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ESTUDO DOS PRECURSORES DE NAD+ - NMN (NICOTINAMIDA MONONUCLEOTÍDEO) E NR (NICOTINAMIDA RIBOSÍDEO) - EM ALIMENTOS VEGETAIS E SUA POTENCIAL CONTRIBUIÇÃO DIETÉTICA PARA A SAÚDE

STUDY OF NAD+ PRECURSORS - NMN (NICOTINAMIDE MONONUCLEOTIDE) AND NR (NICOTINAMIDE RIBOSIDE) - IN PLANT FOODS AND THEIR POTENTIAL DIETARY CONTRIBUTION TO HEALTH

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Study of NAD+ precursors - NMN (nicotinamide mononucleotide) and NR (nicotinamide riboside) - in plant foods and their potential dietary contribution to health

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RESUMO

NAD+ é essencial para todos os organismos vivos, participando em reações cruciais para regulação energética e de sinalização celular. Seus níveis diminuem com a idade e em doenças. A promoção da síntese de NAD+ através de seus precursores NMN e NR, tem mostrado reverter o declínio fisiológico e atenuar diversas patologias como diabetes e Alzheimer. Embora presentes em alimentos, estudos se concentram em suplementos e poucos dados sobre alimentos estão disponíveis. Apenas 1% dos trabalhos publicados sobre NAD+, NMN e NR são da área de ciência e tecnologia alimentos, existindo grandes lacunas e oportunidades de pesquisa nesse campo. Visando ajudar a responder e preencher algumas lacunas, o objetivo desse estudo foi investigar a presença dos novos precursores de NAD+ - NMN e NR - em alimentos vegetais e a sua potencial contribuição dietética para a saúde. Um rastreamento do conteúdo de NAD+, NMN, e NR em alimentos vegetais, através de método desenvolvido e validado para HPLC/UV-DAD, depois confirmado por LC-MS/MS, demonstrou que as concentrações de NMN foi na faixa de 40 a 13.000 μg/100g, e NR na faixa de 62 a 1.600 μg por 100g de vegetais frescos. Os alimentos mais ricos em NMN foram brócolis e vagem, enquanto que para NR foram almeirão, banana e laranja. O teor médio de NAD+ encontrado variou de 58 a 8.800 μg/100g (peso úmido), tendo a taioba roxa (*Xanthosoma violaceum*), taioba (*Xanthosoma sagittifolium*), espinafre e couve as maiores quantidades. PANC (plantas alimentícias não convencionais) também foram analisadas, e dentre elas moringa (*Moringa oleifera*) e taioba roxa tiveram os maiores teores de NR, enquanto que Ora-pro-nobis violácea (*Pereskia Violacea*), foi a que apresentou o maior teor de NMN. Considerando que a Ora-pro-nobis violácea é uma nova espécie de Ora-Pro-nobis com nenhum dado ainda publicado sobre sua composição, também realizamos sua caracterização nutricional e potencial antioxidante. As folhas de Ora-pro-nobis violácea mostraram alto teor de proteínas, fibras, potássio, cálcio e ferro, chegando até ultrapassar a ingestão diária recomendada (IDR) para cálcio. Também conteve compostos fenólicos (de 769,3 a 1.043,6 μg GAE/g de peso seco), que foi significativamente correlacionado com a capacidade antioxidante. Através de um modelo *in vitro* de fermentação colônica constatou-se que NAD+ e seus precursores impactaram minimamente na atividade da microbiota intestinal humana, não alterando significativamente a produção de amônia e ácidos graxos de cadeia curta. Apesar disso, aumentaram a riqueza e alteraram a composição da comunidade microbiana, promovendo a abundância de espécies de Firmicutes em detrimento de Fusobacteriota. Por sua vez, o metabolismo microbiano mostrou desamidar NAD+, NMN,

NR e NAM, liberando outros precursores desamidados, sugerindo sua participação na biodisponibilidade e bioatividade desses compostos. Os extratos das plantas analisadas, particularmente espinafre, contribuíram para aumentar o pool desses metabólitos microbianos precursores da biossíntese de NAD+. NAD+ e seus precursores NMN e NR podem ser mais uma potencial razão para os benefícios à saúde do consumo de frutas e vegetais. Esse estudo contribui para a expansão do conhecimento desse tema na área de ciência de alimentos, fomentando a abertura para futuras pesquisas.

