Programa de Doctorado en Biociencias y Ciencias Agroalimentarias

Valoración de Fórmulas de Nutrición Enteral: Bioaccesibilidad de elementos inorgánicos y comparación con parámetros clínicos de pacientes con alimentación enteral domiciliaria

Assessment of Enteral Nutrition Formulas: Mineral and trace element bioaccessibility and comparison with clinical parameters of patients with home enteral nutrition

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TITULO: *Assessment of Enteral Nutrition Formulas: Mineral and trace element bioaccessibility and comparison with clinical parameters of patients with home enteral nutrition*

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TÍTULO DE LA TESIS: Valoración de Fórmulas de Nutrición Enteral: Bioaccesibilidad de elementos inorgánicos y comparación con parámetros clínicos de pacientes con alimentación enteral domiciliaria

DOCTORANDO/A: María Aurora Iturbide Casas

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(Se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La doctoranda ha desarrollado su tesis doctoral bajo nuestra supervisión directa, cumpliendo holgadamente con lo planificado en su Plan de Formación y en el de Investigación. Para ello, ha llevado a cabo un estudio exhaustivo de las fórmulas más habitualmente empleadas en la nutrición enteral, que ha incluido desde trabajos de investigación básica para estudiar la composición nutricional y bioaccesibilidad de los micronutrientes inorgánicos presentes en estas fórmulas, hasta el desarrollo de estudios clínicos longitudinales que han permitido evaluar la idoneidad de las mismas según los resultados de diversos análisis de supervivencia. La tesis ha dado lugar a tres publicaciones (y otra más en fase de publicación) en forma de artículos científicos en revistas indexadas en el Journal Citation Reports (JCR).

Además, ha realizado una estancia de tres meses en la Facultad de Farmacia de la Universidad de Lisboa (Portugal) bajo la supervisión de la Dra. Cristina Carvalho del Dpto. de Bromatología y Toxicología Alimentaria, donde ha completado su formación sobre metodología de investigaciones que emplean cultivos celulares y el uso de la técnica Western-Blot.

Por otro lado, es preciso destacar la gran capacidad de aprendizaje de la doctoranda, tanto en lo que a conocimientos relacionados con la bioestadística y trabajos de laboratorio se refiere, como a su notable mejora en la redacción de artículos científicos. Estamos convencidos de que, tras la defensa de su tesis doctoral, Doña María Aurora Iturbide Casas continuará desempeñando una brillante carrera científica, haciéndose valer como un importante exponente del nivel investigador de la Universidad de Córdoba.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 24 de septiembre de 2020

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Macronutrients and trace elements in enteral nutrition formulas: Compliance with label, bioaccessibility and contribution to reference intakes through a probabilistic assessment**. *Journal of Food Composition and Analysis,*** (2019) 83, 103250. https://doi.org/10.1016/j.jfca.2019.103250	Food Science & Technology 3.721 (2019)	Q1 (32/139)
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Abstract

Enteral nutrition is a type of nutrition support that has an extensive clinical background, yet its use has recently intensified in the last decades due to the improvements in materials and formulations. Given that enteral nutrition formulas are commonly used as a single nutrition source, or in situations that require a supplementary intake, corroborating the adequacy and sufficiency of these products is critical. To this end, this dissertation addresses both the nutritional composition of formulas at different levels, as well as the effect they have on patients who use them in the short, medium and long term. To approach this, the content of protein, fat, fiber and some micronutrients (Ca, Fe, Zn, Mg, Cu, Mn) of commercial formulations was determined, laboratory data was then compared to label data. The further analysis assessed the contribution of these products to the Dietary Reference Intake (DRI). Then, *in vitro* bioaccessibility assays were performed to measure the dialyzable fraction of such micronutrients, which also led to reveal common interactions between the formulas´ components. Furthermore, bioaccessibility of one trace element was confirmed through a Cloud Point Extraction speciation method developed for its use in nutrition formulas. Complementary to this, to assess bioaccessibility and antioxidant capacity of selenium present in nutrition formulas these parameters were analyzed at a cellular level using human hepatoma cell models. Different effects on the activity and expression of Selenoenzymes were found, depending on selenium´s chemical form and concentration. To elaborate, a retrospective cohort study was carried out with patients who started enteral nutrition in the province of Cordova, Spain from 2012 to 2017. Characteristics of tube-fed patients in the province are described, whereas Kaplan Meier survival analysis and Cox regression model allowed to shed light on the factors associated with higher mortality. In conclusion, the results of this thesis contribute to the field of evidence-based nutrition thanks to a multi-dimensional assessment. Findings should be considered in the decision-making process of enteral feeding indication and when choosing the formula to be prescribed.

Keywords: Enteral nutrition, Enteral nutrition formulas, Food analysis, bioaccessibility, Minerals, Trace elements, survival analysis.

Resumen

La nutrición enteral es un tipo de soporte nutricional que tiene un amplio respaldo clínico. Sin embargo, el desarrollo de nuevos materiales y formulaciones ocurrido en las últimas décadas ha propiciado que su uso se haya intensificado notablemente. Dado que las fórmulas de nutrición enteral frecuentemente se usan como fuente de nutrición única o como una necesidad complementaria, es crítico ratificar la idoneidad y suficiencia de estos productos. Con este propósito, esta tesis aborda tanto el análisis de la composición nutricional de las fórmulas a distintos niveles, como el efecto que tiene en los pacientes que utilizan estos productos a corto, medio y largo plazo. Para ello, se determinó en primer lugar el contenido de proteína, grasa, fibra y algunos micronutrientes (Ca, Fe, Zn, Mg, Cu, Mn) en formulaciones comerciales, comparando los resultados obtenidos en el laboratorio con los datos del etiquetado. Posteriormente se evaluó la contribución de estos productos a la Ingesta Dietética de Referencia (IDR). Luego, se realizaron ensayos *in vitro* de bioaccesibilidad para medir la fracción dializable de dichos micronutrientes, lo que también reveló interacciones comunes entre los componentes de las fórmulas. Además, la bioaccesibilidad de alguno de estos elementos traza fue confirmada mediante un método de especiación ¨cloud point extraction¨ desarrollado para su uso en estas formulaciones. Para evaluar la bioaccesibilidad y la capacidad antioxidante del selenio presente en las fórmulas de nutrición, estos parámetros se analizaron a nivel celular utilizando modelos de células de hepatoma humano. Se encontraron diferencias en el efecto del selenio sobre la actividad y expresión de Seleno-enzimas según la forma química utilizada y la concentración biodisponible. Asimismo, se realizó un estudio de cohorte retrospectivo con pacientes que iniciaron la nutrición enteral entre el 2012 y 2017 en la provincia de Córdoba, España. En este apartado se describen las características de los pacientes alimentados con sonda en la provincia, se realiza un análisis de supervivencia de Kaplan Meier y se evidencian los factores independientes asociados con una mayor mortalidad con el modelo de regresión de Cox. En conclusión, los resultados de esta tesis contribuyen al campo de la nutrición basada en la evidencia gracias a una evaluación multidimensional del tema.

Estos hallazgos se deben tener en consideración al decidir indicar alimentación enteral y al elegir una fórmula determinada.

Palabras clave: nutrición enteral, fórmulas de nutrición enteral, análisis de alimentos, bioaccesibilidad, minerales, elementos traza, análisis de supervivencia.

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Abbreviations and Acronyms

8-HQ	8-Hydroxyquinoline
AAS	Atomic Absorption Spectroscopy
AcH/AcNa	Acetic Acid/Sodium Acetate
$AcNH_4/NH_4OH$	Ammonium Acetate/Ammonium Hydroxide
AI	Adequate Intake
AIC	Akaike Information Criterion
ALC	Absolute Lymphocyte Count
APDC	Ammonium Pyrrolidine Dithiocarbamate
ASPEN	American Society For Parenteral And Enteral Nutrition
BG	Blood Glucose
Bis–Tris	2-[Bis(2-Hydroxyethyl)Amino]-2-(Hydroxymethyl) Propane-1,3-Diol
Ca	Calcium
CG	Cancer Group
CI	Confidence Interval
$ClNH_4/ NH_4OH$	Ammonium Chloride/Ammonium Hydroxide
CPE	Cloud Point Extraction
CRP	C- Reactive Protein
Cu	Cupper
DMA	Dimethylarsenic Acid
DMEM / F12	Dulbecco's Modified Eagle's Medium / F-12 Nutrient Mixture
DRI	Dietary Reference Intake
DRV	Daily Reference Value
DTNB	Ellman's Reagent
EDTA	Ethylenediamine Tetraacetic Acid
EFSA	European Food Safety Authority
EGTA	Ethylene Glycol Tetraacetic Acid
EN	Enteral Nutrition
ENF	Enteral Nutrition Formulas
ESPEN	European Society Of Clinical Nutrition And Metabolism
ETAAS	Electrothermal Atomic Absorption Spectrometry
ETF	Enteral Tube Feeding
EU	European Union
F1/2/3	Formula 1,2,3
FBS	Fetal Bovine Serum
Fe	Iron
FOS	Fructooligosaccharides
FSMP	Foods For Special Medical Purposes
GI	Gastrointestinal

GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
h	Hours
H_2O_2	Hydrogen Peroxide
HBP	High Blood Pressure
HCl	Hydrochloric acid
HDL	High-Density Lipoprotein
HEN	Home Enteral Nutrition
HepG2	Human Hepatoma Cells Lines
HNO_3	Nitric Acid
HPLC	High Performance Liquid Chromatography
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IgG	Immunoglobulin G Antibody
IgG–HRP	Horseradish Peroxidase Conjugated Antibody
IOM	Institute Of Medicine
KM	Kaplan Meier
LCT	Long-Chain Triglycerides
LDL	Low-Density Lipoprotein
LOD	Limit Of Detection
LOQ	Limit Of Quantitation
MCT	Medium-Chain Triglycerides
MES	4-Morpholineethanesulfonic Acid
Mg	Magnesium
MMA	Monomethylarsenic Acid
Mn	Manganese
MNT	Medical Nutrition Therapy
MR	Mortality Risk
MTT	(3,4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MUFAs	Mono-Unsaturated Fatty Acids
NaBH4	Sodium Borohydride
NaCl	Sodium Chloride
NaCOH	Nacoh3
NaF	Sodium Fluoride
NaOH	Naoh
NF	Nutrition Formulas
NG	Neurologic Impairment Group
NGT	Nasogastric Tube
NICE	National Institute For Health And Care Excellence (UK)
NIVs	Nutrient Intake Values

NRV	Nutrient Reference Value
ONS	Oral Nutritional Supplements
PB	Peptide Based
PBS	Phosphate Buffered Saline
PEG	Percutaneous Endoscopic Gastrostomy
PMBP	1-Phenyl-3- Methyl-4-Benzoyl-5-Pyrazolone
PRI	Population Reference Intakes
PUFAs	Polyunsaturated Fatty Acids
RM	Regular Media
S.E.M	Standard Error Of The Mean
SDS–PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Se	Selenium
Sec	Selenocysteine
SFA	Saturated Fatty Acids
SP	Serum Proteins
T2DM	Type 2 Diabetes
Trx	Thioredoxin
TrxR	Thioredoxin Reductase
Zn	Zinc

1. Introduction

As life expectancy has increased, so have the rates of diseases associated with the elderly population. Such is the case of chronic degenerative diseases, which start to increase at 65 years old and escalate at the age of 85 [1]. This aging population and the patients who suffer from neurologic and neurodegenerative diseases often experience difficulties in feeding properly by mouth because of factors such as mobility loss, swallowing difficulties (dysphagia) and loss of appetite, among others. In addition to a deficient diet, patients may suffer from highly prevalent concomitant diseases like type 2 diabetes or cancer, which are emerging at younger ages. Many of these chronic diseases (cancer, chronic obstructive pulmonary disease, and cerebrovascular events) are by themselves the origin of disease-related malnutrition, which deteriorates physical and psychological functions and increases morbidity and mortality [2]. It is estimated that 30 to 50% of hospitalized patients over 60 years old are malnourished at admission, which adds to common hospital conditions that affect food intake, causing further deterioration of the nutrition status. Clinically, malnutrition gives place to a series of medical complications, that involve a higher infection rate manifesting an impaired immune system, or alterations of the cognitive status, wound healing, and increasing pressure ulcers, falls, fractures, intolerance to pharmaceutical treatment and even fatal prognosis. Economically its impact is sturdy too, with increased length of stay, more intensive care admissions, and hospital readmissions [3, 4].

This fundamental role nutrition plays at maintaining and achieving health is well recognized worldwide nowadays. Correspondingly, a timely screening is the first step towards identifying the need for nutrition intervention. Malnourished patients or patients at risk of malnutrition that have oral feeding difficulties or cannot feed by modification or adaptation of their regular diet may be candidates to medical nutrition therapy.

In this way, enteral nutrition (EN) became particularly relevant as it created the possibility to deliver energy and nutrients to patients unable to obtain them orally [5]. This nutrition support feeds patients using tubes that go to the gastrointestinal tract without the need to turn to riskier

and more invasive techniques, such as parenteral nutrition. Besides, its use has proven to decrease the length of in-hospital stay, infection-related complications, and healthcare costs [6–8]. However, before EN indication, candidates undergo a thorough evaluation to assess the risks and benefits of this therapy. Such assessment gathers relevant information needed to create a care plan and help determine the most appropriate administration route, and also to identify potential adverse events, gastrointestinal intolerances and metabolic and fluid disturbances [9]. Afterwards, the patient´s needs of energy, macronutrient, fluids, and micronutrients are estimated, and a suitable product (usually commercially available formulations) for its condition and personal requirements is chosen.

In the last few decades, this therapy and the products related to it have significantly evolved, along with these improvements its use has escalated at home and in hospital facilities. The last national Spanish (voluntary) survey accounts for 90.51 home enterally-fed patients per 1,000,000 habitants in 2015 [10]. Just in the US, 250,000 patients per year use EN in hospitals, additionally to patients at residential care settings and home enteral nutrition (HEN) [9, 11] which has an estimated prevalence of 1385 patients per million inhabitants (2013 data) [12]. The estimated cost is around 266 euros per patient per month (only including feeds/formulas and delivery materials)[13]. The feeds used by tube-fed patients have significantly progressed from their precursory blenderized diets in the last half-century. Current commercially available Enteral Nutrition Formulas (ENF) contain enough macro and micronutrients to cover daily Dietary Reference Intake (DRI) in a determined volume specified in each product [14, 15].

These days there is a wide variety of ENF from which the healthcare team can choose from depending on multiple characteristics like calorie/volume density, macronutrient distribution, macronutrient hydrolysis, or even adapted to specific diseases (kidney, liver and pulmonary diseases, compromised immune system or glycemic control) [16]. When opting for the ENF to be prescribed to a patient, health professionals rely on the information given by pharmaceutical sales representatives, product´s brochures, and the information displayed in the product label [17]. Meanwhile, if the prescription is within the public health network (and

subsidized) availability of products will also be influenced by the trade agreements made with the pharmaceuticals upon acceptance of the national health system [18]. As ENF compositions can be very different when it comes to volume and calorie density, macronutrient distribution or added compounds (i.e. fiber, immunomodulating agents), the indication of the appropriate product for each patient relies on transparent and trustworthy information.

However, not all the relevant information is included in the description of each product. In this case, we are referring to bioaccessibility data. Bioaccessibility refers to the compound fraction that is released from the food matrix and is solubilized in the gastrointestinal tract and therefore, susceptible to being absorbed [19]. That fraction that would be available to be used to perform its physiological role is the bioaccessible fraction. Moreover, the amount of the ingested minerals and trace elements can significantly differ depending on different factors affecting each mineral or trace element, like its chemical form and the synergetic or antagonistic interactions with the rest of the nutrients present [20–22]. As ENF are commonly used as a single nutrition source, assessing the accuracy of the information displayed on the label, as well as their sufficiency, is vital. Hence, this doctoral dissertation was carried out, which is structured as follows.

In **Chapter I**, a thorough analysis of ten different ENF (bought in Spain and Mexico) is presented. This assessment comprises the determination of major nutritionally relevant minerals and trace elements (Ca, Mg, Fe, Zn, Cu, and Mn), as well as their *in vitro* bioaccessibility obtained through laboratory analysis. Similarly, the content of protein, fat and dietary fiber was also determined; then, the obtained measurements of these macro and micronutrients were compared to the information provided by each product´s label to assess compliance. Finally, the contribution of the studied micronutrients to reference intakes was evaluated using a probabilistic model (@Risk). The models were developed from values of total and bioaccessible (dialyzable) trace element content. It should also be noted that the present statistical tool was completed using the variability of inorganic elements present in food as well as the variability of the ENF ingested. Both aspects determine the total amount of inorganic elements ingested [23].

On the other hand, a more in-depth insight of micronutrient analysis is displayed in **Chapter II**, where cloud point extraction and atomic absorption spectroscopy (AAS) are used in a novel preconcentration and speciation method which was then used to distinguish between ionic (free) Mn and bound Mn from the bioaccessible fraction of ENF. The chapter describes the response to three chelating reagents [8-hydroxyquinoline (8-HQ); 1-phenyl-3- methyl-4-benzoyl-5-pyrazolone (PMBP); and ammonium pyrrolidine dithiocarbamate (APDC)], along with a non-ionic surfactant (Triton X-100) in different operating conditions such as pH, temperature and time that were used to develop and optimize the method.

The methodology and findings from further laboratory research can be found in **Chapter III**. However, this time it is to address the effect of Selenium (Se) as an antioxidant enhancer in ENF. Given the importance of selenoproteins in the maintenance of the balance in the redox state (thus, in the cellular and organismal functions), the effect of Se exposure in Selenoproteins´ activity and expression (*Thioredoxin reductase (*TrxR) and *Glutathione peroxidase (*GPx)) was studied using Human hepatoma cells lines (HepG2) (see **Fig. 1**). The bioaccessibility method firstly described in Chapter I was used to obtain the bioaccessible fraction of ENF, which was later used as a medium to treat HepG2 cell cultures. The viability essays (with MTT (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) and the optimization process previously done are also described here.

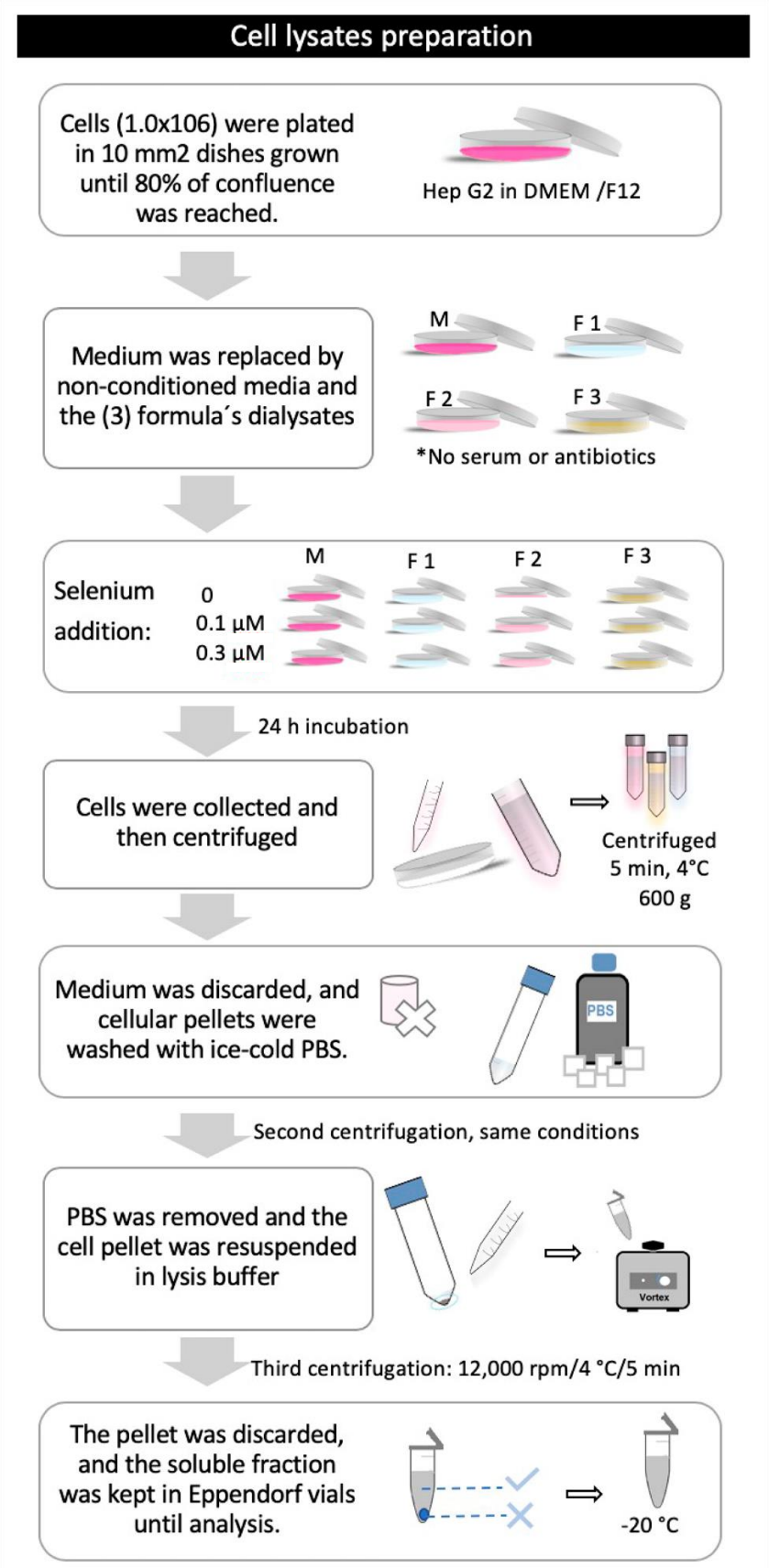

Figure 1. *In vitro process for the evaluation of selenium bioaccessibility in the dialyzable fraction of ENF.*

Nevertheless, after assessing and analyzing multiple aspects concerning ENF composition and its possible effects on patients, the most conclusive results can be obtained when the effect of ENF is studied in real settings and real patients. Hence, in **Chapter IV,** this thesis targets patients fed with Enteral Tube Feeding (ETF) using a retrospective analysis of 377 patients from a tertiary hospital in the city of Cordoba, Spain. Sociodemographic, biochemical, and clinical data were analyzed. Kaplan Meier and Cox regressions were used to assess survival expectancy and baseline characteristics that pose a higher mortality risk, such as underlying disease, present comorbidities, and blood parameters. Differences in the short-, medium-, long-term, and all-time mortality rates and risk factors can be seen there. We expect that this chapter manages to accomplish what was stated by a peer-reviewer, *¨The content in this article will be helpful for clinicians, patients and family members applying patient-centered care at the pivotal point of making decisions about initiation of enteral nutrition. ¨*

2. Conceptual framework

2.1 Enteral Nutrition

In clinical practice and research, the terms enteral nutrition (EN) and enteral tube feeding (ETF) are often used interchangeably, although enteral feeding could also mean oral feeding if commercial nutrition formulas are used. However, by definition, the American Society for Parenteral and Enteral Nutrition (ASPEN) states that EN is ¨the system of providing nutrition directly into the gastrointestinal (GI) tract bypassing the oral cavity¨ [9]. Similarly, The European Society of Clinical Nutrition and Metabolism (ESPEN) defines ETF as ¨nutrition therapy given via a tube or stoma into the intestinal tract distal to the oral cavity¨ [24]. This nutritional support is included as medical nutrition therapy (MNT) together with oral nutritional supplements (ONS), and parenteral nutrition (intravenous). ETF and parenteral nutrition (which were formerly called artificial nutrition) can be used as a single feeding source, complementing each other or supplementing the oral intake [24].

When diet alone is not enough to maintain or restore and adequate nutritional status, the most physiological and less invasive MNT is used. Usually, this would result in supplementing a regular diet with ONS as the first approach. Then, if energy and nutrients needs cannot be met orally (even with the use of ONS) because the patient is unable or unwilling to eat, ETF is indicated if the patient has a functioning gastrointestinal tract [25, 26]. These situations may be brought due to a reduced capacity to feed orally or sufficiently, i.e. neurologic disorders (dysphagia), head and upper GI trauma or injury, and comatose state. Hypermetabolic states (burns, cancer, heart failure, inborn errors of metabolism, Crohn's disease, etc.), and impairments in digestion, absorption and metabolism are other causes of EN prescription [5]. A simple flow diagram of this process is shown in **Figure 2**.

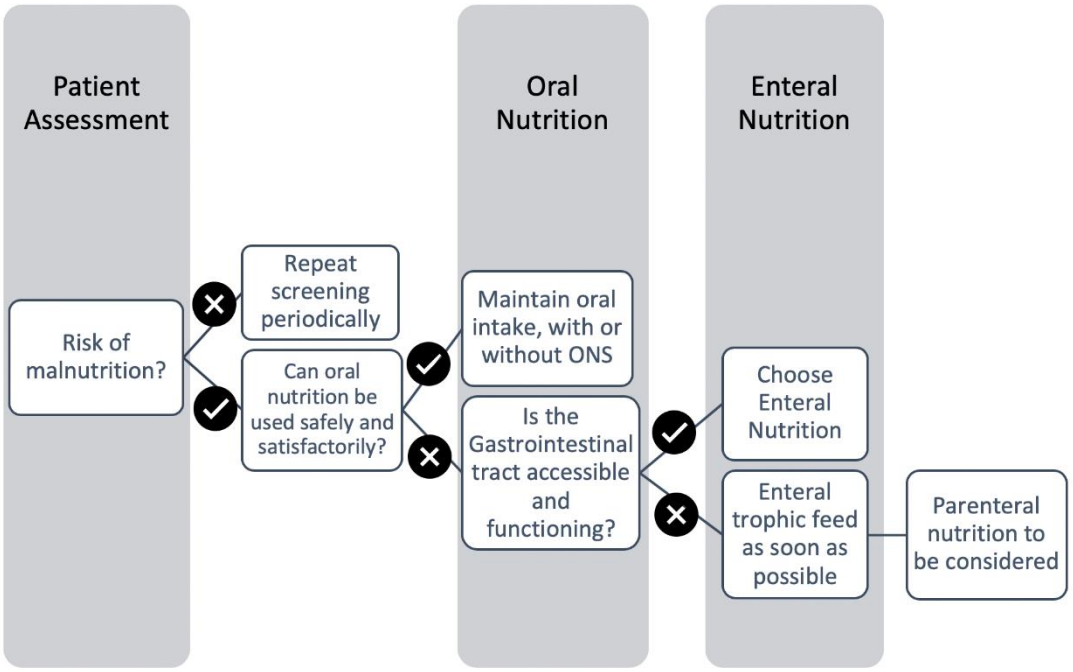

Figure 2. *Nutrition therapy selection process, personal creation based on the ¨Patient Pathway Algorithm¨ from the NICE Guideliness [27].*

However, assessing the appropriateness and safety of EN is a complex multicomponent task that should not be taken lightly. It must include firstly, data on the patient´s history including clinical diagnoses, interventions, medications, and supplements, not to forget its social and religious background, or potential ethical dilemmas, and mental status challenges [27]. Secondly, a nutrition-focused physical evaluation, including aspects such as the gastrointestinal function and symptoms, as well as the skin condition, fluid status, and handgrip strength, must be performed [5] . Meanwhile, anthropometric data may include weight history, body mass index, and nutrition status indicators like calf, arm, or neck circumferences. Lastly, a series of laboratory values reflecting nutrition, inflammation, and hydration status (comprehensive blood panel, urinary tests, or predictive nutritional indexes) should be used. They may be predictors of morbidity and mortality [9, 28].

After a very exhaustive assessment of the EN-candidate has concluded the eligibility of the patient, the feeding route is determined. Then, the needs of fluid volume, energy, protein, and macronutrients are estimated based on the patient's status, and an ENF is chosen [29].

2.2 Nutrition Formulas and Composition

ENF are nutritional products specially formulated to be administered via the gastrointestinal tract under medical supervision. These are intended for the dietary management of patients with a limited, impaired, or disturbed capacity that cannot be fed with ordinary food. For this reason, they are classified as foods for special medical purposes (FSMP) and being under the Regulation (EU) No 609/2013 of the European Parliament and of the Council on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control [30].

Most ENF used nowadays come in a liquid, ready to use format to avoid manipulation and contamination. When EN is used as a single nutrition source, ENF that are nutritionally complete are prescribed. This fact means that when a specific volume of such ENF is administered, it provides enough energy, macro, and micronutrients to cover the DRI. Besides, depending on the formula and its characteristics, the volume needed is around 1,300 – 1,900 mL/day [31]. A standard ENF will meet the nutritional needs of the general population with 1,500 mL. However, at the moment, there is an extensive repertoire of ENF available (see **Fig. 3**). They are most commonly classified by its energy density (low-energy, 0.5-0.8 kcal/mL; standard 0.9-1.2 kcal/mL; and high-energy, > 1.2 kcal/mL), the complexity of their protein structure (intact protein/polymeric/standard, peptides and amino acids/elemental), their protein content (low, 6-8%; standard, around 15%; high, >20%), or its indication for specific diseases (renal, hepatic, pulmonary, glycemic control, severe allergies, and immune-modulating). Besides the mentioned characteristics, fiber-free, milk-free, and low-sodium ENF are also available [32–34].

