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1 **Chelate titrations of Ca²⁺ and Mg²⁺ using microfluidic**
2 **paper-based analytical devices**

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3 20 ABSTRACT
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5 21 We developed microfluidic paper-based analytical devices (μ PADs) for the chelate
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7 22 titrations of Ca^{2+} and Mg^{2+} in natural water. The μ PAD consisted of ten reaction zones
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10 23 and ten detection zones connected through narrow channels to a sample zone located at
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12 24 the center. Buffer solutions with a pH of 10 or 13 were applied to all surfaces of the
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14 25 channels and zones. Different amounts of ethylenediaminetetraacetic acid (EDTA) were
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16 26 added to the reaction zones and a consistent amount of a metal indicator (Eriochrome
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18 27 Black T or Calcon) was added to the detection zones. The total concentrations of Ca^{2+}
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20 28 and Mg^{2+} (total hardness) in the water were measured using a μ PAD containing a buffer
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22 29 solution with a pH of 10, whereas only Ca^{2+} was titrated using a μ PAD prepared with a
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24 30 potassium hydroxide solution with a pH of 13. The μ PADs permitted the determination
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27 31 of Ca^{2+} and Mg^{2+} in mineral water, river water, and seawater samples within only a few
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30 32 minutes using only the naked eye — no need of instruments.
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3 34 1. Introduction
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5 35 Since the first demonstration by Whitesides' group in 2007 [1], microfluidic
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7 36 paper-based analytical devices (μ PADs) have gained a significant amount of attention as
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9 37 an analytical platform. Several publications have recognized μ PADs that are fabricated
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11 38 from paper substrates as suitable for point-of-care testing and on-site analysis [2-4],
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13 39 because they are easy to fabricate, light, inexpensive, disposable, transportable, and
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15 40 instrument-free.
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19 41 As seen in a recent review article on μ PADs [5], several detection methods
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21 42 including colorimetry [6-8], electrochemistry [9-11], fluorometry [12],
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23 43 chemiluminescence [13,14], and electrochemiluminescence [15], have been reported in
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25 44 the past decade. Among them, colorimetry is the most popular detection scheme
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27 45 whereby a scanner or digital camera captures the color image of a μ PAD, followed by a
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29 46 measurement of the color intensity using image-processing software [7,16-19]. For the
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31 47 purpose of point-of-care testing, smart phones are coupled with μ PADs since smart
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33 48 phones are equipped with both a camera and image-processing software in a small
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35 49 package [20].
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39 50 Conversely, the naked eye is a potentially excellent detector, as we demonstrated in
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41 51 a previous study on μ PADs used for acid-base titrations [21]. Using the μ PADs, the
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43 52 endpoint of the neutralization reaction can be visualized by a color change in the
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45 53 detection zone adjacent to the reaction zone containing an equivalent amount of titrant.
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47 54 The first attempt at using the naked eye was in distance-based detection, which was
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49 55 developed by Henry's group [22]. The length-based detection scheme permitted the
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51 56 determinations of glucose, glutathione, nickel ion [22], and lactoferrin [23]. With
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53 57 these methods, the concentration of an analyte was determined by the length of the
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3 58 colored channel that became elongated with increases in its concentration.
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5 59 In our previous research, we demonstrated acid-base titrations on μ PADs consisting
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7 60 of ten reaction zones and ten detection zones [21]. Strong and weak acids and bases
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9 61 could be titrated by selecting an appropriate indicator. The principle would obviously be
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11 62 applicable to other classic titration methods that include chelate titrations, redox
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13 63 titrations, and precipitation titrations. Although the detection scheme is slightly different,
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15 64 iodometry was demonstrated as an example of redox titrations [24]. In addition,
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17 65 acid-base titrations were achieved using another type of paper-based device, which was
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19 66 constructed by stacking two conventional PADs on top of one another and bonding them
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21 67 together [25]. As seen in these articles, μ PAD-based titrations are expected to be an
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23 68 alternative to classic titration techniques since they simplify the operations, reduce
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25 69 consumption of the reagents, facilitate on-site-analysis, and are free from treatment of
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27 70 waste solutions after titrations due to possible incineration disposal.
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34 71 In the present study, we developed μ PADs for chelate titrations of Ca^{2+} and Mg^{2+}
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36 72 using ethylenediaminetetraacetic acid (EDTA) according to the same principle as that
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38 73 previously reported for acid-base titrations. Several factors including selection of the
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40 74 buffer components and the indicator, amounts of chemicals added to the μ PAD, and the
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42 75 channel length were optimized to obtain accurate and precise analytical results. The
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44 76 developed μ PADs were successfully applied to the rapid determinations of Ca^{2+} and
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46 77 Mg^{2+} in mineral water, river water, and seawater samples.
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52 53 79 2. Experimental

