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STUDY PROTOCOL

Extraction of E171 or E172 food additives from sugar-based or lipid-based complex matrices for primary particles identification by electron microscopy

[version 1; peer review: 3 approved with reservations, 1 not approved]

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Abstract

Measurement of primary particle size distributions is fundamental to determining if a material should be classified as a nanomaterial according to the European Commission recommended definition. This is of particular relevance to alimentary products, where current regulation requires that any additives containing nanoforms be explicitly declared as such in the ingredients' list. Currently, Electron Microscopy is the only instrumental technique able to reliably classify materials as nanomaterials. To verify the nanostatus of a material using this technique, it is essential to firstly extract the additives from the matrix in which they are dispersed, and then measure a wide size distribution while avoiding artefacts from the food matrix.

In this operating procedure, extraction protocols, developed and applied for sugar-based or lipid-based food, are described. In particular, case studies for titanium dioxide E171 and iron oxide/iron hydroxide based materials such as E172 are considered. The protocols are based on gentle extraction of the additive materials and could, in principle, be used for other nanomaterials provided they are not soluble in water or ethanol and are resistant to enzymatic reactions. The size distribution of the extracted material can then be determined from images acquired using scanning and/or transmission electron microscopy.

Plain language summary

Measurement of primary particle size distributions is fundamental to determining if a material should be classified as a nanomaterial according to the European Commission recommended definition. In the case of food additives, it is important to use a reliable protocol to extract materials from matrices to obtain samples to be analysed by

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Approval Status ✗ ? ? ?

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version 1 18 Dec 2024	✗ view	? view	? view	? view

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Any reports and responses or comments on the article can be found at the end of the article.

electron microscopy.

Based on results we can assess if nanomaterials are present or not in food, and if their characteristics respect current legislation.

Keywords
extraction, food additives, E171, E172, titanium oxide, iron oxides and hydroxides, electron microscopy



This article is included in the Joint Research Centre gateway.



This article is included in the Food Safety and Waste collection.

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Author roles: PONTI J: Conceptualization, Data Curation, Formal Analysis, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing;

Competing interests: No competing interests were disclosed.

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Introduction

Food additives containing nanomaterials have been used for many years throughout Europe and the rest of the globe. For example, E171 titanium dioxide, now banned as a food additive in the European Union since 2022 (2022/63), is still widely used in food products outside the European Union. Another example, this time still approved for use in the European Union (EU), is the iron based additive E172, which is widely used as a natural colouring agent offering yellow, brown, red, or even black colour to food, drink, supplements, medicine, or cosmetics.

Different instrumental techniques exist to measure particle size distributions (Rauscher *et al.*, 2019), but none can reliably identify the size distribution of primary nanoparticles because they are based on indirect or measures which cannot distinguish single particles from aggregates and agglomerates. In samples, where the presence of agglomerates/aggregate of nanoparticles cannot be excluded, Electron Microscopy (EM) remains the only method which is able to unambiguously confirm the size distribution of the primary particles according to the European Commission recommended definition (2022/C 229/01).

In the case of additives already incorporated into a food matrix, a pre-requisite for reliable EM analysis is avoiding experimental artefacts by minimising or eliminating residues of the food matrix. It is thus critical to use extraction procedures to separate the nanomaterials from food in a way which guarantee an efficient removal of the matrix without damaging the material itself.

The following operating procedures describe fast protocols for the extraction of nanomaterials from: (i) sugar-based confectionary products, containing, according to the manufacturers' declaration, around 80–90% of carbohydrates. This protocol is based on the use of Ethanol and ultrapure water (Geiss *et al.*, 2020); (ii) lipid rich food products, such as tartare sauce, which, according to the manufacturers, contain around 70–80% of fats. The protocol is based on the use of enzymes to sequentially degrade lipids and carbohydrates (Facchetti *et al.*, 2020).

In each case, the final product of the extraction process is a purified, water-based suspension of nanoparticles, which can be deposited on appropriate supports for subsequent analyses by Scanning (SEM) and/or Transmission Electron Microscopy (TEM).

The protocols also detail the additional steps necessary to deposit the suspension on typical silicon wafer support or grid for SEM or TEM, respectively. As a verification of the efficiency of each step, samples of the various wash solutions were examined by EM to confirm the absence of food additives particulate materials. It should be noted that these procedure extract, non-specifically, all particulates, which are not soluble in water, Ethanol or digested by the enzyme. In the case of commercial alimentary products, where mixtures of different particle types may be present, it is recommend that steps be taken to directly verify the identity of extracted particles using EM equipment coupled with chemical analysis, e.g. energy dispersive X-Ray spectroscopy (EDX). Where direct analysis of individual particles is not possible, then bulk elemental analyses by e.g. inductively coupled plasma mass spectrometry (ICP-MS) or Total Reflection X-ray Fluorescence (TXRF) may permit the exclusion of other inorganic or metal-organic particles.