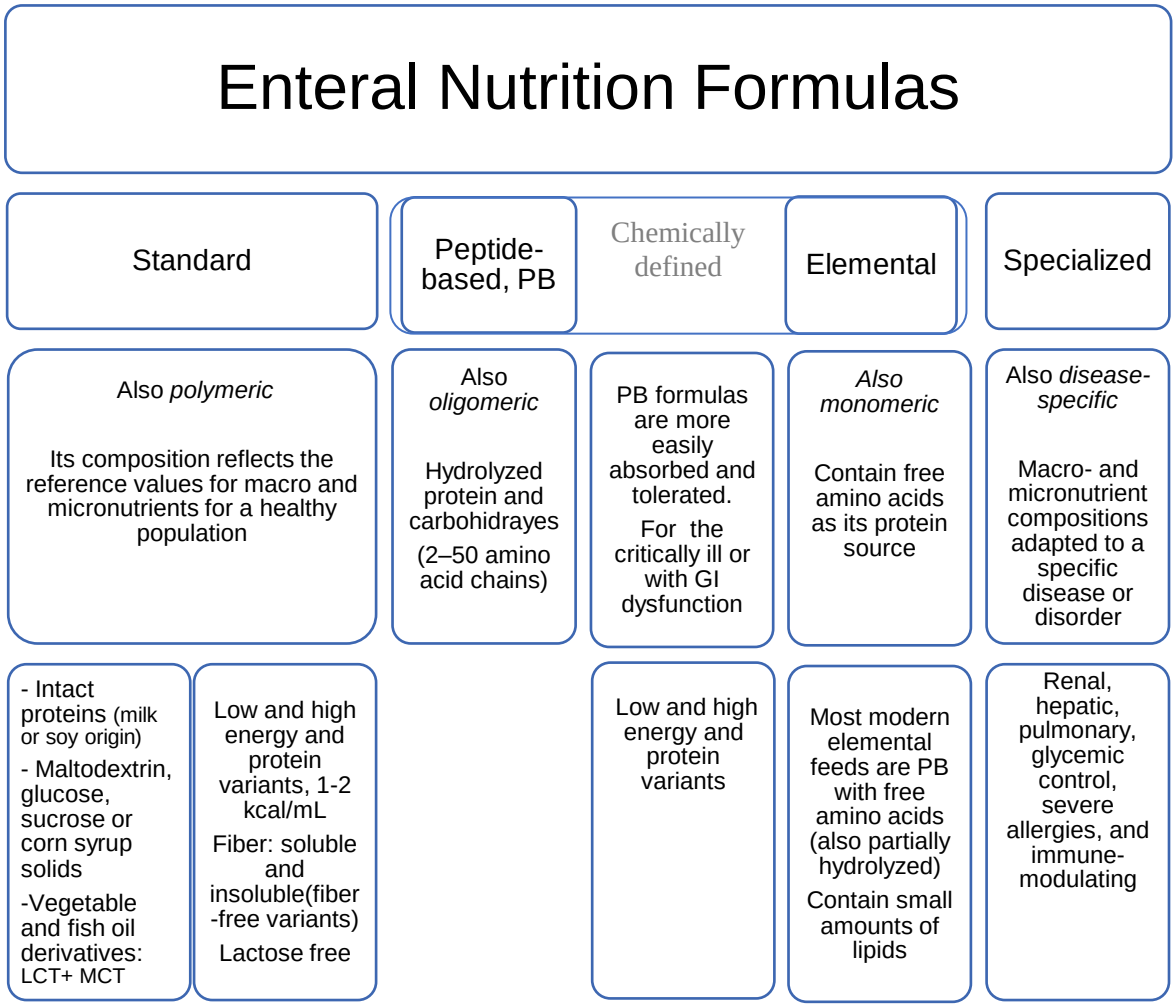

Figure 3*. Classification of commercially available enteral nutrition formulas*

GI, gastrointestinal; LCT, long-chain triglycerides; MCT, middle- chain triglycerides; PB, peptide based

[30, 35, 36].

2.3 Macronutrients in ENF

Proteins´ importance in nutrition is well known mainly because of their structural role; they are essential for the organism´s growth, maintenance, and repair; however, they are also needed to operate as enzymes, carriers, hormones, and membranes. According to the current recommendations of the Institute of Medicine (IOM), the acceptable macronutrient distribution range for proteins may supply from 10 to 35 % of daily energy, or a minimum of 0.8 g/kg/day [35, 36] to replace daily basic nitrogen losses (i.e. urine, fecal matter, sweat, skin) [37]. This

agrees with the European Food Safety Authority (EFSA) [38]. However, this data may be underestimated, especially in an older population or during stress periods, as these groups are at higher risk of experiencing muscle loss, reduced energy intake and physical activity, and comorbidities [39]. Meanwhile, in ENF, proteins can take up from 6 to 25 % of total calories, and they may be added as casein/caseinates, whey, soy, egg whites and peas [5]. Of all these sources, casein protein is one of the most commonly used; others have found that on short-term (1, 3, 5, and 7 days) its effect on serum prealbumin and C-reactive protein is similar to that of ENF with egg-whites [40].

Fats, on the other hand, are mainly known for being a major energy source and aiding on the absorption of fat-soluble vitamins and antioxidants. Nevertheless, fatty acids (obtained by the breakdown of fats) are also needed to act as membrane structures, precursors or ligands for the receptors that regulate adipogenesis, inflammation, insulin action and neurological function [35]. They may be ingested in the form of saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs), or polyunsaturated fatty acids (PUFAs). As the body can synthesize SFAs through the ingestion of other fatty acids, there is not a minimum of them to be ingested. However, there is the recommendation that their intake should be ¨as low as is possible¨ according to the EFSA [41], or less than 10% of daily energy according to the IOM [35]. Likewise, MUFAs lack a dietary reference value as they can also be synthesized by the body [42].

Conversely, PUFAs cannot be synthesized by the body. Therefore, they are considered essential fatty acids; to prevent their deficiency 5 to 10 % of daily energy ingested should be provided by these fatty acids [43]. The recommendation for the general population is that fats provide 20–35 % of daily energy ingested [38]. In ENF, this nutrient can supply from 1.5% (modular products) to 55% of the total calorie content, mostly as long-chain triglycerides (LCTs). As ENF produce higher insulin and glucose responses than regular food, modifying the percentage of fat has been useful in formulations for better glycemic control. Thus, low carbohydrate — high MUFA versions of ENF (for diabetes) have been developed. In these ENF, about 35% of total energy is provided from mono-unsaturated fatty acids [34]. Primary fat sources

used in the formulations are corn, sunflower, and canola oil, although some formulations now contain olive oil. Some standard ENF (and all the elemental ENF) also have medium-chain triglycerides (MCTs) added, with the particularity that these can be absorbed in the portal circulation without enzymatic digestion but lack essential fatty acids. Moreover, some ENF have structured lipids, which are formulated to contain both LCTs (like omega-3 fatty acids) and MCTs, with the properties of both and the advantage of being better tolerated than the combination of LCTs with MCTs previously mentioned [5].

Complementary to this, **carbohydrates** are also used to provide energy, fueling the body's cells is their leading role [35]. There is not an optimal amount of this macronutrient, while on average 100- 130 g /day is enough to maintain the brain´s daily demands (and avoid ketosis), the recommendation for a standard diet is to provide 45–65 % of daily energy from this nutrient [35, 38]. Among the types of carbohydrates available, intake of the ones that are rapidly digested and absorbed (i.e. glucose, fructose, sucrose, lactose, maltose) should be limited and slowly absorbed starchy foods should be the main source. Likewise, in ENF, their content can be variable, providing as much as 30 to 85% of the total energy. They may be added as maltodextrins, cornstarch, corn syrup solids, or combinations of them [5].

Lastly, **fiber** may also be added to ENF, although previously, it was avoided to prevent tube obstruction and because of the idea that a bowel rest would be more beneficial [34]. However, in the last decades, it has been incorporated into multiple ENF, as the beneficial effect of water-soluble (pectins and gums) and -insoluble (cellulose or hemicellulose) fiber, and fructooligosaccharides (FOS, prebiotics) has been recognized [34]. In standard conditions, it is suggested that enterally fed patients meet the fiber recommendation for the general population (14 g/1,000 kcal). However, in ENF, fiber´s purpose is to maintain gut physiology and tolerance, as well as glycemic and lipid control [35, 44]. Even though the use of fiber-enriched ENF in critical care is still inconclusive, it is suggested that it should also be used in the intensive care unit as it causes fewer episodes of diarrhea than fiber-free ENF. Fewer gastrointestinal complications mean less feeding interruptions; hence, less protein-energy malnutrition risk [45].

2.4 Micronutrient relevance in general population and enterally-fed patients

Standard ENF usually have similar compositions, while disease-specific formulas often have a different vitamin and mineral profile adapted to the condition for which they were intended [5]. Nevertheless, as mentioned before, most ENF are nutritionally complete, or in other words, they provide enough micronutrients to satisfy the DRVs for the general (healthy) population. Depending on the country or area, it is also known as Dietary Reference Intake (DRI; the US and Canada), the nutrient reference value (NRV; Australia and New Zealand), or as a global harmonization workshop proceeded by the National Academies of Science recently (2018) agreed on, ¨nutrient intake values¨ or NIVs [46]. The term DRVs involves a group of nutrient reference values, and the EFSA NDA Panel defines it as ¨quantitative reference values for nutrient intakes for healthy individuals and populations which may be used for assessment and planning of diets¨ [47]. Among these reference values, the Population Reference Intakes (PRI) is the one that defines the amount of intake that is adequate for virtually all people in a population group. However, when a PRI cannot be established because there is not enough evidence, the Adequate Intake (AI) is used as a reference [41]. The latter refers to ¨the average observed daily level of intake by a population group (or groups) of apparently healthy people that are assumed to be adequate¨. These DRVs are needed in dietary assessment and planning. Depending on the data available, the recommendations by official organs may be given as any of these values [41].

In this section, the central theme will be minerals and trace elements that are more nutritionally relevant, either because of their body amount or the complications derived from an imbalance of them. Inorganic micronutrients as part of nutritional supplements can be defined as ¨ a group of substances essential for regular metabolism, growth and development, body structure, regulation of cell function, and electrolyte balance in body fluids¨ [48].

To start with, Calcium (Ca) is the most abundant mineral in the human body, most of it carrying a structural role in bones and teeth (as calcium hydroxyapatite), just 1% is used as an

intracellular messenger [41]. Providing a sufficient intake of this mineral (PRI 950 mg/d ;>25 years, male) is essential to preserve the integrity of the bone mass, as blood calcium concentrations are maintained by resorption of Ca from the skeleton when it is not externally provided [41]. Absorption of this mineral in the intestinal lumen can be done actively by transcellular absorption (in the duodenum) when intake is low, or passively as paracellular absorption (in the jejunum and ileum.) when Ca availability is high [49].

Next, magnesium (Mg) is a very relevant earth metal needed in more than 300 enzymatic reactions where it acts as a cofactor, making it vital in the metabolism for the synthesis of carbohydrates, lipids, and proteins [41]. It is stored mainly in the bone and muscle, and a minimal amount is free in the serum. Along with Ca, it is considered a macro element as its daily requirement rises over 100 mg/day; in this case, it would mean daily intake of 350 mg/d (AI, for males). Its absorption takes place in the distal intestine and is aided by the fermentation of soluble fiber [41]. Whereas phytic acid and phosphate reduce its absorption, a marked deficiency of this mineral can lead to neurological and cardiac symptoms [41].

Alternatively, Iron (Fe) is a trace mineral widely recognized for its function of oxygen transport. It is also involved in electron transfers, oxidase activities, and energy metabolism [50]. Seventy-five percent of Fe is present in the blood, and the rest is in the liver, bone marrow, and muscles, adding up to 3 to 5 g in the human body [51]. Its absorption is increased when needed to maintain homeostasis and takes place mainly in the duodenum; it is hugely variable depending on the host and diet-related factors. For instance, it is facilitated by acidic conditions and impaired by phytates and oxalates [51]. A deficit will cause (microcytic) anemia, one of the most common nutritional deficiencies, mainly when there is an impaired absorption or in infection and inflammation. A prolonged deficit can lead to heart failure and can be fatal; to avoid it, the PRI is set to 11 mg/d (>18 years, males) [41, 51].

Zinc, on the other hand, is a trace mineral needed in a variety of primarily intracellular metabolic processes playing a structural and regulatory paper. Furthermore, it is also a catalyst ion in enzymes and forms part of proteins whose biological roles include transcriptional and

translational control/modulation and signal transduction [41]. During illness, it may be supplemented due to its role in immune function, wound healing, and antioxidant properties [52]. Zinc storage sites include prostate, eyes, brain, muscle, bones, kidney, and liver; its concentration in blood plasma depends on its intake [52]. Its absorption occurs in the upper small intestine and can be affected by phytates, influencing the percentage of zinc that is available for absorption [41]. The established PRI for this element ranges from 9.4 to 16.3 mg/d (males, >18) depending on the phytate content of the diet [41]. It is estimated that approximately 17% of the global population does not meet an adequate zinc intake [53]. A deficit of this mineral can create a series of nonspecific metabolic changes that can lead to increased infections and skin lesions, but there are no characteristic signs or symptoms [54].

Copper (Cu) relevance is given by its part as a central component of multiple enzymes needed for neurotransmitter synthesis, energy metabolism, and collagen and elastin cross-linking [41]. Cytochrome c oxidase, superoxide dismutase, and lysyl oxidase are just a few of them [41, 52]. Deficiency, even though rare, can cause neurologic manifestations or hematologic disorders like anemia and neutropenia [55]. As Cu is present in almost every tissue of the body and has a very low daily demand (AI of 1.6 mg/d for males > 18 years), a deficiency would need a long time to develop. But poor absorption of this trace element could be secondary to gastric and bariatric surgery, as well as an excessive Zn ingestion [41, 55]

Another essential trace element involved in amino acid, lipid, and carbohydrate metabolism is Manganese (Mn); it is also involved in neurological, immune, and antioxidant metabolism [56]. Mn makes up relevant metalloenzymes like superoxide dismutase, arginase, and pyruvate carboxylase [37]. Enough supply of this element is needed for the healthy brain performance, the activity of the nervous system and bone structure [49]. An AI around 3 mg/day is estimated based on observed intakes of European adults. The body is capable of adapting quickly to variations on its intake to maintain homeostasis, to the point that there has not been described a Mn deficiency profile [41].

Finally, Se, another key trace element, is a constituent of multiple selenoproteins that carry out a variety of functions in humans. Some of the physiological effects that stand out are the antioxidant defense (glutathione peroxides and thioredoxin reductase), immunomodulating effects, and thyroid and skeletal and cardiac muscle metabolism [41]. Clinically, signs of hair browning, nail whitening, and macrocytic anemia suggest a deficiency of Se [57]. In populations with very low intake, it has also been observed to generate: bone and joint disease, loss of appetite, fatigue, cardiomegaly, congestive heart failure, and cardiac insufficiency, arrhythmia and palpitations [52]. An AI of 70 µg/day for adult men and women is recommended [41]. Although, critically ill patients have shown the benefit of higher doses between 500 - 750 µg /day to be used only during acute periods up to 1 to 3 weeks (depending on the severity of disease)[58]. Its supplementation in Se-deficient populations has also shown less cancer incidence and mortality, but elevated levels of Se have been associated with insulin resistance and diabetes [59, 60]. These divergent effects on health demonstrate the importance of the balance needed when it comes to Se. An adequate intake of Se is needed by selenoproteins to maintain the intracellular redox status. However, an excessive Se concentration has an oxidant effect by generating oxygen free radicals leading to apoptosis [61].

2.5 Bioavailability and bioaccessibility

Knowing that the body does not exploit all the nutrients (in this case, minerals and trace elements) ingested has led to the need to quantify the variations and study the interactions that produce them. Such bioavailability information has been used to establish population DRV´s and as well as helped improve nutrient formulations and dietetic recommendations. The bioavailable fraction of a nutrient refers to the content that reaches the systemic circulation and is available to be absorbed by cells and tissue in the body, and then be stored or used in metabolic functions [62]. As deduced from its definition, bioavailability is determined through in vivo methods (in animals or humans), ergo embodying a series of disadvantages such as high equipment and labor

costs, ethical constraints, lack of certified reference standards, and great assay complexity due to influence of multiple factors and the difficulty to extrapolate from animals to humans [63].

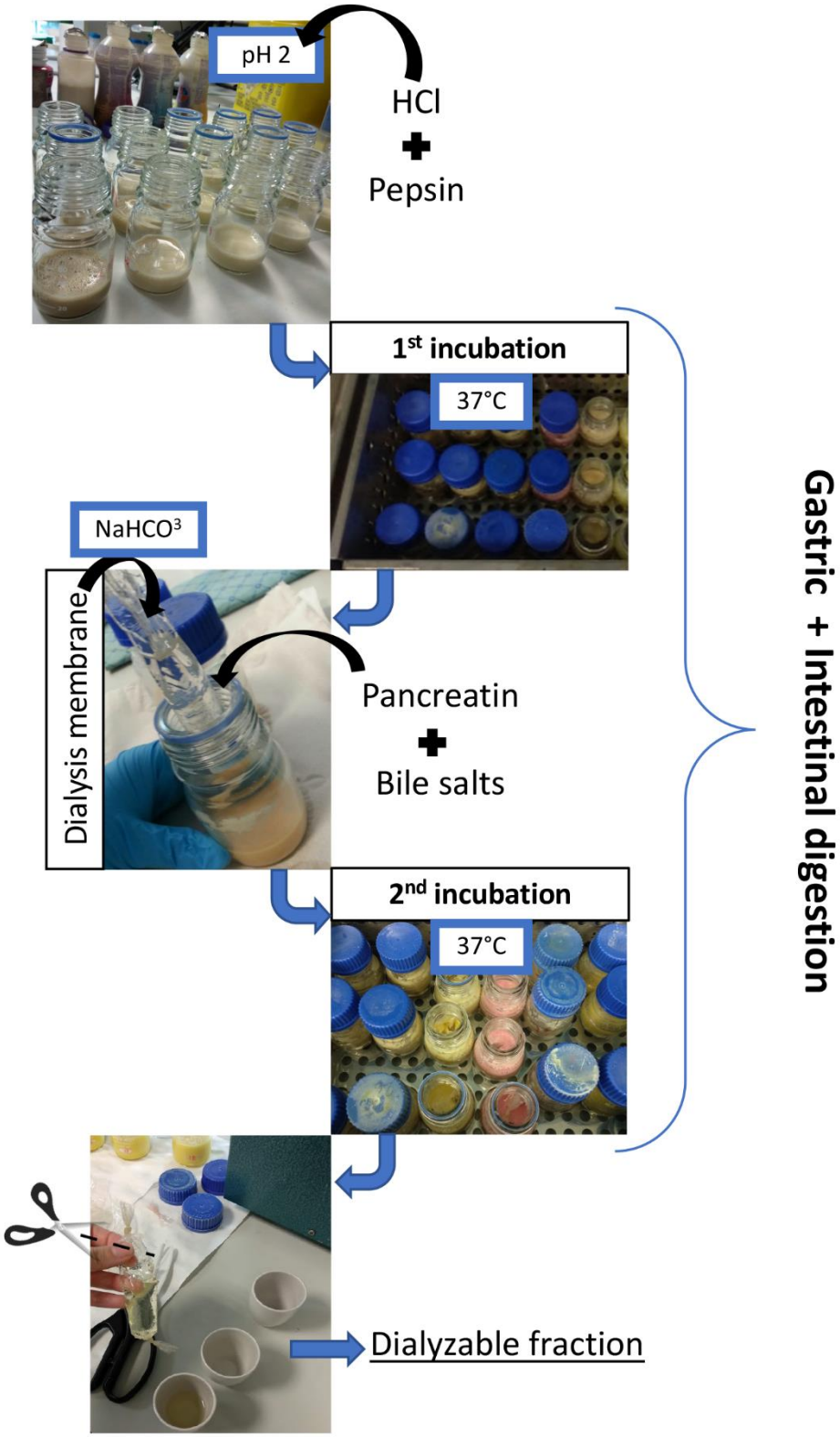

Figure 4. *Bioaccessibility determination through dialysis method as described by Cámara et al. [19]*

Therefore, a useful and practical approach is estimating bioaccessibility, which refers to the fraction of the trace element that is soluble in the gastrointestinal environment and is available for absorption [62]. The later assessment only requires *in vitro* methods which are widely used and happen to have a good correlation to *in vivo* assays. Between these two concepts, the bioaccessible fraction is always equal or higher than the bioavailable [62, 64]. Some of the *in vitro* methods that simulate the biochemical reactions that occur in the human gastrointestinal tract and that are available nowadays are solubility, dispersibility, fractional dialyzability [49, 65]. The process of bioaccessibility determination through dialysis method is shown in **Figure 4**.

Food processing will likely affect nutrient bioavailability. It can help by decreasing anti-nutrient levels, but the exposure to some conditions (i.e., light, moisture, heat, and oxygen) and reaction with other food constituents (i.e., carbohydrate and protein biopolymers) can lower its levels [49]. In such cases, in vitro methods provide reliable knowledge on possible interactions between nutrients, and bioavailability and bioaccessibility [66]. Although said bioavailability depends on multiple factors like digestion, release from the food matrix, the absorption rate of the compound by enterocytes, and its transport to body cells [49], there is an estimated percentage for each essential micronutrient. For example, the absorption of Mg is considered to be 40–50%, although the range can extend from 10 to 70%. Ca and Se, on the other hand, have a high absorption assumed to be 60 and 70%, respectively. Next, according to absorption approximations, are Cu with 50% and Zn with 30%. The expected Fe absorption is 16% in men and 18% in (premenopausal) women, and the lowest absorption value is for Mn, with an estimated < 10% [41].

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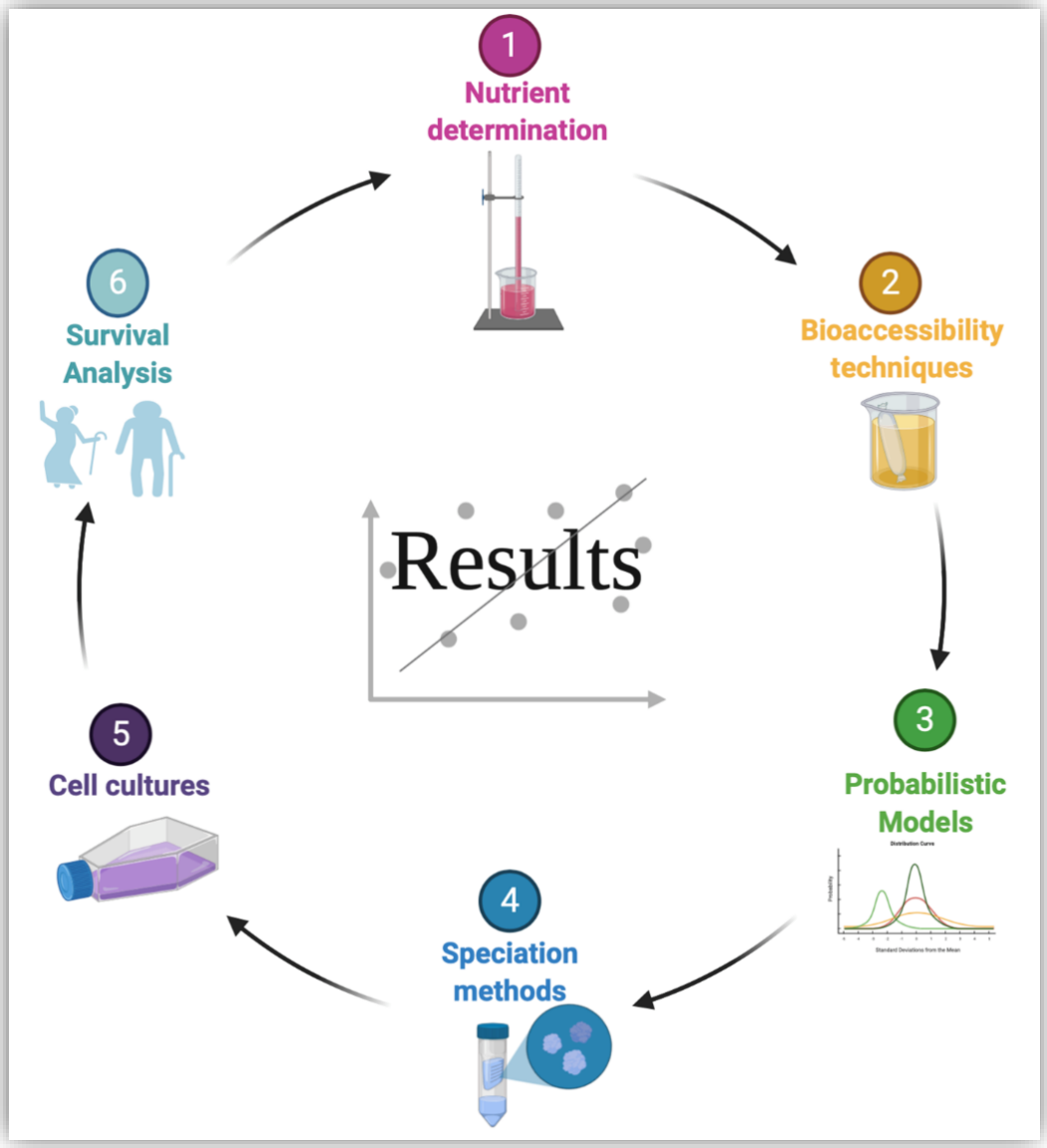
3. Objectives

The overall aim of this dissertation is to contribute to the field of evidence-based nutrition, particularly medical nutrition therapy. To achieve this, a comprehensive multi-dimensional assessment of enteral nutrition formulas is approached through the following objectives:

- Analyze the content of macro and micronutrients present in ENF from different pharmaceutical companies, comparing the values obtained from laboratory analysis to what is stated on the label. Subsequently estimating the contribution of these products to the DRI (Chapter I).
- Determine b of trace elements incorporated in commercial ENF using *in vitro* methods (dialyzability tests). Estimate the contributions to the DRI from bioavailability measurements (Chapter I).
- Develop a speciation method to be used in enteral nutrition formulas for trace element analysis. Assess its influence on the bioavailability data previously obtained (Chapter II).
- Determine the content of heavy metals (Hg) in enteral formulas, as well as their bioavailability. Assessment of its toxicity (Absence of contamination found in Chapter III).
- Compare bioavailability results from ENF with biochemical markers of nutritional status in patients enterally fed. The variables that indicate the nutritional and metabolic state to be used are lipid profile, plasma proteins, glycated hemoglobin, glycemia, Fe, Cu, Zn, Ca, Mg, Mn, and indicators of inflammation such as CRP, Albumin / Globulin ratio (Chapter IV).
- Develop new technologies for determining the bioavailability of micronutrients in enteral nutrition formulas (Chapter III).
- Analyze differences in the nutritional composition of formulas according to the place of commercialization (Spain, Mexico, and both).

4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Chapter I

Chapter I: Macronutrients and trace elements in enteral nutrition formulas: Compliance with label, bioaccessibility and contribution to reference intakes through a probabilistic assessment

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1.1 Abstract

Ten enteral nutrition formulas were analyzed to determine their macro and micronutrient (trace element) composition. Protein and fat content ranged between 3.7 - 8.0 and 1.3 – 11.9 g / 100 mL respectively. Trace element concentrations ranged between 7.2 – 15; 2.0 – 6.8; 0.6 – 1.6; 1.1 – 1.5; 0.2 – 0.3; 0.3 – 1.7 mg / 100 mL for Ca, Mg, Fe, Zn, Cu and Mn respectively; revealing that in many cases they do not match the label values. Trace element bioaccessibility analysis showed dialyzability percentages between 2 – 16 %. Furthermore, a negative correlation was found between protein content and dialyzed Mg content ($r = -0.644$, $p < 0.05$); and between protein content and dialyzed Fe content ($r = -0.679$, $p < 0.05$). In contrast, a positive correlation ($r = 0.801$; $p < 0.05$) was found between dialyzable Ca and dialyzable Mg, displaying a synergistic interaction between both elements. Finally, a probabilistic assessment showed that the analyzed enteral nutritional formulas meet the Dietary Reference Intakes of all micronutrients assessed.

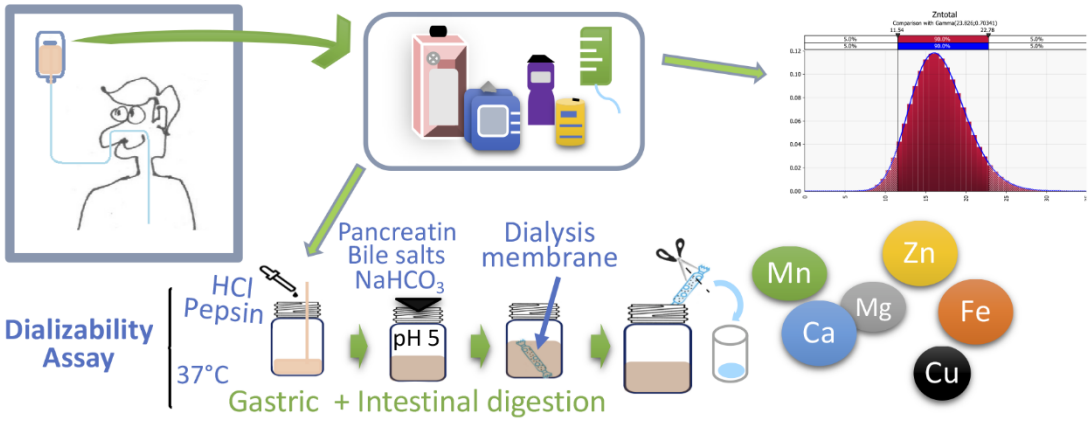

Figure 1. *Graphical Abstract*

Keywords: Trace elements, Minerals, Enteral nutrition, Food analysis, Dietary reference intake, Food composition.