54 55 80 *2.1. Chemicals*

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57 81 Deionized water was prepared by means of an Elix water purification system (Millipore
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3 82 Co. Ltd., Molsheim, France). N-Cyclohexyl-3-aminopropanesulfonic acid (CAPS) was
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5 83 obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Magnesium
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7 84 sulfate, calcium chloride dihydrate, sodium hydroxide, iron(III) standard solution (1000
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9 85 ppm), 1-(2-hydroxy-1-naphthylazo)-2-naphthol-4-sulfonic acid sodium salt (Calcon),
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11 86 and 2-hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthoic acid (NN) were
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13 87 purchased from Wako Pure Chemical Industries (Osaka, Japan). Ammonium chloride,
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15 88 ammonium hydroxide solution, potassium cyanide, and Eriochrome Black T (EBT)
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17 89 were obtained from Kanto Chemical (Tokyo, Japan). Ethylenediaminetetraacetic acid,
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19 90 disodium salt (EDTA · 2Na) and methanol were purchased from Sigma–Aldrich (St.
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21 91 Louis, MO, USA). All indicators were dissolved in methanol at a concentration of 0.1
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23 92 (w/v)%. A buffer solution with a pH of 10 was prepared by dissolving appropriate
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25 93 amounts of CAPS in water and adjusting the pH with a sodium hydroxide solution.
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34 95 ***2.2. Fabrication and preparation of the μ PADs***

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36 96 The structure of the μ PAD employed in this study was similar to that reported
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38 97 previously [21] except for the channel length between the reaction and detection zones
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40 98 (Supplementary Material, Fig. S1). Microsoft Office Power Point 2010 was used to
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42 99 design the μ PADs with a sample zone located at the center and ten reaction and
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44 100 detection zones each arranged radially in a 30 × 30 mm square. According to a method
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46 101 developed by Carrilho and co-workers, the designed μ PADs were printed on a sheet of
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48 102 filter paper (200 × 200 mm, Chromatography Paper 1CHR, Whatman, GE Healthcare
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50 103 Lifesciences, United Kingdom) using a wax printer (ColorQube 8570DN, Xerox, CT,
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52 104 USA) [26] followed by heating at 120 °C for 3 min in a drying machine (ONW-300S,
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54 105 AS ONE Corporation, Osaka, Japan). The back of the printing surface was covered with
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3 106 transparent packing tape to prevent solutions from leaking from beneath the μ PAD.

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5 107 Each μ PAD was cut into a piece that measured 30×30 mm, and then 30 μ L of the
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7 108 buffer solution (pH 10 or 13) was added to the sample zone so as to completely fill the
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10 109 surfaces of the channels and zones of the μ PAD (30 μ L is the volume needed to fill all
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12 110 hydrophilic channels and zones [21]). The μ PAD was completely dried, and then a 1 μ L
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14 111 solution of ten different EDTA concentrations was added to each of the reaction zones
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16 112 since the volume needed to fill a reaction zone was determined to be 1 μ L as reported in
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18 113 the previous paper [21], and 0.5 μ L of a 0.1(w/v)% indicator solution was added to each
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20 114 of the detection zones. To accomplish titration, the μ PAD was placed on an acrylic plate
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22 115 holder that was composed of two 30×30 mm plates with four fins at the corners of the
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24 116 holder in order to avoid bending of the μ PAD (Supplementary Material, Fig. S1).
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27 117 Finally, a micropipette was used to introduce 30 μ L of the sample solution from the
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29 118 center of the μ PAD.
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36 120 ***2.3. Principle of chelate titrations using the μ PADs***

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38 121 The principle of chelate titrations is similar to that of classic chelate titrations for Ca^{2+}
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40 122 and Mg^{2+} with EDTA, wherein the total concentration is determined at pH 10, whereas
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42 123 only Ca^{2+} is titrated at pH 13 since Mg^{2+} is masked with hydroxide ion so as not to react
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44 124 with EDTA [27]. However, in contrast to the classic titrations, the concentrations of
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46 125 Ca^{2+} and Mg^{2+} are directly determined by finding the endpoint from the color change
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48 126 without either calibration or calculation when using the μ PAD method. When a sample
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50 127 solution is introduced into the center of the μ PAD, the sample solution penetrates into
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52 128 ten reaction zones. Then, 1 μ L of the sample solution can react with EDTA at each
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54 129 reaction zone since the volume occupying the reaction zone is 1 μ L, which is the same
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130 as the volume of EDTA solutions added to the reaction zones. So, when the amount of
131 metal ions exceeds that of the EDTA in the reaction zones, uncomplexed metal ions
132 penetrate the detection zones, resulting in a color reaction of blue (free form) to purple
133 (complex with metal ion) from the indicator. The concentration of the metal ion is
134 equivalent to the lowest EDTA concentration of the reaction zone among those adjacent
135 to the detection zones with blue color, which indicates no influx of the uncomplexed
136 metal ions. Therefore, in the proposed method, we need to know only the exact
137 concentrations of EDTA added to the reaction zones without further calibration.

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139 **2.4. Practical samples**

140 Bottles of commercially available mineral water were purchased at a local supermarket.
141 Natural water samples were taken from the Asahigawa River, from Kojima Bay, and
142 from the Shimotsui Fishery Harbor in Okayama Prefecture, Japan. The sample solutions
143 were kept in plastic bottles and were determined in our laboratory. The sample solutions
144 were titrated with EDTA via both the classic titration method and the μ PADs in order to
145 evaluate the accuracy and precision of the μ PADs.