Methods

Extraction of E171 from commercially available sugar-based jelly beans

The details of the following procedure are based on the observation that E171 is principally a component of the jelly beans' external coating, and that the content of TiO_2 material varies with the colour of the confectionary product. It was found that the highest amount of E171 is present in yellow, white and green jelly beans and including material from jelly beans of other colours (e.g. violet, pink, red) is undesirable, as it serves only to increase the proportion of matrix which must be removed. The procedure is developed on a fixed amount (100mg) of external coating of jelly beans containing E171 food additive. Adaptation of reagents' concentrations are allowed, if necessary. Reagents, ultrapure water and absolute Ethanol, are filtered using syringe filter of 0.22µm pores, before use to avoid possible external contamination. Representative steps of the sample preparation are shown in Figure 1.

First, remove 100 mg of the external coating of the jelly beans using blade avoiding the removal of the jelly internal part that is not considered in this sample preparation procedure. Collect the sample in Eppendorf tube of 1.5 mL, add 1 mL of absolute Ethanol, vortex for 30 seconds and ultra-sonicate using ultrasonic bath for 10 minutes. Centrifuge the suspension at 16000 rcf (relative centrifugal force) for 15 minutes, remove the supernatant ant store it in case of need of further analysis. Add 1 mL of absolute Ethanol and repeat the previous centrifugation step once, remove supernatant and add 1 mL of ultrapure water and mix, centrifuge, as previously described, twice. In the end of the procedure, we obtain a clean pellet ready for the preparation of sample for EM analysis after addition of 500 µL ultrapure water and sonication (e.g. vial tweeter 20 minutes, 75% amplitude, 0.5 cycle).

Once sonicated, the sample is enough dispersed to be deposited on the EM support and analysed to assess primary size distribution of E171 food additive.

The sample grid, 200 mesh Cu-Formvar carbon coated grid, or equivalent, for TEM analysis (Figure 2) and silicon wafer for SEM analysis are prepared by deposition of 3 µL of suspension; to improve particle adhesion and suspension distribution on supports, Alcian Blue (Verleysen *et al.*, 2019), glow discharge or equivalent pre-treatment should be used. Samples are left to dry according to equipment needs and analysed.

Even if the pellet results enough clean for EM analysis, it could be that some interferences remain after the drying step, for this reason it is recommended to gentle wash samples on grids or on silicon wafer with absolute Ethanol and completely left to dry, before analysis.

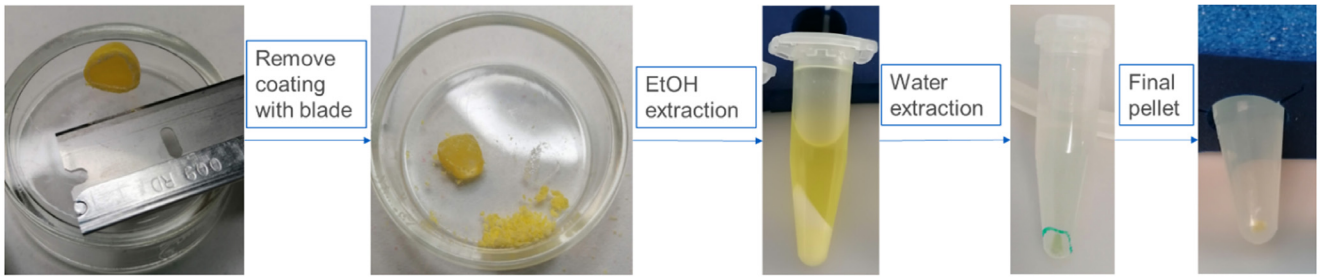


Figure 1. This figure represent different steps of the preparation protocol starting from removal of the external coating of the jelly beans to the final pellet containing TiO_2 particles to be analysed; (EtOH is Ethanol).

