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Activity-Based Sensing of Ascorbate by Using Copper-Mediated Oxidative Bond Cleavage

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Abstract

Ascorbate is an important biological reductant and enzyme cofactor. Although direct detection through ascorbate-mediated reduction is possible, this approach suffers from poor selectivity due to the wide range of cellular reducing agents. To overcome this limitation, we leverage reduction potential of ascorbate to mediate a copper-mediated oxidative bond cleavage of ether-caged fluorophores. The copper(II) complexes supported by a {bis(2-pyridylmethyl)}benzylamine or a {bis(2-pyridylmethyl)}(2-methoxybenzyl)amine ligand were identified as an ascorbate responsive unit and their reaction with ascorbate yields a copper-based oxidant that enables rapid benzylic oxidation and the release of an ether-caged dye (coumarin or fluorescein). The copper-mediated bond cleavage is specific to ascorbate and the trigger can be readily derivatized for tuning photophysical properties of the probes. The probes were successfully applied for the fluorometric detection of ascorbate in commercial food samples, human plasma, and serum, and within live cells by using confocal microscopy and flow cytometry.

Keywords

ascorbate; copper; fluorescent probes; oxidation; sensors

Introduction

Ascorbate (vitamin C) is biosynthesized in plants and animals from a monosaccharide precursor (e.g., glucose or galactose). Interestingly, some primates, including humans, have lost the ability to make ascorbate. For this reason, ascorbate is an essential nutrient for human and has been consumed as a non-prescribed supplement.^[1] Under physiological conditions, ascorbate exists mainly as a monoanion.^[2] One-electron oxidation of ascorbate generates ascorbyl radical, which can be further oxidized to dehydroascorbate (DHA). The

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Conflict of interest

Ho Yu Au-Yeung and Zuo Hang Yu are the inventors of an US patent “Molecular Probes for Ascorbate Detection and Methods of Use” (US 10018,23 B1).

biochemical function of ascorbate is often related to its interconversion between redox forms.^[3] Along with other cellular redox cycles, ascorbate regulates cellular oxidative stress and redox signaling which are both implicated in aging and diseases.^[4] Ascorbate also serves as an enzyme cofactor for collagen^[5] and carnitine biosynthesis,^[6] the amidation of peptide hormones,^[7] and the hydroxylation of dopamine to noradrenaline.^[8] For its diverse occurrence and importance, there are different debates and controversies related to the health benefits and roles of ascorbate in disease treatments and therapies.^[9] As a result, quick, selective and sensitive ascorbate sensing methods are of high importance for both clinical applications and fundamental physiological studies.

Selective detection of ascorbate is challenging in complex biological matrices (e.g., live cells). Conventional HPLC,^[10] electrochemical methods^[11] and plate reader assays^[12] only provide the total ascorbate content (all redox states). Moreover, these methods require sample pretreatment, extraction and derivatization with limited spatiotemporal information. Luminescent techniques, on the other hand, are non-destructive, sensitive and operationally simple.^[13] Additionally, appropriate probe and experiment design can yield information regarding cellular distribution, redox state and interplay with other cellular components. Unfortunately, there are only a limited number of ascorbate luminescent probes with live-cell capabilities. The most common and straightforward strategy for designing ascorbate probes employs the direct reduction of a paramagnetic quencher (e.g., a TEMPO radical).^[14] A key drawback to this approach is the wide redox potential window displayed by cellular oxidants and reductants, which poses concerns regarding undesirable probe activation. It is challenging to overcome these cross-reactivity problems when the responsive moiety is based on a promiscuous redox reaction. To address this, we turned our attention to lytic polysaccharide monooxygenases (LPMOs), a class of copper monooxygenases that can employ ascorbate as a cofactor for the oxidative cleavage of polysaccharides.^[15] By mimicking the catalytic mechanism of the enzyme, it would be possible to couple ascorbate-mediated reduction of a copper complex to induce benzylic oxidation of an ether. Selective coupling of a reporter dye to the responsive element would enable a fluorescent response (Scheme 1). Herein, we report the development of a generalizable responsive element for the detection of ascorbate. The two fluorescent ascorbate probes, AP-cyan and AP-green, employ an ascorbate-induced, copper-mediated C–O ether bond cleavage that is similar to LPMOs. Both probes display good selectivity against a range of redox active biomolecules and are applicable in various complex matrices, such as commercial food samples, human plasma, and serum, by using a plate reader assay, and also in live cells using confocal microscopy and flow cytometry.