Highlights

- Macro and micronutrient laboratory analysis did not match the label data in many cases
- Mineral bioaccessibility results showed mean dialyzability percentages between 2 - 16%
- Protein determination agreed with labels through Dumas and not with Kjeldahl method
- The probabilistic assessment showed sufficiency of all micronutrient Dietary Reference Intakes

1.2 Introduction

Enteral nutrition is the name given to the system that delivers nutrients directly into the gastrointestinal tract. Its purpose is to maintain or achieve a proper nutritional status in patients that have a functional gastrointestinal tract but are unable to meet their nutrient needs with a regular diet. Enteral nutrition can be delivered through the mouth or using a tube or a stoma to feed directly through the stomach, jejunum or even duodenum. The tube´s entry site can be the nostrils, the oral cavity, or an opening through the abdominal wall (Sanz-Paris et al., 2017).

There is a variety of products used in enteral nutrition that are classified as ¨foods for special medical purposes¨. Products of this nature are prescribed to patients afflicted by different pathologies or medical conditions including severe swallowing problems and / or mechanical ventilation, impaired motility of the upper digestive tract, surgery, obstruction and fistulas in the gastrointestinal tract, and elevated risk of malnutrition or cognitive impairment in the elderly. Nowadays, enteral nutrition formulas (ENF) can be found with different profiles, such as standard nutrient profiles (standard enteral formulas) and their high-energy and high-protein versions, or may even be nutrient adapted to certain conditions or pathologies (¨Disease Specific Enteral Formulas¨) (European Food Safety Authority (EFSA), 2015).

Accordingly, when used as a single feeding method ENFs need to be nutritionally complete and deliver the macro and micronutrients that would be obtained through a regular diet in the required amounts (del Olmo – García, 2017). The patient's energy and protein needs, as well as the Dietary Reference Intake (DRI) of micronutrients are usually met with a daily intake

of 1500 mL, if not otherwise stated in the product information. Furthermore, in the case of micronutrients, particularly minerals and trace elements, it is not enough to know the amount of micronutrient present in a given food; assessing the quantity of micronutrient that is released from the food matrix into the intestinal lumen may provide further information of nutritional relevance. This leads to the concept of bioaccessibility, which refers to the fraction of micronutrient, which is solubilized and absorbed in the intestinal lumen. This bioaccessible fraction can then be used by the body for the physiological function for which it is intended.

Due to the good correlation between *in vivo* and *in vitro* studies, these have been extensively applied to assess the bioaccessibility of trace elements present in food, thus demonstrating to be a good alternative to human and animal *in vivo* assays (Marval-León et al., 2014; Minekus et al., 2014; Moreda-Piñeiro et al., 2011). These *in vitro* methods simulate the biochemical reactions that take place in the human gastrointestinal tract, and are then followed by the measurement of the trace element´s soluble fraction, or the fraction that dialyses through a semipermeable membrane of a specific pore size that simulates the intestinal wall (dialyzability assay) (Cámara – Martos et al., 2015).

The mentioned bioaccessibility is affected by multiple factors like biochemical composition of the food matrix, and synergisms and antagonisms between dietary components like protein, fat, fiber and other micronutrients (Galán & Drago, 2014a, 2014b). A vast number of studies have used *in vitro* methods to estimate bioaccessibility of trace elements from different foods, beverages, and even whole meals (Cámara et al., 2005; Moreda-Piñeiro et al., 2011; Ramírez-Ojeda et al., 2018); however, until now the existing information on bioaccessibility of minerals and trace elements in enteral formulas is limited (Bueno, 2012; Galán & Drago, 2014a; Siddiqi et al., 2017) and does not comprise a wide range of minerals and trace elements.

With all the information above, this work´s first objective is to measure the values of a series of macro (protein, fat and dietary fiber) and micro nutrients (Ca, Mg, Fe, Zn, Cu and Mn) present in ten ENFs bought in Spain and Mexico. The analyzed values were compared to those indicated on the labels. Secondly, mineral and trace element bioaccessibility was analyzed. Furthermore, the way such bioaccessibility is affected by other components present in ENFs was

also studied. Finally, a probabilistic model (@Risk) that analyzes the contribution of these minerals and trace elements to the Dietary Reference Intakes (DRI) was developed.

1.3 Material and Methods

1.3.1 Reagents

Only chemicals and reagents of analytical-reagent grade were used throughout the experiments. Ultrapure water (18 MΩ · cm) was obtained from a Milli-Q Reference Water Purification (Millipore, Madrid, Spain). Glassware and crucibles were soaked in 20% nitric acid overnight and rinsed 3 times with deionized water prior to use. Nitric acid (69 %), hydrochloric acid (35 %), and sulfuric acid (98 %) were obtained from Panreac (Barcelona, Spain). Lanthanum chloride and sodium bicarbonate (97 %) were supplied by Perkin Elmer (Madrid, Spain) and Scharlau (Barcelona, Spain), respectively.

Dialysis assays were performed with digestive enzymes and bile salts purchased from Sigma-Aldrich Co. (St. Louis, MO). The pepsin solution consisted of 1.5 g of pepsin (P-7000 from porcine gastric mucosa) dissolved in 9.4 mL of HCl (0.1M)., The pancreatin and bile salts solution was made by dissolving 0.2 g of pancreatin (P-3292 from porcine pancreas) and 1.3 g of bile salts (B-8756 of porcine origin) in 50 mL of 0.mol · L^{-1} NaHCO3. These working solutions were made immediately before use. Before use, dialysis membranes with a pore size (MWCO) of 12-14,000 Å (Size 6 Inf Dia 27/32″–21.5 mm, 30 m, (from Medicell Int. LTD, London, UK), were rinsed several times with distilled deionized water.

For the fiber analysis, enzymes (heat stable α-amylase, protease from *Bacillus licheniformis*; amyloglucosidase from *Aspergillus niger*) from Sigma- Aldrich Co. (St. Louis, MO) were used. For trace element analysis, standard solutions of Fe, Zn, Cu, Mn, Ca and Mg were made before use from 1000 mg L−1 commercial solutions (Scharlau Chemie, Barcelona, Spain).

1.3.2 Samples

Ten enteral nutrition formulas (ENF) with different energy and nutrient profiles (i.e. standard, high – calorie, high protein, diabetes-specific) were used throughout the study (see Table 1 for main characteristics). Three samples of each brand (thirty samples in total) were bought from different pharmaceutical suppliers in the region of Andalusia (southern Spain) and Morelia (Mexico) from February of 2017 to January of 2018.

Table 1
Main characteristics of the ENF used in this work.

Sample	Energy	Protein	Fiber	Characteristic	Origin
F1	High	High	With	Diabetes	Spain
F2	Standard	High	With	Powder/ Diabetes	Mexico
F3	Standard	High	With	Diabetes	Mexico
F4	Standard	Standard	Without	Oral Nutrition Supplement	Mexico
F5	Standard	Standard	Without	Standard	Spain
F6	Standard	Standard	With	Fiber mixture	Spain
F7	High	High	Without	Standard	Spain
F8	High	Standard	Without	Standard	Spain
F9	High	High	With	Standard	Spain
F10	High	High	Without	Hydrolyzed proteins	Spain

High calorie and high protein attributes were displayed following manufacturers criteria, energy higher than 1.2 kcal/mL and protein that contributed to ≥18% of total energy.

1.3.3 Bioaccessibility assay

An *in vitro* simulation of a gastrointestinal digestion based on the one described by Cámara et al. (2005) was used to estimate mineral and trace element bioaccessible fractions (dialyzable fractions) from the formulas. The first stage of the process is a pepsin-HCl digestion that imitates the gastric phase, it starts by adjusting the pH of each aliquot (30 mL) to 2.0 using 6 N HCl. Then, 0.5 g of pepsin were added to each 100 g of sample used (per sample of 30 mL, 0.94 mL of the pepsin-HCl solution described under ¨Reagents¨). After this, the samples were incubated in a shaking water bath at 37°C, 65 rpm for 2 hours.

For the intestinal stage, dialysis membranes (MWCO of 12-14,000 Å, from Medicell Int. LTD, London, UK) were filled with deionized water and the amount of $NaHCO_3$ equivalent to the titratable acidity. The titratable acidity was defined as the number of equivalents of NaOH

Table 2
Instrumental conditions, limit of detection, limit of quantification and analysis of certified references materials.

Element	Wavelength (nm)	Slit Width (nm)	Certified references material (mg kg^{-1})					
			Rice flour NIST – 1568a			Mussel tissue ERM - C278k		
			Certified	Found	Recovery (%)	Certified	Found	Recovery (%)
Fe	248.3	0.2	7.42 ± 0.44	7.58 ± 0.52	102	161 ± 8	174 ± 7	108
Zn	213.9	0.7	19.42 ± 0.26	20.38 ± 0.24	105	71 ± 4	74 ± 4	105
Mn	279.5	0.2	19.20 ± 1.80	18.48 ± 4.20	96	4.88 ± 0.24	5.00 ± 0.21	102
Cu	324.8	0.7	2.35 ± 0.16	2.26 ± 0.34	96	5.98 ± 0.22	6.38 ± 0.66	107
Ca	422.7	0.7	118.4 ± 3.1	116.7 ± 2.1	98	–	–	–
Mg	285.2	0.7	559 ± 10	556 ± 18	99	–	–	–

required to titrate the combined pepsin digest pancreatin – bile salts mixture to pH 7.5. Afterwards, these membranes were placed inside the flasks containing the pepsin digest to be incubated for 30 minutes under the conditions previously mentioned. Then, 6.25 mL of the solution containing pancreatin and bile salts were added to the samples and the incubation continued for 150 min. When the incubation time was over, the dialysis membranes were removed from the flasks and rinsed with distilled deionized water. Lastly, the bioaccessible fraction was transferred to porcelain crucibles to be dry-ashed.

1.3.4 Trace element determination

To estimate total mineral and trace element content, 5 mL of each sample were heated to dryness, which was followed by incineration (at 460 °C for 24 h) in a temperature-programmed muffle furnace (Heraeus M1100/3, Hanau, Germany) to destroy the organic matter. The ashes obtained were bleached by adding 3 mL of 2N HNO_3, heating to dryness and placing in a muffle furnace at 450 ºC for 1 h. Ashes were recovered with 1 mL of HCl and dissolved with deionized water to make up a final volume of 10 mL. Fe, Zn, Mn, Ca and Mg, content was analyzed through flame atomic absorption spectrophotometry (FAAS; model AA-50B Varian Spectra). Instrumental conditions for the determination are shown in Table 2. Electrothermal atomic absorption spectrometry (ETAAS) was used for bioaccessible Cu determination (Drying: 85°C – 5s; 95°C – 40s; 120°C.- 10s; Ashing: 800 °C – 8s; Atomization: 2300 °C – 2.8s; Cleaning: 2300 °C – 2s) Precision and accuracy of the analytical techniques were validated by recovery experiment using Certified Reference Materials (Table 2). Trace element content obtained from the dialysis assay was determined using the same procedure as the samples described above.

1.3.5 Macronutrient determination

Methods from the Association of Officiating Analytical Chemists (AOAC 1995), were used to determine the proximate composition. Samples were lyophilized at -40 °C (Scanvac CoolSafe 55 – 4) for macronutrient analysis. Fat content (920.85) was assessed using 1 g aliquot of the lyophilized samples which were placed on a Soxhlet liquid/solid extractor with petroleum

ether (1 h) and dried in a hot air oven. Fat content was estimated by weight difference. Dietary fiber was measured through an enzymatic digestion -gravimetric method (991.43) (AOAC, 1995) (First step: amylase / T = 95 °C / t = 30 min; Second step: protease / T = 60 °C / t = 30 min; Third step: amyloglucosidase / T = 60 °C / t = 30 min). After these procedures, ethanol was added to precipitate the soluble fiber fraction. Finally, the residue was filtered in crucibles (Alamo SL – 3) with 0.5 g of celite, washed with ethanol (78 % and 95 %) and acetone, and dried overnight before weighing the residue.

Kjeldahl protein analysis (920.87) (AOAC, 1995) was done with 0.5 g of lyophilized samples and 20 mL of sulfuric acid 98% along with a catalyst pellet, which were placed in a digestion flask and heated until the solution was clear. Once cooled, 70 mL of water were added, and the solution was distilled with sodium hydroxide to convert the ammonium sulphate to ammonia. A 0.1 mol · L^{-1} solution of hydrochloric acid was used to capture the ammonia gas. The amount of nitrogen present (as ammonia) was determined by back titration with a 0.1 mol · L^{-1} solution of sodium hydroxide and methyl red was used as pH indicator. Finally, protein content was estimated using a nitrogen conversion factor of 6.25.

Dumas method was also used to determine protein content with an EuroVector Elemental Analyzer EA3000 equipped with Callidus software (EuroVector SpA, Milan, Italy) (Central Service for Research Support of the University of Cordoba). The method is based on the complete combustion of a sample in an oxygen-enriched atmosphere at a high temperature. The protocol used was previously optimized by Serrano et al. (2013). Samples were dried and finely crushed before being individually weighed in tin capsules with a microbalance. Then, each tin capsule was sealed to avoid air inside of it.

Each sample was combusted in a reactor at about 1000 °C in a temporarily enriched oxygen atmosphere (see experimental conditions in Table 3). There, the combustion products were carried by a carrier gas (helium) that passed through a glass packed column together with an oxidation catalyst and a copper reducer. At this temperature, the nitrogen oxides were reduced to nitrogen. Next, the nitrogen was transported to a packed column, separated by gas

chromatograph, and quantified with a TCD detector (Thermal Conductivity Detector) (set at 90 °C). The chromatographic responses to nitrogen were previously calibrated with standards, and the final nitrogen composition was calculated through a calibration curve using the nitrogen chromatographic areas for each sample.

Table 3
Dumas method experimental conditions.

Parameter	Set to	Parameter	Set to
Carrier (kPa)	110	Sample delay (sec)	14
Purge (mL/min)	80	Run time (sec)	120
Oxygen (mL)	7	Front furnace (^{0}C)	1020
ΔPO2 (kPa)	25	Rear furnace (^{0}C)	0
Oxidation time (sec)	4.4	GC Oven (^{0}C)	90
Gain	x1		

1.3.6 Statistics and risk assessment

Data were analyzed using SPSS 15.0 (IBM, Armonk, NY). Normality of the data was validated with the Shapiro-Wilks test. Later, Pearson's correlation (parametric conditions) and Spearman´s correlation (non-parametric conditions) was used for determining the dependence between variables. Significant differences were considered when $p < 0.05$.

A probabilistic model was developed to estimate the intake level for Ca, Mg, Fe, Zn, Cu and Mn derived from feeding with ENF. This model followed a probabilistic approach in which variables were described by probability distributions, they were fitted to concentration data obtained in this study for each element (total element content). Additionally, to estimate the intake level, serving size was considered assuming 1000 to 1500 mL of ENF per day, as suggested by the manufacturers of the ENF used in this study. Daily intake was defined by a uniform distribution in the probabilistic model; meaning that all values in that range had the same probability to occur.

The probability distributions describing the trace element concentration data were fitted using @Risk v7.5 (Palisade, Newfield, NY, USA). The simulation ran with 100,000 iterations per element. The goodness of fit assessed how well the fitted distribution described the data, in this

section the Akaike Information Criterion (AIC) and Chi-square statistical tests were used. Additionally, the visual analysis was considered to assess the fit of the probability distributions to intake data. Data obtained through this probabilistic model was compare to the Spanish DRI for the population over 60 years old (FESNAD, 2010).

1.4. Results and Discussion

1.4.1 Protein, fat and dietary fiber

In this work, Dumas and Kjeldahl methods were used to determine protein content on ENF and as it can be observed in Table 4, agreement between analyzed content and labels data depended on the analytical method used. Results from Dumas analysis agree with data shown on the label of all the formulas, measured values corresponded to 96 ± 5.6 % of the protein content declared on the label (range was from 89.4 to 106 %). On the other hand, when protein from the same ENFs was measured through Kjeldahl methodology, none of the samples meet the label data. In this case, analyzed protein corresponded to values between 44 % in the worst case and 69.5 % on the highest. These percentages of agreement with label data are below the acceptable range of 80 to 120 % previously used by other authors (Abe-Matsumoto et al., 2018; Code of Federal Regulation (USA), 2018).

The main difference between Dumas and Kjeldahl methodology is the way the nitrogen is derived from the measured sample. It should be recalled that foodstuffs contain nitrogen not only from protein and amino acids, but also from other substances such as nucleic acids, amines, urea, ammonia, nitrates, nitrites, phospholipids and nitrogenous glycosides, etc. (Mariotti et al., 2008). Dumas method converts almost all the nitrogen compounds in elemental nitrogen, while the conversion of nitrates, nitrites and other nitrogen compounds to ammonia ions is incomplete with the Kjeldahl method. This translates in to a 1.5 % higher protein content with the Dumas method according to some authors (Thompson et al., 2004).

Table 4
Protein, fat and fiber analyzed content in g / 100 mL (mean ± standard deviation), and content stated on label.

Sample	Protein			Fat		Fiber	
	Analyzed		Label	Analyzed	Label	Analyzed	Label
	Dumas	Kjeldahl					
F1	8.0 ± 0.2	3.7 ± 1.0	7.5	1.8 ± 0.1	2.95	0.6 ± 0.1	1.5
F2	6.2 ± 0.9	3.4 ± 0.2	6.1	11.9 ± 0.2	12.15	3.0 ± 0.5	7.9
F3	5.0 ± 0.0	2.2 ± 0.1	5.0	2.5 ± 0.1	3.80	1.4 ± 0.3	1.3
F4	3.7 ± 0.0	1.8 ± 0.3	3.8	1.8 ± 0.0	2.53	NQ	0
F5	3.8 ± 0.0	2.7 ± 0.0	3.9	1.4 ± 0.1	3.90	NQ	0
F6	4.1 ± 0.0	2.0 ± 0.1	4.0	3.2 ± 0.0	3.90	1.0 ± 0.1	1.5
F7	5.0 ± 0.0	3.5 ± 0.7	5.6	2.3 ± 0.1	3.93	NQ	0
F8	5.5 ± 0.1	3.5 ± 0.1	6.1	3.5 ± 0.6	6.20	NQ	0
F9	7.3 ± 0.1	3.8 ± 0.1	8.1	2.9 ± 0.2	4.30	1.0 ± 0.2	1.5
F10	6.6 ± 0.0	3.2 ± 0.5	6.8	1.9 ± 0.1	2.50	NQ	0

*NQ = Not Quantified.

The differences found between the two methods used highlight the need to consensuate the adequate method to deliver accurate protein information. Especially in elderly adults, both healthy and critically ill, protein has been recognized as a key factor in health maintenance and recovery. Increasing its intake improves quality of life by preventing the onset of chronic diseases, improving muscle function and preventing protein loss (greater protein loss associates with increased morbidity and mortality) (Baum et al., 2016; Hurt et al., 2017; Wolfe, 2012). Concerning its quantity, older patients should meet (and even exceed) the recommended dietary allowance of at least 0.8 g / kg / day (Beasley et al., 2016).

A statistically significant positive correlation (r= 0.707 $p < 0.05$) was found between Dumas protein content (g/100 mL) and Zn total content (mg / 100 mL), when it comes to Kjeldahl method, this positive correlation was also observed but without statistical significance. This correlation has been previously found in previous studies where high protein foods also have a high Zn content (Ramírez-Ojeda et al., 2017). However, in ENF the protein sources are usually added as isolated protein (milk, soy and pea protein), thus, according to label information this protein - Zn correlation has been intentionally maintained by developers. On the other hand, among the analyzed variables, with the Kjeldahl method a significant negative correlation was found between protein content (g / 100 mL) and (bioaccessible) dialyzed Mg content (mg / 100 mL) (r = -0.644, $p < 0.05$) and between protein content (g / 100mL) and dialyzed Fe (mg / 100 mL) (r = -0.679, $p < 0.05$). Among the other elements analyzed (Ca, Zn, Cu and Mn), this negative trend of proteins on trace element dialyzable concentration was also observed, but without statistical significance. This negative effect by protein from plant origin on the bioaccessibility of minerals and trace elements has been previously observed by other authors (Berner & Miller, 1985; Ortiz & Cámara-Martos, 2018).

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added as isolated protein (milk, soy and pea protein), thus, according to label information this protein - Zn correlation has been intentionally maintained by developers. On the other hand, among the analyzed variables, with the Kjeldahl method a significant negative correlation was found between protein content (g / 100 mL) and (bioaccessible) dialyzed Mg content (mg / 100 mL) ($r = -0.644$, $p < 0.05$) and between protein content (g / 100mL) and dialyzed Fe (mg / 100 mL) ($r = -0.679$, $p < 0.05$). Among the other elements analyzed (Ca, Zn, Cu and Mn), this negative trend of proteins on trace element dialyzable concentration was also observed, but without statistical significance. This negative effect by protein from plant origin on the bioaccessibility of minerals and trace elements has been previously observed by other authors (Berner & Miller, 1985; Ortiz & Cámara-Martos, 2018).

Regarding fat analysis (Table 4), except for two samples that showed a very low agreement percentage (24 and 35 %), the rest of the ENF showed an intermediate compliance with label data (56 - 109%, mean value of 76 ± 18 %). The number of ENF whose fat measured content agreed with label data was six out of ten (when the 80 - 120 % range was used). In ENF, lipids usually contribute to about 2 – 55 % of total energy (Ireton-Jones & Krystofiak-Russell, 2017), which matches with the data found on the label of the ENF included in this paper (15 - 45 %).

However, fat analysis performed in the laboratory showed that this macronutrient represented around 14 to 30 % of the total energy (from measured protein and fat, and label carbohydrates). This percentage is slightly inferior, but closer to the recommended percentages for the healthy adult population (20% to 35%) (Vannice & Rasmussen, 2014). In ENF, this macronutrient is important not only because it is a major energy source, but also because it supplies essential fatty acids, improves the absorption of liposoluble vitamins and helps maintain glycemic homeostasis (DeLegge, 2015; Flock & Kris-Etherton, 2013; Gulati et al., 2015). Finally, as with proteins, a negative trend was observed between fat content and the dialyzability of the analyzed trace elements. A possible explanation to this is that a higher fat content may have impaired the enzymes that release low molecular weight compounds, thus reducing their absorption (Marval-León et al., 2014).

Finally, as seen in Table 4, dietary fiber analysis showed that six out of ten formulas matched the label information. Where sample contents of the other four formulas were below 80 % of declared amount of fiber. On the other hand, ENF labeled as fiber- free corresponded to the samples with no quantified fiber. Fiber content showed no effect on the dialyzability of the analyzed trace elements. This agrees with previous observations which show a controversial effect of fiber on trace element bioaccessibility (positive, negative and null effects) (García-Sartal et al., 2013; Moreda-Piñeiro et al., 2013; Ortiz & Cámara-Martos, 2018).

1.4.2 Minerals and trace elements

Total content of minerals and trace elements (Ca, Mg, Fe, Zn, Cu and Mn) from ENF were analyzed and results are shown in Table 5. As in the macronutrient analysis, values found were considered within range when the measured content variability did not exceed 20 % from the one stated in the label. This cut off value was chosen to agree with other authors and regulations (Abe-Matsumoto et al., 2018; CFR, 2018). As it can be observed, except for Cu, most of analyzed values were below labels data, even when 20 % range was considered. This panorama is particularly relevant with Fe, Mg and Mn, where seven, eight and nine ENF, respectively, do not agree with the label. However, Zn and Ca showed less disagreement, with only two out of ten formulas out of the delimited range.

Ca content ranged between 66 – 113 mg / 100 mL, corresponding to the standard/fiber mixture formula and the standard oral nutrition supplement, respectively. Regarding Mg content, analyzed values ranged from 14 mg / 100 mL of a high-calorie / standard / fiber - free ENF to 41 mg / 100 mL from the standard oral nutrition supplement. The higher Ca and Mg content observed in the standard oral nutrition supplement can be due to different purposes of this ENF. This formula is given as a complement to food sources instead of replacing the whole intake with a higher dose of the product. Furthermore, a significant positive correlation ($r = 0.697$; $p < 0.01$) was found between Ca content and Mg studied content from all the ENF studied.

Table 5
Mineral and trace element analyzed content in mg / 100 mL (mean ± standard deviation); besides inorganic element content stated on label.

Sample	Ca		Mg		Fe		Zn		Cu		Mn	
	Analyzed	Label	Analyzed	Label	Analyzed	Label	Analyzed	Label	Analyzed	Label	Analyzed	Label
F1	94.9 ± 0.7	100.0	27.4 ± 0.3	31.0	0.6 ± 0.1	0.9	1.5 ± 0.0	1.7	0.23 ± 0.03	0.11	0.30 ± 0.01	0.44
F2	101.7 ± 3.6	116.7	28.4 ± 1.5	37.5	1.4 ± 0.1	1.8	1.5 ± 0.1	2.3	0.28 ± 0.02	0.22	1.67 ± 0.04	3.10
F3	104.1 ± 6.0	105.5	27.6 ± 2.3	33.7	1.1 ± 0.3	1.9	1.5 ± 0.0	1.6	0.26 ± 0.00	0.20	0.30 ± 0.01	0.40
F4	112.9 ± 5.8	126.6	41.5 ± 1.4	42.2	1.5 ± 0.1	1.9	1.4 ± 0.0	1.6	0.33 ± 0.02	0.21	0.40 ± 0.01	0.51
F5	67.9 ± 1.8	80.0	18.2 ± 0.6	23.0	1.2 ± 0.0	1.6	1.1 ± 0.0	1.2	0.20 ± 0.00	0.18	0.26 ± 0.01	0.33
F6	65.6 ± 2.8	80.0	16.6 ± 0.5	23.0	1.4 ± 0.0	1.6	1.2 ± 0.0	1.2	0.20 ± 0.02	0.18	0.32 ± 0.01	0.33
F7	72.1 ± 0.9	83.0	19.0 ± 1.0	24.0	1.3 ± 0.0	1.8	1.3 ± 0.0	1.5	0.27 ± 0.03	0.20	0.29 ± 0.01	0.40
F8	94.8 ± 3.6	110.0	14.3 ± 0.5	18.0	1.5 ± 0.1	1.6	1.3 ± 0.1	1.5	0.26 ± 0.07	0.22	0.35 ± 0.03	0.36
F9	99.3 ± 6.0	115.0	18.7 ± 0.8	25.0	1.6 ± 0.2	1.6	1.4 ± 0.1	1.7	0.22 ± 0.03	0.20	0.33 ± 0.02	0.40
F10	91.9 ± 3.2	100.0	25.0 ± 0.4	30.0	1.4 ± 0.3	2.0	1.5 ± 0.2	1.8	0.28 ± 0.05	0.24	0.41 ± 0.05	0.50

Table 6
Dialyzable fraction of Ca, Mg, Fe, Zn, Cu and Mn in ENF (mg / 100 mL).

Sample	Dialyzed Ca	Dialyzed Mg	Dialyzed Fe	Dialyzed Zn	Dialyzed Cu	Dialyzed Mn
F1	11.4 ± 0.7	3.8 ± 0.2	0.05 ± 0.01	0.04 ± 0.01	0.004 ± 0.001	0.039 ± 0.002
F2	10.8 ± 1.4	3.4 ± 0.5	0.08 ± 0.02	0.02 ± 0.00	0.004 ± 0.001	0.033 ± 0.011
F3	11.5 ± 0.8	5.5 ± 0.4	0.11 ± 0.02	0.08 ± 0.01	0.006 ± 0.002	0.052 ± 0.004
F4	15.4 ± 2.3	6.8 ± 0.3	0.09 ± 0.01	0.03 ± 0.01	0.005 ± 0.001	0.041 ± 0.010
F5	10.5 ± 0.7	3.6 ± 0.2	0.09 ± 0.01	0.08 ± 0.01	0.005 ± 0.001	0.036 ± 0.003
F6	7.2 ± 0.4	2.8 ± 0.7	0.12 ± 0.01	0.03 ± 0.00	0.006 ± 0.001	0.016 ± 0.001
F7	9.4 ± 1.3	3.1 ± 0.3	0.09 ± 0.03	0.07 ± 0.02	0.007 ± 0.001	0.034 ± 0.005
F8	10.3 ± 1.3	2.0 ± 0.1	0.06 ± 0.00	0.07 ± 0.02	0.009 ± 0.004	0.024 ± 0.005
F9	10.6 ± 1.1	3.1 ± 0.2	0.10 ± 0.02	0.03 ± 0.01	0.004 ± 0.001	0.015 ± 0.001
F10	11.0 ± 1.1	3.5 ± 0.2	0.08 ± 0.01	0.04 ± 0.01	0.004 ± 0.001	0.042 ± 0.011

Regarding bioaccessible mineral content (dialyzable content), Ca and Mg content ranged from 7.2 to 15 mg / 100 mL and 2.0 – 6.8 mg / 100 mL, respectively (see Table 6). In both cases the lower content was found in the standard/ fiber mixture formula, and the highest values corresponded to the standard oral nutrition supplement. Dialyzability percentages were around 12 and 16 % for Ca and Mg, respectively. These values agree with those reported by Galán and Drago (2014a) who analyzed twenty ENF and found slightly inferior values of Ca dialyzability (4.21- 16.5 %). However, this kind of hospital nutrition (enteral nutrition) showed considerably inferior dialyzability of Ca and Mg when compared to a standard diet (either solid or blended). The hospital meals (breakfast, lunch, and dinner) showed dialyzability percentages of 37.8 % and 50.4 % for Ca and Mg, respectively (Velasco-Reynold et al., 2010).

