | 0.7490         |
| water samples           |                                  |                         |                           |                |
| Drinking water          | 0.4401 ± 0.0047 0.4311 ± 0.0149 | 0.0089                  | 1.2840                    | 0.2378         |
| Tap water               | 0.2431 ± 0.0087 0.2291 ± 0.0071 | 0.0140                  | 1.8180                    | 0.2106         |
| Fisandun dam water      | 0.1366 ± 0.0031 0.1358 ± 0.0050 | 0.0007                  | 0.1638                    | 0.8849         |
| Ultra pure water        | 0.1169 ± 0.0010 0.1189 ± 0.0030 | −0.0019                 | −1.3047                   | 0.3219         |
| Fruit juice samples     |                                  |                         |                           |                |
| Orange juice            | 0.2530 ± 0.0115 0.2599 ± 0.0026 | −0.0068                 | −1.0103                   | 0.4186         |
| Cherry juice            | 0.2137 ± 0.0057 0.2130 ± 0.0012 | 0.0007                  | 0.2799                    | 0.8057         |
| Apple juice-1           | 0.1656 ± 0.0047 0.1622 ± 0.0032 | 0.0034                  | 0.7960                    | 0.5094         |
| Tomato juice            | 3.5641 ± 0.0208 3.5811 ± 0.0117 | −0.0169                 | −1.282                    | 0.3282         |
| Apple juice-2           | 0.5365 ± 0.0055 0.5300 ± 0.0063 | 0.0065                  | 1.7410                    | 0.2238         |
| Fruit juice (mixed fruit–flavoured) | 0.7224 ± 0.0150 0.7200 ± 0.0052 | 0.0024                  | 0.3282                    | 0.7739         |
| Alcoholic beverage samples |                                  |                         |                           |                |
| Beer                    | 0.3285 ± 0.0087 0.3314 ± 0.0066 | −0.0028                 | −2.2070                   | 0.1580         |
| Red wine                | 0.2671 ± 0.0075 0.2617 ± 0.0043 | 0.0054                  | 0.9072                    | 0.4600         |

Null Hypothesis: mean1=mean2 = 0  
Alternative Hypothesis: mean1=mean2 <0  
At the 0.05 level, the difference of the population means is NOT significantly different with the test difference (0)
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