

## Article

# Direct Determination of Readily Accessible Quercetin-Reactive Aluminum and Ascorbate-Reducible, Ferrozine-Reactive Iron in Cosmetic Emulsions

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## Abstract

Aluminum in antiperspirants is relevant to formulation control and compliance, whereas reactive iron can promote oxidative instability in emulsions. We developed smartphone-based colorimetric assays relying on metal–reagent complexation directly in oil-in-water (O/W) emulsions, without digestion or extraction. The aluminum assay measures quercetin-reactive aluminum from aluminum chlorohydrate (ACH). Calibration data were fitted by linear regression ( $R^2 \geq 0.971$ ). Acceptable accuracy (relative bias within  $\pm 8.5\%$ ) and intermediate precision ( $CV \leq 9.4\%$ ) were obtained upon quality control (QC) analysis (above the LLOQ). Relative bias in commercial antiperspirants and corresponding in-house preparations was within  $\pm 15.2\%$ . The iron assay measures ascorbate-reducible, ferrozine-reactive iron. Its calibration data were also fitted by linear regression ( $R^2 \geq 0.981$ ), while QC analysis showed acceptable accuracy (relative bias within  $\pm 13.7\%$ ) and intermediate precision ( $CV \leq 12.6\%$ ). Comparison with portable energy-dispersive X-ray fluorescence (EDXRF) yielded a regression slope of 1.14 and an intercept of  $0.40 \mu\text{g/g}$ , suggesting a proportional difference and a possible constant component. Common chelators and certain metals interfered with both assays. The effect of interferents in commercially relevant concentration was, however, minimal (relative bias within  $\pm 15.2\%$  for Al and at  $4.5\%$  for a Co/Cu/Fe-fortified model emulsion) after matrix matching or dilution. Although mostly inferior in terms of technical characteristics compared to established instrumental methods, this methodology is sufficiently accurate, precise and selective under the defined conditions.

**Keywords:** digital image colorimetry; cosmetics analysis; antiperspirants; X-ray fluorescence; extraction-free emulsion analysis; quercetin; ferrozine

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## 1. Introduction

Aluminum salts are widely used in cosmetics as antiperspirant actives [1]. Despite their long-standing, widespread use, concerns regarding potential toxicity persist. They are suspected of acting as genotoxic agents [2,3] and of interfering with estrogen receptor-mediated signaling in breast cancer cells, potentially influencing cancer growth [4]. A link to Alzheimer’s disease has also been proposed [5]. However, these toxicological claims remain a subject of debate [6]; the German Federal Institute for Risk Assessment (BfR)

states that current studies have not established a conclusive causal link between antiperspirant use and disease [6].

The 2020 SCCS opinion concluded that cosmetic exposure did not add significantly to the systemic aluminum burden from other sources [7]; the SCCS considered aluminum compounds safe in deodorants and antiperspirants at equivalent elemental Al concentrations up to 6.25% in non-spray products and 10.60% in spray products.

Due to its potential toxicity and safety limits, the quantification of aluminum in cosmetics is important for ensuring product safety and regulatory compliance. With few notable exceptions relying on electrochemical or paper-based colorimetric methods [8–10], aluminum is commonly quantified by high-performance liquid chromatography (HPLC) [11], atomic absorption spectroscopy (AAS) [12], or inductively coupled plasma optical emission spectrometry (ICP-OES) [13], often after substantial sample pretreatment. Although the latter methods offer high sensitivity and selectivity, they also require expensive equipment and trained personnel. Alternatively, aluminum can be quantified colorimetrically through the formation of colored aluminum–quercetin complexes [14].

Iron oxides are widely employed in cosmetics as pigments and can provide photoprotective effects [15]. These are considered safe for cosmetic use when they meet specific purity requirements [16]. Soluble or weakly bound, redox-active iron can promote oxidative instability and rancid off-odors [17]. Potential adverse effects of lipid oxidation products have been reviewed in the context of dietary exposure [18] and are relevant to food emulsion stability. Total iron is commonly determined by AAS, ICP-OES or X-ray fluorescence (XRF) [19–23]. AAS and ICP-OES generally require digestion, whereas XRF involves limited pretreatment. Nevertheless, these techniques require specialized instrumentation; XRF may also exhibit matrix effects and comparatively high quantification limits in low-iron samples. Ferrozine colorimetry is a sensitive and inexpensive alternative for soluble or reducible iron, but can be affected by chelating agents [24].

We propose and validate adapted methodologies for the quantification of readily accessible, quercetin-reactive aluminum from aluminum chlorohydrate (ACH) and of ascorbate-reducible, ferrozine-reactive iron. The novelty lies in the adaptation of established chromogenic reactions to direct, extraction- and digestion-free analysis within O/W emulsions, using widely available smartphone imaging. The assays have lower analytical performance than established instrumental techniques and require matrix-matched calibration or dilution. They measure the specified reactive fractions, not total elemental content. The proposed workflow is thus designed as a fit-for-purpose screening and quality control tool for the specified reactive fractions.

## 2. Materials and Methods

### 2.1. Materials and Stock Solutions

All experiments used water deionized through sequential cation- and anion-exchange columns (1665 Ion Exchanger, Osmo Membrane Systems GmbH, Leonberg, Germany). Stock solutions were stored at room temperature and in the dark. Iron salt solutions were freshly prepared on the day of the assay. Aluminum chlorohydrate (ACH) was supplied as a 50% *w/w* aqueous solution (Chlorohydrol) by Elementis Specialties (Ascot, UK) and contained 12.2 to 12.7% *w/w* aluminum (Al). In this manuscript, all ACH concentrations are expressed on a dry-active basis, using an Al content of 24.8% *w/w*. An O/W base cream (BC), was used as the matrix for all standards and dilutions: Aqua 81.7% *w/w*, Paraffinum Liquidum 10% *w/w*, Cetearyl Alcohol 4.0% *w/w*, Glyceryl Stearate (and) PEG-100 Stearate 3.0% *w/w* (PEG, polyethylene glycol), Phenoxyethanol 1.0% *w/w*, Xanthan Gum 0.2% *w/w*, Ethylhexylglycerin 0.1% *w/w*. All BC preparations had viscosities in the range of 270,000–400,000 mPa·s, pH in the range 5.1 to 5.7 and a density of 1.0 g/mL. Commercial products were manufactured in-house under contract and had known nominal

metal concentrations. Quercetin 95% (UV)/ 98% (HPLC) was a *Sophora Japonica* Extract from Kingherbs Limited (Changsha, China). Quercetin stocks in ethanol (HPLC grade, Fisher Scientific, Schwerte, Germany) were 3.33 mg/mL for microwell assays and 6.66 mg/mL for paper assays. The iron/ferrozine kit from Biosis (Athens, Greece), stored at 4 °C, included Reagent R1 (an ascorbic acid solution at pH 5) and Reagent R2 (a ferrozine solution). The reagents reduce accessible  $\text{Fe}^{3+}$  and detect  $\text{Fe}^{2+}$  that becomes available under the assay conditions. American Chemical Society (ACS)-grade iron(III) chloride hexahydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) from Merck (Darmstadt, Germany) was used to prepare iron standards from a 2.47 mg/mL aqueous stock solution.