Results and Discussion

Design of the copper-based ascorbate probes

LPMOs are a class of monooxygenase that can employ ascorbate as a cofactor for dioxygen activation at a mononuclear copper site. The resulting metal center is then poised for oxidation of the substrate (e.g., C–H hydroxylation or oxidative cleavage of glycosidic bonds). The active-site structure of LPMOs was recently characterized as a mononuclear copper coordinated by two histidine imidazoles and one peptidyl NH₂ in a T-shaped

geometry, named the ‘histidine brace’.^[15b,f,g,16] It has been established that ascorbate is an efficient electron donor required for dioxygen activation and substrate oxidation by LPMOs, and specificity for the electron source has also been demonstrated in different classes of LMPOs.^[17] We reasoned that a functional mimic of LPMOs could be exploited as a new activity-based sensing platform for ascorbate detection (Scheme 1). Indeed, similar metal-based oxidation strategies have been successful for the development of selective metal and reactive oxygen species probes.^[18,19] Collective evidence suggests that the metal reactivity towards the fluorophore’s oxidative release is specific to the environment in both the first and secondary coordination sphere and the metal electronic structures. For this reason, the ligand geometry and denticity, nature of coordination donors, and secondary groups can be systematically varied to tune the reactivity and selectivity of probes towards different target analytes.

Our design of a bond cleavage-based ascorbate probe consists of a fluorophore linked to an ascorbate-responsive unit. For example, **1-Cu** contains a {bis(2-pyridylmethyl)}benzylamine coordinated Cu^{II}, designed as a synthetic analogue of the ‘histidine brace’ where it contains two sp² and one sp³ nitrogen donor, is linked to a fluorescein in its non-emissive form. When the fluorescein is caged as an ether, π -conjugation of the xanthene moiety is disrupted and renders the compound weakly absorbing and weakly emissive. An ether bond cleavage and keto-enol tautomerization of the released phenol will restore the π -conjugation and the uncaged fluorescein will become emissive. Similar fluorescence off-on control by caging the phenol group of coumarin- and xanthene-based fluorophores as an ether has also been exploited for developing photocaged fluorescent dyes and probes for metal ions and reactive species.^[18–20] It is worth noting that the present strategy is different from most other copper-based fluorescent probes because the fluorescence enhancement is not due to the removal of the paramagnetic Cu²⁺ quencher, but to the specific chemical reactivity of ascorbate with the copper. This key difference provides the necessary ascorbate selectivity by preventing false positive via simple Cu²⁺ reduction, competitive ligand exchange or other non-bond cleavage-leading reactions.^[21] On the other hand, because of the irreversible nature of the bond cleavage, the cleaved fluorophore can be accumulated to result in a fluorescent signal despite potential competitive binding to the copper by other molecules, which further distinguishes the current design from other metal-based probes that produce a fluorescent response via a simple reversible binding with the target analyte.

Treatment of **1-Cu** with ascorbate resulted in a 114-fold fluorescence enhancement, showing the histidine brace mimic is indeed responsive to ascorbate. Next, the responsive element was modified to contain oxygen ligands to mimic the subset of LMPOs that contain an axial tyrosine coordination in addition to the histidine brace.^[15f] Consistent with previous reports,^[18,19] the reactivity of the copper complex was sensitive to the coordination environment where **3-Cu** (with a {bis(2-pyridylmethyl)}(2-hydroxybenzyl)amine ligand) and **4-Cu** (with a (2-pyridylmethyl){bis(2-hydroxyethyl)}amine ligand) had no significant fluorescent response (Figure 1 and S1).^[22] On the other hand, an increased level of fluorescence turn-on was observed for **2-Cu**, which contains a {bis(2-pyridylmethyl)}(2-methoxybenzyl)amine ligand. More detailed studies are necessary to elucidate the roles and effect of different oxygen donors on the ascorbate reactivity. To determine whether the counter ion played an

effect on the fluorescence enhancement, **1-Cu** was prepared from copper(II) chloride, perchlorate or tetrafluoroborate, where no effect was observed (Figure S2). Finally, the ascorbate reactivity of the copper(II) complex was evaluated when the green emitting fluorescein dye ($\lambda_{em}=510$ nm) was replaced by the cyan emitting 3-(benzothiazole-2-yl)-7-hydroxycoumarin dye ($\lambda_{em}=488$ nm) (**5-Cu** and **6-Cu**). The two coumarin-based compounds were also found to be responsive towards ascorbate, with a strong fluorescence turn-on, suggesting that this activity-based sensing strategy is modular (Figure 1). Together, these experiments demonstrate that the bioinspired oxidation is an effective strategy for fluorescent ascorbate sensing. It is worth noting that the selective triggering of metal-based oxidative reactivity by neither the metal nor the oxidant is indirect and non-trivial; thus, this further distinguishes the present ascorbate sensing from most other current designs of activity-based sensing that feature metal reactivity.