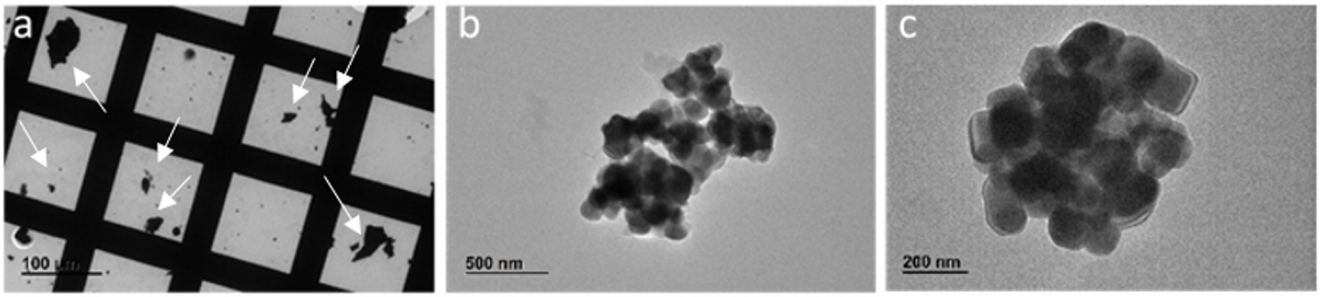


Figure 2. These figures show images obtained by TEM JEOL-JEM 2100 at 120 kV. The grid appear with some residues of matrix (arrows) (**a**) that do not interfere with the final analysis (**b**) where particles, at higher magnification (**c**), can be visualised and measured.

Extraction of E172 from commercially available sugar-based red pearls

The hereafter described procedure is based on the observation that E172 is mainly contained and distributed on the red pearls' external coating. The advantage of E172 colour based material, in this case red, is that we can optically see the phase with higher probability to contain the material. In addition, since the nanomaterial concentration is quite low, for the EM analysis, the ultrapure water volume used is 1 mL for 35–40 red pearls, to avoid sample dilution. Reagents, ultrapure water and absolute Ethanol, are filtered using syringe filter of 0.22µm pores, before use to avoid possible external contamination. Adaptation of reagents' concentrations are allowed where necessary.

Please note that even if this protocol remove most of the sugar-based matrix, some could remain and appear as white part of the pellet. Repeat the washing steps, hereafter described, indicatively 2 times each, if the sugar matrix is still visible. Observe that in the end of the extraction procedure, the major part of the sample is red and the white part of the matrix is negligible. Relevant steps of the protocol are shown in Figure 3.

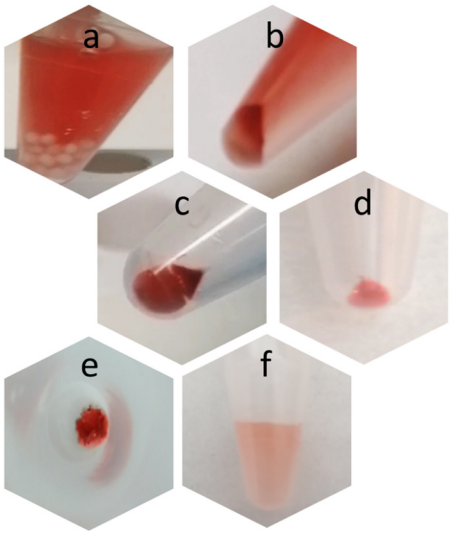


Figure 3. Images of the results obtained after different steps of the extraction protocol: **a**) 30 seconds after water addition, red pearls become white; **b**) after first centrifugation; **c**) after first Ethanol wash; **d**) after second Ethanol wash and **e**) same sample upper view; **f**) after water addition and sonication.

First, collect 35–40 red pearls in a 1.5 mL Eppendorf tube, add 1 mL of ultrapure water and gently shake for 30 seconds. Remove supernatant, transfer in 1.5 mL Eppendorf tube and discard the remaining part of the pearls. Centrifuge the suspension at 16000 rcf (relative centrifugal force) for 5 minutes, remove 500 µL of the supernatant and store it in case of need of further analysis.

Observe the pellet in which you can see a white and a red part, remove this last only using the 500 µL of supernatant present in the tube. Transfer this volume in a clean tube and add 500 µL of ultrapure water reaching 1 mL volume in total. Centrifuge the suspension at 16000 rcf for 2 minutes and if the white part is still visible in the pellet, repeat the last steps (from remove 500 µL point), twice.

If not, remove the supernatant, add 1 mL of absolute Ethanol, centrifuge suspension at 16000 rcf for 2 minutes and remove the supernatant leaving, as described above, 500 µL of Ethanol. Again, observe the pellet and remove the red part only. Transfer the 500 µL in a new tube and add 500 µL of absolute Ethanol reaching 1 mL volume in total. Centrifuge the suspension at 16000 rcf for 2 minutes. Remove the supernatant, add 100 µL of ultrapure water, and sonicate (e.g. vial tweeter 20 minutes, 75% amplitude, 0.5 cycle).