The water-soluble organic interferents tested were potassium sorbate > 99% (Nantong Acetic Acid Chemical Co. Ltd., Nantong, China; 12.5% *w/v* stock), sodium gluconate > 99% (Jungbunzlauer Suisse AG, Basel, Switzerland; 8.3% *w/v* stock) sodium benzoate > 99% (Lanxess Chemical B.V., Rotterdam, The Netherlands; 8.7% *w/v* stock), citric acid monohydrate > 99.5% (Laiwu Taihe Biochemistry Co., Ltd. Jinan, China; 13.5% *w/v* stock), ethylenediaminetetraacetic acid disodium salt dihydrate (sodium EDTA dihydrate)  $\geq$  99.0% (Shijiazhuang Jackchem Co., Ltd., Shijiazhuang, China; 8.6% *w/v* stock), PURAC® lactic acid 80% (Corbion N. V., Gorinchem, The Netherlands; 3.3% *w/v* (active lactic acid) stock) and acetic acid  $\geq$  99.8% (VWR S.r.l., Milano, Italy; 3.3 *w/v* stock). An additional interferent tested was a mixture of fatty acids with the commercial name Palmera B1802 (51.0% hexadecanoic acid, 47.6% octadecanoic acid, 0.8% eicosanoic acid, 0.2% dodecanoic acid and 0.4% tetradecanoic acid) from KKK OLEO (Petaling Jaya, Malaysia) which was dissolved in ethanol (at 6.55% *w/v*). Moreover, in the Fe assay the following interferents were tested, using stocks prepared in water: Magnesium sulfate heptahydrate  $\geq$  99.5% (Honeywell, Charlotte, NC, USA; 12.0% *w/v* stock), potassium chloride European Pharmacopeia (Ph. Eur.) (Carlo Erba Reagents, Cornaredo, Italy; 5.9% *w/v* stock) sodium chloride > 99.4% (Kalamarakis-Kalas S.A. Athens, Greece; 4.7% *w/v* stock), sodium sulfate 99.8% (Lenzing AG, Lenzing, Austria; 5.6% *w/v* stock), zinc sulfate heptahydrate  $\geq$  99% (Merck, Darmstadt, Germany; 13.7% *w/v* stock), calcium chloride dihydrate  $\geq$  99% (Solvay Chemicals, Brussels, Belgium; 10.9% *w/v* stock), magnesium carbonate Ph. Eur. (J. T. Baker, Phillipsburg, NJ, USA; 0.15% *w/v* stock), zinc chloride > 98% (Merck, Darmstadt, Germany; 4.5% *w/v* stock), ACS grade cobalt(II) nitrate hexahydrate (Merck Darmstadt, Germany; 0.1% *w/v* stock) and ACS grade copper(II) sulfate pentahydrate (Sigma, Burlington, MA, USA; 0.5% *w/v* stock).

## 2.2. Instrumentation and Equipment

Aluminum assay images were captured using an iPhone 6s (Apple Inc., Cupertino, CA, USA), while iron assay images were captured with a Galaxy S21 FE (Samsung Electronics Co., Ltd, Gyeonggi-do, South Korea). Both cameras provided 12 megapixel images ( $4032 \times 3024$  pixels). Photographs were taken inside a white photographic box (cube of an edge of 23 cm from Shenzhen PULUZ Technology Limited, Shenzhen, China) equipped with an opening at the top and two light-emitting diode (LED) strips, positioned along opposite edges. Emulsions were prepared using a Silverson L5M-A homogenizer equipped with a Square Hole High Shear Screen (homogenization for 10 min at 7500 rpm). Creams were diluted with BC by manual mixing or a handheld mini mixer. pH was measured with an Inolab pH Level 1 meter and WTW Sentix 41 electrode (both from Xylem Analytics, Weilheim, Germany), calibrated with standard buffers. Viscosity was measured at 20 °C and 0.6 rpm with a Brookfield DV-E viscometer and S94 Helipath T-bar spindle, both from Ametek Brookfield (Middleborough, MA, USA).

## 2.3. Assay in Microwells

The direct digital image colorimetry workflow for aluminum and iron quantification in cosmetic emulsions is presented in Figure S1 (Supplementary Materials).

### 2.3.1. Determination of Readily Accessible Quercetin-Reactive Aluminum

Standards (corresponding to nominal concentrations of 0, 39, 62, 70, 98, 131, 196  $\mu\text{g Al/g}$  emulsion) or quality controls (QCs) were prepared by diluting a 4.4% *w/w* ACH stock emulsion in BC, using BC as diluent. Nominal aluminum concentrations were calculated from the amount of ACH added and its aluminum content. Unknown samples were prepared by diluting commercial products using BC as diluent, to bring the measured color intensity within the linear calibration range. Water was added to facilitate homogenous incorporation. For example, for a 1:250 dilution, 100 mg of sample was briefly mixed with 7.14 mL of deionized water before incorporation into 17.76 g of BC. Standards and QCs were prepared from the 4.4% stock emulsion, using the same mixing sequence and water-to-emulsion ratio. Materials were weighed using a calibrated analytical balance, and mixtures were thoroughly homogenized with a handheld mini mixer. Subsequently, 1 g of each standard, QC or diluted unknown sample was weighed into vials, to which 240  $\mu\text{L}$  of a 3.33 mg/mL quercetin solution in ethanol and 100  $\mu\text{L}$  of deionized water were added to facilitate mixing. After thorough blending, samples were transferred to detachable microwells and excess cream was removed with a plastic spatula to level the surface.

### 2.3.2. Determination of Ascorbate-Reducible, Ferrozine-Reactive Iron

To assess linearity, twelve iron standards corresponding to 0.8, 1.2, 1.8, 2.4, 2.8, 3.2, 3.6, 4.0, 4.6, 5.0, 6.6, and 8.0  $\mu\text{g Fe/g}$  emulsion plus the blank were prepared in BC from a 2.47 mg/mL  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  stock solution. Iron concentrations represent nominal amounts added as  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , expressed as elemental Fe. QCs were prepared in BC, and, when necessary, diluted with BC to bring the measured color intensity within the linear calibration range. Subsequently, 1 g of each standard or QC was weighed into a vial. Each sample received 200  $\mu\text{L}$  of R1/R2 (1:2) and was mixed thoroughly with a plastic spatula. A portion of the resulting cream was used to fill a detachable well. The surfaces were leveled with a plastic spatula, and the detachable well strips were arranged in the microtiter plate frame for photo capturing.

### 2.4. Aluminum Assay on Filter Paper

A total of 12  $\mu\text{L}$  of a quercetin stock solution (6.66 mg/mL in ethanol) was applied in duplicate on filter paper strips; a thin layer of emulsion was then applied on the quercetin spots and was pressed firmly using a cover glass to spread the emulsion into a thin, even layer. Excess emulsion around the spots was cleared using the cover glass. This process has been described in detail elsewhere [25–27]. Photo capturing of the colored spots followed, to quantify aluminum, as below.

### 2.5. Photo Capture, Image Analysis and Data Processing

For photo capturing, the mobile phone was placed over the opening at the top of the white photographic box, at a distance of 23 cm from its base. Images were captured using default camera settings without flash. The standards and samples, whether inside the detachable wells or on the filter paper, were placed flat inside the box, in a straight line and parallel to the LED light source, and never directly under the lights.