Ascorbate reactivity and selectivity

The ascorbate selectivity of **1-Cu**, **2-Cu**, **5-Cu** and **6-Cu** were evaluated in more detail. Solutions of all the four complexes are weakly emissive in aqueous buffer (pH 7.4) but showed a rapid fluorescence turn-on upon treatment with ascorbate. For example, **2-Cu** showed a ca. 65-fold and 153-fold fluorescence turn-on after 2 min and 30 min incubation with ascorbate, respectively (Figure 2). In all cases, the ascorbate response is fast with ca. 90% of the maximum fluorescence enhancement obtained within 10–15 minutes under all the studied conditions. For comparison, some activity-based probes for metal sensing could have a reaction time as long as 1 to 2 hours.^[18] Considering that the reaction rate will be dependent on the local concentrations of both the probe and ascorbate in different samples, a common 30-minute reaction time was adopted in subsequent studies. In addition, all of the four compounds displayed good selectivity against a panel of biological and inorganic reducing agents, including NADH, lipoic acid, α -tocopherol (vitamin E), and uric acid. Incubation with other common cellular or food components, such as sugars, amino acids, other vitamins, and organic acids also resulted in no appreciable fluorescent response (Figures 2 and S3). Importantly, no fluorescence turn-on was observed when the probes were treated with DHA, demonstrating a good selectivity for the reduced form (ascorbate).

In addition to the tuning of the metal reactivity, varying the coordination ligand in the ascorbate-responsive unit also gives probes of different stability. For example, **5-Cu** and **6-Cu** differ significantly in their stability against copper(II) dissociation, despite their similar ascorbate reactivity and selectivity. Liquid chromatography mass spectrometry (LCMS) showed complete copper(II) dissociation from **5-Cu** under the acidic chromatographic conditions. On the other hand, **6-Cu** elutes as an intact complex with only partial copper(II) dissociation (Figure S14). The higher stability of **6-Cu** can be attributed to the weakly coordinating methoxy group that chelates to the metal. Isothermal titration calorimetry experiments showed that binding of the {bis(2-pyridylmethyl)}(2-methoxybenzyl)amine ligand to Cu^{2+} is strong, with a measured K_d value of ca. 170 nM, suggesting ca. 90% of the probe remained as the copper(II) complex in a 10 μM probe solution (Figure S51). This result highlights the fact that careful design of the coordination ligand can result in other favorable properties beyond sensitivity and selectivity. Subsequent studies were therefore focused on **2-Cu** (AP-green) and **6-Cu** (AP-cyan) because of their better stability. To further

evaluate the suitability of AP-cyan and AP-green for fluorescent ascorbate detection in complex matrix, their fluorescence response towards ascorbate was also measured in the presence of other biological reducing agents. As shown in Figure S4, a strong fluorescence enhancement was observed when AP-cyan or AP-green was treated with 20 equiv. of ascorbate in the presence of other biological redox agents such as NADH, α -tocopherol, CoQ₁₀ and dopamine, albeit with a slight reduction of the overall fluorescence turn-on. The fluorescence response can be interfered by a high concentration of GSH (2 mM), but a strong and statistically significant ascorbate-induced emission enhancement can still be observed for both AP-cyan (16-fold) and AP-green (10-fold), and these fluorescence turn-on are indeed comparable with other activity-based probes that have been demonstrated to be applicable in cells (Figure S5). The interference from GSH could probably be due to a competitive copper(II) coordination and/or a short-circuiting of the electron transfer in the reaction between ascorbate and the probe. It will therefore be important to carefully characterize the analytical performance of the probe in different samples.