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147 **3. Results and Discussion**

148 **3.1. Selection of the reagents**

149 In a classic chelate titration of Ca^{2+} and Mg^{2+} with EDTA, the total concentration is
150 determined using an ammonia buffer at pH 10, whereas only Ca^{2+} is titrated at a pH of
151 13 that is adjusted with KOH to buffer the pH and mask the Mg^{2+} . However, volatile
152 ammonia is unsuitable for use in μ PADs since reagent solutions added to the μ PAD
153 must be dried before use, which results in a change in the pH value due to the

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3 154 volatilization of ammonia.
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5 155 A buffer that would be suitable for use in μ PADs should have a pK_a value that is near
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7 156 the selected pH and should be a stable solid at room temperature. We selected the CAPS
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9 157 buffer to accomplish a pH of 10 since the pK_a value of CAPS is 10.6, whereas KOH was
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11 158 employed for the buffer at pH 13. These buffer components are solid so that the pH
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13 159 could be adjusted to 10 and 13, although exposure to CO_2 under air should be avoided
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15 160 in order to maintain an alkaline pH. Thus, the μ PADs should be stored with either
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17 161 vacuum-sealed or nitrogen-substitution packaging.
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22 162 In a classic chelate titration for Ca^{2+} and Mg^{2+} , EBT is used as the indicator at pH 10,
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24 163 while NN permits the determination of only Ca^{2+} at pH 13 [28]. Both EBT and NN
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26 164 change from blue to purple via the formation of a complex with Ca^{2+} and Mg^{2+} in
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28 165 different pH regions because of their different pK_a values. Therefore, EBT and NN are
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30 166 employed as the indicators at pH 10 and 13, respectively, in classic titrations.
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34 167 In the preparation of the μ PAD for the chelate titration at pH 10, we added 0.1 M
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36 168 CAPS buffer to completely fill the channels and zones. When 0.1% EBT was dropped
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38 169 into the detection zones containing 0.1 M CAPS buffer, the EBT showed a clear blue
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40 170 color. Thus, EBT worked well as the indicator in the presence of the CAPS buffer, pH
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42 171 10, when using the μ PAD. However, when 0.1% NN was dropped into the detection
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44 172 zone of a μ PAD with the pH adjusted to 13 using 0.1 M KOH, no color was observed,
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46 173 despite the presence or absence of metal ions (Fig. 1) — i.e., the NN was unsuitable due
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48 174 to its low color intensity when using the μ PAD. Therefore, instead of NN, we examined
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50 175 EBT and Calcon at pH 13 since their structures are similar to NN, with the exception of
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52 176 a substituted functional group (Supplementary Material, Fig. S2).
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57 177 Surprisingly, the blue color of EBT without Ca^{2+} persisted on the filter paper even in
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3 178 the presence of 0.1 M KOH (Fig. 1), despite a purple color in solution. The purple color
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5 179 of EBT at pH 13 was quite reasonable since the pK_{a2} value was 11.6 [27], because more
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7 180 than 98% of EBT is a trivalent anionic species (purple color). When adding Ca^{2+} and
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9 181 Mg^{2+} into the solution, the purple color persisted, which meant that EBT was unsuitable
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12 182 as an indicator in the solution with a pH of 13.

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15 183 The fact that EBT exhibited a blue color in the presence of 0.1 M KOH implied
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17 184 some interaction between EBT and the paper substrate. One possible explanation could
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19 185 be the interaction of the dissociated hydroxyl group of EBT with the hydroxyl groups of
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21 186 the paper substrate, cellulose. The color of EBT should change from purple to blue
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24 187 when one of the dissociated hydroxyl groups is protonated. Therefore, on the paper
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26 188 substrate, the dissociated hydroxyl group may have interacted with the hydroxyl groups
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29 189 of the cellulose. As a result, EBT may show a blue color on filter paper containing 0.1
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31 190 M KOH, although its color was purple in a solution with a pH of 13.

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34 191 Unfortunately, EBT was unsuitable for use at a pH of 13 since it changed color in
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36 192 the presence of Mg^{2+} (Fig. 1), which could have been due to the complex formation of
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38 193 Mg^{2+} with EBT. Conversely, a change in the color of Calcon was observed only when
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40 194 adding Ca^{2+} , whereas its color remained blue in the presence of Mg^{2+} . Calcon has a pK_{a2}
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42 195 value of 13.5 [27]; i.e., more than 50% of Calcon exists as a blue-colored species at pH
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44 196 13. Therefore, it was quite reasonable that Calcon showed a clear color change from
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46 197 blue to purple in the presence of Ca^{2+} , as noted in Fig. 1. These results suggest that
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48 198 Calcon is the most suitable indicator for the measurement of Ca^{2+} at pH 13 from among
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50 199 these three indicators.