Aluminum assay images in microwells were captured 8–10 min after quercetin addition to the first sample, to allow for sample processing. In the filter assay, images were captured around 5 min after emulsion application to the reagent-loaded paper. Measurements within 20 min after addition/application also gave satisfactory results. Iron assay

images were captured 35 min after ferrozine addition to accommodate the slower complexation reaction; measurements between 35 and 65 min also gave satisfactory results.

Digital images were processed using ImageJ software (version 1.54) by red–green–blue (RGB) analysis of a homogeneous and uniform area in the center of the well or a spot of approximately 400–600 total pixels. RGB values were transferred to Excel to construct color intensity calibration curves and calculate the analyte content in unknown samples. The blue channel was generally used for aluminum determination, though green or average components were also employed. For iron determination, the red channel was found to be optimal, while blue and average components frequently also provided satisfactory linearity. Standards, QCs and unknowns were positioned side-by-side in the same image under fixed LED illumination; therefore, a new within-image, matrix-matched calibration was generated for each acquisition rather than transferring a calibration equation between devices or sessions.

## 2.6. Method Validation

Method performance was evaluated according to the International Council for Harmonization (ICH) Q2(R2) principles for analytical-procedure validation and fitness for intended purpose [28]. Acceptance criteria of  $\pm 15\%$  for accuracy and  $\leq 15\%$  for intermediate precision, relaxed to  $\pm 20\%/\leq 20\%$  at the LLOQ, were adopted as fit-for-purpose criteria.

In the microwell assay, each individual calibration curve included one measurement per standard concentration. Replication was assessed across calibration curves prepared on different days. Each calibration curve used at least five standards in BC and was fitted by linear regression in Excel. Calibration curves were used to calculate unknown sample concentrations when  $R^2 \geq 0.967$  for Al and 0.981 for Fe, and the accompanying QC results met the specified acceptance criteria. Calibration-model performance was assessed from the relative bias of back-calculated standard concentrations and residual analysis. Intermediate precision was evaluated using independent analytical determinations of QC samples performed in separate analytical runs on different days and was expressed as a coefficient of variation (CV%), where  $CV (\%) = 100 \times SD/\text{mean}$  and SD is standard deviation. Each determination comprised a separate aliquot subjected independently to reagent addition, mixing, well filling, image acquisition, and image analysis. The reported  $n$  represents the total number of independent determinations at each QC concentration. Accuracy was evaluated using independently prepared fortified QC and recovery samples and expressed as relative bias (%), where  $\text{relative bias } \% = 100 \times (\text{mean measured concentration} - \text{nominal concentration})/\text{nominal concentration}$ . Agreement of the iron assay with an established analytical method, XRF, was, furthermore, evaluated separately by regression analysis. To determine the sensitivity of the assay, theoretical Limits of Detection (LOD) and Quantification (LOQ) were initially calculated based on the standard deviation of the y intercept and the absolute slope of the linear regression curve, using the Regression function of Microsoft Excel:  $LOD = 3.3 \times \text{standard deviation of the y-intercept/slope}$  and  $LOQ = 10 \times \text{standard deviation of the y-intercept/slope}$ . Multiple independent calibration curves were employed in the calculation. In contrast, the experimental Lower Limit of Quantification (LLOQ) was the lowest spiked concentration added into the emulsion matrix that met the criteria for accuracy (bias within  $\pm 20\%$ ) and intermediate precision ( $CV \leq 20\%$ ) across multiple analytical runs on different days. It defined the lower end of the working range of the assay.

### 2.7. XRF Analysis

For portable energy-dispersive X-ray fluorescence (EDXRF), approximately 200 mg of cream was spread as a thin layer over a Prolene® membrane on an XRF cup. Triplicate preparations at each iron concentration were dried at room temperature for 48–72 h, yielding approximately 60–65 mg and a surface density <13 mg/cm<sup>2</sup>; these were treated as thin targets [29]. Samples were irradiated using a portable EDXRF system (Amptek's Experimenter's XRF Kit; Amptek Inc., Bedford, MA, USA), consisting of an X-123 complete spectrometer with a silicon positive-intrinsic-negative (Si-PIN) detector (25 mm<sup>2</sup>, resolution 0.160 keV at 5.9 keV (Mn K $\alpha$ )) and a Mini-X Universal Serial Bus (USB)-controlled X-ray tube (50 kV/100  $\mu$ A) with a silver (Ag) anode. A fixed 45° XRF geometry was employed, with a 1 cm distance between the sample and the experimental setup. A 2 mm collimator and a 25  $\mu$ m Al filter (in the X-ray tube window) were used. Measurement conditions were set at 40 kV and 20  $\mu$ A, with acquisition times ranging from 1000 to 1500 s. The mean of three analytical replicates was reported, with combined standard uncertainty of approximately 8%–10% [29]. The SPECTRW v 83-1 © 2023 software was used for spectral analysis [30].

The comparison was performed for iron only since the portable EDXRF configuration used here was unsuitable for aluminum determination at the concentrations of interest, since aluminum is a light element and its low-energy fluorescence is strongly attenuated by air, windows, filters, and sample matrix.

## 3. Results and Discussion

### 3.1. Determination of Readily Accessible Quercetin-Reactive Aluminum

#### 3.1.1. Preliminary Studies

We tested direct aluminum–quercetin complexation in emulsions using detachable microwells, to measure aluminum made accessible from ACH under the assay conditions. Published cosmetics protocols employing this complexation include pre-column derivatization HPLC after extraction [11] and smartphone colorimetry after acid digestion [31]. The assay showed a linear relationship between nominal aluminum concentration in the emulsion standards and reflected light RGB intensity (Figure 1). Nominal aluminum concentrations were calculated from the amount of ACH added and its aluminum content. This linearity was observed across at least seven replicate curves prepared on different days, yielding  $R^2 \geq 0.971$ . Of the 25 calibration points at or above the LLOQ, 23 met the predefined back-calculation acceptance criteria. Two points exceeded these limits: one above the LLOQ showed an absolute relative deviation of 18.6% and one at the LLOQ of 21.6%. These were treated as isolated deviations and did not lead to rejection of the corresponding calibration curves. The linear range extended from zero up to 0.079% *w/w* ACH (corresponding to 0–196  $\mu$ g Al/g of emulsion), suggesting the potential for quantifying aluminum directly within emulsions. These values are much lower than the Al concentrations considered safe by the SCCS (6.25% *w/w* for non-sprays and 10.60% *w/w* for sprays) [7]. The practical significance of this is that any commercial emulsion must be significantly diluted in a suitable cream matrix for analysis. This dilution effectively shifts the quantitative determination into the diluent's matrix, thereby minimizing any potential interferences from the original emulsion matrix. Residual plots (Figure S2—Supplementary Materials) show concentration-dependent patterns in several datasets, suggesting possible departures from linearity. The limited number of observations per curve restricts, however, definitive interpretation. The practical significance is that linearity should be assessed not simply on the basis of  $R^2$ , but also through independent QC performance.