On the other hand, a significant positive correlation ($r = 0.801$; $p < 0.005$) was found between dialyzable Ca and dialyzable Mg (mg / 100 mL). These interaction reveals a synergistic interaction between both elements which have already been demonstrated in previous studies with other food matrix such as legumes (Ramírez-Ojeda et al., 2018) and hospital meals (Velasco-Reynold et al., 2010).

When it comes to Fe, total content analyzed ranged from 0.6 to 1.6 mg / 100 mL (Table 5). These values corresponded to a high-calorie / high-protein / diabetes formula and a high-energy / high-protein / standard / fiber-containing formula, respectively. On the other hand, bioaccessible content values (dialyzable Fe) went from 0.05 – 0.12 mg / 100 mL (Table 6), which correspond to an overall dialyzability percentage of 7 %. These findings agree with those obtained from the analysis of twenty ENF (0.36 - 5.48 % Fe dialyzability) (Galán & Drago, 2014b); but are higher than the ones reported by Bueno et al. (2012) (0.80 %) who measured a multiple nutrient formulation developed by them, and others from Indian ENF (0.23- 2.52 %) (Siddiqi, et al. 2017). Similar percentages of dialyzable Fe (4.81 %) were found in other feeding options available in hospitals such as whole meals (breakfast, lunch, dinner) (Velasco-Reynold et al., 2009). From this, it can be inferred that Fe dialyzability is equally effective with ENF as with whole meals. This matter is of interest as anemia prevalence is associated with longer hospital stays and poor recovery (Reade et al., 2010).

As for Zn, analyzed content among the formulas demonstrated little variation, results ranged between 1.1 mg / 100 mL that corresponded to a standard / fiber - free ENF and the highest value 1.5 mg / 100 mL (Table 5) was found in four of the analyzed formulas (high-calorie / high – protein / diabetes formula, high – protein / diabetes / powder formula, high -protein / diabetes formula, and high – calorie / peptide ENF). Alternatively, dialyzable Zn ranged from 0.02 to 0.8 mg / 100 mL (Table 6,) which represents a mean 3.7 % bioaccessibility. These results agree with the findings of Galán and Drago (2014a) (0.22 – 12 %) in their analysis of twenty ENF but are considerably lower than Zn dialyzabilty in standard hospital meals (25.23 %) (Velasco-Reynold et al., 2008a). On the other hand, a positive correlation was found between total Zn and total Ca content (mg / 100 mL) ($r = 0.757$; $p < 0.05$). Concerning the Zn and Ca interaction, even though a positive correlation was found in this paper there is not much evidence to support this interaction between these elements according to a similar study with ENF (Galán & Drago, 2014a).

Cu analysis showed that the total content of this trace element was relatively homogeneous, it ranged between 0.20 and 0.33 mg / 100 mL (Table 5); while its dialyzable content ranged from 0.004 to 0.009 mg / 100 mL (Table 6). These values corresponded to a 2.1 % dialyzability, which is very low, especially when compared to other studies performed on hospital meals with mean values around 26 % (Velasco-Reynold et al., 2008b). Considering that dialyzability percentages of this trace element in more complex meals (Cámara et al., 2005; Ramírez-Ojeda et al., 2017) is usually higher (about 20%), a concern about improving Cu dialyzability in ENF must be raised. Even though Cu deficiency is not common and may be asymptomatic, it may cause anemia, neutropenia, myeloneuropathy, paresthesia and even affect cardiac rhythm (Ben-Hamouda et al., 2017; Palm & Dotson, 2015).

Lastly, regarding Mn, there was one ENF with a higher content than the others (high – protein / diabetes / powder ENF), mean of 1.67 mg / 100 mL, while the other formulas ranged between 0.26 and 0.41 mg / 100 mL (see Table 5). On the other hand, the dialyzable content (Table 6) showed less difference among the analyzed samples, with values of 0.15 – 0.52 mg / 100 mL. In this case, the formula with the highest Mn content was not the one with the highest

dialyzable content. Its overall dialyzability was 13 %, which compared to other feeding methods at the hospital did not show great differences (19-9 - 23.1 %) (Velasco-Reynold et al., 2008c).

1.4.3 Probabilistic Assessment

As specified in the section of ¨materials and methods¨, a probabilistic model was developed to estimate the intake levels for minerals and trace elements derived from consumption of 1000 - 1500 mL of the ENF analyzed in this paper. In this assessment, only the ENF that can be used as a single feeding source are considered, thus, the results do not consider other foods being consumed. Even though ENF sufficiency is estimated using the reference intake of men aged 19-50 years (as specified on product description), the latest report on home enteral nutrition in Spain shows mean age of population fed with ENF was 73 years old (59 – 83) (Wanden-Berghe et al., 2017); because of this, the DRI for the elderly (from the Spanish population) was chosen to compare adequacy.

To develop this model, only total content data was used, as the dietary reference values already take into consideration nutrients´ bioaccessibility (Hurrell & Egli, 2010; Perignon et al., 2018). Nevertheless, it should be noted that the dialyzability assay used only uses passive diffusion process by which many trace elements are absorbed, which does not include the active transport processess that take place *in vivo*. The developed probabilistic model showed that the analyzed ENF meet the DRI of all micronutrients assessed; this is not observed in other cases where single foods are studied, such as cereals, legumes and even weaning foods (Ortiz & Cámara-Martos, 2018; Ramírez-Ojeda et al., 2018, 2017).

In this way, Ca total mineral concentrations were fit to risk gamma distribution (AIC= 139522.4; Chi-square= 525.3). The results derived from the simulation of the probabilistic model, indicated that the intake of Ca from these ENF would be equivalent to at least 1073 mg / day to 50 % of the population (50^{th} percentile). When compared to the DRI of 1000 mg / day, the results show that half of the population fed with ENF consume 100 % of the recommended value (Fig. 2A). Even if the 5^{th} percentile is considered, Ca intake would be 686 mg (69 % of DRI), which

shows that ENF provide Ca in enough amounts. Concerning Total Mg, measured contents were fit to a risk Pearson distribution (AIC = 117448.0; Chi – square = 316.98) which shows an intake of 270 mg / day in the 50th percentile that corresponds to 77 and 85 % of DRI for males and females, respectively (Fig 2B).

In the case of Fe, assessed total concentrations were fit to a risk LogLogistic distribution (AIC= 55711.2; Chi – square = 453.05). The estimated intake to 50 % of the population is 14.8 mg / day, corresponding to 148 % of the DRI of this trace element (Fig 2C). In this case, even the population on the 10th percentile would meet 100 % of the DRI (10.1 mg / day). This information is especially relevant when considering that not only the excess Fe is associated to increased oxidative stress, as it is commonly known, but Fe deficit may have the same effect (Alvarez-Hernandez et al., 2017).

Zn assessment (see Fig. 2D) (gamma distribution, AIC = 52691.72; Chi – square = 378.7) showed an intake of 11.6 mg / day in the 5th percentile, meaning that all the population fed with these ENF would meet the established DRI (it should be noted that even in the 1st percentile, ENF provide 9.8 mg / day). The importance of an adequate Zn intake in healthy and ill patients, is related to its various roles such as: maintaining the immune function, preventing the appearance of skin lesions, and aiding on wound healing (Lin et al., 2018).

Meanwhile, Mn analysis (Pearson distribution; AIC = 44843.7; Chi – square = 82.76) predicted an intake of 3.75 mg / day (50th percentile). These values represent 163 and 208 % of the male and female DRI, respectively (see Fig 2F). Lastly, Cu assessment exhibited a predicted intake of 3.38 mg / day for the 50th percentile (Fig. 2F; gamma distribution; AIC = 22379.6; Chi -square = 464.83) which is considerably higher than the established DRI of this trace element (1.1 mg / day), for both male and female populations. Even the population on the 1st percentile have an intake higher than the DRI (2.04 mg / day).

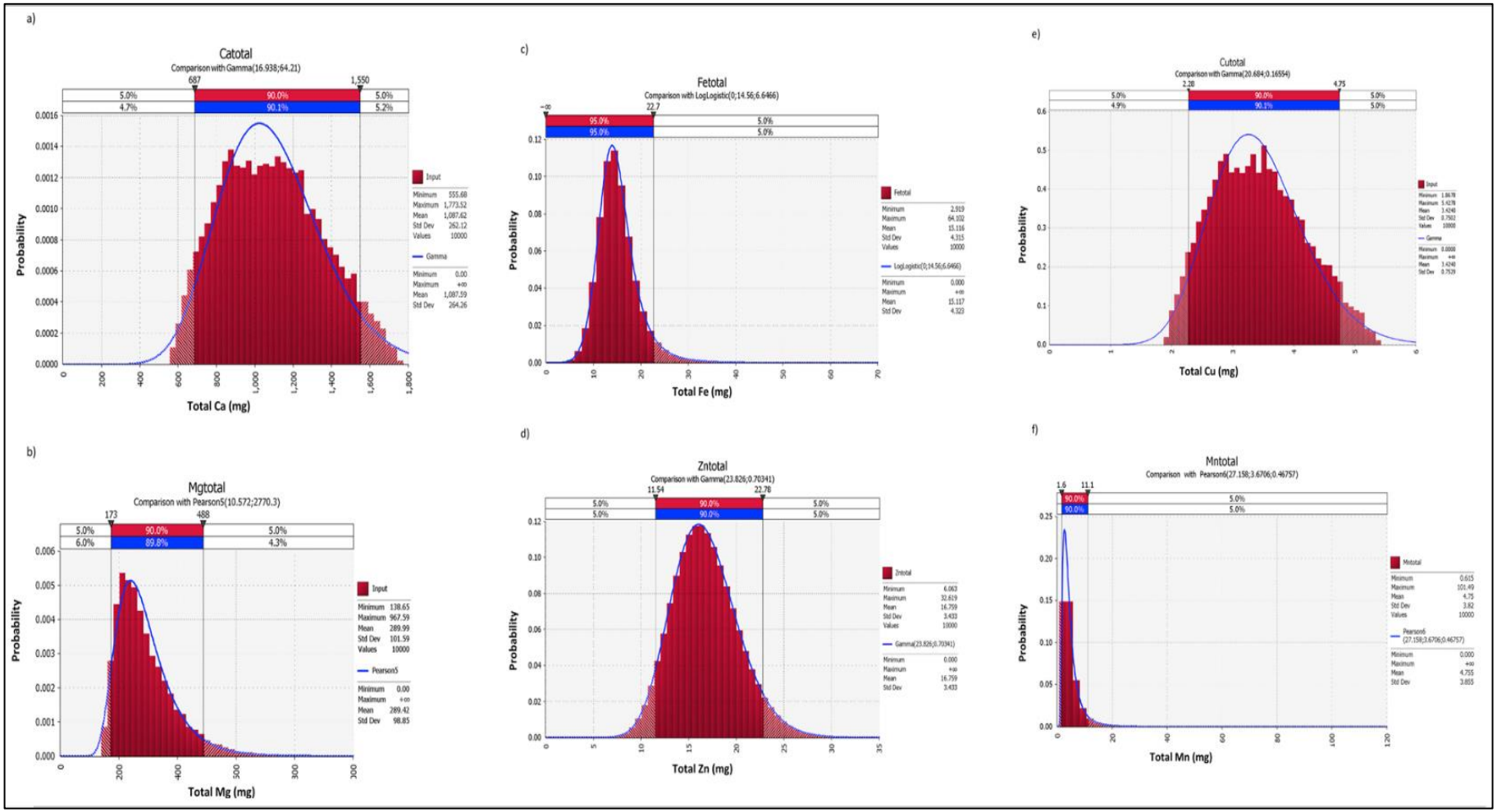

Figure 2. *Simulated data and fitted probabilistic distribution for total trace element content.*

1.5 Conclusions

The analytical methodologies used in the present research allowed analysis of major components of ENF, as well as mineral total content and mineral bioaccessibility. The data obtained through these procedures, along with the probabilistic model developed, provided further knowledge about the composition and adequacy of ENF. As a summary, it shows that even though ENF sometimes have a lower mineral content than the one stated in the label, according to the model based on the mineral content measured in the laboratory, patients fed with this nutritional support still get the nutrients established in the DRI (for the elderly Spanish population), consequently could be considered sufficient in the population considered.

Conflict of interest

Declarations of interest from all the authors: none.

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1.6 Supplementary material

This project aimed to include a variety of enteral nutrition formulas to be studied, although the number of products ultimately included depended on the availability of different products at the beginning of this study. As it can be observed, and as a reflection of the market, the products display several differences in their composition, characteristics, and ingredients. In the following table, the reader can find multiple aspects of interest such as calorie density, protein content, macronutrient sources, fiber-content, disease-specificity, and country of origin (see Table 1.7).

The following comparison shows more items from Spanish origin, this is a consequence of the smaller variety of commercially available formulations found in Mexico. To start with, even if some manufacturers are established in both countries, they do not have the extensive product repertoire in Mexico as in Spain, as seen in the fieldwork previous to this study. For example, from the four products bought in Spain from Abbott®, only one of them was available in Mexico. Additionally, other internationally renowned brands failed to provide enteral nutrition products aimed at adults and their pathologies in Mexico. Such is the case of Nutricia® and Nestle Health Science®, which catalogs are more inclined towards children´s products such as specific enteral nutrition products and infant formulas.

Another difference found, is that ENF from Mexico used in this study (and in general) are not usually found in ¨ready to hang¨ RTH formats, such as bottles and bags. In that scenario, portion-sized cans and bottles, or powdered formats that need to be diluted are used. Perhaps, economic limitations influence their availability, which agrees with previous personal experience in Mexican public hospitals (city of León, Guanajuato). Where EN patients at hospitals and discharge were fed with ¨Homemade Blenderized¨ diets, and commercial products (portion sized) were seldomly used depending on availability, which was secondary to donations. Thus, many do not feed entirely by commercial ENF and are more commonly used as supplements (through enteral tubes or orally).

Table 7 *Comprehensive comparison of all the formulations studied*

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
Description											
Energy	High	Standard	Standard	Standard	Standard	Standard	High	High	High	High	Standard
Protein	High	High	High	Standard	Standard	Standard	High	Standard	High	High	Standard
Fiber	With	With	With	Without	Without	With	Without	Without	With	Without	With
Kcal/mL	1.5	1.1	1.0	1.0	1.0	1.0	1.2	1.6	1.3	1.5	1.0
Content, g / 100 mL											
Proteins	7.5	6.1	5.0	3.8	3.9	4.0	5.6	6.1	8.1	6.8	4.3
Fat	7.5	3.3	3.8	2.5	3.9	3.9	3.9	6.2	4.3	5.5	4.2
Carbohydrates	12.8	14.7	11.4	16.9	12.3	12.3	15.8	19.3	14.2	18.4	11.3
Sugars	6.7	6.6	0.3	9.7	4.6	0.8	0.4	1.7	0.7	3.5	2.3
Energy distribution, %											
Protein	20	22	20	14	16	16	18	16	25	18	17
Fat	45	27	34	21	35	34	29	36	30	33	37
Carbohydrates	34	52	45	64	49	47	52	49	45	49	44
Macronutrient sources											
Protein	Milk proteins soy protein isolate	Soy protein isolate, soy protein concentrate	Caseinates (sodium, calcium)	Milk proteins, soy protein isolate	Cow's milk protein caseinate	Whey protein, caseinate, pea protein, soy protein	Milk proteins	Milk proteins (calcium caseinate, sodium caseinate)	Milk proteins, soy protein isolate	Hydrolysed milk proteins	Soy protein

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
Carbohydrates	Isomaltulose, corn maltodextrin, sucromalt	Polydextrose, fructose, corn syrup solids, dextrose, malt extract	Maltodextrin	Sucrose, maltodextrin	Maltodextrin, sucrose	Maltodextrin, oligofructose	Maltodextrin	Maltodextrin	Maltodextrin	Maltodextrin, sucrose	Starch (tapioca), fructose
Lipids	High oleic safflower oil, canola oil,	Vegetable oil, not specified	Vegetable oil (safflower), canola oil	Soy oil, corn oil, canola oil, high oleic sunflower oil	Rapeseed and sunflower oils	Sunflower oil, rapeseed oil, MCT oil (coconut oil, palm kernel oil))	High oleic sunflower oil, canola oil, corn oil, MCT oil from palm kernel oil	Rapeseed and sunflower oils, MCTs	High oleic sunflower and canola oil, MCT from palm kernel oil	Vegetable oils (MCT oil from palm kernel, canola)	Sunflower oil, rapeseed oil
Selenium	Sodium selenate	Sodium selenite	Sodium selenite	Se aminoacid chelate	Sodium selenite	Sodium selenite	Sodium selenite	Sodium selenite	Sodium selenate	Sodium selenate	Sodium selenite
Flavoured	Yes	Yes	Yes	Yes	Yes	Neutral	Neutral	Yes	Neutral	Yes	Yes
Specification	Diabetes	Powder/ diabetes	Diabetes	Oral nutrition supplement	Standard	Fiber mixture	Standard	Standard	Standard	Hydrolyzed proteins	Diabetes
Suggested use	Both	Supplement	Supplement	Supplement	RTH	RTH	Both	Both	RTH	Both	Both
Origin	Spain*	Mexico	Mexico	Mexico	Spain	Spain	Spain	Spain	Spain	Spain	Spain
Manufacturer	Abbott	Estrategias modernas	Victus inc	Branded by walmart	Nutricia	Nutricia	Abbott	Nestlé	Abbott	Abbott	Nutricia

*Ready to hang, RTH. Both, RTH and supplement. *Also available in Mexico.*

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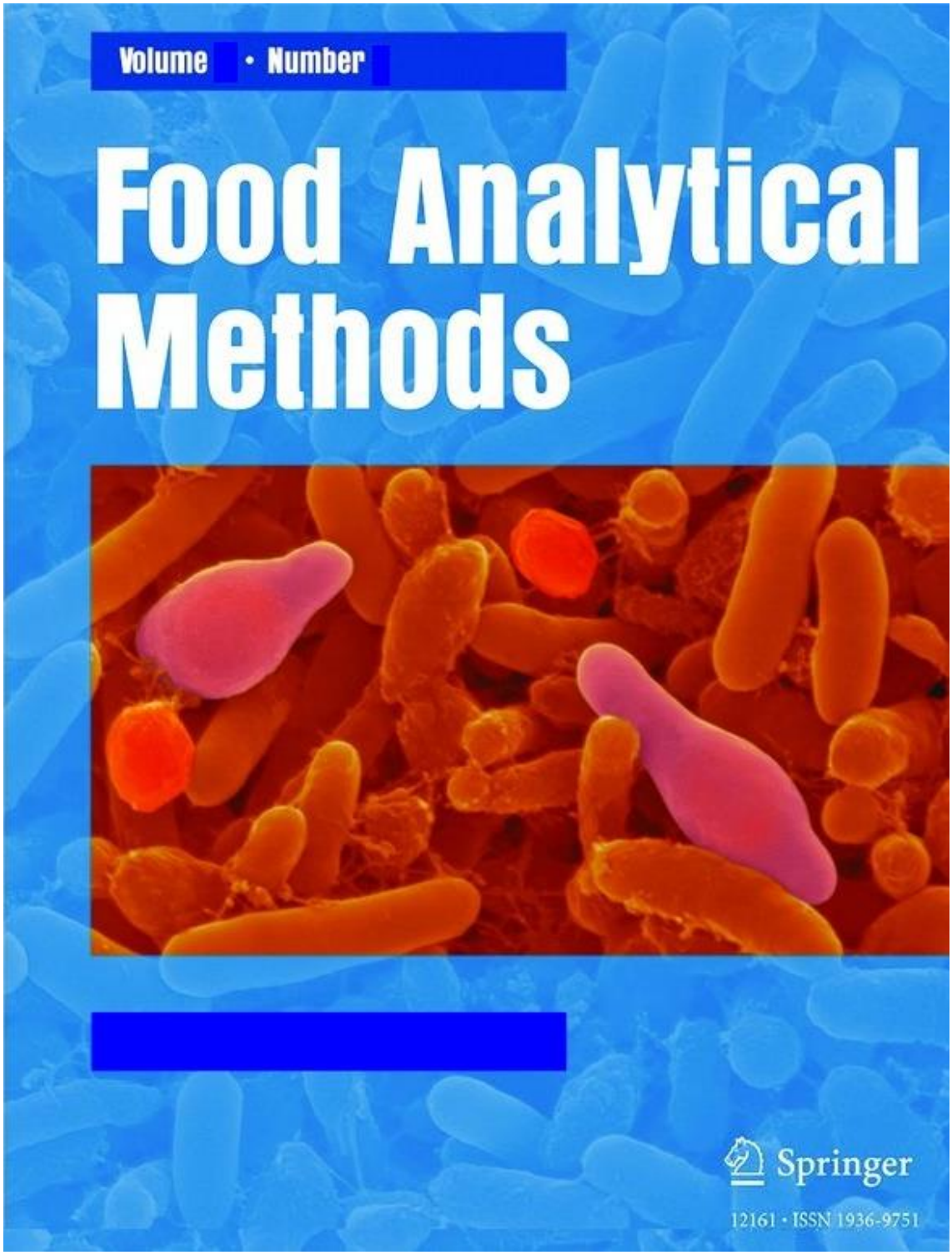
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Chapter II

Chapter II: Manganese Preconcentration And Speciation In Bioaccessible Fraction Of Enteral Nutrition Formulas By Cloud Point Extraction (CPE) And Atomic Absorption Spectroscopy

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2.1 Abstract

The present study develops a new method for manganese (Mn) preconcentration and speciation in bioaccessible fraction (soluble and dialyzable fraction) of enteral nutrition formulas by Cloud Point Extraction (CPE), and atomic absorption spectroscopy (AAS). For this purpose, a non-ionic surfactant (Triton X – 100) and three chelating reagents [8 – hydroxyquinoline (8-HQ); 1 – phenyl – 3 – methyl – 4 – benzoyl – 5 –pyrazolone (PMBP); and ammonium pyrrolidine dithiocarbamate (APDC)] were used with different operating conditions. The results showed that an efficient extraction of ionic Mn could be achieved with the following conditions: pH = 10; chelating reagent concentrations of [8-HQ] = 0.36 M, [PMBP] = 0.09 M, [APDC] = 0.09 M; surfactant Triton X – 100 at 25% (w/v), and equilibration temperature – time of 85^{o}C and 30 min.Limit of detection and quantitation were 0.015 and 0.050 mg L^{-1} respectively. Furthermore, when analyzing the bioaccessible fraction of the enteral nutrition formulas, the proposed approach can be used to distinguish between ionic (free) Mn, and Mn that has been previously bound to existing substances obtained from enzymatic digestion. These Mn speciation findings can be used to validate results obtained from the bioaccessibility assays.

Keywords: Cloud point extraction, manganese, speciation, bioaccessibility, food, enteral nutrition formula

2.2 Introduction

Bioaccessibility determination of a given micronutrient in food is one of the principal methods to evaluate its nutritional characteristics. The term bioaccessibility refers to the amount of the micronutrient that is found soluble in intestinal lumen after an *in vitro* simulated digestion; therefore, this micronutrient is available to be absorbed by the enterocytes. In the case of trace elements, this bioaccessibility will be influenced by the presence of other components present in the food matrix (fiber fractions, peptides from protein digestion, vitamins, etc.) as well as the chemical form the element has at the moment of absorption (Cámara-Martos et al. 2015).

Trace element speciation studies performed directly on the bioaccessible fraction of food are scarce. Nevertheless, Cámara et al. (2005b) have developed a spectrophotometric method using bathophenantroline as colorimetric reagent to speciate iron [Fe (II), Fe (III) and heme-Fe] in the bioaccessible fraction of typical meals from Spanish school menus. Similarly, Rosas-Castor et al. (2016) developed another method to speciate minerals, in this case with arsenic [As (III), As (V), dimethylarsenic acid (DMA) and monomethylarsenic acid (MMA)] from corn and rice samples using cloud point extraction (CPE) and atomic fluorescence spectrometry. Meanwhile, other more expensive methodologies like anion-exchange HPLC-ICP-MS, have been used to identify the selenomethionine as the main source of selenium in the bioaccessible fraction of mushrooms (Bhatia et al. 2013). As it can be observed, what makes these studies valuable is that they make it possible to identify which chemical forms of the studied element will have greater bioaccessibility.

Cloud Point Extraction (CPE) is a preconcentrating method widely used in the analysis of inorganic elements. The procedure is based on the principle that, in most aqueous solutions containing non-ionic surfactants, the monomers are found scattered in the aqueous solvent as part of a single phase. However, above certain micellar concentration, or as consequence in changes in Ph, temperature, or surfactant concentration, these monomers can begin to spontaneously associate with each other, reducing the solubility of the surfactant in water and therefore, resulting

in the separation of a single isotropic micelle phase into two phases. One of the resulting phases isrich in small volume surfactant molecules and the other is an aqueous phase (Rubio and Pérez-Bendito 2003; Bezerra et al. 2005; Filik and Aksu 2012; Soylak et al. 2012). The addition of a chelating agent, capable of creating a stable complex with the metal and with higher affinity (solubility) to the surfactant phase, will allow the desired preconcentration.

In the CPE approach, the key reagents are the surfactant and the chelating agent (Pytlakowska et al. 2013). Most of the surfactants used in CPE are nonionic, such as Triton X-114 (TX-114) and Triton X-100 (TX-100); this later has been less studied than the first. When choosing the chelating agent, it is sought that the organic ligand has particular affinity for the trace element to be analyzed. Within the wide variety of these compounds used for research some are to be highlighted; such is the case of 8 – hydroxyquinoline (8-HQ). This is a very versatile molecule, due to a of basic nitrogen and phenolic hydroxyl group in its structure, it has been used in the determination of Al (III),Cd (II), Co (II),Cu (II), Fe(III), Gd (III), La (III), Mn (II), Ni (II), Pb(II), Zn (II) among others (Draye et al. 2005; Farajzadeh and Fallahi 2006; Bavili Tabrizi 2007; Yalçin et al. 2012). Also,1 – phenyl – 3 – methyl – 4 – benzoyl – 5 –pyrazolone (PMBP), a complex metric organic reagent, is capable of establishing complexes with a wide variety of metal ions and has found numerous applications in trace element separation and preconcentration by solvent extraction (Sun et al. 2006; Liang et al. 2006). Also used are dithiocarbamates like ammonium pyrrolidine dithiocarbamate (APDC), which is capable of stabilizing a variety of oxidation states and coordination geometries from a wide spectrum of di- and trivalent metals (Giokas et al. 2004; Donati et al. 2006; Baig et al. 2009; Wang et al. 2017).

The current paper studies the influence of these three chelating agents (8-HQ, PMBP and APDC) and a non-ionic surfactant (TX-100) in the development of a method to preconcentrate and to speciate manganese (Mn) from the bioaccessible fraction of food samples. Among trace elements, Mn stands out for the role it plays in the human body as part of many enzymes, in the development of bone structures, and in normal function of the brain and nervous system (Gouda 2014; Ávila D.S. et al. 2016). The development of analytical technologies to identify the chemical

form in which it is present at the moment of absorption will be very relevant to justify its bioaccessibility. Besides, within the procedures applied to preconcentrate and speciate Mn and other trace elements, cloud point extraction (CPE) has proven to be the most efficient and environmentally friendly technique (Meeravali and Jiang 2009).

2.3 Materials and Methods

Samples

For the optimization of the developed method, three enteral nutrition feeding formulas were used (with fiber; high protein with fiber; high calorie). These enteral nutrition formulas should be nutritionally complete when used as a single feeding source and should have the same nutrients as a regular diet (Waitzberg DL and Torrinhas RS 2015). The ingredients that make up the different formulas studied are shown in Table 1.

Chemicals and reagents

All chemicals and reagents were of analytical-reagent grade. Ultrapure water (18 MΩ · cm) obtained from a Milli-Q Reference Water Purification (Millipore, Madrid, Spain) was used for the analysis. All glassware and plastic containers were soaked in 20% nitric acid overnight and rinsed 3 times with deionized water prior to use. Nitric acid (69 %), hydrochloric acid (35 %), sodium chloride, and ammonium chloride were obtained from Panreac (Barcelona, Spain). Manganese sulfate trihydrate (96 %) from Acros (Geel, Belgium), and manganese permanganate from Quimivita, (Barcelona, Spain).

Acetic acid, ammonium hydroxide and sodium acetate were purchased from Carlo Erba Reagents Group (Rodano, Italy), and ammonium acetate from VWR International (Leuven, Belgium). Non-ionic surfactant Triton X-100 (TX-100) was purchased from VWR International (Leuven, Belgium). Chelating agents 8-Hydroxyquinoline (8-HQ), 1-Phenyl-3- Methyl-4-Benzoyl-5-Pyrazolone (PMBP), and ammonium pyrrolidine dithiocarbamate (APDC) were

purchased from Fisher Scientific (Geel, Belgium), Alfa Aesar (Karlsruhe, Germany), and Sigma-Aldrich (St. Louis, U.S.A.), respectively. Pharma grade ethanol (96%) was used to dissolve the surfactant and chelating agents.