Photostability, aqueous stability and effect of pH

The photostability of AP-cyan and AP-green was also evaluated. A 5 μ M solution of the probes was irradiated at 254 nm using a 6 W UV lamp for 30 minutes, and then the fluorescence response towards ascorbate was measured. The photo-irradiated probe solutions were found to give similar ascorbate-induced emission turn-on as from probe solutions that are prepared freshly, showing a good photostability of both AP-cyan and AP-green (Figure S6). In addition, similar fluorescence response was also observed from solutions of AP-cyan and AP-green that were prepared and stored under ambient conditions for three days (Figure S7). Moreover, no significant difference in the ascorbate-induced fluorescence response of AP-green was observed when the measurement was conducted at pH 5 to pH 8, showing that the probe can be applied in these biologically relevant pH values (Figure S8). Of note, a significant drop in the fluorescence intensity was observed from AP-cyan below pH 6, which is probably due to the non-emissive, protonated form of 3-(benzothiazole-2-yl)-7-hydroxycoumarin ($pK_a=7.02$).^[23] Nevertheless, the high modularity of the copper-based ascorbate trigger will allow the facile incorporation of fluorescent reporters to give probes of different photophysical, chemical and biological properties for use in specific experiments with different requirements.

Mechanistic studies

The ascorbate-induced ether bond cleavage was studied in more detail using AP-cyan. First, the proposed reaction products were confirmed via LCMS, wherein ascorbate-treated AP-cyan yielded the uncaged coumarin ($m/z=296.2$; retention time=4.7 min; yield=44%) along with some other non-emissive oxidation products (Figure S16). This confirms that the observed fluorescence response was due to an ascorbate-induced ether bond cleavage. Next, molecular oxygen was identified as the terminal oxidant: no fluorescence turn-on was observed when AP-cyan was treated under anoxic conditions (Figure S9). Importantly, the fluorescence enhancement could be rescued upon re-introduction of air to the reaction mixture (Figure S10). In addition, the oxidative cleavage is dependent on the copper(II) center, because treatment of the ligand-fluorophore conjugate 6 with ascorbate did not yield any fluorescent response (Figure S11). From these results, we hypothesize that the ether

bond cleavage is due to a copper-based oxidant resulting from a reaction between the probe, ascorbate and molecular oxygen. To support this hypothesis, compound 6 was treated with reactive oxygen species (ROS) and no fluorescent response was observed (Figure S11). Moreover, incubating AP-cyan with different ROS gave no significant turn-on response (Figure S12). Indirect oxidation of the benzylic position was ruled out because no ROS were detected with different ROS probes under the same conditions (Figures S17 and S18). The presence of catalase or superoxide dismutase, which rapidly removes hydrogen peroxide and superoxide, also had no significant effect on the ascorbate-induced fluorescence turn-on of AP-cyan (Figure S13). These observations are consistent with the ether bond being cleaved by a copper-based oxidant rather than any free ROS generated by a Fenton-like reaction.

Although further mechanistic studies are warranted, it has been suggested that substrate oxidation by synthetic LMPO mimics may involve a copper(II) hydroperoxo species. This intermediate has been characterized by a weak *d-d* transition at ca. 650 nm and a strong LMCT at ca. 375 nm.^[24] The strong absorption of the coumarin and fluorescein fluorophores at ca. 350–470 nm may however preclude the characterization of any high-energy absorption below 450 nm. Nevertheless, UV/Vis analysis of ascorbate-treated AP-cyan also showed a weak absorption at 680 nm ($\epsilon = 50 \text{ m}^{-1} \text{ cm}^{-1}$), which is consistent with a copper(II) species in our system (Figure S23). A reversible redox couple at -212 mV (vs. the normal hydrogen electrode (NHE)) was characterized by cyclic voltammetry for the model complex **7-Cu**,^[25] indicating that an electron transfer from ascorbate to the copper(II) is thermodynamically feasible (Figure S26).^[26] Under aerobic conditions, AP-cyan displayed a second order reaction kinetics (first order with respect to both ascorbate and AP-cyan). This suggests that electron transfer from ascorbate to the probe is possibly involved in the reaction. Indeed, HRMS-ESI analysis of an immediate reaction mixture of AP-cyan and ascorbate also showed a new peak at $m/z = 689.1269$, which can be assigned as a one-electron reduced product of the probe (Figure S19). Although the detailed mechanism is likely to be complicated by the specific environment of the reacting ascorbate and copper in different samples,^[27] the involvement of multiple redox-active species and the possibility of different electron-transfer pathways (e.g., one- versus two-electron transfer, proton-coupled electron transfer),^[28] it is clear that the {bis(2-pyridylmethyl)}benzylamine coordinated copper(II) can effectively and selectively trigger the oxidative cleavage of the ether bond upon reacting with ascorbate.