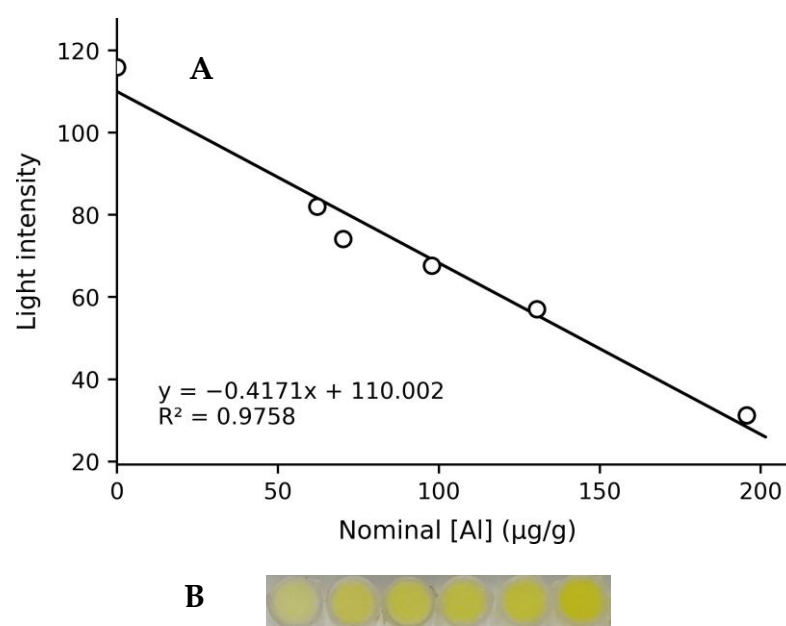


**Figure 1.** (A). Linear relationship between reflected blue color intensity and Al concentration in emulsion BC, determined using the detachable wells assay format. Al concentrations represent nominal amounts added as ACH, calculated from its Al content. The coefficient of determination ( $R^2$ ) and the equation of the linear regression are provided. (B). An image of the corresponding wells is shown where Al concentration increases from left (0 μg Al/g of emulsion) to right (196 μg Al/g of emulsion).

We consistently maintained an aluminum/quercetin molar ratio above 0.5. We recommend a final quercetin concentration within the 0.6–1.5 mg/mL range. Exceeding this concentration range limits the width of the linear range. Furthermore, we concluded that the diluent's pH should be maintained between 5.1 and 5.5 at 25 °C. Aluminum has poor solubility at pH values higher than 5.5, with solubility becoming much more limited around pH 6.0 [32]. Indeed, at a pH of 5.9, we observed a limitation of linearity to a shorter span of concentrations, which we presume is due to a lack of aluminum solubility at these higher concentrations.

### 3.1.2. Interferences

Other aluminum-binding ligands may compete with quercetin and interfere with the assay. Tests on BC preparations containing 0.022% *w/w* ACH indicate that EDTA, citric acid, and a mixture of fatty acids markedly reduced the aluminum–quercetin signal (Figure 2). Complexation of aluminum by EDTA and citric acid and the formation of metal fatty acid salts have been reported [33–35]. Gluconates, lactic acid, and acetic acid showed moderate signal reduction at the concentrations used; aluminum complexation with gluconate, lactate and acetate has also been reported [33,34]. These signal reductions may reflect decreased aluminum availability for reaction with quercetin due to binding by the interferences under the assay conditions. Conversely, benzoates and sorbates increased the signal (Figure 2). This may reflect matrix effects, such as changes in pH or the emulsion's optical properties and does not necessarily indicate increased aluminum–quercetin complex formation. The mechanism was not established.

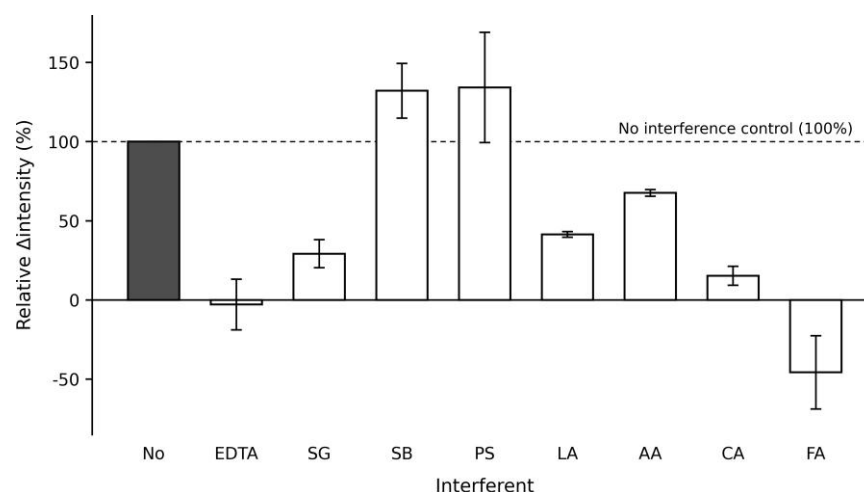


**Figure 2.** Effects of interferent on the Al-dependent decrease in reflected light intensity in a cosmetic emulsion. The decrease in reflected light intensity attributable to Al ( $\Delta$ intensity) was calculated as the difference between the light intensity of the no-ACH/no-interferent control and that of the ACH-containing emulsion:  $\Delta$ intensity = intensity<sub>no-ACH/no-interferent control</sub> – intensity<sub>ACH sample</sub>.  $\Delta$ intensity was then expressed relative to that of the aluminum-containing emulsion where no interferent was added, with the  $\Delta$ intensity value for this no-interferent control (filled bar) defined as 100%. The dashed reference line identifies the 100% no-interferent response. The assay was conducted using an emulsion containing 0.022% *w/w* ACH and a potential interferent at commercially relevant or deliberately elevated challenge concentration. Data represent the average of at least three independent analytical determinations per condition, with error bars denoting one standard deviation. Abbreviations and corresponding concentrations (% *w/w*): No: no interferent; EDTA: ethylenediaminetetraacetic acid disodium salt (1.0%); SG: sodium gluconate (1.3%); SB: sodium benzoate (0.5%); PS: potassium sorbate (1.5%); LA: lactic acid (0.25%); AA: acetic acid (0.25%); CA: citric acid (2.0%); FA: fatty acid mixture (1.0%).

Furthermore, since quercetin can form coordination compounds with various transition metals [36], the presence of these metal ions in the emulsion matrix could also introduce interference. This would be detectable as a change in the blank reading. Given that aluminum is the dominant metal in antiperspirants, however, its important molar excess is expected to minimize interference from trace metals. Altogether, these findings underscore the necessity of using a metal-free, chelator-free dilution matrix, avoiding organic acids for pH adjustment because they may interfere with aluminum–quercetin complexation.

### 3.1.3. Assay Validation in Microwells

Through regression analysis, the calculated LOQ and LOD for the aluminum microwell assay were 50  $\mu$ g Al/ g of emulsion and 16.5  $\mu$ g Al/ g of emulsion, respectively. To further validate the adapted assay and establish its technical characteristics, several quality control (QC) samples were prepared in the BC matrix with known, spiked concentrations of ACH (Table 1). They covered the entire linear range starting at the LOQ and included an additional QC containing 13,695  $\mu$ g Al/g of emulsion (1.37% *w/w*), i.e., commercially relevant levels. The latter was diluted 250-fold into BC, to lower its concentration within the linear range before analysis.