Table 1 *Ingredients of enteral nutrition formulas used, as labeled by the manufacturer*

Formula	Ingredients
With fiber (F)	Water, maltodextrin, vegetable oils (sunflower, canola, MCT oil), dietetic fiber (soy polysaccharides, resistant starch, inulin, arabic gum, cellulose, oligofructose), whey protein (from milk), caseinate (from milk),pea protein, soy protein, emulgent (soy lecithin), citric acid, sodium chloride, potassium hydroxide, fish oil, tricalcic phosphate, dipotassium phosphate, potassium chloride, carotenoids (contains soy) (beta-carotenoids, lutein, lycopene), choline chloride, calcium hydroxide, potassium citrate, magnesium hydroxide, sodium L-ascorbate, magnesium phosphate, ferrous lactate, zinc sulfate, nicotinamide, retinyl acetate, DL-alpha-tocopherol, copper gluconate manganese sulfate, sodium selenite, calcium D-pantothenate, chromium chloride, cholecalciferol, D-biotin, thiamine chlorhydrate, pteroylmonoglutamic acid, pyridoxine hydrochloride, riboflavin, potassium iodine, sodium fluoride, sodium multidate, phytomenadione, cyanocobalamin.
J High protein with fiber (HPF)	Water, maltodextrin, sodium and calcium caseinates (milk proteins), milk protein isolate, high oleic sunflower oil, canola oil, FOS, soy protein isolate, MCT from palm kernel, minerals (potassium citrate, sodium citrate, magnesium chloride, potassium chloride, dibasic magnesium phosphate, calcium carbonate, ferrous sulfate, zinc sulfate, manganese sulfate, cupric sulfate, sodium molybdate, chromium chloride, potassium iodide, sodium selenate), oat fiber, soy polysaccharides, emulsifier: soy lecithin, arabic gum, choline chloride, cellulose, sodium carboxymethyl cellulose, vitamins (ascorbic acid, DL-alpha tocopherol acetate, niacinamide, calcium pantothenate, pyridoxine chlorhydrate, vitamin A palmitate, thiamine chlorhydrate, riboflavin, beta carotene, folic acid, biotin, phylloquinone, D_3 vit., cyanocobalamin), taurine, L-carnitine, stabilizer: gellan gum.
V Hypercaloric (H)	Water, maltodextrin, hydrolyzed milk proteins, vegetable oils (MCT from palm kernel, canola), sucrose, minerals, calcium carbonate, magnesium phosphate dibasic, dibasic potassium phosphate, potassium chloride, sodium citrate, potassium citrate, ferrous sulfate, zinc sulfate, manganese sulfate, copper sulfate, sodium molybdate, chromium chloride, sodium selenate, potassium iodide, flavoring, emulsifier: E472e, stabilizers (E460, E407, E466), choline chloride, vitamins (C, E, niacinamide, calcium pantothenate, vitamin A palmitate, B_6, B_1, B_2, folic acid, K1, biotin, D_3, cyanocobalamin), L-carnitine, taurine, sweetener: E955

Throughout the dialysis assays, digestive enzymes and bile salts from Sigma-Aldrich Co. (St. Louis, MO) were used. For the pepsin solution, 1.5 g of pepsin (P-7000 from porcine gastric mucosa) were dissolved in 9.4mL of HCl (0.1M). Meanwhile, the pancreatin and bile salts solution was made by dissolving 0.2 g of pancreatin (P-3292 from porcine pancreas) and 1.3 g of bile salts (B-8756 of porcine origin) in 50 mL of 0.1M NaHCO3. These working solutions were prepared immediately before use. Dialysis membranes, with a pore size (MWCO) of 12-14,000

Å (Size 6 Inf Dia 27/32″–21.5 mm, 30 m, from Medicell Int. LTD, London, UK), were rinsed several times with distilled deionized water before use.

Standard solutions for interference assays (Fe, Zn, Ca and Cu) were prepared immediately before use by dilution of a 1000 mg/L standard solution (Titrisol, Merck, Darmstadt, Germany) with distilled deionized water.

Apparatus

A model AA—50B Varian Spectra atomic absorption spectrophotometer equipped with standard air-acetylene flame and single element hollow cathode lamps (Varian Spectra) without background correction were used throughout measurements. A Precisterm thermostatic bath (J. P Selecta, S.A., Barcelona, Spain), maintained at the desired temperature, was used for CPE experiments, and phase separation was assisted by a Jouan C41 Centrifuge (Thermo Electron Corporation, Massachusetts, U.S.A.). Finally, pH values were measured with a bench meter with Ph/temperature digital probe 230VAC (Hanna Instruments, Inc, RI, USA). *In vitro* digestions were performed using a shaking water bath (HSB-2000 Shaking Bath; E-Chrom Tech CO., LTD, Taipei, Taiwan).

CPE optimization method

Ionic Mn preconcentration CPE [Mn (II) and Mn (VII)] was done with 1.5 mL of the bioaccessible fraction of enteral nutrition formulas. To validate the method´s accuracy, recovery tests were carried out by addition of different amounts of Mn (II) or Mn (VII) to the bioaccessible fraction. For this purpose, stock solutions of Mn (II) and (VII) [0.2, 0.5, 1 and 2 mg/L] were prepared by dissolving the appropriate amounts of their respective salts (manganese sulfate trihydrate and manganese permanganate) in distilled deionized water.

Then, the amount of buffer needed to reach a volume of 3.5 mL was added. The buffers used to set pH of the solutions were the following: Acetic acid/Sodium acetate (AcH/AcNa) (pH 2-5), Ammonium acetate/Ammonium Hydroxide (AcNH4/NH4OH) (pH 6-8), and Ammonium chloride/Ammonium Hydroxide (ClNH4/ NH4OH) (Ph 9-12). Finally, 0.5 mL of sodium

chloride 5% (m/v), 0.5 mL of chelating agent (8-HQ, PMBP or APDC), and 0.5 mL of surfactant (TX-100) were added to the 64ioaccessible tubes of 15mL (Deltalab, Barcelona, Spain).

As it will be further described in the Results and discussion section, three different chelating agents were assessed at varying concentrations before establishing the concentration to be used in the upcoming tests. These chelating agents were: 8-HQ (0.03M-0.36M), PMBP ($1.2x10^{-3}$- 1.2M), and APDC (0.03 -0.36M). The same process was then followed by the surfactant TX-100, in which the concentrations tested were 5 to 50%.

The resulting solution was heated in a thermostatic bath up to 85°C to incubate (15, 30, and 45 min). Then, it was centrifuged for 5 minutes at 4000 rpm to accelerate separation of the two phases. After cooling down in an ice bath for 10 minutes, the surfactant rich phase became viscous and adhered to the bottom of the tubes, while the upper aqueous phase was discarded by inverting the tubes. In order to enable sample handling and reduce viscosity of the surfactant rich phase, which contained the complexes, sample dissolution was carried out by adding HCl/ethanol (90:10) 2M until a 5 mL volume was met. Mn content in the final solution was analyzed by flame atomic absorption spectroscopy (FAAS) under the following conditions: 279.5nm wavelength, a slit width of 0.2 nm, standard air-acetylene flame (3.50 L/min-1.50 L/min), and reading time of 4 seconds.

Bioaccessibility assays

The bioaccessible fraction (dialyzable fraction) of enteral nutrition formulas was obtained through an *in vitro* process of gastrointestinal digestion simulation based on the one described by (Cámara et al. 2005a). The procedure started by taking 30Ml of the enteral nutrition formulas and adjusting the pH of each to 2.0 using 6N HCl. The first stage of the process required a pepsin-HCl digestion, in which 0.5 g of pepsin (in the pepsin-HCl solution described under ¨Chemicals and Reagents¨) were added for each 100g of sample used (0.94 mL of solution per sample of 30 mL); the samples were then incubated for 2 hours in a shaking water bath at 37°C.

Afterwards, dialysis membranes (molecular mass cut-off value 10–12,000 Da) were filled with deionized water and the amount of $NaHCO_3$ equivalent to the titratable acidity. These membranes were placed inside the flasks containing the pepsin digest, and then were incubated under the conditions mentioned previously. After 30 minutes, 6.25 mL of the solution containing pancreatic and bile salts were added to the samples and the incubation continued for 150 minutes. When the incubation time was over, the dialysis membranes were removed from the flasks and rinsed with distilled deionized water. After this, the membranes were cut open and the transparent liquid from the inside (bioaccessible fraction) was kept for future assays.

To obtain the soluble fraction from the enteral nutrition feeding formulas, a solubility assay was performed, in which a pepsin-HCl digestion has taken place like the one previously described at the beginning of the dialysis-based assay. After this, the intestinal digestion was carried out by setting the pH to 5 by the addition of 1M NaHCO3. Then, the intestinal stage, with the pancreatin and bile salts mixture was done as before. Finally, the Ph of the samples was set to 7.2 with 0.5M NaOH. After this, aliquots of the digested sample were transferred to polypropylene centrifuge tubes (CTSP-050-050 model, Labbox Labware, S.L. Barcelona, Spain), and these were centrifuged for 1 h at 4000 rpm and 4 °C (Eppendorf Centrifuge 5810 R). Then, the supernatant (soluble fraction) was collected, to be used in further CPE assays.

2.4 Results and Discussion

Effect of pH

One crucial step in CPE is the formation of a stable compound with the chelating agent, followed by the migration and solubilization of this hydrophobic chelate to the non-ionic phase made up of aggregated surfactant micelles (Bezerra et al. 2005). In this process, pH plays a fundamental role; therefore, pH influence (2 – 12) on the recovery percentage of Mn was studied on the bioaccessible part (dialyzable fraction) with the three chelating agents previously mentioned [8-HQ 0.36M; PMBP 0.12M; APDC 0.18M]. It can be seen in Fig. 1 that lower pH values do not favor the recovery of ionic Mn. Meanwhile, extraction was quantitative for Mn II

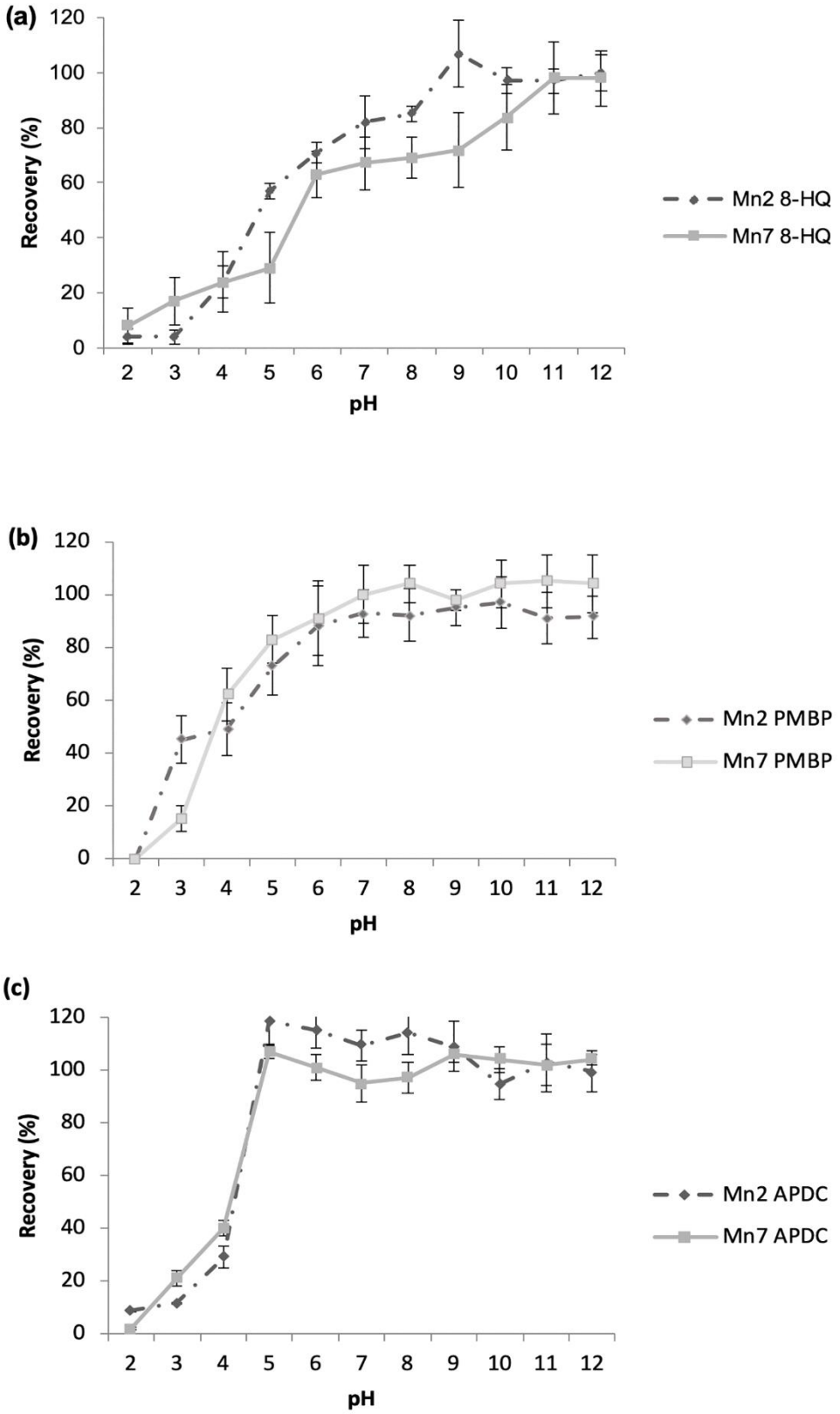

Fig. 1 Effect of pH on the recovery of Mn(II) and Mn(VII) using 8-HQ [0.36 M] (a); PMBP [0.12 M] (b); and APDC [0.18 M] (c). The other conditions established for these assays were Triton X-100 50% (w/v), NaCl 5% (w/v), temperature 85 °C, and incubating time 45 min

and Mn (VII) from pH = 9, pH = 8 and pH = 5 with 8 – HQ, PMBP and APDC respectively. No significant difference between Mn (II) and Mn (VII) was found, meaning that the three chelating agents are equally able to chelate both ionic species of Mn, in order to preconcentrate and speciate Mn a pH of 10 was selected for subsequent assays.

Effect of the chelating agents

To improve separation of metallic ions by CPE, the formation of an insoluble, or relatively insoluble complex, in water is needed (Pytlakowska et al. 2013). Hence, several concentrations of three different chelating agents at pH = 10 were used to study their capacity to extract ionic Mn. In all the assays performed, the chosen concentration of the chelating agent was at least 100 times more than the ionic Mn expected in the bioaccessible fraction (dialyzable fraction). This way the capacity to chelate would not be a limiting factor.

In particular, 8-hydroxyquinoline (8-HQ) is a reagent widely used in chemical analysis due to its amphoteric properties. It has been used as chelating agent with several metallic ions such as lanthanides (III), V (III) and Mn (II) among others (Favre-Reguillon et al. 2004; Khan et al. 2010; Yalçin et al. 2012). In this study, the range of concentrations of 8-HQ assayed was from 0.03M to 0.36M. It can be observed (Figure 2a) that for both metallic ions, Mn (II) and Mn (VII), the recovery percentage increased from 40% with the lowest 8-HQ concentration, to 100% with the highest concentration. These findings contrast with what was found by Yalçın et al. (2012) where concentrations of 8-HQ as low as 0.005M were enough to obtain 100% recovery of ionic Mn in tea samples, using pH=9. In the present study, concentration of 8-HQ 0.36M was set for further assays.

Additionally, the efficiency of another organic reagent 1-Phenyl-3- Methyl-4-Benzoyl-5-Pyrazolone (PMBP) was tested. The ability of PMBP to interact with determined metal ions depends on the highly hydrophobic nature of its metal complexes, and this is given by the ketone functional group on its structure (Manzoori and Abdolmohammad-zadeh 2007). Varying concentrations of PMBP starting from $1.2x10^{-3}$. Figure 2b shows a high recovery percentage of ionic Mn around 100% for all the concentrations studied. Liang et al. (2005) reported an optimal

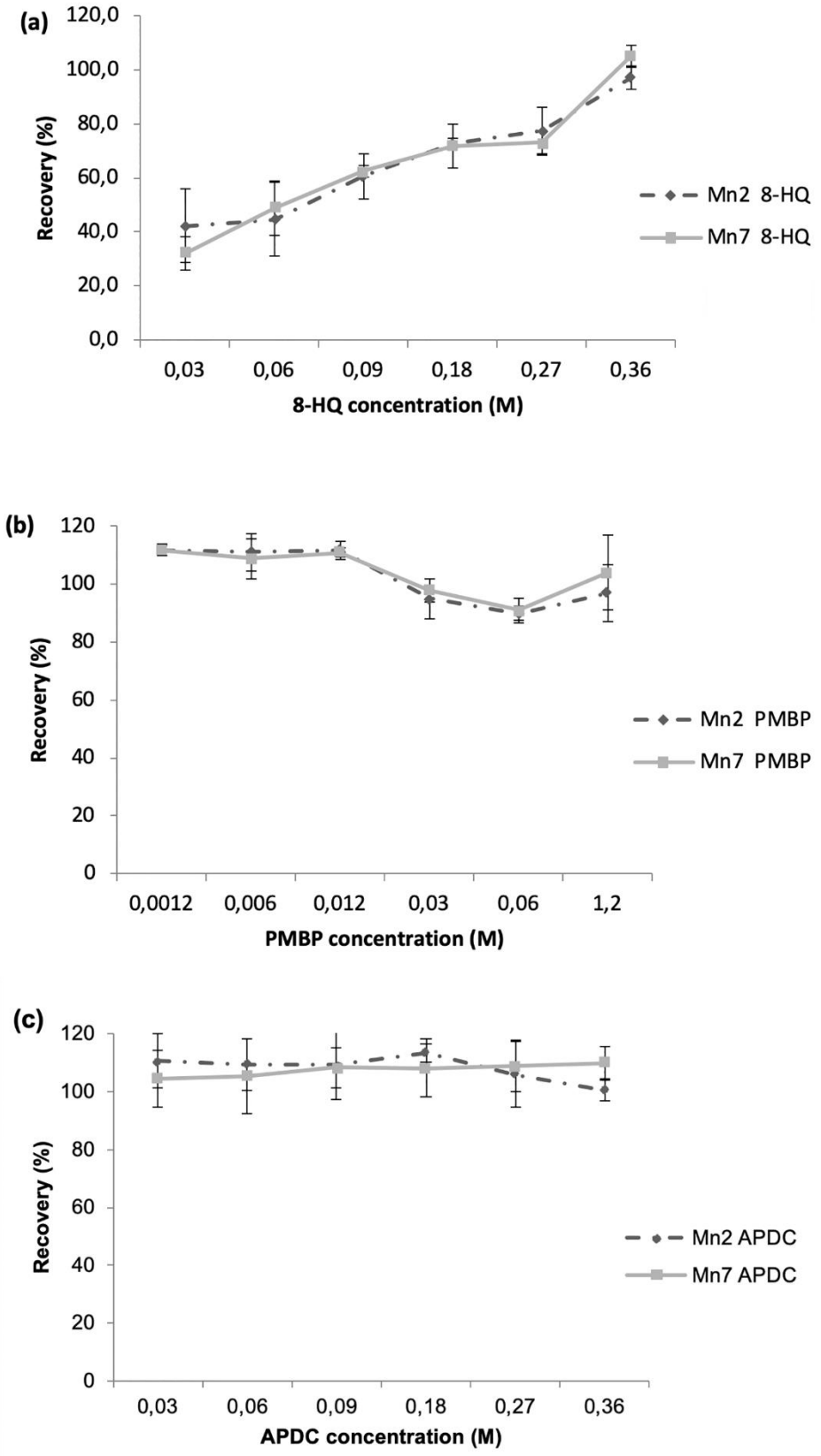

Fig. 2 Effect of concentration of complexing agent on the recovery of Mn(II) and Mn7(VII) using 8-HQ [0.03–0.36 M] (a); PMBP [1.2 × 10^{-3} to 1.2M] (b); and APDC [0.03–0.36M] (c). Conditions: pH 10, Triton X- 100 50% (w/v), NaCl 5% (w/v), temperature 85 °C, and incubating time 45 min

PMBP concentration of $8x10^{-4}$M to achieve an efficient extraction of Cd using PMBP in tap water. to 1.2M were tested. In our assay, the addition of 0.5mL of PMBP $1.2x10^{-3}$M to a final volume of 5mL results in a similar concentration to that reported in the study mentioned above ($1.2x10^{-4}$ M). However, to avoid the concentration of chelating agent to be a limiting factor in the recovery of Mn in the following experiments, concentration of PMBP slightly superior (0.09M) was chosen.

Dithiocarbamates, as ammonium pyrrolidine dithiocarbamate (APDC), are known as some of the most efficient chelating reagents commonly used to preconcentrate metal ions other than Mn (Giokas et al. 2004; Donati et al. 2006; Baig et al. 2009; Wang et al. 2017). As it has been previously discussed, this happens as consequence of being capable of reacting with many di- and trivalent metallic ions. To assess the optimal APDC concentration, assays with varying concentrations of the chelating agent (0.03,0.06, 0.09, 0.18, 0.27 to 0.36M) were performed. As shown in Figure 2c, following the same trend of PMBP, the recovery of ionic Mn with APDC was approximately 100% in all the experiments, with both Mn oxidation states. For that reason and to ensure sufficient efficacy, a concentration of 0.09M was employed in further experiments.

Effect of TX-100 Concentration

The success of the CPE technique lies in the capacity to maximize the extraction efficiency through minimizing the phase volume ratio (ratio between the volume of the aqueous phase and the final volume of the surfactant rich phase), consequently taking advantage of the most of its concentrating ability (Giokas et al. 2002). In this aspect, the surfactant chosen plays a fundamental role. It has to be able to not only extract the ion with the chelating ag but also to easily separate the aqueous phase after centrifugation.

In the present study, the surfactant chosen to study modifying its concentration was TX-100. This is because of its commercial availability, relatively low price, low toxicity, and high density of the surfactant rich phase.

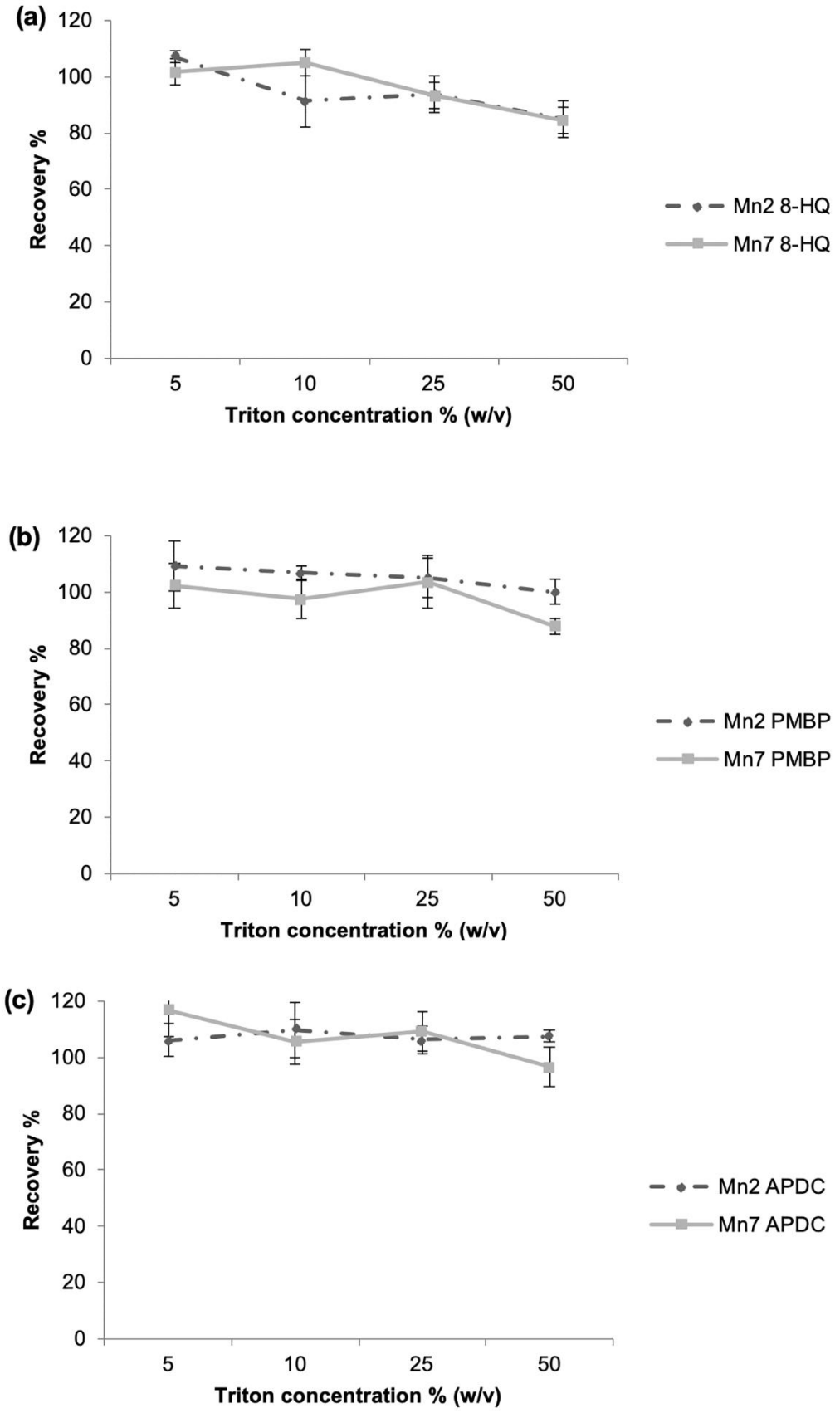

Fig. 3 Effect of Triton X-100 concentration on the recovery efficiency using 8-HQ (a), PMBP (b), and APDC (c). Conditions: pH 10, [8-HQ] = 0.36 M; [PMBP] = 0.09 M; and [APDC] = 0.09 M, Triton X-100 [5– 50%], NaCl 5% (w/v), temperature 85 °C, and incubating time 45 min

The concentrations (m/v) assayed were the following: 50%, 25%, 10% and 5%. Concentration of the chelating agents and pH values were already established in the section above (pH=10; [8-HQ] =0.36M; [PMBP]=0.09M; and [APDC]=0.09M). It was observed that the recovery percentage of ionic Mn was around 100 in all the four concentrations (Figures 3 a-c). Nevertheless, the lowest TX-100 concentration, failed to allow visual separation between viscous and aqueous phases for separation after centrifugation. Because of this, a TX-100 concentration of 25% (m/v) was employed in further studies.

Equilibration temperature and time

Other variables needed to be tested in CPE are equilibration temperature and time. It is known that temperature in which TX-100 attains the cloud point is around 65°C (Hinze and Pramauro 1993). On the other hand, it is desirable that incubation time in CPE is no longer than needed to speed up lab processes. The influence on the extraction recovery of equilibration time was studied at 85°C for 15, 30 or 45 minutes at pH 10 using [APDC]= 0.09M; and TX -100 25%. The results from the optimization experiments showed that incubation time of either 15, 30 or 45 minutes were adequate for quantitative complexation of both species, as no difference in percentage recovery was found between them (see Fig 4).

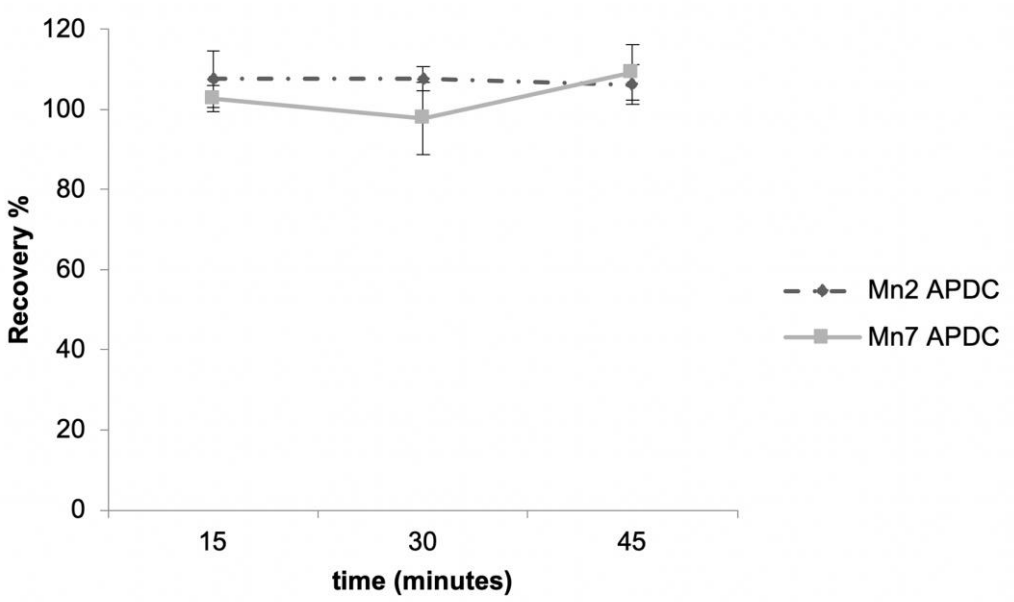

Fig. 4 *Effect of time on the recovery efficiency*

Cationic interferences

It is known that the concentration of the chelating agent must compensate sufficiently for any consumption of the reagent by other metals (Stalikas 2002). Therefore, once the optimum conditions were chosen (pH=10, APDC 0.09M, T-X100 25%, t=15min), possible interferences in the preconcentration step were studied to demonstrate sufficiency of the chelating agent.