Once sonicated, the sample is enough dispersed to be deposited on the EM support and analysed to assess primary size distribution of E172 food additive.

The sample grid, 200 mesh Cu-Formvar carbon coated grid, or equivalent, for TEM analysis (Figure 4) and silicon wafer, for SEM analysis are prepared by deposition of 3 µL of suspension; to improve particle adhesion and suspension distribution on supports, Alcian Blue (Verleysen *et al.*, 2019), glow discharge or equivalent pre-treatment should be used. Samples are left to dry according to equipment needs and analysed.

Even if the pellet results enough clean for EM analysis, it could be that some interference remain after the drying step, for this reason it is recommended to gentle wash samples on grids or on silicon wafer with absolute Ethanol and completely left to dry, before analysis.

Extraction of E171 from lipid-based tartare sauce

The hereafter described operating procedure is developed to extract E171 single particles and/or agglomerates from food product rich in lipid content such as tartare sauce, where around 70–80% of fats, are generally declared. The protocol is based on the use of enzymes to degrade lipids and carbohydrates. The aim of this procedure is to give detailed instructions on how to prepare sample, using relatively low amount of tartare sauce (500 mg), removing most of the matrix effect, and deposit drop of final obtained sample on grids for EM analysis. Adaptation of reagents' concentrations are allowed, if necessary. The protocol cannot guarantee a complete removal of matrix, but can produce a sample suitable for E171 detection and measurement of primary size distribution.

Before staring the extraction, prepare solutions as follow: (i) 0.2 M Na-Cacodilate (CAS 6131-99-3; Sigma Aldrich C4945-25G) buffer solution (pH 6-8 to 7.4), weigh 4.88 g of Na-Cacodilate powder and dissolve in 100 mL of ultrapure water, measure pH and adjust if necessary, store at 4°C; (ii) prepare 0.05 M of Na-Cacodilate buffer (pH 6-8 to 7.4) with 50 mL Na-Cacodilate 0.2 M + 3 mL HCl 0.2 N + 147 mL ultrapure water and adjust the pH after adding 2–3 mL HCl, if necessary; (iii) prepare 1 mL of lipase from Aspergillus niger (200 UI, CAS 9001-62-1; Sigma Aldrich 62301-1G-F) weight 2 mg of lipase powder and add 1mL of 0.05 M Na-Cacodilate, if necessary adjust pH at 6.8–7.4.

Remove 500 mg tartare sauce from the original envelop with a clean spatula (to avoid external contamination) taking care of removing the creamy part, only, put in 1.5 mL Eppendorf tube and add 1 mL of absolute Ethanol. Vortex for 30 seconds and incubate in the ultrasonic bath for 10 minutes. A solid part will appear in the vial, remove it using tweezers or spatula or directly remove the solvent using a pipette. Centrifuge the solvent at 16000 rcf (relative centrifugal force) for 15 minutes, remove supernatant and store it, in case needed for further analysis. Add 1mL absolute ethanol to the pellet, mix and centrifuge at 16000 rcf for 15 minutes, again remove supernatant and store it, in case needed for further analysis.

Add 1mL of 50 mL Na-Cacodilate 0.05 M, transfer the suspension in a 20 mL glass vial and add 1 mL of lipase, incubate

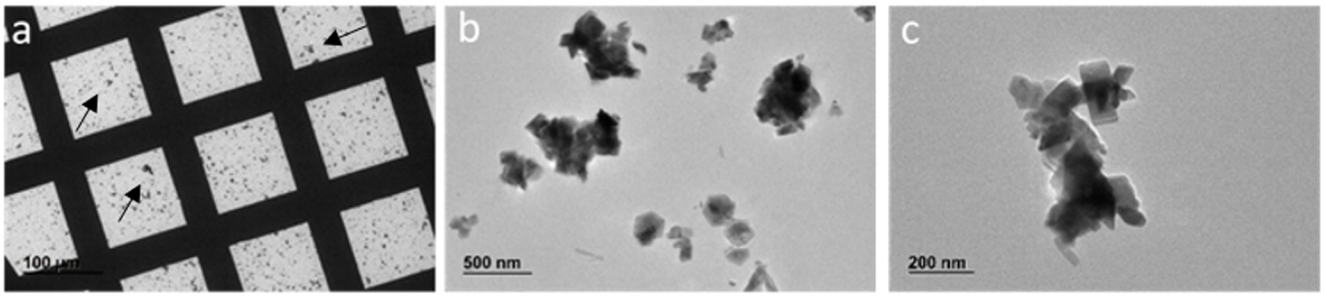


Figure 4. Images obtained by TEM JEOL-JEM 2100 at 120 kV. The grid appear with some residues of matrix (arrows) (**a**) which do not interfere with the final analysis (**b**), where particles, at higher magnification (**c**), can be visualised and measured.