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55 200 Consequently, 0.1 M CAPS buffer was used to attain a pH of 10 and 0.1 M KOH
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57 201 was selected to adjust the pH to 13. For indicators, EBT and Calcon were employed at

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3 202 pH 10 and 13, respectively. Using these reagents, we prepared two μ PADs, one for
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5 203 determining the total concentration of Ca^{2+} and Mg^{2+} (hardness) at pH 10, and the other
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7 204 for determining the concentration of Ca^{2+} at pH 13.
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11 12 206 **3.2. Optimization of the design**

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14 207 In the preliminary stages, we employed the same design as that of the μ PADs for
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16 208 acid-base titrations. Initially, 30 μL of 0.1 M CAPS buffer was introduced into the
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18 209 center of the sample zone to completely fill the channels and zones of the μ PAD. After
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20 210 drying the μ PAD completely, 1 μL each of the 0.01 to 0.1 M EDTA solutions were
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22 211 added to the ten reaction zones in 0.01 M intervals, and 0.5 μL of 0.1% EBT was added
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24 212 to each of the ten detection zones. The titrations showed no clear endpoint for 0.03 M
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26 213 Ca^{2+} solution, e.g., the color changed from blue to purple in the zones for amounts from
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28 214 0.01 to 0.03 M (Supplementary Material, Fig. S3a) while the other zones, however, only
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30 215 showed partial color changes, which led to incorrect results. The reading error became
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32 216 most apparent at the level of 0.08 M of Ca^{2+} where the detection zones up to 0.1 M
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34 217 should have changed color in like manner to the detection zones at the levels of 0.01 to
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36 218 0.06 M (Supplementary Material, Fig. S3b).

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38 219 The incorrect color change was attributed to an incomplete reaction between Ca^{2+} and
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40 220 EDTA due to a slow dissolution rate of the reagents including CAPS and EDTA. In the
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42 221 acid-base titrations using the μ PAD, the acid analyte accelerated the dissolution rate of
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44 222 the base in the reaction zones, and vice-versa, i.e., the dissolution rate of the reagent in
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46 223 the reaction zone was fast enough to react with the sample solution. However, the
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48 224 dissolution rate of the reagents (CAPS and EDTA) was too slow to react with EDTA
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50 225 sufficiently in the chelate titration since the sample solution did not accelerate the
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3 226 dissolution of the reagents.
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5 227 To complete the complex formation in the reaction zones, we delayed the influx of
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7 228 the sample solution from the reaction zone into the detection zone. This was achieved
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9 229 by increasing the channel length between the reaction zone and the detection zone. The
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12 230 channel length between the reaction zone and the detection zone was 0.8 mm in the
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14 231 μ PAD reported previously, whereas it was increased to 1.4, 2.0, and 3.0 mm. The time
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17 232 required to flow from the reaction zone to the detection zone was increased with
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19 233 increases in the length, and was 23.6 ± 2.9 s for the original design compared with 72.2
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22 234 ± 2.9 s for the 3.0 mm length. Thus, the sample solution was impounded in the reaction
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24 235 zone for a longer period of time in the improved design with 3.0 mm than in the original
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26 236 version, which resulted in an increase in the time for dissolution and reaction in the
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29 237 reaction zones.
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31 238 The holder of the μ PAD was also modified to reduce the flow rate of a sample
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33 239 solution. In the acid-base titrations, we employed a holder that covered the surface of
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36 240 the μ PAD and accelerated the flow rate of the sample solution due to the formation of
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38 241 an open channel between the μ PAD and the acrylic cover plate similar to the same
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41 242 principle as the method to accelerate the flow rate in μ PADs using razor-crafted open
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43 243 channels [29]. Conversely, in the chelate titration, we needed to reduce the flow rate
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45 244 to obtain a sufficient amount of time to dissolve the reagents in the reaction zone.
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48 245 Therefore, the design of the holder was changed so as not to cover the flow channels of
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51 246 the μ PAD (Supplementary Material, Fig. S1).
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54 248 **3.3. Determination of Ca^{2+} and Mg^{2+}**

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57 249 Using the improved design, the μ PAD for the chelate titrations of Mg^{2+} and Ca^{2+}
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3 250 was prepared by completely filling the channels and zones with 30 μL of 0.1 M CAPS
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5 251 at pH 10, and then by adding 1 μL of an EDTA solution in increments of 0.01 to 0.1 M
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7 252 to the reaction zones and 0.5 μL of 0.1% EBT to the detection zones. Two mixtures, 10
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10 253 mM Mg^{2+} + 10 mM Ca^{2+} and 30 mM Mg^{2+} + 30 mM Ca^{2+} , were titrated using the
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12 254 μPADs , as shown in Figs. 2a and 2b. The endpoints were observed in the detection
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14 255 zones by the blue color adjacent to the reaction zones containing the lowest
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16 256 concentration of EDTA, which corresponded to 20 mM, as shown in Fig. 2a and 60 mM
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19 257 in Fig. 2b. These results indicated that the μPADs permitted successful titrations for the
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21 258 determination of the total concentrations for Mg^{2+} and Ca^{2+} . In this study, the limits of
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23 259 quantification (LOQ) was defined as the lowest concentration determined by the μPADs
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26 260 whereas the limits of detection (LOD) was defined as the lowest concentration that
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29 261 changes the color of the detection zone adjacent to the reaction zone without EDTA.
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31 262 The LOD and LOQ were 0.5 mM for a total concentration of Mg^{2+} and Ca^{2+}
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33 263 (Supplementary material, Fig. S4). The μPADs for the titration of Ca^{2+} at pH 13 were
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36 264 prepared by completely filling the channels and zones with 0.1 M KOH instead of 0.1
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38 265 M CAPS buffer, and then by adding 1 μL of an EDTA solution in increments of 0.01 to
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41 266 0.1 M to the reaction zones and 0.5 μL of 0.1% Calcon to the detection zones. The same
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43 267 sample solutions in Figs. 2a (10 mM Mg^{2+} + 10 mM Ca^{2+}) and 2b (30 mM Mg^{2+} + 30
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45 268 mM Ca^{2+}) were titrated using the μPAD , and the results are shown in Figs. 2c and 2d,
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48 269 which show that the sample solutions contained 10 mM Ca^{2+} and 30 mM Ca^{2+} ,
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51 270 respectively, and both were determined using two different μPADs — one for total
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53 271 Mg^{2+} and Ca^{2+} and the other only for Ca^{2+} .