Ascorbate detection in drinks, human plasma and serum samples by AP-cyan

Ascorbate is often added to consumer products (e.g., fruit juice, vitamin water, etc.) because of its antioxidizing and nutritional properties. Moreover, ascorbate levels are clinically monitored to prevent scurvy or contaminations in the quantification of other bioanalytes.^[29] To test the performance of the ascorbate probe in a plate reader assay, we chose to employ AP-cyan in real sample matrices. AP-cyan (limit of detection = $0.14 \mu\text{M}$ by 3σ method) was selected over AP-green (limit of detection = $0.48 \mu\text{M}$ by 3σ method) because of its superior sensitivity. AP-cyan was first tested in two commercial drink samples. As shown in Table 1, the measured ascorbate concentrations matched well with the values provided on the nutrition labels.^[30] The probe was then applied in human plasma and serum samples. Under both conditions, the measured ascorbate concentrations were consistent with reported

physiological concentrations.^[31] The good recovery upon spiking with known concentrations of ascorbate indicates that the fluorescent measurement do not experience significant matrix effects. Together, these results show that AP-cyan can be used for a fast, sensitive, selective and high-throughput measurement of ascorbate concentrations in real samples. Yet, with the very different compositions in different samples, especially the presence of competitive copper binders and redox agents, proper characterization and control experiments will be needed when applying the probes in ascorbate quantification in real samples.

Fluorescent imaging of ascorbate uptake in live cells

As previously discussed, current strategies for ascorbic acid detection are limited for use in living systems. Due to the stability, selectivity, and sensitivity of AP-cyan, we were interested in evaluating its performance in live cells. Prior to performing live-cell imaging, we assessed the biocompatibility of AP-cyan where no significant cytotoxicity was observed under typical imaging conditions (Figure S27). A cellular ascorbate uptake model was selected for the imaging experiment because the vitamin cannot be synthesized in human cells. Although ascorbate uptake pathways are cell-type dependent and poorly characterized, transport of DHA via glucose transporters (e.g., GLUT1 or GLUT3) has been proposed due to the structural similarity of the sugar and DHA.^[32] Following cell uptake, one would expect that DHA would be reduced to ascorbate upon entering the reducing environment of the cell. Previous studies have employed radioisotope labelling and HPLC to study DHA transport.^[33] While successful, these methods may be technically challenging and have no specificity on the ascorbate redox state. Fluorescence imaging is an attractive alternative to radiolabeling approaches because it allows direct visualization of ascorbate within cells. Moreover, the fluorescence enhancement reports on the activity of the ascorbate rather than strictly the concentration, which should more closely parallel the biological effects.^[34]

First, HeLa cells were stained with AP-cyan and then treated with media enriched with variable concentrations of DHA (0, 0.5, and 1.0 mM) for 15 min. Under these conditions, we observed a dose-dependent response to DHA (Figure 3). Motivated by these results, we were interested in confirming whether GLUT1 and GLUT3 are responsible for DHA uptake. To address this question, HeLa cells were stained with AP-cyan followed by incubation with 1 mM DHA supplemented media either in the presence of 10 mM D-glucose or D-fructose. Co-incubation of 1 mM DHA and 10 mM D-glucose decreased the fluorescence by 41%, presumably via competitive inhibition at the glucose transporters.^[32c] D-Fructose, which is not a ligand for glucose transporters, had less of an effect on the observed fluorescence (Figure 3). These experiments establish AP-cyan as a suitable tool for imaging ascorbate in live cells and provides additional experimental evidence for ascorbate trafficking via GLUT uptake of DHA followed by intracellular reduction.

Application of AP-green in flow cytometry

Finally, intracellular detection of ascorbate was performed using flow cytometry. Flow cytometric analyses are complementary to confocal microscopy because they enable rapid detection of large cell populations but lack spatial resolution. AP-green ($\lambda_{\text{ex}}=488$ nm, $\lambda_{\text{em}}=510$ nm) was selected for these experiments due to good spectral overlap with the

ubiquitous FITC excitation source. A large shift in fluorescence was observed for HeLa cells that were cultured in a DHA-enriched media as compared to the untreated control (Figure S28). Importantly, this experiment demonstrates the advantage of the bond cleavage detection strategy in which the modular trigger allows for facile tuning of the photophysical properties of the probe. More generally, one could envision the modular optimization of other properties (e.g., analytical or biological) due to the uncapping approach.