under agitation, if possible, at 50°C in the hoven, overnight (for glass vial smaller than 20 mL, it is recommended to leave the cap slightly open). The day after add 100 µL amylase solution (α-Amylase from Bacillus sp. (CAS 9000-85-5; Sigma Aldrich A4862-250 mL) for 2 hours at 50°C in the hoven, under agitation.

Then, transfer the obtained suspension in 2 Eppendorf tubes of 1.5 mL each and centrifuge at 16000 rcf for 15 minutes, remove supernatant and store it, in case needed for further analysis. Add 1mL ultrapure water to each tube and mix, centrifuge at 16000 rcf for 15 minutes, remove supernatant, twice.

Add 100 µL ultrapure water and sonicate (e.g. vial tweeter 20 minutes, 75% amplitude, 0.5 cycle). Representative steps of the protocol are summarised in Figure 5.

The sample grid, 200 mesh Cu-Formvar carbon coated grid, or equivalent, for TEM analysis (Figure 6) and silicon wafer, for SEM analysis are prepared by deposition of 3 µL of suspension; to improve particle adhesion and suspension distribution on supports, Alcian Blue (Verleysen *et al.*, 2019), glow discharge or equivalent pre-treatment should be used. Samples are left to dry according to equipment needs and analysed.

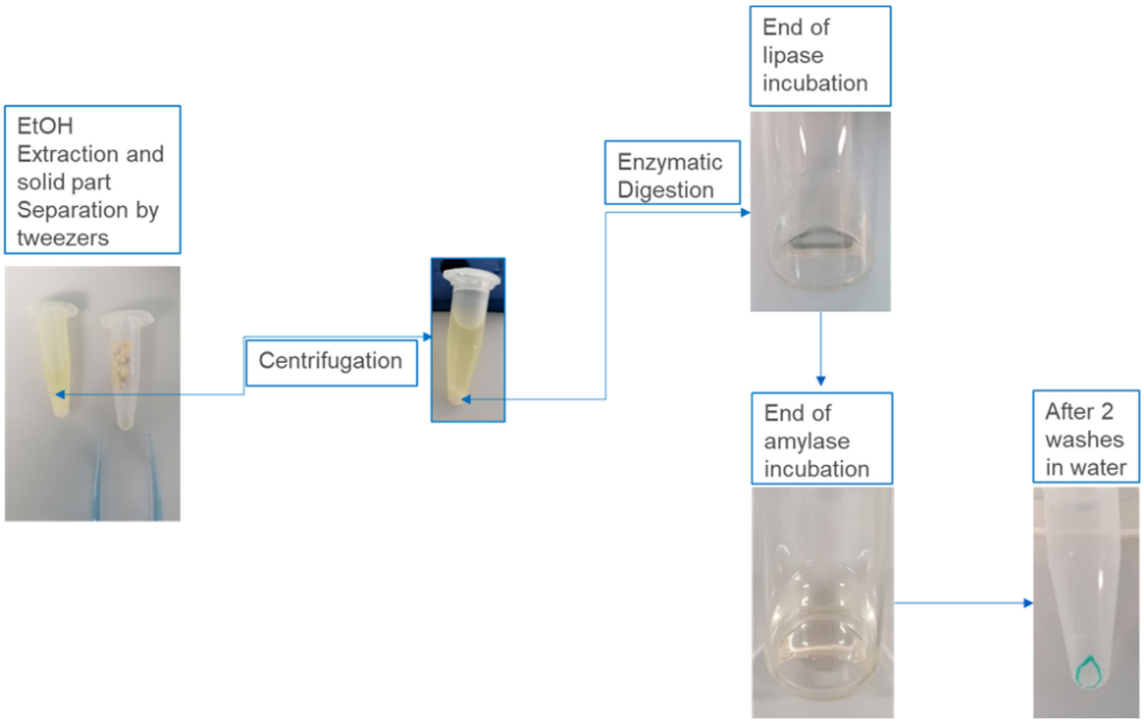


Figure 5. Images of the results obtained after different steps of the extraction protocol, as described in the corresponding legends.