Across days, QC accuracy and intermediate precision met the criteria specified in Section 2.6 (Table 1). This QC analysis established the experimentally determined Lower Limit of Quantification (LLOQ) at 51.5  $\mu$ g Al/g of emulsion, thereby confirming a useful analytical range between 52 and 196  $\mu$ g Al/g of emulsion.

**Table 1.** Technical characteristics of the proposed assay for Al quantification directly in quality control emulsions. Nominal Al concentrations were calculated from the amount of ACH added and its Al content. The QC of original concentration 13,695 µg Al/ g emulsion was measured after 250-fold dilution into BC.

| Intermediate Precision |              | Accuracy            |                   |                 | Linearity      |                               |
|------------------------|--------------|---------------------|-------------------|-----------------|----------------|-------------------------------|
| Mean [Al], µg/g        | CV % (n)     | Measured [Al], µg/g | Spiked [Al], µg/g | Relative Bias % | R <sup>2</sup> | Linear Range, µg/g            |
| 53.9                   | 19.5 (n = 2) | 53.9                | 51.5              | +4.5            | ≥0.971         | 0–196                         |
| 99.4                   | 9.4 (n = 3)  | 99.4                | 97.4              | +2.0            |                | Useful analytical range, µg/g |
| 137.2                  | 9.3 (n = 4)  | 137.2               | 129.9             | +5.6            |                |                               |
| 178.3                  | 5.4 (n = 4)  | 178.3               | 194.9             | −8.5            |                |                               |
| 13,919                 | 7.1 (n = 4)  | 13,919              | 13,695            | +1.6            |                | 52–196                        |

*n* represents independent analytical determinations of the QC sample.

To evaluate the aluminum assay in formulated products, three commercial antiperspirants, manufactured by Anaveris, were examined. Two additional lotion samples were prepared in-house using the corresponding commercial formulations, with only the ACH concentration reduced, giving five test samples in total. Nominal aluminum concentrations were calculated from the known formulation amounts of ACH and its aluminum content and refer to the samples before dilution with BC. The *n* values indicate the number of independent analytical determinations (Table 2). Products were diluted up to 250-fold in BC to bring aluminum within the linear range of the assay, and standards were prepared in the same BC matrix. Bias ranged from −6.81% to +15.18%; only the latter marginally exceeded the ±15% acceptance limit. Notably, antiperspirant lotion 1 contained 3.5% *w/w* of the fatty acid mixture, while antiperspirant lotion 2 contained sorbate, citrate and benzoate salts (total concentration 0.002% *w/w*), which were previously identified as assay interferences. The generally acceptable bias suggests that extensive dilution with BC helped limit matrix interference under the tested conditions. No independent AAS, ICP-OES, or XRF comparison was performed for aluminum; this is a limitation of the present validation.

**Table 2.** Accuracy of the proposed assay for Al quantification directly in commercial antiperspirant matrices. Nominal Al concentrations were calculated from the amount of ACH present and its Al content.

| Matrix                  | Sample Condition                      | Nominal [Al] (mg/g) | R <sup>2</sup> | Relative Bias (%) |
|-------------------------|---------------------------------------|---------------------|----------------|-------------------|
| Antiperspirant lotion 1 | commercially supplied                 | 13.7                | ≥0.967         | −6.81 (n = 2)     |
| Antiperspirant lotion 1 | In-house preparation with reduced ACH | 9.14                | 0.995          | +15.18 (n = 1)    |
| Antiperspirant cream    | commercially supplied                 | 9.14                | ≥0.967         | +8.76 (n = 3)     |
| Antiperspirant lotion 2 | commercially supplied                 | 10.88               | 0.967          | +8.00 (n = 1)     |
| Antiperspirant lotion 2 | In-house preparation with reduced ACH | 9.14                | 0.995          | −2.55 (n = 1)     |

*n* represents independent analytical determinations of the sample.

### 3.1.4. Assay on Filter Paper

We additionally explored a paper format, following approaches validated for other chromogenic assays [26,27]. We observed a linear relationship between the reflected light RGB intensity of spots on paper strips and the concentration of ACH in applied emulsions. Two replicate standard curves achieved R<sup>2</sup> values of at least 0.982, with linearity maintained between zero and at least 0.013% *w/w* ACH, corresponding to zero to 32.4 µg Al/g

of emulsion (Figure S3—Supplementary Materials). Further studies are required to examine the analytical validity of this alternative approach.

A published Alizarin S paper test using diffuse-reflectance measurement, offers lower LOD and LOQ, better repeatability and a comparable working range [10]. The preliminary quercetin paper format studied here retains the intended scope of eliminating digestion or extraction while using paper and smartphone imaging.

### 3.2. Determination of Ascorbate-Reducible, Ferrozine-Reactive Iron

#### 3.2.1. Preliminary Studies

The proposed assay uses the well-established complexation of  $\text{Fe}^{2+}$  ions with ferrozine, generating a magenta-colored coordination compound, whose absorbance is linearly related to iron concentration [24]. We adapted this reaction for direct analysis in emulsions and integrated digital colorimetric monitoring. Standards were prepared in emulsion BC, with concentrations ranging from zero to  $8.0 \mu\text{g Fe/g}$  of emulsion. These concentrations represent iron added as  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , expressed as elemental Fe. Following the addition of the ferrozine R1/R2 mixture, to achieve both reduction of accessible  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  and complexation, the colored standards were photographed in microwells. Linear regression of reflected light RGB intensity (over most of the examined concentration range) against nominal Fe concentration yielded  $R^2 \geq 0.981$  (Figure 3A,C) across at least eight standard curves prepared on different days. Of the 67 calibration points at or above the LLOQ evaluated during validation, 65 met the predefined back-calculation acceptance criteria. Two points exceeded these limits: one above the LLOQ showed an absolute deviation of 16.7%, and one at the LLOQ showed an absolute deviation of 22.4% from the nominal concentration. These were treated as isolated deviations and did not lead to the rejection of the corresponding calibration curves. Residual plots showed concentration-dependent patterns in several calibration datasets, suggesting possible departures from linearity (Figure S4—Supplementary Materials). The practical suitability of the linear calibration therefore requires assessment alongside independent QC performance. Using the same procedure, standards were also prepared in two other commercial emulsions of very different composition, and linear regression was applied ( $R^2 \geq 0.980$ , in both cases). The pH across the three emulsions ranged between 5.1 and 5.9, while viscosity ranged between 72,000 and 420,000 mPa·s, highlighting the flexibility of the assay in terms of matrix pH or viscosity.

#### 3.2.2. Interferences

Since the proposed assay relies on the complexation of  $\text{Fe}^{2+}$  with ferrozine, any substance that can complex with  $\text{Fe}^{2+}$  ions may interfere, leading to inaccurate results. Moreover, since ferrozine readily forms coordination compounds with other transition metals—cobalt and copper [37]—the presence of these metal ions in the matrix could also introduce interference.