In these tests, 5 mL of solution containing 0.5mg/L of Mn (II) or Mn (VII) were mixed with ascending amounts of different interferent ions. An ion was considered as interferent when it caused a variation in the absorbance in the sample greater than 5%. The tolerance limits of various added ions are given in table 2. The results show that excess amounts of several cations have no obvious influence on CPE of ionic Mn under the studied settings.

Table 2 *Tolerable limits of added ions*

Interferent ion	Foreign ion to analyte ratio (foreign ion/Mn)
Zn^{2+}	1000
Cu^{2+}	1000
Ca^{2+}	800
Mg^{2+}	500
K^{+}	500
Na^{+}	500
Pb^{2+}	200
Fe^{2+}	100

Characteristics of the method

To validate the optimized method, the CPE approach was used with the optimum conditions (pH=10, APDC 0.09M, TX-100 25%, t=15min) with 1.5mL of the dialyzable fraction of enteral feeding formula. Simultaneously, the same amount of dialyzable fraction was incinerated by dry-ashing dissolved with HNO_3 0.1 M, and measured by FAAS. Results from the CPE technique showed a Mn concentration of 0.117 ±0.010 mg/L versus 0.116 ± 0.001 mg/L. This demonstrates an adequate recovery percentage of the method developed. The limit of detection (LOD) (blank $\pm 3\sigma$) and limit of quantitation (LOQ) (blank $\pm 10\sigma$) of the proposal method

were determined. The obtained values of LOD and LOQ based on the optimized parameters were 0.015 and 0.050 mg L^{-1}.

Determination of complexed Mn in bioaccessible fraction

The CPE approach has been used not only to preconcentrate ions from liquid samples but also to speciate between the different chemical forms in which the trace element can be found. According to our previous results, it was not possible to use it to distinguish between Mn (II) and Mn (VII) because bonding of both ions with the three chelating agents studied was equally effective. However, CPE can be used to differentiate between ionic Mn and Mn that has been previously bound to substances obtained from enzymatic digestion of other components, such as peptides, fiber fractions, polyphenols, etc.

Accordingly, Yalcin et al. (2012) have already developed a separation method to distinguish between flavonoid bound Mn and free Mn in tea infusions. In the methodology described by them, bound Mn was extracted from samples that incorporated neither a chelating agent nor a buffer solution. On that account, the previously optimized method described in this paper was adapted following a similar methodology. This adapted method was used to separate Mn bound to different compounds (or chelated Mn) and ionic Mn from the bioaccessible fractions. In this later assay, the bioaccessible fraction used was the soluble fraction instead of the dialyzable fraction, because more bound Mn can be extracted from this soluble fraction. This is due to the size of the chelated compounds, which generally is greater than the size of the pores of the dialysis membrane. Therefore, the chelated complexes are not able to get through them into the dialyzable fraction.

Therefore, to achieve Mn speciation (between bound Mn and ionic Mn), the optimized CPE approach was used with the soluble fraction of three enteral feeding formulas. First, to determine bound Mn, no chelating agent nor a buffer solution were used. Secondly, to determine total Mn (bound Mn and ionic Mn), the CPE method developed was used in the soluble fraction in this case, with the chelating agent (APDC), the buffer (pH 10), and the rest of conditions

previously optimized. Finally, ionic Mn was determined by estimating the difference between total Mn and bound Mn. The results are shown in table 3, it can be observed that, in the samples studied, the proposed method successfully distinguishes between bound Mn and ionic Mn. Differentiating between Mn forms may be of special interest when bioaccessibility of Mn is being assessed. As with the dialyzable fraction, to validate these assays, the results were compared to the Mn present in the soluble fraction obtained by dry-ashing.

Table 3 Speciation of Mn [complexed and ionic] in soluble fraction of enteral nutrition formulas (mean standard ± deviation)

Sample	Mn ionic by CPE-AAS (mg/L)	Mn complexed by CPE-AAS (mg/L)	Mn total by CPE-AAS (mg/L)	Mn total by AAS (mg/L)
With fiber	0.18 ± 0.01	0.22 ± 0.05	0.40 ± 0.09	0.45 ± 0.03
High protein with fiber	0.11 ± 0.01	0.39 ± 0.03	0.50 ± 0.04	0.54 ± 0.01
Hypercaloric	0.18 ± 0.02	0.57 ± 0.02	0.75 ± 0.05	0.81 ± 0.04

2.5 Conclusions

The present study uses CPE -FAAS methodology to preconcentrate and speciate Mn in soluble fraction of enteral formulas. The parameters, refined until an optimal preconcentration of total Mn in bioaccessible fraction was achieved, were: pH=10, [chelating agent; (APDC)] = 0.09M, [surfactant agent; (TX-100)] = 25%, and incubation time = 15min. Enteral formulas were chosen to develop this assay is because they supply the same main nutrient classes (carbohydrates, fat and proteins) and micronutrients that a standard diet would. It should be highlighted that most speciation studies done with CPE have been developed in simpler samples such as water and tea. Besides Mn preconcentration, this method was used to speciate bound Mn, using the same surfactant concentration and time as for total Mn, but without the chelating agent and buffer (pH 10).

The results from this paper show that, even though the initial optimization procedure to establish the methodology used is time consuming, CPE is a practical and viable method to determine and speciate free and bound Mn in these food samples; offering a method that is faster, cheaper, less polluting, and more accessible than others.

The technique in this paper has the advantage of being able to determine the chemical forms in which many nutrients are found in the intestinal lumen while, at the same time, avoiding the incineration process by dry-ashing. Using this information, bioaccessibility can be improved as needed.

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Conflicts of interest: None declared

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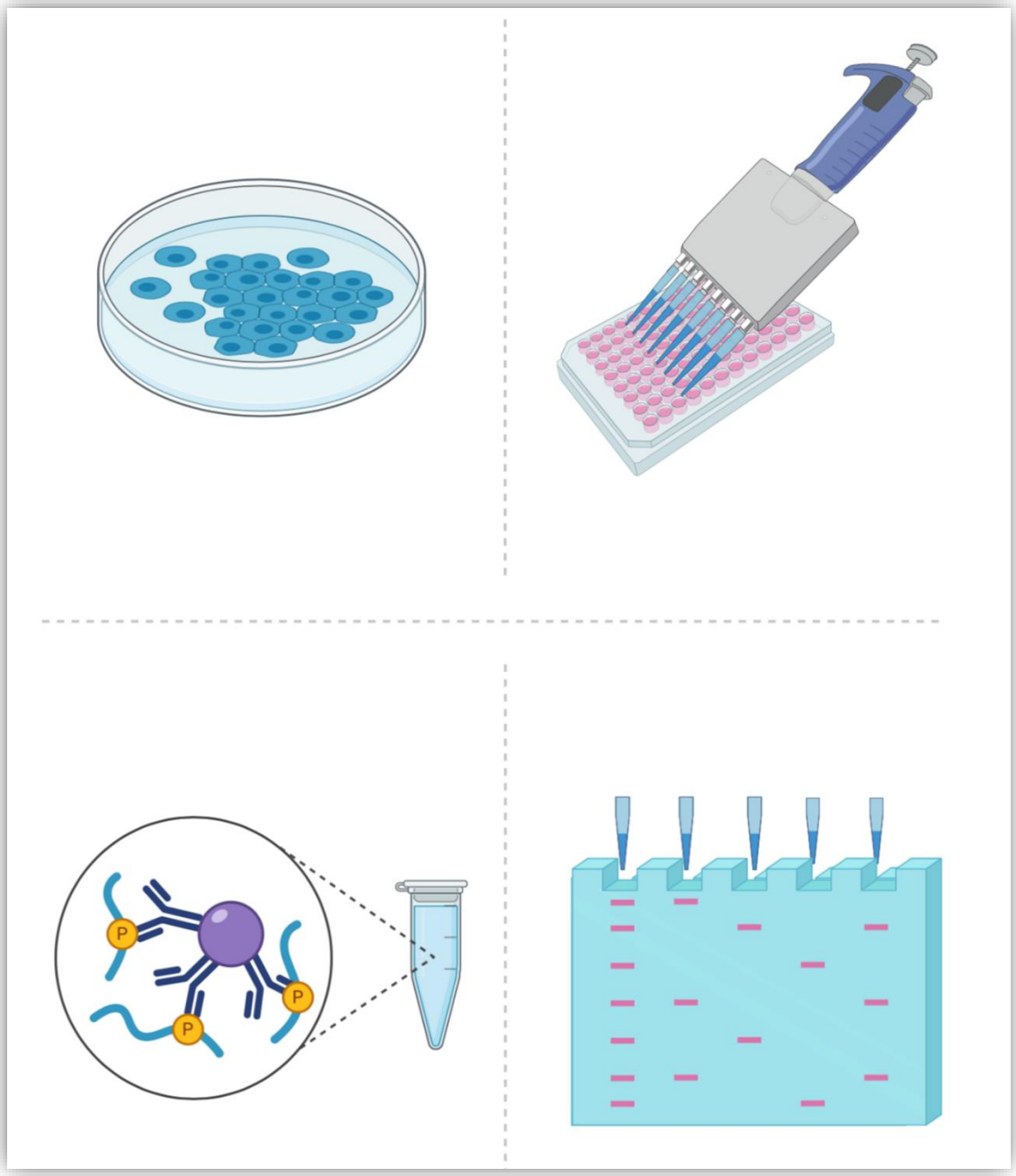

Chapter III

Chapter III: In Vitro Evaluation Of Selenium Bioaccessibility From Enteral Nutrition Formulas: Effect Of Supplementation Over Selenoenzyme Activity And Expression

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3.1 Abstract

Selenium (Se) dependent enzymes such as thioredoxin reductase (TrxR) and glutathione peroxidase (GPx) are major systems needed to maintain the redox balance. Diet provides such needed Se, though patients unable to eat a regular diet supplement it or replace it through nutrition formulas (NF) to provide daily nutrients. To evaluate the antioxidant capacity of NF through the activity of TrxR and Gpx in human hepatoma cells using NF dialysates obtained through in vitro bioaccessibility methods. Further effect of Se supplementation was also studied. Cells were cultured in dialysates of three different NF (the fourth treatment was DMEM/F12 media as the control). Se was added at final concentrations of 0; 0.1 and 0.3 μM. TrxR activity was measured through the end-point insulin assay specific and GPx activity was assessed by reduction of H_2O_2.Western blot analysis was used to quantify expression of both proteins. Se supplementation had different effect on TrxR activity depending on the cell culture condition. A significant increase on its activity occurred in Regular Media (RM) and formula 1 (F1) (175 and 51% respectively, $p < 0.05$). Contrastingly, formula 2 (F2) and formula 3 (F3) did not differ from their non supplemented control, due to initial Se levels in F2 and the fiber content of F3. The activity of GPx was only affected in cells cultured in RM, and no effect was detected in any of the nutrition formulas ($p > 0.05$). Overall results demonstrate that some NF seem to provide sufficient Se to carry out its antioxidant role, while some commercially available supplements provide a form of Se that lacks enough evidence to support its use. This should be taken into account to protect patients ´condition especially in elderly people.

Keywords Selenium; Thioredoxin reductase; Glutathione peroxidase; Bioaccessibility; Enteral nutrition formulas

3.2 Introduction

The inability to maintain the redox-balance generates irreversible cellular damage which relates to chronic diseases [1]. Alternatively, antioxidants such as vitamins, mineral compounds, polyphenols and enzymatic antioxidants act at multiple stages regulating oxidant levels [2]. Two major redox system found in several metabolic pathways are the thioredoxin and glutathione systems. The thioredoxin system comprises the selenoenzyme thioredoxin reductase (TrxR), which reduces thioredoxin and several other substrates such as, lipoic acid, dehydroascorbate, selenocysteine, ubiquinone, protein disulfide isomerase and peroxides [3,4]. The glutathione system includes several glutathione (GSH) dependent enzymes such as the seleno-glutathione peroxidases (GPx), which reduce peroxides preventing oxidative damage [5]. Selenium (Se) integrates the catalytic site of both TrxR and GPx enzymes in the form of selenocysteine (Sec). Inorganic (selenite, selenate) and organic (selenomethionine and Sec) forms of Se from the diet are converted to selenide to then synthesize Sec [6,7].

Acknowledging that frequent chronic diseases such as hypertension and diabetes enhance oxidative stress, dietary intake of Se becomes more relevant. When some patients cannot eat normally or sufficiently to provide all the needed nutrients, nutrition formulas (NF) supplement or replace their diet. Hence, patients with high malnutrition risk but with functional gastrointestinal tract obtain essential macro and micronutrients through NF [8].

Given the oxidative processes occurring in these patients, NFs´ antioxidant capacity should be reviewed, which includes assessing Se bioaccessibility. This refers to the fraction that is solubilized and absorbed in the intestinal lumen and therefore available to perform its physiological function.

In this study we analyzed Se bioaccessibility *in vitro*, replicating the biochemical reactions occurring in the gastrointestinal tract and obtaining the element´s dialyzable fraction. These fractions were then tested in HepG2 cells as a culture media and used to evaluate how Se supplementation to NF affects selenoenzymes (TrxR and GPx) activity and expression.

3.3 Materials and Methods

Materials

All chemicals and reagents used were of analytical-reagent grade. Ultrapure water (18 MΩ/SCF) prepared with a Milli-Q Reference Water Purification (Millipore, Madrid, Spain) was used throughout experiments. All glassware and plastic containers were soaked in 50% nitric acid overnight, then 20% hydrochloric acid for an additional 24 h, and rinsed three times with de-ionized water prior to use. Hyperpure nitric acid (65%) and hydrochloric acid (35%) were obtained from Panreac (Barcelona, Spain).

Three NF/supplements were bought in Mexico; from which 2 were aimed to patients with diabetes, and all had similar selenium content declared on the label, see **Table 1** for more information about each product.

Table 1 *Nutrition Formulas: composition and general characteristics*

	Per 100mL			**Per serving**		
	F1	F2	F3	F1	F2	F3
Serving size, mL				237	237	240
Energy, kcal	105.3	101	113.4	250	240	272.2
Protein, g	3.8	5	6.125	9	12	14.7
Fat, g	2.5	3.8	3.3	6	9	8
Carbohydrates, g	16.9	11.4	14.7	40	27	35.3
Sugars, g	9.7	0.3	6.6	23	0.8	15.9
Total fiber, g	0	1,3	7.9	0	3	19.0
Se, µg	7.4	7.4	7.9	17.5	17.5	18.9
Other characteristics						
Selenium form	Se aminoacid chelate	Sodium selenite	Sodium selenite			
Specification	Standard	Diabetes	Diabetes			

AI, Adequate Intake; Se, selenium.

Cell culture

Human hepatoma cells (HepG2) used throughout the experiment were generously gifted by Dr. Elsa Dias (National Health Institute Dr. Ricardo Jorge), and were cultured in a 1:1 solution of Dulbecco's modified Eagle's medium : F-12 Nutrient Mixture (DMEM / F12) supplemented with

10% (v/v) fetal bovine serum (FBS), and 1% (v/v) antibiotics (penicillin/streptomycin). Cells were kept at 37 °C in a humidified atmosphere (5% CO_2) and medium renewal was done twice per week and cell passed upon reaching 70% confluence. Medium, FBS and antibiotics were acquired from Gibco.

Dialysis (bioaccessibility) assays

Working solutions were prepared immediately before use with digestive enzymes and bile salts from Sigma-Aldrich Co. (St. Louis, MO). The pepsin solution consisted in 1.5 g of pepsin (P-7000 from porcine gastric mucosa) dissolved in 9.4mL of HCl (0.1M). Meanwhile, the pancreatin and bile salts solution was made by dissolving 0.2 g of pancreatin (P-3292 from porcine pancreas) and 1.3 g of porcine bile salts (B-8756) in 50 mL of 0.1M NaHCO3. Dialysis membranes with a pore size (MWCO) of 12-14,000 Å (Size 6 Inf Dia 27/32″–21.5 mm, 30 m, from Medicell Int. LTD, London, UK), were rinsed several times with distilled deionized water before use.

The bioaccessible fraction (dialyzable fraction) of the NF was obtained through an in vitro process of gastrointestinal digestion simulation based on the one described by Cámara et al. [9]. First, the pH of NF (30mL aliquots) was set to 2.0 with 6N HCl. Then a pepsin-HCl digestion took place, for which 0.5 g of pepsin were added for each 100mL of sample used (per each sample of 30mL, 0.94 mL of the pepsin-HCl solution previously described); the samples were then incubated for 2 hours in a shaking water bath at 37°C.

The second part of the in vitro digestion starts by placing in each sample a dialysis membrane (molecular mass cut-off value 10–12,000 Da) filled with deionized water and the amount of NaHCO3 equivalent to the titratable acidity. After 30 minutes of incubation under these conditions, 6.25 mL of the solution containing pancreatin and bile salts were added to the samples and the incubation continued for 150 minutes. When the incubation was over, the dialysis membranes were removed from the jars, rinsed with distilled deionized water, and the membranes were cut open. The dialysate from the inside of the membrane (bioaccessible fraction) was kept to be used the next day

in the assays with HepG2 cells. Prior to adding the dialysate to the cells, pH was adjusted to 7.4 and the solutions were sterilized using 0.20 μm sterile filters (VWR).

Cell viability assay

Cell viability was determined using the MTT ((3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich Co. , St. Louis, MO) assay first described by Mosmann [10] and then adapted by Carvalho et al. [11]. Cells ($5x10^3$) were left to attach for 24 hours in 96-well plates (Nunc®) with triplicate wells for each exposure condition. Afterwards, conditioned media was replaced by solutions of non-conditioned media (with 0, 1, 2, 5, and 10% FBS) and the three NF dialysates (with 0, 1, 2, 5, and 10% FBS each). At time 0 and after 24, 48 and 72h after the solutions were applied, each well was added with the amount of MTT necessary to achieve a concentration of 400 μg/mL and left to incubate for 4 h at 37 °C. To measure absorbance, the microplates were added a guanidine-HCl solution (6 M, pH 8.0) and were shaken for 15 min. Lastly, absorbance was measured at 550 nm using a microplate reader (Zenyth 3100, Anthos Labtec Instruments). Results are expressed as percentages relative to the non-treated control, representing the mean ± standard error (S.E.M.) of quadruplicate experiments.

Cell lysates preparation

Cells ($1.0x10^6$) were plated in 10 mm^2 culture dishes and grown until 80% of confluence was reached. At this point medium was replaced by non-conditioned media (furtherly referred as regular media, RM) and the dialysates from each formula, and Se was then added in different concentrations (0, 0.1 and 0.3 μM), see **Figure 1**. Selenite stock solutions were freshly prepared for each intervention from sodium selenite (214485 Sigma-Aldrich Co.). After 24 h of incubation, cells were collected and centrifuged for 5 min at 4°C and 2315 rpm (600 g), after which the medium was removed, and cellular pellets washed with ice-cold PBS (phosphate buffered saline). After a second centrifugation PBS was removed and the final cell pellet was resuspended in lysis buffer (100 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 20 mM NaF, 1 mM sodium orthovanadate, 20 mM sodium pyrophosphate;

20 mM sodium β-glycerophosphate, 0.5% Triton X-100 in 25 mM Tris–Cl pH 7.5) containing a protease inhibitor cocktail (Roche, 1 tablet per 10 mL of buffer). Homogenates were centrifuged at 12,000 rpm, 4 °C for 5 min. The pellet (cellular debris) was discarded and the soluble fraction (supernatant) was transferred to Eppendorf vials and kept at -20 °C until analysis.

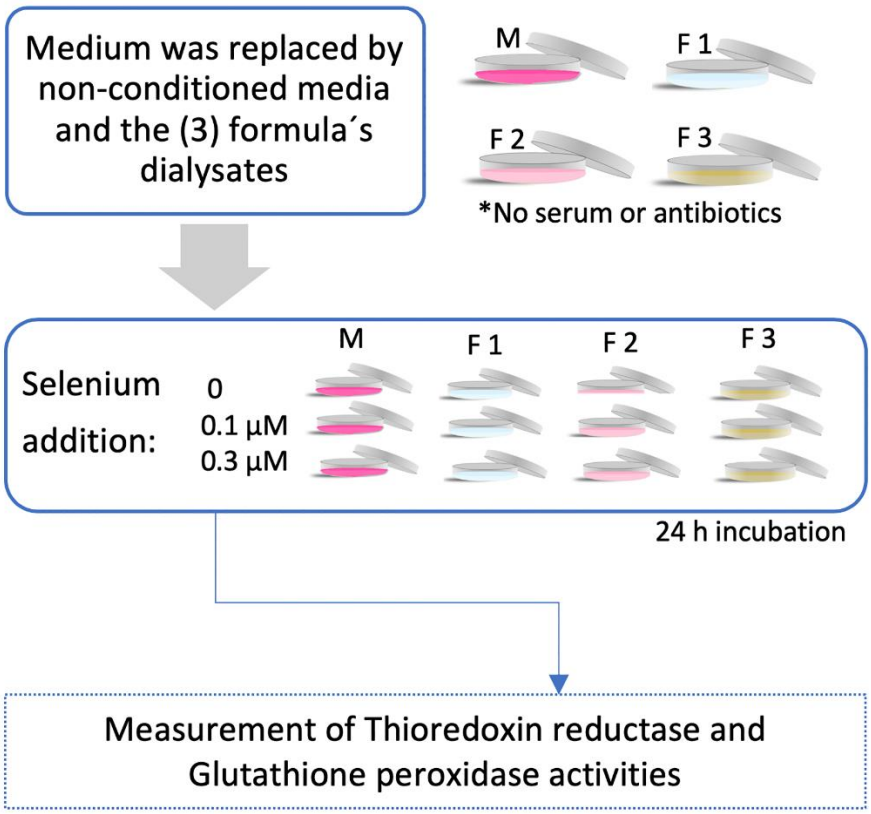

Figure 1. *Diagram showing the experimental conditions and Se supplementation*

M, Regular Medium (DMEM /F12); F, Formula dialysate; h, hours

Soluble protein determination

Bradford´s method [12] was used to quantify protein content. To do this, samples were incubated with Coomassie dye (Bio-Rad; 5 times dilution) in 96-well plates and absorbance measured at 595 nm in the microplate reader. Protein concentration was quantified using a calibration curve made with BSA (bovine serum albumin) as a standard.

Thioredoxin reductase assay

Thioredoxin reductase (TrxR) activity was measured through the end-point insulin assay specific for complex biological samples developed by Arnér and Holmgren [13]. Briefly, 30 μg of soluble protein were incubated in Hepes buffer (85 Mm, pH 7.6) with a master mix (0.3 mM insulin, 660 μM NADPH, 3 mM EDTA) and 3 μM of fully reduced human Trx1 (IMCO Corp., Sweden) for 20 min at 37ºC. Following incubation, the reaction was stopped with a DTNB/Guanidine (pH 8.0) solution and reduced thiols quantified at 412 nm. In the same 96-well plate, for each sample, wells with the same reagents but excluding Trx were prepared to measure TrxR activity by quantifying the difference in absorbance to the wells containing Trx.

Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was assessed by following the method described by Esworthy et al. [14], which measures seleno-dependent GPx reduction of H_2O_2 by evaluating NADPH consumption at 340 nm. The method encompasses mixing of samples (30 μg of soluble protein) with GSH (10 mM), NADPH (2 mM), sodium azide (1.125 mM), and GR (100 U/ml) in sodium phosphate buffer (50 mM, pH 7.0), using a 5mM H_2O_2 solution as the substrate. The reaction was followed for 5 min at 37°C.

Western blot analysis

Cell lysates (30 μg of protein) were separated by SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) under reducing conditions with a 4–12% Bis–Tris gel (2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl) propane-1,3-diol) using MES running buffer (4-morpholineethanesulfonic acid, Invitrogen). Following electrophoresis, proteins were transferred to a nitrocellulose membrane (2 h at 30 V), blocked with a 5% skimmed milk solution and probed with the appropriate primary and secondary antibodies diluted at 1:500 and 1:2000, respectively. The following antibodies were used: anti-human TrxR1 rabbit polyclonal IgG (sc-20147, Sta. Cruz), anti-

human GPx1/2 mouse monoclonal IgG (sc-133160, Sta. Cruz), anti-human GAPDH rabbit polyclonal IgG (sc-25118, Sta. Cruz), goat anti-rabbit IgG–HRP (sc-2004, Sta. Cruz) and goat antimouse IgG–HRP (sc-2005; from Sta. Cruz). Protein bands were visualized using an enhanced chemiluminescence detection system (Biorad, Chemidoc) according to the manufacturer's protocol. Densitometric analysis was performed using Quantity One software and Ponceau Red (Sigma-Aldrich) as staining marker.

Mercury determination

A Leco AMA-254 mercury analyzer was used to determine the presence of mercury in the three NF as described by Costley et al. [15].

Total Selenium determination

Se content in pellets was measured with a continuous Flow Hydride Generation-Atomic Fluorescence Spectrometer (FHG – AFS) with a selenium hollow cathode lamp (PSA Millennium Merlin with 20.400 Autosampler, PS Analytical Ltd., GB). Sodium borohydride (106371 Merck, Spain) stabilized with sodium hydroxide (106498 Merck, Spain) was used as reductant (0.7% m/v NaBH4/ 0.1M NaOH). Measuring instrument conditions were: delay time, 15 s; analysis time, 30 s; memory time: 40 s. Samples was weighed accurately into clean digestion vessels. They underwent a two-stage digestion with HNO_3 (300 µL, concentrated) and H_2O_2 (100 µL) at 120°C for 1 h each. Then, the solutions were transferred carefully into sample bottles and made up to the 5 mL. Following the digestion procedure, the digests were transferred to clean digestion vessels along 2 mL of concentrated HCl. The samples were then heated at 120 °C for 1 h to reduce Se (VI) to Se (IV), and when cooled they were diluted to 10 mL with deionized water. Method´s accuracy was tested with mussel certified reference material (ERM-CE 278K Certified: 1.62 ± 0.12 µg/g; Found:1.50 ± 0.07 µg/g; Recovery: 94%). Standard solutions for measuring the element Se were prepared immediately before use by dilution with distilled deionized water of 1000 mg/L standard solutions (Certipur – Merck, Darmstad, Germany).

Statistical analysis

Differences between groups and treated and non-treated samples were evaluated using Mann- Whitney Tests and considered significant at $p<0.05$. For all the analysis, precision was expressed with the standard error of the mean (S.E.M.).

3.4 Results and Discussion

Cell viability

Dialyzed formulas' nutrients supported cells viable for 24 and 48h (Figure 2). With the objective of reducing the presence of FBS, a universal growth supplement of cell culture media that also contains Se, different concentrations of this supplement were added to the different formulas.

In general, cells´ survival was not affected by different concentrations of FBS (as shown in Fig. 2, $p > 0.05$). Variation in cell viability for each formula is shown relatively to its corresponding 10 % FBS form. Although controls (10% FBS) of F1 and F2 did not vary from the cell culture media, F3´s control showed 20% lower viability than the rest ($p < 0.05$). Still, the 4 cultures tested contained the sufficient amount of factors required for cell attachment, growth, and proliferation [16], even when the survival conditions were not the same depending on the culture used. Given the sustainable cell growth without FBS, this component was not included in the following cell cultures to avoid variations in the culture's Se content due to unknown amounts in FBS. An incubation period of 24 h was selected for following assays, this incubation period has also been satisfactory in previous assays with HepG2 cells performed by our research under different conditions [17].

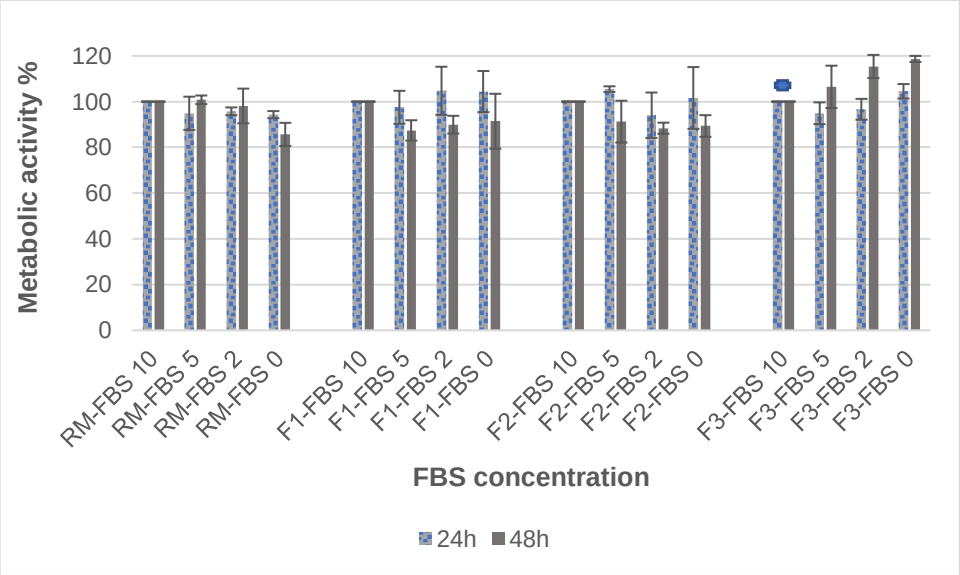

Figure 2. *Cell viability at 24 and 48 hours. FBS concentrations at 0, 2, 5 and 10 %.*

Variation was calculated considering each media/formula control with 10% FBS as 100% activity. FBS, fetal bovine serum. ⬬ *Lower viability relatively to the remaining conditions (*$p < 0.05$*).*

Total Selenium Quantification

Total Se content in pellets was determined to evaluate total Se uptake by the cells in the different scenarios used (Table 2). Initially, the non-treated cell cultures were compared, and there was a significant difference between the controls of RM and F2 and F3 (higher and lower levels than RM, respectively; $p < 0.05$). In contrast, there was no difference between the non-supplemented controls from RM and F1, showing that minor and similar Se concentrations are present in both cases. When changes in each culture were assessed RM, F1 and F3 showed statistically significant differences according to Se treatment (none, 0.1 and 0.3µM; $p < 0.05$). Meanwhile, F2 failed to show this effect, indicating that F2 already has a sufficient amount of Se, thus no major chance is observed after its supplementation. In contrast, the other 3 cell cultures showed benefit from Se supplementation, as RM and F1 start with minimal Se amounts, and F3 had an unviable Se source (Se aminoacid chelate).