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55 272 The μPADs for measuring 1 to 10 mM of Ca^{2+} with an interval of 1 mM were also
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58 273 prepared using KOH to adjust the pH to 13, and by adding 0 to 9 mM of EDTA

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3 274 solutions to the reaction zones with Calcon as the indicator. When samples containing 1
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5 275 to 5 mM Ca^{2+} were measured using the μPADs , the solutions containing 1 to 3 mM Ca^{2+}
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7 276 showed a color change only in the detection zone adjacent to the reaction zone
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10 277 containing no EDTA. Conversely, 4 mM and 5 mM Ca^{2+} solutions did exhibit the
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12 278 correct color change in the detection zones, i.e., the interval of 1 mM of Ca^{2+} was
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14 279 measurable in a range of more than 4 mM, even though 1 to 3 mM of Ca^{2+} could not be
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17 280 determined (Supplementary material, Fig. S5). The LOQ (4 mM) of the μPADs for a
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19 281 titration at pH 13 was slightly poorer than that for a titration at pH 10 since the
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21 282 measurable lowest concentration of Ca^{2+} was 4 mM, which was 8-fold higher than its
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23 283 measurable concentration at pH 10 (0.5 mM). Therefore, the μPADs can determine the
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25 284 total concentration of Mg^{2+} and Ca^{2+} in an amount higher than 1 mM, whereas Ca^{2+}
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27 285 concentrations lower than 4 mM are immeasurable. The LOD at pH 13 was 1 mM
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30 286 which was also slightly larger than 0.5 mM at pH 10 due to adsorption of Ca^{2+} on the
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36 288 It was noteworthy that concentrations of more than 4 mM were distinguishable at an
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38 289 interval of 1 mM, and we were able to discriminate between the solutions containing 4
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40 290 mM and 5 mM of Ca^{2+} at pH 13. This fact made it seem quite strange that the interval
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42 291 of 1 mM of Ca^{2+} was measurable in the range of more than 4 mM, although 1 to 3 mM
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44 292 of Ca^{2+} could not be determined. To clarify this phenomenon, we added 0.5 μL of
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46 293 Calcon to the sample, reaction and detection zones at pH 13, and then solutions of 1~5
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48 294 mM of Ca^{2+} were introduced into the sample zone while EDTA was absent from the
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50 295 reaction zones. In the sample zone only, 1 mM Ca^{2+} showed a color change, i.e., 30 μL
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52 296 of 1 mM Ca^{2+} (30 nmol of Ca^{2+}) was completely retained in the sample zone
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55 297 (Supplementary Material, Fig. S6). The color changes spread to the reaction and
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3 298 detection zones as the concentration of Ca^{2+} was increased(Supplementary Material, Fig.
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5 299 S6). These results indicated that Ca^{2+} could not reach the detection zones at
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7 300 concentrations lower than 3 mM at pH 13 even when the reaction zones contained no
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9 301 EDTA. Therefore, free Ca^{2+} was adsorbed onto the paper substrate at a pH of 13, which
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11 302 was probably due to the dissociated hydroxyl groups of cellulose (the pK_a value of
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13 303 glucose is known to be 12.28) [30], although the dissociation constant is unknown. The
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15 304 adsorption of Ca^{2+} in the sample zone and in the reaction zones would have been
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17 305 saturated by the 3 mM of free Ca^{2+} since concentrations higher than 4 mM were
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19 306 measurable at pH 13.
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24 307 The μPADs employed in Fig. 2 were prepared for determining a concentration range
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26 308 of 10 to 100 mM. The μPADs need to be prepared so as to allow the sample
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28 309 concentration to fall into the range of the EDTA concentrations added to the reaction
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30 310 zones. When we measure a few mM of a sample, 1 to 10 mM EDTA solutions must be
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32 311 added to the reaction zones, resulting in data with 1 significant figure. Conversely, if the
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34 312 concentration of the sample contains 10 to 20 mM of the metal ion, we can prepare the
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36 313 μPAD using the EDTA solutions with 10 to 20 mM at an interval of 1 mM. In this case,
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38 314 we can determine the concentration of the sample to 2 significant figures. Therefore the
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40 315 concentrations obtained by the μPADs are digital values restricted by the concentration
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42 316 interval of EDTA added to the reaction zones. This feature of the μPAD requires no
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44 317 skill in order to find the endpoint and avoid misreading in the titrations since the
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46 318 concentration can be judged only by the position of the detection zone indicating the
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48 319 endpoint.
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55 320 The developed μPADs have several advantages superior to commercially available
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57 321 paper strips for measuring the total hardness of water. With paper strips, the hardness of
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3 322 a sample is measured by a color intensity generated by the formation of a colored
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5 323 complex. To determine the hardness, we have to compare the result with a reference
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7 324 which represents the relationship between the color intensity and the concentration
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10 325 range, e.g., a paper strip reports that the hardness of the sample is <55, >90, >180, >270,
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12 326 >360, or >445 ppm. This method could cause mistakes originating from the subjective
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14 327 view of an observer. In addition, a paper strip cannot determine the concentrations of
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16 328 Ca^{2+} and Mg^{2+} independently. Conversely, the μPADs permit the determination of the
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18 329 exact concentrations for both Ca^{2+} and Mg^{2+} and lead to no reading errors since the
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20 330 judgement of the color change is clearer than the comparison of the color intensity in
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22 331 the paper strip.
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28 333 **3.4. Interference**