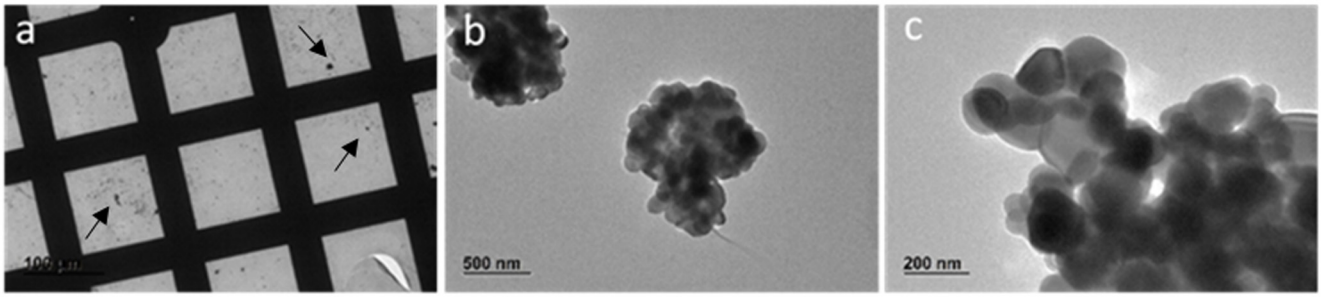


Figure 6. Images obtained by TEM JEOL-JEM 2100 at 120 kV. The grid appear with some residues of matrix (arrows) (**a**) that do not interfere with the final analysis (**b**) where particles, at higher magnification (**c**), can be visualised and measured.

Even if the pellet results enough clean for EM analysis, it could be that some interferences remain after the drying step, for this reason it is recommended to gentle wash samples on grids or on silicon wafer with absolute Ethanol and completely left to dry, before analysis.

Discussion and conclusion

Extracting food additives from complex matrices is a multifaceted challenge that requires careful consideration of extraction methods, analytical techniques, and the inherent complexities of food compositions. Ongoing advancements in extraction technologies and a growing emphasis on sustainability are paving the way for more efficient and effective methodologies. As food safety and quality continue to be paramount concerns, further research in this field is essential for developing standardized, reliable extraction protocols that meet regulatory requirements and consumer expectations.

The three protocols developed and here described are simple and relatively fast, they are based on the use of low amount of material and few reagents. They aim is to provide detailed instructions list that can be applied for qualitative and quantitative analysis of food additives presence in food matrices. The reagents used, gently extract the material of interest without damaging it or without creating changes in their chemical composition, size and morphology.

Protocols are designed to give to the operator the possibility to obtain suspensions to be analysed by electron microscopy, but also by other techniques, if needed. Additional steps to better purify the obtained suspensions can be added and optimised based on specific requirements.

Ethics and consent

Ethical approval and consent not required

Data availability

Reporting guidelines

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Data set available:
https://data.jrc.ec.europa.eu/dataset/8b6b9e2d-82c0-4165-a75b-fa445fd11edc

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Reference Source

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Reference Source

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PubMed Abstract | Publisher Full Text

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Reference Source

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PubMed Abstract | Publisher Full Text | Free Full Text

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Current Peer Review Status: ✗ ? ? ?

Version 1

Reviewer Report 20 January 2025

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Katrin Loeschner
Technical University of Denmark, Lyngby, Denmark

The manuscript presents protocols for the extraction of titanium dioxide (E171) and iron oxide / iron hydroxide (E172) from sugar-based or lipid-based food for following analysis by electron microscopy. Three case studies are presented: E171 in commercially available sugar-based jelly beans, E172 in commercially available sugar-based red pearls and E171 in lipid-based tartare sauce. The protocols are described in sufficient detail and supported by helpful figures.

My main criticism is that the described protocols are written for three specific commercial products, whose exact nature is unknown to the reader. It is unclear how the protocols can be applied for “sugar-based or lipid-based complex matrices” in general (as the title of the protocol implies). If the authors think that the protocols from the case studies can be applied to other products, it would be highly beneficial to generalize the protocols or at least to add more general considerations after the protocols. There is also some information missing regarding the development of the protocols despite the authors referring to other published works. My specific comments are listed in the following and divided by each section of the manuscript.

Abstract:
Please explain what you mean “... and then measure a **wide** size distribution” in the abstract (focus on the word “wide”).

Introduction:
Consider revising this sentence / paragraph in the introduction “Different instrumental techniques exist to measure particle size distributions (Rauscher *et al.*, 2019), but none can reliably identify the size distribution of primary nanoparticles because they are based on indirect or measures which cannot distinguish single particles from aggregates and agglomerates”. This sentence says that there is no instrumental technique at all. The next sentence says that EM is the only technique. I assume that you want to say “no other technique than EM exists”.