Table 2 *Selenium content in cell pellets before and after Se supplementation at 0.1µM and 0.3µM.*

Se supplementation	Se content (µg/g)
RM	
0	1.03 ± 0.04
0.1µM	1.74 ± 0.49
0.3µM	2.99 ± 1.00
F1	
0	1.11 ± 0.50
0.1µM	1.69 ± 0.37
0.3µM	1.91 ± 0.28
F2	
0	1.55 ± 0.33
0.1µM	1.86 ± 0.71
0.3µM	1.31 ± 0.49
F3	
0	0.34 ± 0.04
0.1µM	1.01 ± 0.16
0.3µM	1.09 ± 0.46

SEM, Standard error of the mean; RM regular media; F, Formula.

Activities and expression of antioxidant selenoenzymes

Thioredoxin reductase activity in lysates

The use of RM and F1 as cell cultures led to similar levels of TrxR activity, whereas values of F2 were higher, and lower for F3 (Figure 3, 0 added Selenium) ($p < 0.05$). This observation agrees with the results obtained in Se quantification (Table 2)

Supplementation of dialysates with Se had different effects over thioredoxin reductase (TrxR) activity depending on the formula used (RM or NF). After 24h of exposure to Se at a concentration of 0.1 µM, cells cultured in RM showed the highest rise in TrxR activity, translating to an increase of 175% ($p < 0.05$). F1 was the only formula that also showed an increase of its TrxR activity in comparison to their non-supplemented control (51%; $p < 0.05$), whereas, cells cultured in F2 and F3 showed a tendency to decrease TrxR activity although this change was not statistically significant ($p > 0.05$) (Figure 3).

The different effect that Se supplementation showed in each cell culture, may be explained by the composition of each growing medium/formula. From what could be observed in the first intervention, RM and F1 start with low available Se, therefore being more responsive to 0.1 µM Se supplementation. Even though the three NF had similar Se amounts according to the label (7.4 µg /100 mL), Se in F2 and F3 was present as sodium selenite, and in F1 it was bound to an amino acid (¨selenium amino acid chelate¨). It should be noted that the later form of Se lacks enough evidence to support its use. Accordingly, in 2009 the EFSA declared that the safety and bioavailability of Se from this substance cannot be assessed due to the lack of an adequate dossier supporting its use [18]. In another study that aimed to determine and compare Se in multiple supplements forms, results were not quantitative for Se chelates in the two methods used [19]; these results would agree with our results that show that this form of Se is less effective. On the other hand, studies concerning sodium selenite bioavailability have shown mixed results. Indeed, some studies like the one that compared Belgian Se-enriched food supplements, found lower bioaccessibility of the selenite-based supplements when compared to selenate-based and Se-yeast [20]. On the contrary, another study that assessed Se uptake and bioaccessibility after supplementing Se in mushrooms´ cultivating substrates, found that sodium selenite had the highest utilization efficiency when compared to Se-enriched yeast and sodium selenate [21]. Even if sodium selenite has shown different bioaccessibility results depending on the conditions, it has been widely used and studied, and is categorized as a safe and efficacious source of Se for all animal species [14]. Contrary to in vivo situations, studies in cell lines are not affected by bioaccessibility, thus sodium selenite is extensively used.

We can infer that as the Se form in F1 is poorly absorbed, cells respond positively to Se supplementation (with 0.1 µM in the form of selenite), as also observed in RM, which has no selenium source in its composition. The extent at which Se supplementation rises TrxR activity in RM more than in F1, is likely because of its less complex configuration, consisting only of glucose, amino acids, and vitamins which is better suited to enhance cellular metabolism.

Regarding the different behavior found between F2 and F3, further analysis on NF composition (Table 1) shows that both formulas have the same Se source (selenite), and similar

protein and fat content. However, F3 shows higher levels of fiber in comparison with F2 (7.9 vs 1.3/100mL), which is mainly soluble fiber. This higher fiber content in F3 may compromise Se absorption and utilization, as it is known that fiber, along with the other components like carbohydrate, fat, protein, oligoelements and toxic metals determine and affect Se bioavailability [23]. Research on the effect of fiber on micronutrient absorption has shown contradictory effects [24–26], many times as a consequence of not discerning between different types and amounts of fiber. The effect of fiber on Se bioavailability seems to depend on the type of fiber and its concentration, more promising results on minerals have been observed with soluble fiber. Recent evidence has noted that the binding effect that fiber has on minerals is only observable *in vitro* and not in animal and human studies. Dietary fiber *in vitro* as well as *in vivo* can form insoluble complexes or increase viscosity impairing mineral bioavailability in the small intestine. However, bacterial fermentation in the colon releases previously bound minerals and can enhance its absorption. As commonly used *in vitro* studies lack microbial fermentation, the positive effect of soluble fibers is not perceived in this case, resulting in less mineral absorption [25,26]. Thus, in this assay the low Se bioaccessibility in F3 translated to in a lower TrxR activity (Figure 3).

Further Se supplementation, to a concentration of 0.3µM, showed that in comparison to their non-supplemented control, only the RM had relevant changes on TrxR activity ($p<0.05$). No change was observed in F1, and although the activity of F2 and F3 seemed to increase at the concentration of 0.3µM, this change in the three formulas tested was not statistically significant ($p \geq 0.2$). From these results, it can be inferred that greater concentration of Se (0.3 µM) does not increase TrxR activity, this may be due to the enzyme saturation that does not need higher Se concentration. Similar behavior has been displayed in TrxR activity from rat livers, where saturation of selenocysteine-based enzymes seem to show little effect when a high Se diet (1000 mg Se/kg group)was given, showing similar results to activity of the control diet (240 mg Se/kg); on the other hand, the rats with a very low Se showed a significantly reduced activity [27]. As no further benefit is provided with high Se doses and an excess of this element may result in additional toxicity, a more conservative approach would be prudent [4].

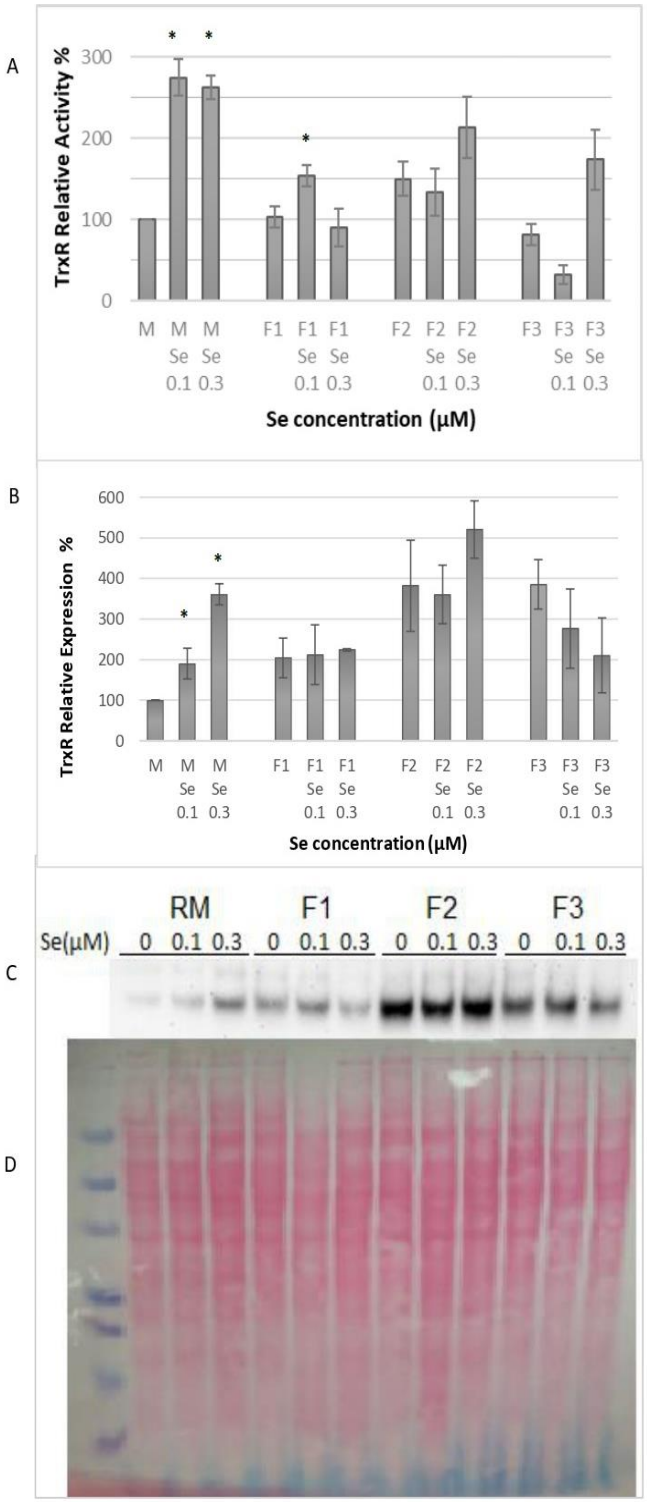

Figure 3. *RM and dialysates of NF with and without Se and its effect on TrxR activity and expression on HepG2 cells.*

*Cells were exposed to Se at concentrations of 0.1 µM and 0.3 µM for 24 h and its effect was analyzed on TrxR activity (A) and expression (B). Additionally, the effect of Se supplementation on TrxR expression was analyzed by Western Blot (C), the membrane was incubated with respective antibodies for TrxR detection (see Materials and Methods for details). Ponceau staining of protein loading (D). Variation was calculated considering the regular media control as 100% activity. Data are representative of at least 3 independent experiments, $p < 0.05$ showed in RM and F1 and increased expression of RM. * $p < 0.05$ significance of increased activity from its control.*

Thioredoxin reductase expression

Similarly to what was observed with the TrxR activity, increasing Se concentrations displayed different TrxR expression levels of cells cultured in RM ($p<0.05$) and no significant changes were found in the three NF ($p > 0.05$) (Fig. 4). Interestingly, the highest protein expression is observable in F2, where the three Se conditions show high protein expression. This confirms previous results for TrxR activity, where enough Se was already provided in the NF, thus there was no improvement by further Se supplementation. However, in this case, there is not an evident increase of TrxR expression when F1 is supplemented, that could be because of the greater variability seen in this assay. Furthermore, F3 showed high basal TrxR expression, interestingly, as this formula contained the lowest amount of Se, and that its basal activity is similar to basal RM (that has no added Se in its composition). This kind of behavior could indicate that the extremely low Se on this NF is completely prioritized to TrxR synthesis given the vital role of this enzyme in cell survival [29]. However, either because of its amount or its source, Se in F3 is inadequate, which in the longer-term impacts cell survival as also seen on the cell viability study where viability in F3 falls after 48h. More studies addressing this subject would be needed for a better understanding of these mechanisms.

Glutathione peroxidase activity in lysates

As can be seen in Figure 5, samples cultured in NF dialysates (F1 – F3) in which cells were treated with Se to reach a concentration of 0.1 and 0.3 µM resulted in overall little or no significant increase of the GPx activity. The only cell culture that showed difference from its control after Se supplementation was RM ($p < 0.05$). This scenario persisted at Se concentrations of 0.1µM (increase 26.4 %) and 0.3µM (increase of 41.2%). The influence of Se addition (0.1 and 0.3µM) to GPx activity on the three NF was not enough to be considered relevant. The increase observed in GPx activity of RM follows the response observed in diets that had reestablished Se intake to adequate levels leading to a clear increase in GPx1 activity [30]. Nevertheless, in our study this activity was increased but to a minor extent than TrxR, and not replicable to the NF. The way in which TrxR and GPx react after the same amounts of supplemented Se sometimes is not equivalent, as also observed while assessing

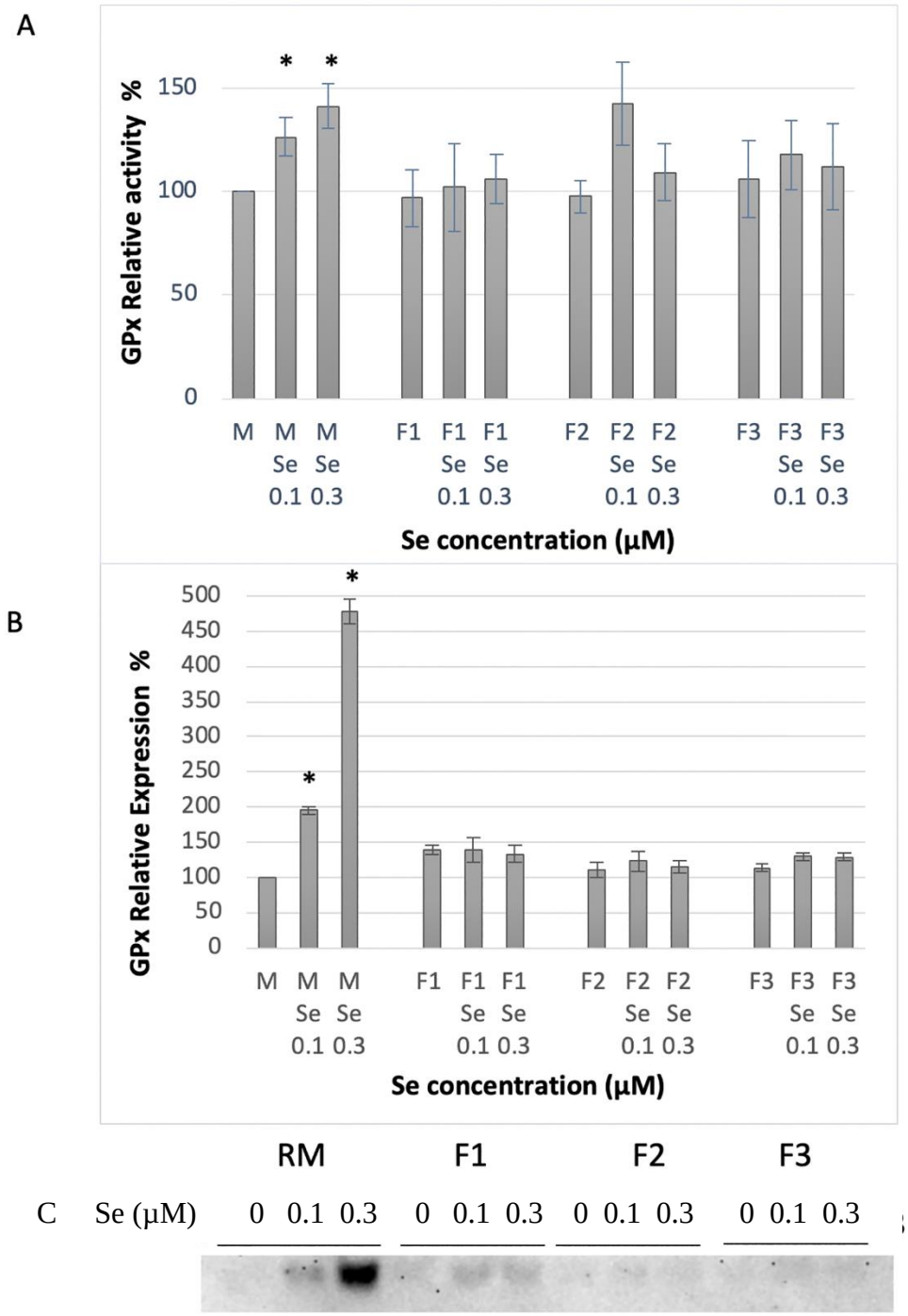

Figure 4. *RM and dialysates of NF with and without Se and its effect on GPx activity (A) and expression (B) on HepG2 cells.*

*Cells were exposed to Se at concentrations of 0.1 µM and 0.3 µM for 24 h. Cell lysates were analyzed by Western Blot (C), after TrxR development with chemiluminescent substrate the membrane was treated with H_2O_2 and incubated with the appropriate antibodies for GPx detection (see Materials and Methods for details). Ponceau staining of protein loading is shown in Fig. 3 (D). Variation was calculated considering the regular media control as 100% activity. Data are representative of at least 3 independent experiments, * $p < 0.05$ significant increase on activity and expression from its control.*

its defensive role against toxic compounds like mercury (Hg) [31]. However, in this case, Hg content in the three studied formulas was negligible (data not shown), therefore heavy metal contamination from this compound was discarded. Indeed, TrxR is a selenoenzyme of high priority due to its major role as part of the thioredoxin system in regulating several essential cellular functions [29,32] and thus available Se is likely channeled to its de novo synthesis. On the other hand, GPx1 is not a priority enzyme since several alternative pathways exist for H_2O_2 reduction in cells such as catalase and Trx-system dependent peroxiredoxins [33,34].

Glutathione peroxidase expression

Se supplementation only showed an effect on the expression of GPx when the cell culture used was RM (see Fig 6), and it did not significantly affect the three NF ($p>0.5$). In this case, GPx expression follows the same pattern observed in GPx activity, with very similar results among all the EF. The response observed in RM follows what is described in literature about the expression of GPx1 that is supplemented with Se after being in a deficient state [30]. Correcting Se deficit translates in an increased expression of GPx1, this fold stimulation is larger when observed in western blots as has been reported by recent studies comparing four different cell lines and five complementary analytical methods [35]. As western immunoblotting in GPx is highly sensitive to Se concentration variation, and the three NF used did not show an increase on its expression, in this case it seems like the supplementation was not efficient to provide enough Se to improve this parameter.

3.5 Conclusions

The findings of the current study point out the intricacy of the process involving nutrient absorption until its cellular utilization. As observed with TrxR activity, some NF seem to provide sufficient Se to allow full activity of this enzyme, while other products in the market restrain the activity of redox systems by providing species of Se or amounts that hinder selenoproteins activities. Indeed, the low bioavailability of some micronutrients such as Se may in the mid to long term may further aggravate the health status of patients undergoing NF treatment. Reinstating the importance

of label assessment before selecting or prescribing commercial formulations. On the other hand, supplementation of NF failed to achieve an effect on GPx (activity and expression), as opposed to its clear increase in cells cultured in regular media. The complexity of commercial formulations and the possible presence of non-disclosed components (e.g. trace metals) may limit Se bioavailability and metabolic use. This is apparent in the distinct effect of Se supplementation over selenoprotein activity/expression in cells cultured with the different NF and stresses the need to evaluate and create a strategy of Se supplementation case by case, that is, no general Se supplementation strategy can be established for all NF.

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Conflicts of interest: None declared

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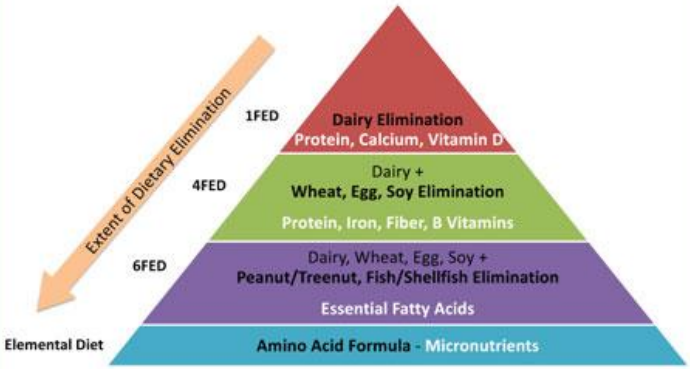

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Cover Art Note: This issue of *JPEN* presents a multidisciplinary approach to manage eosinophilic esophagitis and illustrates the nutrients at risk among various elimination diets (EDs). Each level of the pyramid depicts the foods eliminated and specific nutrient deficiencies of the levels above it, where the empiric diet elimination therapies are categorized by number of food types eliminated: single food (1FED), 4 food (4FED), and 6 food (6FED).

Chapter IV

Chapter IV: Survival analysis of enterally fed patients: prognosis and mortality risk according to baseline characteristics

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4.1 Abstract

Background

Enteral nutrition is widely used. However, its benefits remain unclear in specific conditions like dementia. This study assesses the survival of enterally-fed patients and the baseline characteristics associated with higher mortality.

Methods

A retrospective analysis of biochemical and clinical data from 377 patients (age 77.5 ± 13.8) who received enteral tube feeding (ETF) at a tertiary hospital in Spain was performed. Kaplan Meier and Cox regressions were used to analyze survival expectancy and mortality risk. Risk was evaluated for overall survival, and in 30/180 days and up to 5 years.

Results

The most recurrent individual diagnoses leading to ETF prescription were: dementia (37.9%) and head/neck/upper gastrointestinal tract cancer (17.5%). Comorbidities (high blood pressure and/or diabetes) were present in 72.4% of patients. The first 30 days after tube placement showed the highest mortality rate, corresponding to 85.4% of patients that did not continue being tube-fed. Multivariate Cox analysis ($p < 0.05$, CI 95%) showed high blood pressure and glycemia to be predictive of overall (HR = 1.600; HR = 1.756) and long- term (HR = 3.092; HR = 4.539) death. In the short-term, only glycemia showed an increased mortality risk (HR = 1.572).

Conclusions

This enterally-fed population showed a noticeably high initial mortality rate. Despite official recommendations against it, ETF is very common in advanced dementia. Baseline characteristics are useful for identifying patients that would be less benefited by the intervention. Accordingly, families should be informed about realistic outcomes and risks derived from this procedure.

Keywords:

Enteral nutrition, enteral feeding, medical nutrition therapy, survival analysis, dementia, mortality.

4.2 Introduction

Enteral nutrition is a type of nutrition support that delivers nutrients through the gastrointestinal tract. Though this could imply oral feeding too, the term is more frequently used to refer to enteral tube feeding (ETF), which uses nasogastric (NGT), nasoenteral or percutaneous tubes (i.e. PEG, percutaneous endoscopic gastrostomy). ETF is indicated for patients unable to maintain an adequate oral intake either because it is unsafe or insufficient, or they are unwilling to eat. During the last decades, the use of this support inside and outside hospital facilities has rapidly increased partly due to the improvement in tube placing techniques, their material and the nutritional formulations commercially available [1].

Depending on the pathology, morbidity and mortality can be improved by ETF. This has already been established with some types of cancer and some neurologic conditions such as acute stroke, motor neuron disease and multiple sclerosis [2,3]. Another common condition that impairs oral intake is dementia particularly in the advanced stages of the disease, when the patient loses the ability to hold their head up and to swallow. However, due to the unclear benefits of ETF in advanced dementia, it is discouraged in clinical guidelines [4–7].

Overall, ETF is a physiologic medical nutrition therapy that has been found to be cost-effective in certain patient groups [8,9]. However, when indicating this resource, it should be taken into consideration that it is an invasive technique comprising certain risks, along with its expense and maintenance [8]. Therefore, when deliberating about ETF prescription each case should be studied to assess its adequacy, which would depend on each patient's characteristics. Dealing with this issue, the present paper aims to assess the impact of patient nutritional status before ETF initiation on short-, medium- and long-term survival. Thus, this research sought to identify the characteristics associated with higher survival rates and differentiate among the patients who would benefit from the intervention and those that would benefit the most from avoiding it.

4.3 Methods

Study cohort

A retrospective cohort study was undertaken with adult patients prescribed ETF at the Reina Sofia University Hospital, a third-level public hospital in Cordoba, Spain. ETF could have been initiated during hospitalization or as an ambulatory procedure. All the electronic medical records of patients who received placement of a feeding tube for the first time from July 2012 to May 2017 were reviewed. Patients being exclusively fed commercially available enteral nutrition formulas (supplied by the Spanish public health service) were included. Exclusion criteria were being in the Intensive Care Unit or transplant receptors, a lack of analytical parameters in their medical records, and not being exclusively tube-fed.

This research was approved by the Cordoba Bioethical Committee, in the Health Department (Regional Government of Andalusia, Act. No. 256, ref. 3549).

Data collection

The following data were obtained from each patient´s chart when available: primary diagnosis, gender, age at the start of ETF, date of tube placement, duration of treatment, date of death (if deceased), nutrition formula name, feeding route (NGT or PEG), and biochemical parameters (blood glucose (BG), hemoglobin, hematocrit, serum proteins (SP), absolute lymphocyte count (ALC), platelets, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, and triglycerides). Known comorbidities such as high blood pressure (HBP) and type 2 diabetes (T2DM) were also recorded.

Patients with dysphagia due to any kind of dementia, stroke, cerebral palsy, or amyotrophic lateral sclerosis, were classified into the neurologic impairment group (NG). All types of cancer were labeled as CG (Cancer Group); while a third group included patients whose main diagnosis was neither neurologic nor cancer (Esophageal achalasia, anorexia, Crohn´s disease, human immunodeficiency virus). See patient selection process in Figure 1.

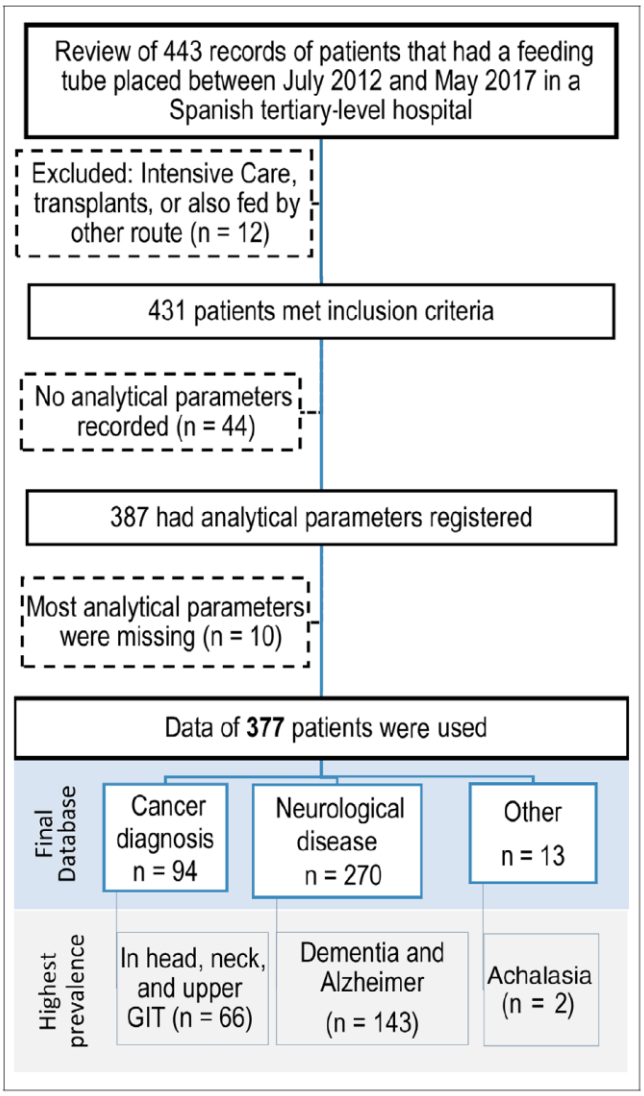

Figure 1. *Patient selection flow diagram*

GIT, Gastrointestinal tract

Statistical Analysis

A statistical analysis was carried out using IBM SPSS Statistics V.25.0 software (SPSS, IBM, Chicago, Illinois, USA) and Epidat V.4.2. (Department of Sanidade, Xunta de Galicia, Spain). Continuous data are presented or mean values, SD and 95% CI. Categorical data are shown as frequency counts and percentages.

Survival was analyzed by Kaplan Meier (KM) estimation and a descending stepwise Cox regression model was used to assess variables as independent hazards. Date of death from any cause

was considered as a primary end point. Analytical data were analyzed in their Napierian logarithm form.

The KM estimates of comparative survival in the global cohort and per strata, defined by age, sex, comorbidities, and blood parameters, were calculated. Cox's proportional survival risk model was applied to estimate the relative mortality risks (MR) using as independent adjustment variables age, sex and prior disease linked to dysphagia.

The level of statistical significance was fixed in all the contrasts for an α error of below 5%, and the CI was calculated with a 95% level of confidence.

4.4 Results

Demographics and clinical features

A total of 443 patients that had placement of a feeding tube at the hospital were reviewed. After verifying exclusion criteria 377 patients remained. Mean age was 77.5 (± 13.8) years old, 55.9% females. The NG accounted for 71.6% of patients with ETF, while 24.9% corresponded to the CG. Furthermore, the most frequent diagnoses were: dementia (and Alzheimer's disease) that required tube feeding (37.9%), cancer at head/neck/upper gastrointestinal tract (17.5%), and stroke (15.4%); no other condition accounted for more than 10% of the population. Regarding comorbidities, 72.4% of patients suffered from at least one of the studied comorbidities while 27. 3% suffered from both (see Table 1).