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31 334 In the classic titrations, heavy metal ions influence the titration results since several
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33 335 metal ions form chelates with EDTA and the indicator. Therefore, the interference of a
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35 336 heavy metal was investigated using Fe^{3+} as a possible interference in the determination
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37 337 of natural water samples. Sample solutions with 1, 10, and 100 ppm Fe^{3+} were applied
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39 338 to the μPADs with 0 to 90 mM of EDTA in the reaction zones at 10 mM interval using
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41 339 EBT as the indicator (pH 10). The solutions of 1 ppm Fe^{3+} changed the color of EBT in
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43 340 the detection zone when the reaction zones contained no EDTA, despite a much lower
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45 341 concentration (corresponding to 18 μM) of Fe^{3+} than the LOD of Ca^{2+} and Mg^{2+} (0.5
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47 342 mM). Obviously, Fe^{3+} is a serious interfering substance for the μPAD .
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53 343 In the classic titration, KCN is frequently employed for masking many heavy metal
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55 344 ions [27]. Thus, we added 30 μL of 10% KCN in all channels and zones after drying 0.1
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57 345 M CAPS. Sample solutions of 40 mM Ca^{2+} with 100 to 500 ppm Fe^{3+} were titrated
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3 346 using the μ PADs treated with KCN. In the presence of KCN, no interference was
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5 347 observed up to 500 ppm (8.95 mM) of Fe^{3+} . To apply the μ PADs to the determination of
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7 348 practical samples, especially to natural water samples, the addition of KCN will be
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9 349 effective to exclude the interference of heavy metal ions since the concentration levels
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12 350 in natural water are known to be as low as 0.5–50 μM [31].
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16 17 352 **3.5. Practical analysis**

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19 353 In order to demonstrate practical applicability of the μ PADs, commercially available
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21 354 mineral water, river water, and seawater were titrated using both a classic chelate
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23 355 titration and the μ PADs. The results are summarized in Table 1. The total
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25 356 concentrations of Mg^{2+} and Ca^{2+} in the mineral water samples were successfully
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27 357 determined at 3.2 and 19 mM using classic titration and 3 and 20 mM via the μ PADs,
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29 358 which was in good agreement. Using the μ PADs, the concentration of Ca^{2+} in mineral
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31 359 water 1 was below the detectable concentration limit at pH 13, and the concentration of
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33 360 Mg^{2+} in mineral water 2 were also undetectable due to its low concentration (both the
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35 361 hardness and the concentration of Ca^{2+} were found to be 20 mM).

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39 362 The river water samples were taken at three different sites near the estuary of the
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41 363 Asahigawa River: at a point 0.2 km away from the estuary (Site 1), at the estuary (Site
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43 364 2), and at the bay (Site 3). The titration results using the μ PADs are shown in Fig. S7 of
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45 365 the Supplementary Material. For these samples, the total concentrations obtained by the
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47 366 μ PADs (5 mM) were also consistent with those determined by classic titration (Table 1),
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49 367 although the concentrations of Ca^{2+} were too low to be determined by the μ PADs.
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55 368 The results for the determination of the seawater sample collected at the Shimotsui
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57 369 fishery harbor are shown in Fig. 3. As shown in Fig. 3a, when using the μ PAD at pH
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3 370 10 without KCN, the color in all the detection zones changed to purple. To prevent
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5 371 interference from heavy metal ions, we added 30 μL of 10% KCN, as described in the
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7 372 section 3.4. Fig. 3b shows the titration at pH 10 by the μPAD containing KCN. The
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9 373 endpoint was clearly found at a concentration of 40 mM, which meant the total
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11 374 concentration of Mg^{2+} and Ca^{2+} was 40 mM. As shown in Table 1, the total
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13 375 concentration of Mg^{2+} and Ca^{2+} obtained by classic titration were 44 mM, which was in
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15 376 good agreement with the results shown in Fig. 3b. The concentration of Ca^{2+} determined
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17 377 via the μPAD was 10 mM (Fig. 3c), which also was consistent with the results of classic
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19 378 titration (11 mM). Furthermore, we prepared the μPAD for a concentration range of
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21 379 from 4 to 12 mM in order to precisely determine the concentration of Ca^{2+} , and the
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23 380 result at 11 mM (Fig. 3d) was similar to that via classic titration. The titrations were
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25 381 attempted 5 times, and four of these resulted in 11 mM with one resulting in 12 mM
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27 382 (mean value, 11.2 mM). These results suggest that the μPADs are reliable for the
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29 383 determination of Ca^{2+} and Mg^{2+} in practical samples since the average concentrations of
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31 384 Ca^{2+} and Mg^{2+} in ocean water are 10.3 and 53.2 mM, respectively [31].