It is said that the protocol for sugar-based confectionery products is based on the use of Ethanol and ultrapure water (Geiss *et al.*, 2020). No ethanol is used in the provided reference. It should be

explained why ethanol is used instead of ultrapure water.

The protocol for lipid rich food products refers to Facchetti et al., 2020. This work is about the detection of metal-doped fluorescent PVC microplastics in freshwater mussels. The authors should argue why they think that the protocol is also the best protocol for inorganic food additives in lipid rich food products. In the presented manuscript it is stated: “The protocol is based on the use of enzymes to degrade lipids and carbohydrates”. The reference also used enzyme (papain) to degrade protein. Why was this modification done? The presented protocol uses Na-Cacodilate buffer which is not mentioned in Facchetti et al., 2020. Please explain the modifications.

The authors claim that “The following operating procedures describe fast protocols for the extraction of nanomaterials”. This should be supported with an estimate of the time required for each protocol. Particularly the protocol for lipid-based tartar sauce appears to require some time.

Methods:

The protocols for jelly beans and red pearls are examples for sugar-based matrices. How can this knowledge be used for other sugar-based matrices? How much material (pearls, beans, …) should one use to obtain a representative subsample? How many jelly beans correspond to 100 mg coating? When does the external coating need to be removed with a blade? When can the core be dissolved together with the coating or when can it be discarded after dissolution of the coating? When does one need to use water and when ethanol? In which case of sugar-based matrices might the protocol not work (certain glazing agents, …)? Etc. For jelly beans and red pearls “observations” are described. These can probably be used to identify a list of necessary pre-studies.

The issue of the different colors of jelly beans containing different amounts of E171 and some of the colors being excluded could also be discussed in a more general way. It probably depends on the question one wants to answer – the E171 in the whole product or in individual items (of a certain color). Is there a guarantee that the same E171 was used for all colors? When is it better to remove certain items (e.g. certain colors of jelly beans) and when not?

The protocol for tartar sauce, which is described as “lipid-based” also uses amylase. This should be explained. How can the specific protocol be applied to other lipid-based matrices? Which types can it be used for, which not? Is amylase always required?

The authors claim that the protocols are also suitable for further analysis by SEM. However, no SEM images are provided to support this claim. To the best of my knowledge, the presence of organic residues on particles is more critical in SEM analysis.

Figure 2c, 4c, 6c – The particles appear to be heavily agglomerated. Can the authors comment on how easy it is to determine the primary particle size in these agglomerates?

Typos:
It should be noted that these procedure extract, non-specifically, all particulates, which are not soluble in water, Ethanol or digested by the enzyme.

The day after add 100 µL amylase solution (α-Amylase from Bacillus sp. (CAS 9000-85-5; Sigma

Aldrich A4862-250 mL) for 2 hours at 50°C in the hoven, under agitation.

Is the rationale for, and objectives of, the study clearly described?
Yes

Is the study design appropriate for the research question?
Partly

Are sufficient details of the methods provided to allow replication by others?
Partly

Are the datasets clearly presented in a useable and accessible format?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: material science, analytical chemistry, food chemistry, nanomaterials

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 11 January 2025

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Abdullah Taner Bişgin
Niğde Ömer Halisdemir University,, Niğde, Turkey

The manuscript entitled "Extraction of E171 or E172 Food Additives from Sugar-Based or Lipid-Based Complex Matrices for Primary Particle Identification by Electron Microscopy" is a valuable study. The authors investigated in detail the entire extraction procedure for E171 and E172. The developed extraction method was thoroughly emphasized, adhering to all scientific principles. Furthermore, evidence (photos taken during the experiments) was provided in the manuscript. Therefore, I have no further comments to add for the review. I have carefully read the manuscript. My decision is accept after following minor revisions.

Review Report
1) Keywords should be given in alphabetical order.

2) The author should use “rpm” (revolutions per minute) instead of “rcf” (relative centrifugal force). Most centrifuge devices are designed based on rpm. The readers should be able to understand it

easily.

3) The author should consider modifying the title of the manuscript as follows:

“Extraction of E171 and E172 Food Additives from Sugar-Based or Lipid-Based Complex Matrices for Primary Particle Identification Using Electron Microscopy”

Is the rationale for, and objectives of, the study clearly described?
Yes

Is the study design appropriate for the research question?
Yes

Are sufficient details of the methods provided to allow replication by others?
Yes

Are the datasets clearly presented in a useable and accessible format?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Analytical Chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 10 January 2025

https://doi.org/10.21956/openreseurope.20207.r49210

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Gustavo Moreno-Martín
Universidad Complutense de Madrid, Madrid, Community of Madrid, Spain

The article describes in detail three protocols for the analysis of primary particles contained in the food additives E171 and E172 by TEM.