Palavras-chave: Precursores de NAD+; nicotinamida mononucleotídeo; nicotinamida ribosídeo; vegetais; microbiota intestinal humana

ABSTRACT

NAD+ is essential for all living organisms, participating in crucial reactions for energy regulation and cellular signaling. Its levels decrease with age and in diseases. Promoting NAD+ synthesis through its precursors NMN and NR has been shown to reverse physiological decline and mitigate various pathologies such as diabetes and Alzheimer's. Although present in foods, studies focus on supplements and limited data on foods are available. Only 1% of published works on NAD+, NMN, and NR are from the food science and technology field, indicating significant research gaps and opportunities in this area. To help address and fill some of these gaps, this study aimed to investigate the presence of the new NAD+ precursors - NMN and NR - in plant foods and their potential dietary contribution to health. Screening of NAD+, NMN, and NR content in plant foods, using a developed and validated HPLC/UV-DAD method, confirmed by LC-MS/MS, demonstrated that NMN concentrations ranged from 40 to 13,000 µg/100g, and NR from 62 to 1,600 µg per 100g of fresh vegetables. The richest sources of NMN were broccoli and green beans, while for NR were wild chicory, banana, and orange. The average NAD+ content ranged from 58 to 8,800 µg/100g (wet weight), with purple malanga (*Xanthosoma violaceum*), malanga (*Xanthosoma sagittifolium*), spinach, and collard having the highest quantities. Non-conventional food plants (PANC) were also analyzed, and among them, moringa (*Moringa oleifera*) and purple malanga had the highest NR levels, while Ora-pro-nobis violacea (*Pereskia Violacea*) showed the highest NMN content. Considering that Ora-pro-nobis violacea is a new species with no published data on its composition yet, we also conducted its nutritional and potential antioxidant characterization. Ora-pro-nobis violacea leaves showed high protein, fiber, potassium, calcium, and iron content, even exceeding the recommended daily intake (RDI) for calcium. They also contained phenolic compounds (from 769.3 to 1,043.6 µg GAE/g dry weight), which were significantly correlated with antioxidant capacity. Through an *in vitro* colonic fermentation model, it was found that NAD+ and its precursors minimally impacted human intestinal microbiota activity, without significantly altering the production of ammonia and short-chain fatty acids. However, they increased microbial richness and altered microbial community composition, promoting the abundance of Firmicutes species at the expense of Fusobacteriota. Microbial metabolism performed NAD+, NMN, NR, and NAM deamidation, releasing other deamidated precursors, suggesting their involvement in the bioavailability and bioactivity of these compounds. Extracts from analyzed plants, particularly spinach,

contributed to increasing the pool of these microbial precursor metabolites for NAD+ biosynthesis. NAD+ and its precursors NMN and NR may be yet another potential reason for the health benefits of fruit and vegetable consumption. This study contributes to expanding knowledge in the food science field, fostering opening for future research.

Keywords: NAD+ precursors; nicotinamide mononucleotide; nicotinamide riboside; plant foods; human gut microbiota

SUMÁRIO

INTRODUÇÃO GERAL

A molécula de nicotinamida adenina dinucleotídeo (NAD+) é essencial para todos os organismos vivos, pois, participa de múltiplas reações enzimáticas que regulam processos biológicos. Além de seu papel vital como coenzima no metabolismo energético, transferindo hidrogênio em reações de oxidação-redução, a função de NAD+ se expandiu para co-substrato de enzimas como CD38, poli adenosina difosfato ribose polimerase (PARPs) e sirtuínas (SIRTs), para várias importantes vias de sinalização que medeiam essenciais processos celulares para homeostase do organismo, tais como reparo do DNA (Herceg & Wang, 2001), apoptose (Pittelli et al., 2011), sobrevivência celular (Alano et al., 2010), regulação do tempo de vida (Houtkooper et al., 2012; H. Zhang et al., 2016), ajustes metabólicos (Houtkooper et al., 2012), inflamação (Cameron et al., 2019) e infecção (Fehr et al., 2020) como por SARS-COV-2 por exemplo (Heer et al., 2020; Omran & Almaliki, 2020).