Potential interferents were examined in iron-containing BC, using the detachable well assay. Disodium EDTA and  $\text{ZnSO}_4$  markedly reduced the iron-dependent response (Figure 4). EDTA interference in ferrozine-based iron assays has previously been reported [38].  $\text{Zn}^{2+}$  can coordinate with ferrozine to form a light-yellow complex [39].  $\text{Zn}^{2+}$  may therefore interfere by reducing ferrozine availability for  $\text{Fe}^{2+}$  complexation.  $\text{MgSO}_4$  and  $\text{Na}_2\text{SO}_4$  served as sulfate-containing controls ( $\text{MgSO}_4$  was at almost equimolar sulphate concentrations with  $\text{ZnSO}_4$ ); their limited effects suggest that sulfate alone does not explain the  $\text{ZnSO}_4$  response. Potassium sorbate also reduced the response, although to a lesser extent; the mechanism underlying this effect was not established. Smaller response changes were also observed with magnesium- and calcium-containing preparations (Figure 4). Overall, the mechanisms remain unresolved; possible contributors include ligand

competition, altered iron availability, and matrix effects at the tested additive concentrations.

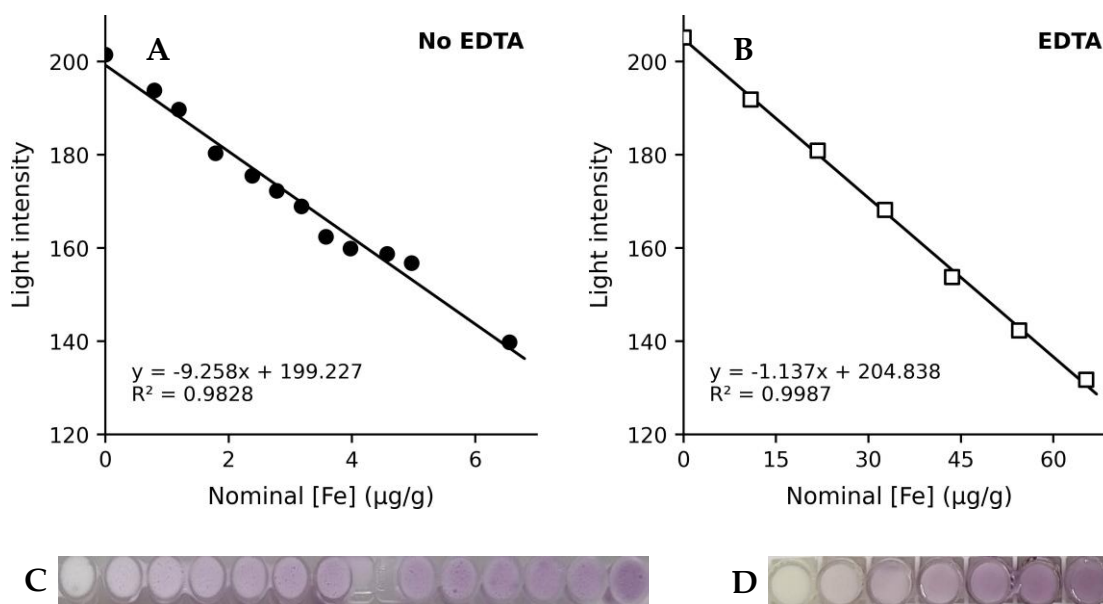


**Figure 3.** Linear relationship between reflected red color intensity and Fe concentration in the emulsion, in the absence of EDTA (full circles—(A)) and in its presence (open squares—(B)). Fe concentrations represent nominal amounts added as  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , expressed as elemental Fe. The coefficient of determination ( $R^2$ ) and equation of each linear regression are provided. Images of the corresponding wells are shown, where Fe concentration increases from left (0  $\mu\text{g Fe/g}$  emulsion) to right (6.55  $\mu\text{g Fe/g}$  emulsion) in the absence of EDTA (C) and between 0  $\mu\text{g Fe/g}$  emulsion (left) and 65.3  $\mu\text{g Fe/g}$  emulsion (right) in the presence of EDTA (D).

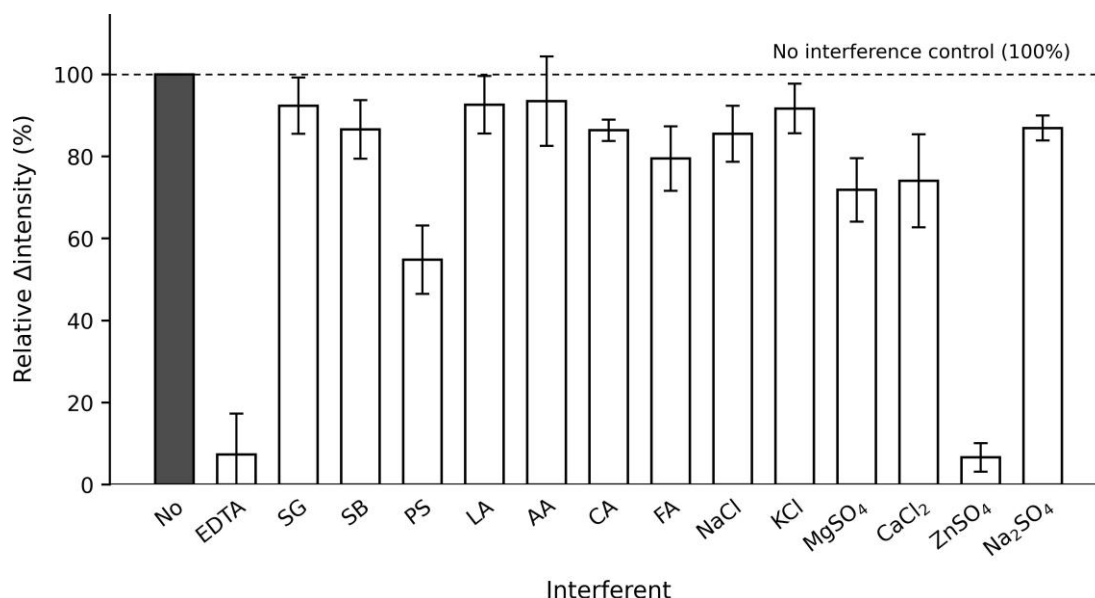


**Figure 4.** Effects of interferent on the Fe-dependent decrease in reflected light intensity in a cosmetic emulsion. The decrease in reflected light intensity attributable to Fe ( $\Delta$ intensity) was calculated as the difference between the light intensity of the no-iron/no-interferent control and that of the iron-containing emulsion:  $\Delta$ intensity = intensity<sub>no-Fe/no-interferent control</sub> − intensity<sub>Fe sample</sub>.  $\Delta$ intensity was then expressed relative to that of the iron-containing emulsion where no interferent was added, with the  $\Delta$ intensity value for this no-interferent control (filled bar) defined as 100%. The dashed reference line identifies the 100% no-interferent response. The assay was performed in BC emulsion

containing 18 µg Fe/g emulsion (added as Fe<sup>3+</sup>) and a potential interferent at commercially relevant or deliberately elevated challenge concentration. Data represent the average of at least three independent analytical determinations per condition, with error bars denoting one standard deviation. Abbreviations and corresponding concentrations (% w/w): No: no interferent (untreated control); EDTA: ethylenediaminetetraacetic acid disodium salt (1.0%); SG: sodium gluconate (1.3%); SB: sodium benzoate (0.5%); PS: potassium sorbate (1.5%); LA: lactic acid (0.25%); AA: acetic acid (0.25%); CA: citric acid (2.0%); FA: fatty acid mixture (1.0%); NaCl (0.74%); KCl (0.95%); MgSO<sub>4</sub>·7H<sub>2</sub>O (2.1%); CaCl<sub>2</sub>·2H<sub>2</sub>O (1.9%); ZnSO<sub>4</sub>·7H<sub>2</sub>O (2.4%); Na<sub>2</sub>SO<sub>4</sub> (0.9%).