Conclusions

A novel bond cleavage strategy was reported for the activity-based sensing of ascorbate. Contrary to the reducing nature of ascorbate, a copper-mediated oxidative ether bond cleavage is exploited for the release of a covalently caged fluorescent reporter. AP-cyan and AP-green display good sensitivity and selectivity and maintain their functionality in complex matrices (e.g., commercial food samples, human plasma, serum, and live cells). By utilizing a fluorescent readout, both probes are compatible with plate reader fluorimeters, confocal microscopy, and flow cytometry for high-throughput quantification, high-resolution detection, and detection in large cell populations. In addition, the ascorbate responsive unit can be modularly attached to different fluorophores without affecting the reactivity and selectivity, providing a versatile strategy for tuning a variety of properties. Finally, the selective metal-mediated bond cleavage could be a versatile strategy for developing other analyte-responsive sensors. This would represent a new direction in activity-based sensing that relies on complementary chemical reactivity between multiple species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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O-glycosidic bond cleavage by LPMOs *C*-O ether bond cleavage in ascorbate probe **Scheme 1.**

LPMOs employ ascorbate as a cofactor for O_2 activation and *O*-glycosidic bond oxidative cleavage of polysaccharide substrate (top). The LPMOs inspired design of fluorescent ascorbate probes featuring an ether bond cleavage for fluorophore uncaging (bottom).

Figure 1. Structures of copper(II) complex–fluorophore conjugates and their fluorescent response towards twenty equivalents of ascorbate after a reaction time of 30 min.

Figure 2.

Fluorescent response of 5 μM AP-green (top panel) and AP-cyan (bottom panel) in 50 mM HEPES at pH 7.4 at 298 K. Left: Time-dependent fluorescent response towards twenty equivalents of ascorbate at 0, 4, 8, 12, 16, 20, and 30 min. Middle: Dose-dependent fluorescent towards 1–10 μM ascorbate for 30 min. Right: Selectivity against a panel of reducing agents, sugars, amino acids, and vitamins (20 equiv., except for GSH: 2 mM). AP-green and AP-cyan were excited at 470 and 450 nm, and the relative fluorescence intensity was measured at 510 and 488 nm, respectively. Data are reported as mean $\pm$ standard deviation ($n = 3$).

Figure 3.

Confocal microscopy images of HeLa cells treated with AP-cyan (50 μ M, 15 min) then a) 0 mM DHA (vehicle control); b) 1 mM DHA; c) 1 mM DHA with 10 mM D-glucose; or d) 1 mM DHA with 10 mM D-fructose for 15 min at 37 $^{\circ}$ C. Quantification of e) DHA concentrations and f) GLUT transporter inhibition studies. The probe was excited at 488 nm and emission was collected between 493–735 nm. The scale bar represents 20 μ M. Mean fluorescence is reported as the mean of biological replicated $\pm$ standard deviation. Statistical analysis was performed by using a two-tailed Student's *t*-test: * = $p < 0.05$ ($n = 3$).

Table 1.

Ascorbate analysis by using AP-cyan on drinks, human plasma, and serum samples.

Samples	Ascorbate concentration		
	diluted sample ^[a]	original sample	reported
Nice 100% apple juice	$6.81 \pm 0.07 \mu\text{M}$	$1.36 \pm 0.01 \text{ mM}$	$1.70 \text{ mM}^{[b]}$
Glacéau vitamin water power-C	$6.63 \pm 0.19 \mu\text{M}$	$1.32 \pm 0.04 \text{ mM}$	$1.31 \text{ mM}^{[c]}$
plasma	$0.24 \pm 0.04 \mu\text{M}$	$48 \pm 8 \mu\text{M}$	$46.5 \mu\text{M}^{[30]}$
serum	$0.32 \pm 0.03 \mu\text{M}$	$64 \pm 6 \mu\text{M}$	$51.4 \mu\text{M}^{[31]}$

^[a] $n = 3$; dilution factor = 200

^[b] value given in the food label = 0.30 mgmL^{-1}

^[c] value given in the food label = 0.23 mgmL^{-1} .