Devido seu papel chave em patofisiologias, principalmente as relacionadas ao envelhecimento, o metabolismo de NAD+ passou a ser um tópico de interesse renovado nos últimos anos (Covarrubias et al., 2021; N. Xie et al., 2020; Yaku et al., 2018; Yang & Sauve, 2016).

NAD+ é constantemente sintetizado, catabolizado e reciclado na célula para manter níveis estáveis. Porém, elementos como envelhecimento (Guest et al., 2014; Massudi et al., 2012) e supernutrição (Seyedsadjadi et al., 2018; J. Yoshino et al., 2011), aceleram seu gasto, e/ou afetam a síntese, e estão associados à diminuição de seus níveis. Os baixos níveis de NAD+ são uma das marcas do declínio fisiológico e do aparecimento de doenças associadas à idade, como neurodegenerativas, metabólicas e oculares (Imai & Guarente, 2014; Johnson & Imai, 2018). Da mesma forma, a depleção de NAD+ foi relacionada a complicações e agravamento da doença Covid-19 (Heer et al., 2020; Huizenga, 2020), particularmente em idosos.

A perda de NAD+ em muitos desses cenários pode ser atribuída, pelo menos em parte, à atividade elevada de PARPs e CD38, que implica na redução da expressão e/ou atividade de SIRTs (Camacho-Pereira et al., 2016; Omran & Almaliki, 2020). Enquanto que o aumento do conteúdo de NAD+ pode reforçar as funções antivirais de PARPs e apoiar a imunidade ao SARS-CoV-2.

Dessa maneira, compostos que podem aumentar rapidamente a concentração de NAD+ têm estimulado pesquisas para avaliar a atenuação do declínio fisiológico, prevenção e tratamento de doenças (Imai, 2010; Martens et al., 2018; J. Yoshino et al., 2011).

A síntese de NAD+ pode ocorrer a partir de triptofano e vitamina B3 (niacina e niacinamida) (Elvehjem, 1949). Mais recentemente, nicotinamida ribosídeo (NR) e nicotinamida mononucleotídeo (NMN) também foram identificados para promover a síntese de NAD+ (Bieganowski & Brenner, 2004; Revollo et al., 2004).

Com base na capacidade em elevar os níveis de NAD+, estudos têm demonstrado que NMN e NR podem melhorar várias patologias e sintomas relacionados ao envelhecimento em modelos animais (Diguet et al., 2018; Mills et al., 2016; Trammell et al., 2016; J. Yoshino et al., 2011).

NMN demonstrou diversos efeitos benéficos em diabetes tipo 2 (J. Yoshino et al., 2011; M. Yoshino et al., 2021), na doença de Alzheimer (Yao et al., 2017), e na insuficiência renal aguda (Guan et al., 2017). Teve ação cardio e vasoprotetora (de Picciotto et al., 2016; Yamamoto et al., 2014), evitou a disfunção endotelial (Mateuszuk et al., 2020), e reverteu alterações pró-inflamatórias e pró-aterogênicas no perfil de expressão de MicroRNAs (miRNA) relacionadas à idade na aorta de ratos (Kiss et al., 2019). Promoveu efeitos neuroprotetores da degeneração em fotorreceptores da retina (Chen et al., 2020), e do estresse oxidativo por meio de reforço mitocondrial (Ito et al., 2020).

NR mostrou proteger do envelhecimento muscular ao melhorar a função mitocondrial, prolongando a vida útil em ratos (H. Zhang et al., 2016). Também protegeu dos sintomas clássicos de Alzheimer como perda cognitiva (Gong et al., 2013), melhorando aprendizagem, memória e função motora (Hou et al., 2018). Em humanos, diminuiu a pressão arterial em indivíduos pré-hipertensos (Martens et al., 2018), e os níveis de citocinas inflamatórias circulantes em homens idosos (Elhassan et al., 2019).

Além disso, foi demonstrado que a suplementação de NMN e NR modulou a microbiota intestinal em camundongos, aumentando a abundância de certas bactérias benéficas e reduzindo a abundância de certas bactérias nocivas (Huang et al., 2021, 2022; Peluso et al., 2023). Considerando que microbiota intestinal desempenha um papel fundamental na digestão dos alimentos, produzindo metabólitos que afetam o estado de saúde do hospedeiro (Agus et al., 2018; Koeth et al., 2013; Roager & Dragsted, 2019; Tang et al., 2013), a interação entre a microbiota intestinal e os precursores de NAD+ sugere um potencial eixo para os benéficos efeitos fisiológicos observados.