A separate experiment assessed which common metals generated colored products with ferrozine and a reducing agent in iron-free BC. Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>2+</sup>, Zn<sup>2+</sup>, and Ca<sup>2+</sup> produced no visible color, whereas Co<sup>2+</sup> and Cu<sup>2+</sup> produced colored emulsions, consistent with earlier reports [24,37]. Tested concentrations were 3.9% w/w NaCl, 5.0% w/w KCl, 0.05% w/w MgCO<sub>3</sub>, 1.5% w/w ZnCl<sub>2</sub>, 0.004% w/w Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, and 0.05% w/w CuSO<sub>4</sub>·5H<sub>2</sub>O.

We performed a literature survey on the metals content of 63 non-color cosmetic emulsions [20,21]. This revealed that iron is in important excess, with the mass ratios of Fe/Co and Fe/Cu exceeding 40 and 8, respectively, in 96% and 82% of the samples surveyed, respectively. A median iron concentration of 56 µg Fe/g emulsion was present in these samples.

To evaluate the potential effect of Co and Cu interference, a recovery study was initiated utilizing a representative matrix (BC) spike. A blank cosmetic emulsion was fortified to realistic baseline concentrations of 41.7 µg Fe<sup>3+</sup>/g emulsion and 1.05 µg Co<sup>2+</sup>/g emulsion (corresponding to a Fe/Co ratio of approximately 40), and 4.99 µg Cu<sup>2+</sup>/g emulsion (corresponding to a Fe/Cu ratio of approximately 8.3). We subsequently analyzed the spiked matrix by the proposed assay, after a suitable dilution of 1: 10 (BC as diluent), to lower iron levels within the linear range. This analysis yielded an accurate iron quantification (bias: + 4.5%). This finding suggests that at such commercially realistic mass ratios, the presence of concomitant cobalt and copper ions exerts limited interference effects on the quantification of ascorbate-reducible, ferrozine-reactive iron by the proposed assay in spiked/model emulsions.

When the sample matrix is available, interference is best minimized by preparing standards within that exact same matrix. For instance, the concentration of iron standards prepared in an emulsion containing 0.1% EDTA were accurately back-calculated (with a bias within ± 0.8%) when a standard curve in the EDTA-containing matrix was prepared (Figure 3B,D).

### 3.2.3. Assay Validation

Seven calibration curves in BC were fitted by linear regression over 0–6.86 µg Fe/g emulsion ( $R^2 \geq 0.981$ ). LOQ and LOD were calculated through regression analysis to be 1.38 and 0.46 µg Fe/g of emulsion, respectively (seven replicates, CV:10.5%). To further evaluate its technical characteristics, matrix-matched QCs (spiked with Fe<sup>3+</sup> at LOQ, or at low, medium and high levels within the linear range and at a commercially relevant level) were analyzed (up to six runs on five different days). Acceptable assay accuracy (relative bias %) and precision values (CV%), satisfying the adopted criteria, were observed for concentrations higher than 1.25 µg Fe/g of emulsion (Table 3). This QC analysis established the limit of 1.87 µg Fe/g emulsion as a practical lower quantification boundary, thereby confirming a useful analytical range between 1.87 and 6.86 µg Fe/g of emulsion.

**Table 3.** Technical characteristics of the proposed assay for ascorbate-reducible, ferrozine-reactive Fe quantification directly in quality control emulsions. Fe concentrations represent nominal amounts spiked as FeCl<sub>3</sub>·6H<sub>2</sub>O, expressed as elemental Fe. The QC of original concentration 41.7 µg Fe/g emulsion was measured after 1:10.4 dilution into BC.

| Intermediate Precision |              | Accuracy            |                   |                 | R <sup>2</sup> | Linearity                     |
|------------------------|--------------|---------------------|-------------------|-----------------|----------------|-------------------------------|
| Mean [Fe], µg/g        | CV % (n)     | Measured [Fe], µg/g | Spiked [Fe], µg/g | Relative Bias % |                | Linear Range, µg/g            |
| 1.29                   | 20.3 (n = 6) | 1.29                | 1.25              | +3.4            | ≥0.981         | 0–6.86                        |
| 2.02                   | 12.6 (n = 4) | 2.02                | 1.87              | +7.9            |                |                               |
| 2.84                   | 6.9 (n = 6)  | 2.84                | 2.50              | +13.7           |                | Useful Analytical range, µg/g |
| 4.09                   | 5.9 (n = 6)  | 4.09                | 3.74              | +9.2            |                |                               |
| 4.63                   | 4.2 (n = 6)  | 4.63                | 4.78              | −3.3            |                |                               |
| 5.04                   | 4.8 (n = 6)  | 5.04                | 5.20              | −3.0            |                |                               |
| 6.39                   | 6.7 (n = 4)  | 6.39                | 6.86              | −6.9            |                | 1.87–6.86                     |
| 45.1                   | 12.2 (n = 6) | 45.1                | 41.7              | +8.3            |                |                               |

*n* represents independent analytical determinations of the QC sample.

Iron assay performance was then compared to portable EDXRF because both workflows involve limited chemical pretreatment. The comparison was performed for iron only; no XRF aluminum measurement or aluminum validation is claimed. QCs within the colorimetric working range were below the quantification capability of the portable EDXRF configuration. Higher QCs were, therefore, prepared in another emulsion containing 0.1% *w/w* disodium EDTA dihydrate, which maintained a linear ferrozine response. EDXRF determines elemental iron largely independently of its chemical form. Therefore, complexation of iron by EDTA does not directly inhibit detection, and EDTA does not contribute to iron fluorescence. A linear relation was established between EDXRF signal normalized to surface density, and spiked iron (Fe<sup>3+</sup>) concentration (Figure S5—Supplementary Materials).

Iron results from the two methods were compared by regression (Figure 5). The comparison yielded a slope of approximately 1.14, with an intercept of 0.40 µg/g, suggesting a proportional difference between the methods and a possible constant component. The cause was not established and may include drying and thin-target preparation, surface-density estimation, matrix-dependent XRF response, and uncertainty in either method. For non-critical applications such as the one envisaged here, this difference represents an acceptable compromise for a workflow with no requirement for specialized equipment or expertise. Because EDXRF measures elemental iron and colorimetry measures the ascorbate-reducible, ferrozine-reactive fraction, this comparison does not establish equivalence between the methods.