The article is well written and structured. However, for the extraction of E171, details on the sonication in the ultrasonic bath (e.g. bath power and temperature) are missing. Also, in the extraction of E171 it would be missing to indicate the reagent with which the pH of the Na-Cocodilate buffer should be adjusted if necessary. In this same protocol it is not specified whether the sonication is with a bath or with an ultrasound probe.

The discussion section is rather poor since no numerical value is given for the size, nor is there any mention of morphology or EDX samples. Therefore, it cannot be stated that the reagents used do not affect the physicochemical properties of the analyzed particles.

Is the rationale for, and objectives of, the study clearly described?
Yes

Is the study design appropriate for the research question?
Yes

Are sufficient details of the methods provided to allow replication by others?
Partly

Are the datasets clearly presented in a useable and accessible format?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Characterization of metal and metalloid nanoparticles in complex matrices (food, sludges, plants...). Study of the transformation of selenium nanoparticles after exposure to living organisms (plants and bacteria).

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 08 January 2025

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In the abstract and introduction to the article, the author highlights the complexity and analytical challenges dealing with the characterization of inorganic particles and nanoparticles in foodstuffs in order to meet regulatory requirements.

The work focuses on the extraction of two food additives, titanium dioxide and iron oxide particles (E171 and E172, respectively) from sweet and fatty matrices for analysis by electron microscopy approaches. Two extraction protocols are presented here, selected and adapted on the basis of works available in the literature. These protocols have the advantage of being relatively quick and

easy to implement, which represents a benefit for laboratories in charge of this type of control. This paper clearly highlights this aspect.

However, a number of points not covered in the article are currently lacking to make it a reference work.

- First, the choice of the various extraction media (solvent, ultrapure water, ethanol, enzymes, etc.) and steps (centrifugation, sonication, etc.) is poorly justified in relation to all the data available in the literature (surfactant, stabilizing agent, alkaline digestion, hexane, bath or probe sonication, filtration or ultrafiltration, etc.). The biographical section needs to be strengthened in this respect, in order to understand these different aspects and highlight the choice of protocols, mainly for reasons of implementation (speed, resources).
- The paper does not clearly highlight the analytical developments implemented and therefore the originality of the work. The discussion section deserves to be more detailed, as the paper is more concerned with the implementation of existing protocols.
- The justification of the efficiency of the various extraction steps is mainly based on visual observations of the tubes with the naked eye or on microscopic images of the deposits on the grids. A more detailed quantitative approach (e.g. determination of titanium or total iron in raw samples and extracts at various steps, before and after centrifugation) using ICP-OES or ICP-MS would seem necessary to estimate recovery rates and thus demonstrate a representative extraction of particles from foodstuffs. This quantitative aspect is necessary to ensure the reliability of microscopic observations and granulometric data, which can be measured by SEM or TEM, for example.
- Since the aim is to propose methodological approaches for preparing and measuring particle size distribution (PSD), results obtained on mean and median sizes, %<100 nm, etc. could be presented.
- the method paragraph is too developed (page 3 to 7) compared to the discussion part (1 paragraph page 7)
- In the conclusion, it is pointed out that these protocols can be used for microscopy as well as other equipment. Would it be possible to obtain data in this respect (comparison with spICPMS, DLS, A4F-MALS, etc.)?
- One final point deserves special attention. In fact, observations alone are not sufficient to confirm that extraction does not lead to degradation or modification of the native PSD. To do this, it would be necessary, for example, to compare the PSD of the extracted particles from foodstuffs with those of the raw materials (E171 and E172) of the corresponding batches used for the formulation. Another option is to spike food samples with raw E171 and/or E172 particle, determine the PSD and estimate the influence of the matrices.

Although the amount of work seems substantial, without additional information or data as presented above, the paper clearly lacks the relevance and impact to be indexing without major modifications.

Is the rationale for, and objectives of, the study clearly described?

Partly

Is the study design appropriate for the research question?

Partly

Are sufficient details of the methods provided to allow replication by others?

Yes

Are the datasets clearly presented in a useable and accessible format?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Analytical chemistry - R&D - Validation - Nanomaterial characterization in consummer products and raw materials - Sample preparation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.