Esses achados têm despertado o interesse no potencial terapêutico de NMN e NR como uma intervenção para mudanças benéficas na fisiologia humana (J. Yoshino et al., 2018), e sido alvo de estudos clínicos a fim de determinar sua segurança e aplicabilidade (Conze et al., 2019; Irie et al., 2020; Martens et al., 2018; Okabe et al., 2022).

No entanto, todos os estudos disponíveis até o momento foram feitos com suplementos de NMN e NR, a doses relativamente altas (por volta de 300 a 500 mg/kg em animais e de 500mg a 2g ao dia para humanos), sejam administrados oralmente ou através de injeção intraperitoneal (em casos de estudos em animais). Nos Estados Unidos, NMN foi proibido de ser vendido como suplemento dietético devido à sua classificação, sob investigação, como um novo medicamento (Food and Drug Administration, 2022). No Brasil, NMN e NR são comercializados como suplementos mas ainda a um preço relativamente elevado, enquanto que obtê-los através da dieta seria uma alternativa mais acessível.

NMN e NR são naturalmente presentes em vegetais, carne e leites (Mills et al., 2016; Ummarino et al., 2017). Dos poucos dados de mensuração de NMN e NR em alimentos que estão disponíveis, dentre os alimentos analisados a maior quantidade de NMN foi encontrada em edamame (0.47–1.88 mg/100g) e avocado (0.36–1.60 mg/100g) (Mills et al., 2016). Além disso, NMN e NR foram determinados em leite de diferentes espécies (Ummarino et al., 2017). Desde que NR foi descoberto pela primeira vez no leite (Bieganowski & Brenner, 2004), só tinha sido medido em leites, com bovino tendo o maior conteúdo de NR (0,5–3,6 μM), e o leite humano mais rico em NMN (2,1–9,8 μM) (Ummarino et al., 2017). Mais recentemente, também foi demonstrada a presença de NR e NMN em cervejas artesanais (Garofalo et al., 2021).

Tendo em vista a escassez de dados sobre NMN e NR em alimentos, e da relevância dos seus efeitos fisiológicos na proteção e reversão de doenças, a investigação do conteúdo destes novos precursores de NAD+ em fontes alimentares e sua potencial contribuição dietética para a promoção da saúde oferece um novo e ainda inexplorado campo de pesquisa para a ciência de alimentos e nutrição.

OBJETIVOS

Objetivo Geral

Investigar a presença dos novos precursores de NAD+ - NMN e NR - em alimentos vegetais e a sua potencial contribuição dietética para a saúde.

Objetivos Específicos

- Desenvolver e validar um método para separar, identificar e quantificar os precursores de NAD+-NMN, NR presentes em alimentos vegetais através de HPLC-DAD;
- Determinar o conteúdo de NAD+, NMN e NR em alimentos vegetais, dos comumente consumidos a bioprodutos como cascas, bem como PANC (plantas alimentícias não-convencionais);
- Avaliar o efeito do tratamento térmico (calor úmido, calor seco) dos vegetais no teor de NAD+, NMN e NR;
- Realizar caracterização de PANC cultivada na floresta amazônica que contenha NAD+, NMN, e/ou NR, mas ainda não tenha estudos publicados sobre sua composição;
- Avaliar a modulação da microbiota intestinal humana pelos precursores de NAD+, NMN e NR presentes nos principais alimentos fontes encontrados;
- Avaliar a biotransformação dos precursores de NAD+, NMN e NR dietéticos e os possíveis metabólitos gerados pela microbiota intestinal humana

CAPÍTULO I – REVISÃO BIBLIOGRÁFICA

NAD+ precursors nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR): potential dietary contribution to health

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Abstract

Purpose of review

NAD+ is a vital molecule that takes part as a redox cofactor in several metabolic reactions besides being used as a substrate in important cellular signaling in regulation pathways for energetic, genotoxic, and infectious stress. In stress conditions, NAD+ biosynthesis and levels decrease as well as the activity of consuming enzymes rises. Dietary precursors can promote NAD+ biosynthesis and increase intracellular levels, being a potential strategy for reversing physiological decline and preventing diseases. In this review, we will show the biochemistry and metabolism of NR (nicotinamide riboside) and NMN (nicotinamide mononucleotide), the latest findings on their beneficial physiological effects, their interplay with gut microbiota and the future perspectives for research in nutrition and food science fields.