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34 385 It should be emphasized that the results of the μPADs represent excellent
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36 386 reproducibility as noted by the standard deviations in Table 1 of zero with the exception
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38 387 of the concentration of Ca^{2+} in the seawater sample. Even for the seawater sample, only
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40 388 one in five measurements showed a 1 mM difference, although the origin of the error is
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42 389 unclear. The μPADs certainly make the operations of the titrations for Ca^{2+} and Mg^{2+}
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44 390 easy and reduce the experimental errors due to simple detection of the endpoint.
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55 392 4. Conclusions

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57 393 We demonstrated successful titrations of Ca^{2+} and Mg^{2+} using μPADs prepared

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3 394 for measurements at pH 10 and pH 13. The μ PADs were superior to a commercially
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5 395 available paper strip for a measurement of the hardness in water samples, because we
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7 396 were able to determine the exact concentrations of both Ca^{2+} and Mg^{2+} using the μ PADs
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9 397 while the commercially available paper strip permitted only a range of hardness that
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11 398 corresponded to the total concentration of Ca^{2+} and Mg^{2+} . In addition, the results of the
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13 399 μ PADs were digitized, which lessened the chances for a misreading.
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17 400 The results of this research underscored several unique characteristics of filter paper.
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19 401 (i) The amount of time required for dissolution of the buffer components and chelating
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21 402 reagents deposited in the zones was a key to the success of the titrations, and increasing
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23 403 the channel length between the reaction and detection zones efficiently dissolved the
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25 404 reagents and helped complete the complex formation reaction. (ii) At pH 13, the EBT
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27 405 showed a blue color, even though it was purple in solution, which could be attributed to
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29 406 the interactions between EBT and the hydroxyl groups of cellulose. (iii) The adsorption
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31 407 of Ca^{2+} occurred at pH 13 due to the partial dissociation of the hydroxyl groups of
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33 408 cellulose under the strong alkaline conditions, and, as a consequence, it was difficult to
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35 409 determine the concentrations of Ca^{2+} that were lower than 4 mM.
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41 410 We also successfully analyzed practical samples of mineral water, river water, and
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43 411 seawater. The concentrations determined by the μ PADs were in good agreement with
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45 412 those obtained by classic titrations when KCN was employed as a masking agent.
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47 413 Therefore, the μ PADs would be more convenient than classic titration for
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49 414 determinations of Ca^{2+} or Mg^{2+} in terms of lightness, less expense, transportability, and
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51 415 ease of operation.
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57 417 **Acknowledgements**
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513 Table 1. Determination of Ca²⁺ and Mg²⁺ in practical samples

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		Classic Titration*	μPAD*
Mineral water 1	Mg ²⁺ + Ca ²⁺ / mM	3.2±0.0089	3±0
	Ca ²⁺ / mM	2.2±0.0089	Undetectable
Mineral water 2	Mg ²⁺ + Ca ²⁺ / mM	19.1±0.0084	20±0
	Ca ²⁺ / mM	16±0.011	20±0
River water (site 1)	Mg ²⁺ + Ca ²⁺ / mM	5.3±0.0071	5±0
	Ca ²⁺ / mM	1.2±0.011	Undetectable
River water (site 2)	Mg ²⁺ + Ca ²⁺ / mM	5.3±0.0055	5±0
	Ca ²⁺ / mM	1.2±0	Undetectable
Sea water (site 3)	Mg ²⁺ + Ca ²⁺ / mM	5.5±0.0089	5±0
	Ca ²⁺ / mM	1.4±0.0071	Undetectable
Sea water	Mg ²⁺ + Ca ²⁺ / mM	44±0.0071	40±0
	Ca ²⁺ / mM	11±0.0045	11.2±0.45

515 *Mean value ± standard deviation of five titrations.