Patient classification according to follow-up time showed significant differences when matched with diagnosis ($p<0.05$), mortality ($p<0.01$), albumin ($p<0.01$) and age group ($p<0.05$). In all the follow-up time groups neurologic conditions were found to be the predominant diagnosis. Also, in all the time groups, most patients fell into the low albumin category (<3.5 g/dL); and the age group with the highest percentage of the population was that of over 80 years old.

Table 1. Population Characteristics According to Enteral Tube Feeding Duration.

	Total Sample		Over Time, n (%)				
		%	<30 d	<90 d	<180 d	>180 d	*P*
Patients (n)	377		101	66	71	139	
Sex (female)	210	55.9	58 (58.0)	33 (50.0)	36 (50.7)	83 (59.7)	NS
Age (mean)	77.5						
Over 65 yo	300	79.6	89 (88.1)	50 (75.8)	51 (71.8)	110 (79.1)	NS
Diagnosis							<.05
Cancer	94	24.9	20 (19.8)	24 (36.4)	23 (32.4)	27 (19.4)	
Neuro	270	71.6	76 (75.2)	41 (62.1)	44 (62.0)	109 (78.4)	
Other	13	3.4	5 (5)	1 (1.5%)	4 (5.6)	3 (2.2)	
Comorbidities							NS
HBP (only)	136	36.1	41 (40.6)	17 (25.8)	34 (47.9)	44 (31.7)	
T2DM (only)	34	9.0	11 (10.9)	5 (7.6)	3 (4.2)	15 (10.8)	
Both	103	27.3	29 (28.7)	22 (33.3)	15 (21.1)	37 (26.6)	
None	104	27.6	20 (19.8)	22 (33.3)	19 (26.8)	43 (30.9)	
Enteral access							NS
Ostomy	52	13.8	10 (9.9)	8 (12.1)	9 (12.7)	25 (18.0)	
Nasal tube	325	86.2	91 (90.1)	58 (87.9)	62 (87.3)	114 (82.0)	

Patients from each time interval are independent, not cumulative. Period of >180 days ends at 5 years and 4 months (end of study).
HBP, high blood pressure; neuro, neurologic conditions patient group; NS, nonsignificant; T2DM, type 2 diabetes; yo, years old.

Survival analysis

Patient follow-up ranged from 1 day to over 5 years (longest record of 1968 days). Survival of all the patients remaining at the end of the study was equivalent to almost half of the population (49.1%). Median and average survival of the population was 421 days (14 months) and 678.7 (± 56.8) days (23 months), respectively (see Fig. 2a for overall survival curve). KM analysis showed a significant association ($p<0.01$), between survival and BG, ALC, age, dementia diagnosis (with impaired oral feeding) (Fig. 2c) and comorbidities (Fig. 2d). The mean survival time for the group without comorbidities doubled the survival time of having both, i.e. 1016.6 days versus 449.8 days (33.4 and 14.8 months), respectively. On the other hand, median survival was almost 5 times longer if patients had no comorbidities than if having both. Mean survival of patients without comorbidities was 29 months versus 6.2 months of patients with HBP and T2DM. Meanwhile, in patients that only had either HBP or T2DM, the latter group showed the lowest median survival equivalent to 8.5 months versus 10.8 months of HBP.

No difference was found between survival and: formula composition, feeding route, gender, albumin, SP, hemoglobin, hematocrit, cholesterol (total and HDL), triglycerides, and platelets.

Short-term, medium-term, and long-term mortality

The cumulative incidence of death at short (30 d), medium (180 d) and long (5 y) term corresponded to 23.1%, 39.8% and 50.9%, respectively. However, when mortality was compared between periods of time, the first 30 days after tube placement stood out with the highest mortality rate. Of all the patients lasting fewer than 30 days with ETF, 86.2% of them stopped because they did not survive. This rate then decreased at 90 and 180 days (66.7% and 26.8%, respectively) (see Table 2).

Table 2. Mortality Rate at Short Term, Medium Term, and Long Term (<30 Days, 90 Days, 180 Days, and 5 Years).

	Total No.	Nonsurvivors	Mortality (%)	*P*
30 d				.053
Cancer	20	14	70.0	
Neurologic	76	68	89.5	
Overall	101	87	86.16	
90 d				.065
Cancer	24	13	54.2	
Neurologic	41	30	73.2	
Overall	66	44	66.7	
180 d				.139
Cancer	23	4	17.4	
Neurologic	44	15	34.1	
Overall	71	19	26.8	
End of study[a]				.152
Cancer	27	5	18.5	
Neurologic	109	37	33.9	
Overall	139	42	30.2	

Comparison of mortality rates at each cutoff point (patients from each time interval are independent, not cumulative). Highest mortality rate is observed during the first 30 days; and between the 2 main diagnoses, the highest mortality is seen in the neurologic group in all the time periods. The "other diagnoses" group is not displayed, as the sample is only 13 throughout the whole study.
[a]This time period lasts for up to 5 years and 4 months.

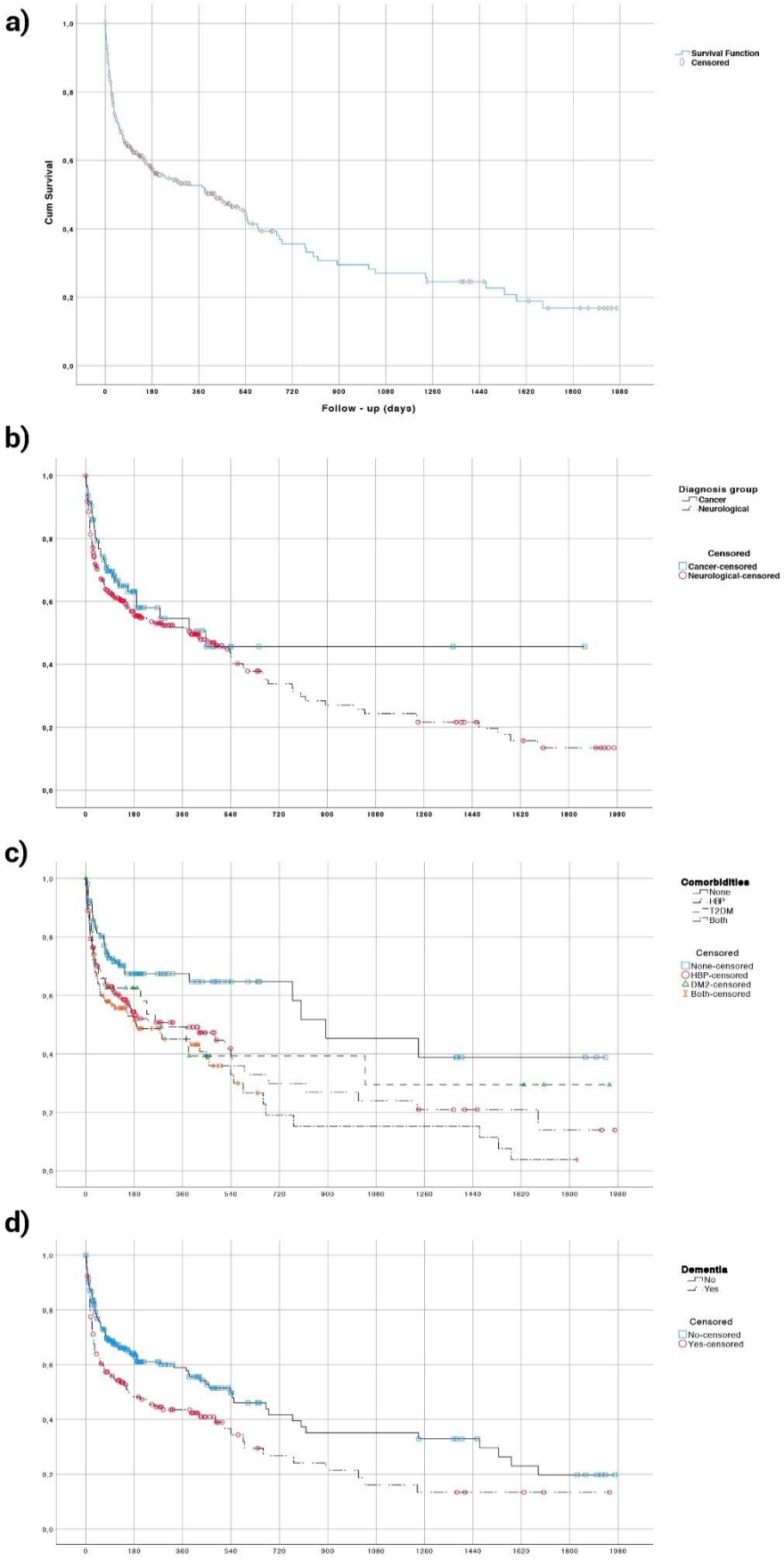

Figure 2. *Kaplan-Meier curves of (a) overall survival, survival according to (b) main diagnosis groups, (c) dementia diagnosis and (d) comorbidities. *Dementia with oral abilities impaired.HBP, high blood pressure, T2DM, type 2 diabetes.*

Due to the reduced sample of the patient group with ¨other¨ diagnoses, Table 2 is focused on neurologic and cancer mortality. There, it can be observed that, in all the four time periods considered, the mortality rate was always higher in the NG (an average of 18% higher), even though the KM test did not show any significant difference per patient group (Fig 2b). On the other hand, if only dementia (with impaired oral feeding) was compared with all the other conditions, mortality was significantly different in the first 30 days ($p < 0.05$) and in the period between 90 to 180 days ($p < 0.01$), when dementia had a 93.9% and 52.6% mortality rate, respectively.

Prognostic factors for survival

Each factor of interest was individually assessed using Cox's analysis to determine its prognostic value, the relevant variables are shown in Table 3. The following factors were identified as potential predictors of survival outcome ($p < 0.05$): age, HBP, T2DM, BG, SP, albumin and dementia (that required ETF). These factors were placed in a multivariate Cox regression model to ascertain their independent effects on survival. In that analysis the presence of HBP, BG and dementia at the beginning of treatment was associated with a poor outcome (Table 3). Regarding comorbidities, even though HBP and T2DM showed an increased mortality in the bivariate analysis, the multivariate analysis only preserved HBP (and BG, but not T2DM).

Table 3. Bivariate and Multivariate Cox Analysis (All-Time, n = 377).

	Bivariate Analysis			Multivariate Analysis		
Variable	HR	95% CI	*P*	HR	95% CI	*P*
Age (Ln)	3.351	1.511–7.431	.003			
Over 65 yo	1.634	1.080–2.471	.020			
HBP	1.696	1.237–2.324	.001	1.585	1.149–2.187	.005
T2DM	1.406	1.056–1.871	.019			
BG (Ln)[b]	1.931	1.358–2.746	.000	1.688	1.186–2.403	.004
SPs (Ln)[b]	0.209	0.045–0.962	.045			
Serum albumin level[b]	0.228	0.074–0.703	.010			
ALC[b]	0.616	0.395–0.961	.003			
Dementia[a]	1.535	1.155–2.039	.003	1.439	1.080–1.917	.013

ALC, absolute lymphocyte count; BG, blood glucose; CI, confidence interval; HBP, high blood pressure; HR, hazard ratio; Ln, napierian logarithm / natural logarithm; SP, serum protein; T2DM, type 2 diabetes; yo, years old.
[a]Dementia with oral abilities impaired.
[b]Analytical parameters such as BG, SP, serum albumin level, and ALC were used in all the cases when data were available, corresponding to n = 375, 199, 156, and 268, respectively.

Furthermore, differences between short- and long-term risk factors were also assessed. Here we found that the only variable that represented a short-term risk was BG. However, in the long term (> 180d), T2DM, HBP, ALC and BG were initially identified as risk factors (Table 4). Then, the Cox proportional hazards ratio multivariate analysis showed that the last three factors were seen to be significantly predictive of death (see Table 5).

Table 4. Bivariate Analysis for Short-Term (<30 Days of Survival) and Long-Term (>180 Days of Survival) Risk Factors.

	Bivariate Analysis					
	<30 d (n = 101)			>180 d (n = 139)		
	HR	95% CI	*P*	HR	95% CI	*P*
HBP				2185	1.096–4.356	.026
T2DM				2208	1.200–4.065	.011
BG (Ln)[a]	1572	1.008–2.451	.046	2513	1.216–5.192	.0013
ALC level[a]						.017
Very low				0.359	0.032–4.070	.408
Low				0.087	0.008–0.975	.048
Normal				0.079	0.015–0.401	.002

ALC, absolute lymphocyte count; BG, blood glucose; CI, confidence interval; HBP, high blood pressure; HR, hazard ratio; Ln, napierian logarithm / natural logarithm; T2DM, type 2 diabetes.
[a]Analytical parameters such as BG (short term, n = 100; long term, n = 139) and ALC (short term, n = 68; long term, n = 100) were used in all the cases where data were available.

Table 5. Multivariate Analysis for Short-Term and Long-Term Risk Factors.

	Multivariate Analysis					
	<30 d			>180 d		
	HR	95% CI	*P*	HR	95% CI	*P*
HBP				3.092	1.010–9.468	.048
T2DM						
BG (Ln)[b]				4.539	1.564–13.170	.005
ALC level[a,b]						.014
Very low						>.5
Low				0.073	0.006–0.842	.036
Normal				0.072	0.013–0.408	.003

ALC, absolute lymphocyte count; BG, blood glucose; CI, confidence interval; HBP, high blood pressure; HR, hazard ratio; Ln, napierian logarithm / natural logarithm; T2DM, type 2 diabetes.
[a]High level used as reference.
[b]Analytical parameters such as BG (n = 375) and ALC (n = 268) were used in all the cases with that data available.

4.5 Discussion

This retrospective analysis of patients initiating ETF in a Spanish hospital was carried out to identify the characteristics associated with higher survival rates aiming to improve future care. We found that neurologic diagnosis **(**particularly dementia), was the most prevalent diagnosis leading to ETF, as has been seen in other settings [10]. These results were not surprising as dementia causes resistance and indifference to food, as well as dysphagia. On the other hand, stroke, the third most frequent diagnosis in this study can also cause dysphagia, although these patients are likely to recover oral feeding [11].

Though ETF is used in dysphagia to prevent malnutrition and aspiration pneumonia, its use in dementia [12] has failed to prove any reduction in this complication because it does not prevent oral secretions and regurgitated material from entering the lungs. In fact, some studies show that ETF is associated with increased pneumonia and MR in elderly patients at different dementia stages [6,7,9]. This increased risk may be because gastroesophageal reflux and pharyngeal secretions are increased in ETF [13].

However, abstaining from medical nutrition therapy in patients unable to meet their nutritional requirements orally can become an ethical dilemma for healthcare professionals and the patient's family, as it may be perceived as starving the patient to death. Nevertheless, in end-stage dementia, neither thirst nor hunger are perceived, indeed, refusal to eat is a sign of an advanced degree of this fatal disease. Thus, oral intake by careful hand feeding is advised to avoid tube-complications, agitation and the need for restraints associated with ETF [5,13].

With regard to feeding routes, in this research, NGT was the preferred practice though its use is recommended for not more than 4-6 weeks; however no difference in mortality was found between the feeding routes in this and other studies [8]. NGT use for extended periods is also common in other countries, particularly in older patients; the easiness of the procedure and the advantage of avoiding more invasive placements in frail adults may encourage this choice [14].

On the other hand, since our cancer group included patients from all stages, palliative stage individuals may have diminished the usually higher survival rate expected in this group [15]. These patients showed an initial high mortality rate, which became stabilized in subjects that survived more than a year. Nevertheless, though many participants from this group requiring ETF, presented upper body lesions (head/neck/esophageal), it is well-known that some of these cancers are more common in younger and healthier populations, who thus have a greater probability of surviving and returning to the oral route [16].

Mortality and Risk Factors

Overall, the primary cause of ETF being discontinued was death, similarly to what was found in a nationwide home-ETF Spanish study [10]. After 14 months of treatment half of the population in our study had died; however, we hypothesize that, in both studies, the mortality has been underestimated as some patients' decease status might not have been updated at data collection time. Even though patients from the NG showed a higher mortality rate (15-20% more per period), that group did not prove to be a significant risk factor by itself, but dementia did.

Mortality studies frequently assess nutrition status, age and critical illness [15,17,18], but in our study the presence of comorbidities also proved relevant. Our patients with (both) HBP and T2DM showed the worst prognosis, whereas those having neither the best one ($p< 0.01$). The influence of HBP and other comorbidities on survival rates has also been observed in breast cancer patients, showing that 5-year survival of patients without concomitant diseases was 82% (versus 72%) [19]. Meanwhile, studies on patients with a PEG tube have revealed that comorbidities are common and that the risk of adverse events (like infection) is proportional to the number of comorbidities. Similar results have been found with a comorbidity index that gives scores to diseases, i.e. the higher the score, the higher the MR [20,21].

It is known that the nutritional status before ETF is related to complications and survival [18,22]. However, in our study, a bivariate analysis showed age, HBP, T2DM, BG, SP, albumin, ALC and dementia as overall mortality predictors. Nevertheless, after adjusting for cofounding variables, the

multivariate analysis confirmed that HBP, BG and ALC were still significantly predictive of overall and long-term (over 180 days) mortality. ALC is a known protein-nutrition (and immune function) indicator [23,24]. On the other hand, the T2DM role in mortality has contradictory findings, even showing a protective role in sepsis, while hyperglycemia did increase MR [25,26]. ALC and BG also predicted in-hospital MR in patients from different populations [27,28]. Meanwhile, HBP is known to contribute to cardiovascular morbidity and mortality, particularly in elderly patients with cognitive impairment [29].

However, short-term, only BG showed a predictive ability. This could be an indication of inflammation and stress due to acute illness resulting in a greater MR [30,31]. Similarly, CRP (C-Reactive Protein) predicts MR in patients with a PEG-tube. In these situations, the recommendation is to evaluate ETF initiation after (if) surviving the acute condition [32,33].

Besides CRP, hemoglobin, cholesterol, albumin, and weight loss are useful for assessing MR [34,35] although, in our study, few patients had those data available for us to obtain significant results.

Further Considerations

First, despite ETF having proved to be beneficial in multiple situations, its suitability for each patient and its psychosocial impact should be assessed individually. In addition to its possible complications, ETF affects aspects related to the quality of life like: body image, social activities, and everyday comfort [8,36]. The distress caused by ETF adds to any previous suffering caused by underlying conditions (depression, anxiety or apathy), which can be disabling, have a negative influence on recovery, reduce the quality of life and lead to the caregiver´s exhaustion, as has been seen in stroke patients [37].

Dementia has been shown to be the most frequent diagnosis in this study, which indicates ETF as being employed to address the oral impairment encountered at its advanced stages. It should be noted that the recommendation to avoid ETF in these cases has not been strictly complied with [7,8,10,38]. It can be inferred that current practices carry an unnecessary economic burden that in many

countries (Spain included) is assumed by the Social Security System [9,39]. Although feeding and delivery material is only estimated to cost 266 euros/patient/month in Spain [40], the increased cost of ETF derives from acute and hospice care [39,41].

Despite all the evidence against ETF in advanced dementia, why is it still common practice in Spain and other countries to feed non-viable patients? To start with, the unrealistic belief that a better nutritional status could be achieved and therefore positively affect dementia course and survival, is a major influence [38,42]. Then, the cultural background also impacts the likeliness to prescribe ETF as a reflection of the relationship with food and the meaning it embraces (Spain, Japan, Australia and African American physicians in the US) [43–45]. Even if most doctors were to prescribe ETF for their patients, they would avoid it for themselves if they were in that situation [46,47]. In contrast, in the Netherlands, they are more likely to opt for palliative care in dementia withdrawing hypothetically life-prolonging treatment, including ETF, hydration and medication [48].

As a retrospective study, this work also has its limitations; indeed, the lack of data in the electronic medical records was one of the main difficulties. Patients with NGT placement without initial blood tests had to be left out of the study from the beginning. We did not find any standardized assessment (analytical, clinical, or dietetic) for tube-fed candidates. Most patients lacked common nutrition assessment tools [1,49] like blood tests (lipid profile, C-Reactive protein, SP, albumin, pre-albumin, creatinine, glycated hemoglobin, etc.); and anthropometric information like Body Mass Index, weight history, handgrip strength or arm/calf circumferences, that would have helped identify malnutrition risk, as well as strengthening our findings. However it should be emphasized that analytical markers should not be used by themselves to assess nutritional status [50].

4.6 Conclusions

The use of enteral feeding is well known to be beneficial in multiple populations of patients who are at risk of malnutrition, and also as an aid to maintain and support life in acute, and chronic conditions. However, we have corroborated that it gives a very high initial mortality rate, and that not all populations show a clear benefit from this therapy. Despite recommendations to the contrary, ETF is still widely spread in patients with severe dementia. Regarding this issue, the implementation of standardized evaluations that include clinical and analytical parameters (found to be useful in this and other studies) of all ETF candidates referred to the hospital, could help identify patients who could benefit from the intervention. As the healthcare team has a major influence on the decision to start ETF, it should be a priority for them to be well informed on the tangible quality of life outcomes that can be expected, depending on the patient's characteristics; and to be aware that the prolongation of life could become an unnecessary prolongation of the dying phase. Furthermore, given the paramount role of health care practitioners in the decisions of families, offering realistic information on the risks and benefits of ETF, and on other alternatives should be a priority.

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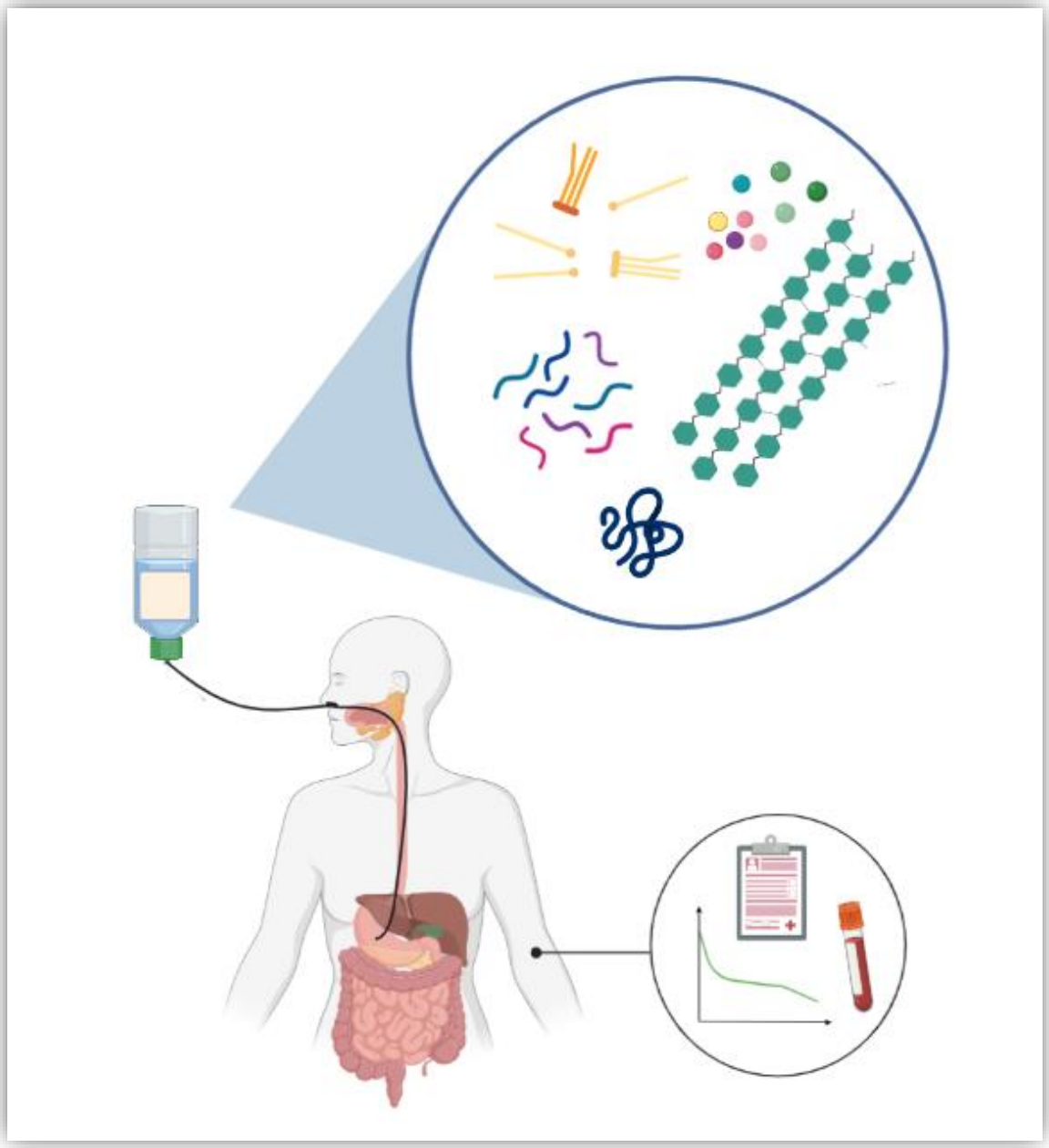
5. Conclusions



The main conclusions obtained in this dissertation are:

FIRST: According to macronutrient laboratory analysis of ENF, protein, as declared in labels, has exceptional compliance to label information if the Dumas method is used. However, Kjeldahl protein determination method demonstrated substantially lower protein content, demonstrating an overestimation of this nutrient by using the method that considers non-protein Nitrogen to estimate total protein. These outcomes prevailed in ENF from Spanish and Mexican origin. More importantly, such findings raise concern over unsuspected protein deficiency in patients consuming such prescribed feeds in their calculated dose (Chapter I).

SECOND: Mineral and trace element determination revealed that discrepancies between ENF composition and labels are not uncommon. Except for Cu, mineral content showed a tendency to contain less than the label data and its accepted variation. Particularly, Fe, Mg, and Mn were more likely to be present in lower amounts than reported, and this was less frequent for Zn and Ca (Chapter I).

THIRD: The application of Risk Assessment methodology through the development of a probabilistic intake assessment indicates that ENF provide sufficient micronutrients to cover Dietary Reference Intakes, which already consider bioaccessibility variations. This, despite containing lower amounts of trace elements than expected. However, practitioners should consider that nutrients´ needs will be altered according to existing pathologies, just meeting the DRI established for a healthy adult population may not be enough for a spectrum of patients (Chapter I).

FOURTH: The dialyzability assays performed confirm previous data indicating that ENF have substantially less bioaccessibility of Ca, Mg, Zn, and Cu than regular meals. Even when bioaccessibility of Fe and Mn is not affected in comparison to other food sources. In addition, synergistic interactions between the dialyzability of Ca and Mg were found, whereas negative correlations between protein content and dialyzed Mg and Fe were also found (Chapter I).

FIFTH: The development of a method to preconcentrate and speciate Mn showed that Cloud Point Extraction (CPE) techniques could be used in complex multi-nutrient samples like ENF. Even though

the optimization process to establish the methodology is time and effort consuming, once it is established, it provides a faster, cheaper, less polluting, and more accessible method. In this case, CPE allowed us to confirm that bioaccessible Mn was present as free-Mn (ionic form) (Chapter II).

SIXTH: In vitro evaluation of Se bioaccessibility from enteral nutrition formulas provides new insight into the importance of the chemical form in which trace elements are present in supplements. One of the Se sources ¨Se amino acid chelate¨ demonstrated to be an unviable form that is scantily absorbed and therefore hinders selenoproteins activities. This evidence stresses the need for practitioners to read and understand ENF labels before selecting the commercial formulations to prescribe (Chapter III).

SEVENTH: The complexity of commercial formulations and the possible interactions between the multiple components that make them up may strain the utilization of some trace elements at the expected levels as demonstrated by the different responses of GPx and TrxR activities upon sodium selenite supplementation. In this case, heavy metal contamination from mercury was discarded to influence these results as its content was negligible in the analyzed ENF (Chapter III).

EIGHTH: Research from the province of Cordoba clearly illustrates that dementia, the leading cause of tube feeding prescription in the region (and replicated on a national scale) is not recommended nor sustained by international organizations, as also demonstrated in this study by the increased mortality displayed in this patient population. Professional awareness and family counseling be aware of realistic outcomes and risks derived from this procedure could avoid such unnecessary use (Chapter IV).

NINTH: While the lack of a standardized and multicomponent assessment preceding EN initiation limits the availability of multiple parameters of nutritional interest for further or broader research. It also denotes an area of improvement in the clinical practice setting, in which appropriateness of medical nutrition therapy should be determined based on established criteria that each patient should meet before the intervention (Chapter IV).

TENTH: The multivariate Cox analysis applied in enterally fed patients enables a better understanding of how a thorough evaluation of comorbidities and baseline patient characteristics are necessary to identify major risk factors associated with worst outcomes, as demonstrated by the higher risk possed by HBP, glycemia, and dementia (Chapter IV).

ELEVENTH: Measured risks in this study could be used along with further studies to develop a nutritional assessment tool to be used in clinical practice, such as other standardized nutritional assessment instruments (Chapter IV).

Finally, this work was able to explore EN through a spectrum of analysis that contributes to theoretical and applicable knowledge of medical nutrition therapy. This approach provides new insight into the complexity of the formulations and interactions that take place within its components and the relevance of being aware of them. Equally important, it brings to light, regular practice areas that can be improved towards patient care and to avoid interventions where the potential benefit does not outweigh the risk. Overall, considering that understanding of ENF is only one part of the treatment, being the other the patient and its characteristics, this work also brings to light the importance of professional with a deep understanding of both elements, such as the dietitian.