Concluding, portable EDXRF was not sufficiently sensitive at the lower colorimetric working concentrations, as reported previously [19]. The established methods of AAS after wet digestion [21,22] and ICP-OES [23] for iron analysis have mostly superior technical characteristics, including a much lower LOQ for AAS only (LOQ determined as 54 ng/g [22] and 100 ng/g for AAS [21], and as 6.28 mg/L for ICP-OES [23]). Under the defined conditions, the proposed assay nevertheless meets routine cosmetic quality control requirements.

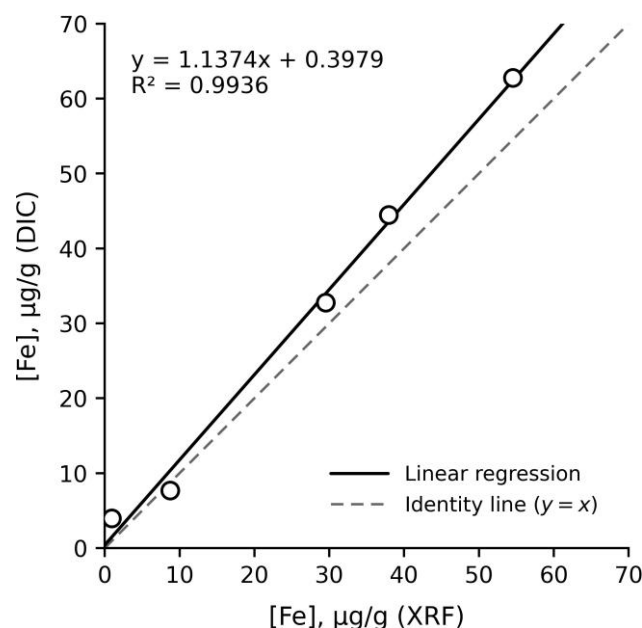


**Figure 5.** Linear correlation between Fe levels in QC emulsion samples determined by the proposed Digital Image Colorimetric (DIC) method and the XRF reference method. An identity line ( $y = x$ ) has been drawn (dashed line). The fitted linear regression (full line), its equation and  $R^2$  are also being displayed.

#### 4. Conclusions

Digestion- and extraction-free digital image colorimetric assays were established for two operationally defined fractions in O/W cosmetic emulsions: quercetin-reactive aluminum made accessible from ACH and ascorbate-reducible, ferrozine-reactive iron. Both use smartphone imaging with matrix-matched standards and unknowns in the same image.

The assays have not been validated for water-in-oil systems, which require separate validation.

For aluminum, QC bias ranged from  $-8.5\%$  to  $+5.6\%$ , intermediate precision above the LLOQ ranged from  $5.4\%$  to  $9.4\%$  (expressed as CV), and the useful working range was  $52\text{--}196\text{ }\mu\text{g Al/g emulsion}$ . Bias in diluted commercial antiperspirants and corresponding in-house preparations ranged from  $-6.81\%$  to  $+15.18\%$ . The assay does not determine insoluble alumina used as an abrasive or pigment. No independent AAS, ICP-OES, or XRF comparison was performed for aluminum, which remains a validation limitation.

For iron, QC bias ranged from  $-6.9\%$  to  $+13.7\%$ , intermediate precision was CV  $4.2\text{--}12.6\%$ , and the useful working range was  $1.87\text{--}6.86\text{ }\mu\text{g Fe/g emulsion}$ . Portable EDXRF comparison was performed for iron only and showed a regression slope of approximately  $1.14$ , with an intercept of  $0.40\text{ }\mu\text{g/g}$ , suggesting a proportional difference and a possible constant component. Their origin was not established, while the two methods measure different operational quantities.

Both assays remain sensitive to matrix composition and competing ligands. Matrix-matched calibration or sufficient dilution into a controlled matrix is therefore required.

Within these defined conditions, the methods provide fit-for-purpose measurements of the stated reactive fractions.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/analytica7040078/s1>, Figure S1: Direct digital-image colorimetry workflow in detachable wells for aluminum and iron quantification in cosmetic emulsions; Figure S2: Residuals versus nominal aluminum concentration for the independent aluminum calibration datasets; Figure S3: Linear relationship between reflected blue color intensity and Al

concentration in the oil-in-water base cream (BC), determined using the paper-based spot assay; Figure S4: Residuals versus nominal iron concentration for the independent iron calibration datasets; Figure S5: Linear relationship between the XRF signal/surface density and the spiked iron concentration in emulsion samples.

**Author Contributions:** G.E.T.: Conceptualization, methodology, validation, investigation, formal analysis, supervision, writing—original draft, writing—review and editing; D.D.: validation, investigation, formal analysis, G.B.: validation, investigation, formal analysis, M.P.: methodology, validation, investigation, formal analysis. writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

## Abbreviations

The following abbreviations are used in this manuscript:

|                |                                                                                                     |
|----------------|-----------------------------------------------------------------------------------------------------|
| AA             | Acetic Acid                                                                                         |
| AAS            | Atomic absorption spectroscopy                                                                      |
| ACH            | Aluminum chlorohydrate                                                                              |
| ACS            | American Chemical Society                                                                           |
| BC             | Base cream used as the calibration and dilution matrix                                              |
| BfR            | German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung)                   |
| CA             | Citric acid                                                                                         |
| CV             | Coefficient of variation                                                                            |
| DIC            | Digital Image Colorimetry                                                                           |
| EDTA           | Ethylenediaminetetraacetic acid; tested as its disodium salt dihydrate                              |
| EDXRF          | Energy-dispersive X-ray fluorescence                                                                |
| EMA            | European Medicines Agency                                                                           |
| FA             | Fatty acid mixture                                                                                  |
| HPLC           | High-performance liquid chromatography                                                              |
| ICH            | International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use |
| ICP-OES        | Inductively coupled plasma optical emission spectrometry                                            |
| LA             | Lactic acid                                                                                         |
| LED            | Light-emitting diode                                                                                |
| LLOQ           | Lower limit of quantification                                                                       |
| LOD            | Limit of detection                                                                                  |
| LOQ            | Limit of quantification                                                                             |
| O/W            | Oil-in-water                                                                                        |
| PEG            | Polyethylene glycol                                                                                 |
| Ph. Eur.       | European Pharmacopeia                                                                               |
| PS             | Potassium sorbate                                                                                   |
| QC / QCs       | Quality control / quality controls                                                                  |
| R1             | Ascorbic acid reagent                                                                               |
| R2             | Ferrozine reagent; distinct from R <sup>2</sup>                                                     |
| R <sup>2</sup> | Coefficient of determination                                                                        |
| RGB            | Red–green–blue                                                                                      |

|                    |                                                         |
|--------------------|---------------------------------------------------------|
| rpm                | Revolutions per minute                                  |
| SB                 | Sodium benzoate                                         |
| SCCS               | Scientific Committee on Consumer Safety                 |
| SD                 | Standard deviation                                      |
| SG                 | Sodium gluconate                                        |
| Si-PIN             | Silicon positive–intrinsic–negative detector            |
| USB                | Universal Serial Bus                                    |
| UV                 | Ultraviolet                                             |
| w/v                | Mass per volume                                         |
| w/w                | Mass per mass                                           |
| XRF                | X-ray fluorescence                                      |
| n                  | Number of independent analytical determinations         |
| $\Delta$ intensity | Metal-dependent difference in reflected light intensity |

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