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5 518 Figure 1. Color of indicators at pH 13 in solution and on a μ PAD. (a) The
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7 519 concentrations of the indicators were 0.1(w/v)% in methanol. Initially, 30 μ L of KOH
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9 520 was applied to the μ PAD so as to completely fill the channels and zones. After drying,
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11 521 0.5 μ L of NN, EBT, or Calcon was added to each of the detection zones, and then 0.5
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13 522 μ L of either a 10 mM Ca^{2+} solution or a 10 mM Mg^{2+} solution was dropped into the
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15 523 detection zones.
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22 525 Figure 2. Chelate titration of Ca^{2+} using the μ PAD. The distance between the reaction
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24 526 zone and the detection zone was 3 mm. (a) 10 mM Ca^{2+} + 10 mM Mg^{2+} at pH 10, (b) 30
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26 527 mM Ca^{2+} + 30 mM Mg^{2+} at pH 10, (c) 10 mM Ca^{2+} + 10 mM Mg^{2+} at pH 13, (d) 30
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28 528 mM Ca^{2+} + 30 mM Mg^{2+} at pH 13. EDTA solutions with 0 to 90 mM were added to the
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30 529 reaction zones at the interval of 10 mM. The numbers of the zones indicate the
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32 530 concentrations (mM) of EDTA solutions added to the reaction zones. Buffer, (a) and (b)
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34 531 0.1 M CAPS, (c) and (d) 0.1 M KOH; indicator, (a) and (b) EBT, (c) and (d) Calcon.
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36 532 The color of each detection zone is indicated by B (blue) or P (Purple).
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43 534 Figure 3. Titrations of a seawater sample. Conditions: (a) pH 10 without KCN, indicator,
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45 535 EBT; (b) pH 10 with KCN, indicator, EBT; (c) and (d) pH 13 with KCN, indicator,
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47 536 Calcon. In (c), EDTA solutions with 0 to 90 mM were added to the reaction zones at the
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49 537 interval of 10 mM. In (d), EDTA solutions with 0 to 12 mM were added to the reaction
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51 538 zones in intervals of 1 mM. The numbers of the zones indicate the concentrations (mM)
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53 539 of EDTA solutions added to the reaction zones. The concentrations of EDTA added to
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55 540 the reaction zones are indicated by the number printed on the μ PADs. The color of each
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3 541 detection zone is indicated by B (blue) or P (Purple).
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Figure 1

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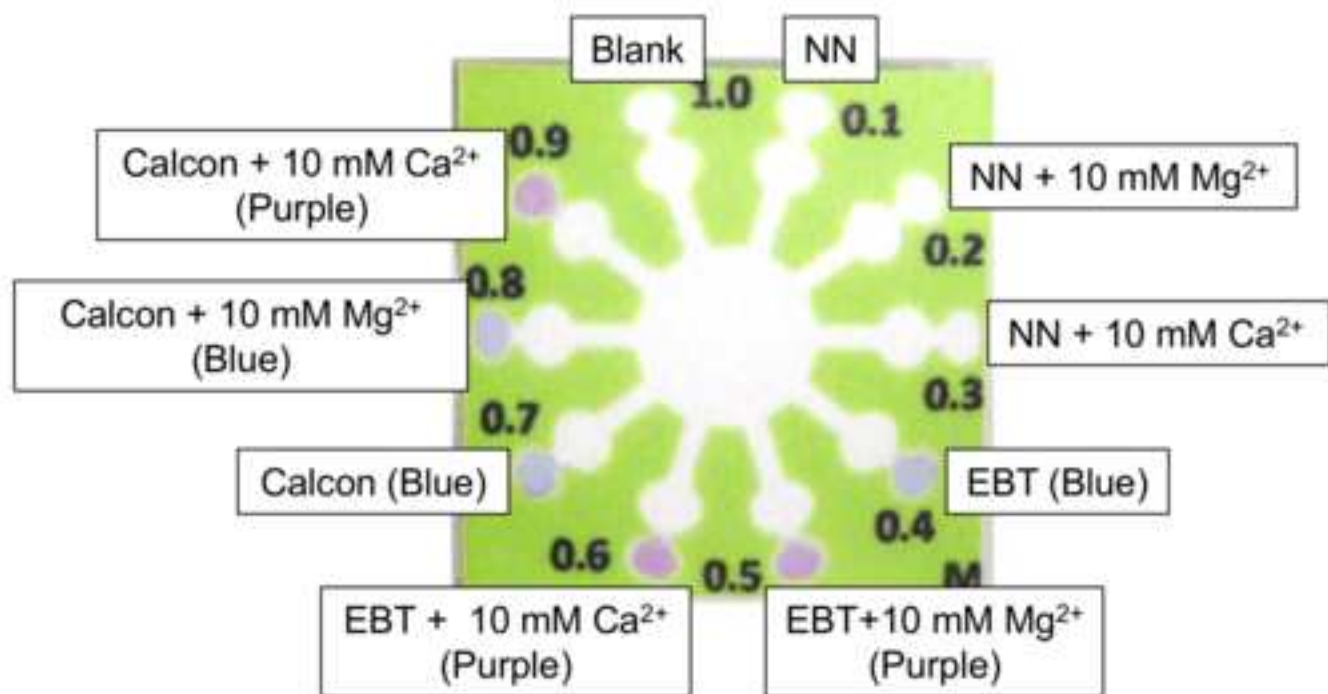


Figure 3
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