Recent findings

NMN and NR demonstrated protect against diabetes, Alzheimer disease, endothelial dysfunction, and inflammation. They also reverse gut dysbiosis and promote beneficial effects at intestinal and extraintestinal levels. NR and NMN have been found in vegetables, meat, and milk, and microorganisms in fermented beverages can also produce them.

Summary

NMN and NR can be obtained through the diet either in their free form or as metabolites derivate from the digestion of NAD+. The prospection of NR and NMN to find potential food sources and their dietary contribution in increasing NAD+ levels are still an unexplored field of research. Moreover, it could enable the development of new functional foods and processing strategies to maintain and enhance their physiological benefits, besides the studies of new raw materials for extraction and biotechnological development.

Keywords: NAD+ precursors; nicotinamide mononucleotide; nicotinamide riboside; pyridine derivatives; promoting health

1. Introduction

NAD+ is the oxidised form of nicotinamide adenine dinucleotide, a molecule essential for living organisms for maintaining cellular health. It has been shown to promote several health benefits, including enhancing energy metabolism, cardio and neuroprotection, DNA repair, and anti-inflammatory and anti-aging effects (1–6).

In addition to its key role in energy metabolism as a coenzyme that accepts electrons for catabolic reactions, NAD+ also participates as a co-substrate in signaling pathways of intracellular calcium mobilization and post-translational protein modification (7). The regulation of these important cellular processes mediated by NAD+ confers protection of mitochondrial function, redox homeostasis control, anti-inflammatory action, attenuation of age-related dysfunctions, cell differentiation, genomic stability, and epigenetic modulation among others (1,8–10).

NAD+ is constantly synthesized, catabolized, and recycled in the cell to sustain stable levels. Disturbances such as aging (11) and overnutrition by high-fat and -protein intake (12,13) affect NAD+ synthesis and are associated with reduced levels of this important molecule. Low NAD+ levels is one of the hallmarks of physiological decline and the onset of age-associated diseases, such as neurodegenerative, metabolic, and ocular (14,15). Likewise, NAD+ depletion was related to complications and the worsening of coronavirus disease (Covid-19) (16,17). In summary, NAD+ decline can result from reduced NAD-synthesizing enzymes, increased NAD-consuming enzymes, or a combination of both.

Boosting NAD+ levels throught biosynthesis precursors has the potential to prevent or alleviate a wide range of diseases such as metabolic and age-related disorders. Based on their ability to elevate NAD+ levels, nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN) have been shown to mitigate the physiological decline, diabetes and diabetic neuropathy (1,13,18–20); protect against hepatic steatosis (18,21); decrease various pathological features of Alzheimer's disease (22–24); protecting neuronal cells from oxidative stress (25) and preserving cognition (26,27). In addition, they have demonstrated beneficial pharmacological activities in acute renal failure (28), anti-inflammatory (29–31), cardio and vasoprotective actions (32–35), telomere lengthening (36,37), extend lifespan and promote health in various organisms, from yeasts to mammals (10,14,15,38).

The NMN and NR supplements have been the subject of clinical trials to assess their safety and applicability in humans (34,38–40). However, these NAD+ precursors are

also present in foods and may have a potential dietary therapeutic role, similar to that of phenolic groups and other bioactive food compounds. Evidence from NMN and NR presence in vegetables, meat, and milk reinforce their natural occurrence (1,41,42), although there is still limited data on NMN and NR content in foods. While vegetal sources, such as edamame, avocate and broccoli, have shown slightly higher content until now, research is essential to identify the best food sources and determine whether a normal diet provides a sufficient amount of these precursors to increase NAD+ levels. The distribution of these novel NAD+ precursors in food sources presents an exciting new research field, particularly regarding their potential contribution to health promotion through diet.

Additionally, the potential impact of link between gut microbiota and NAD+ metabolites on overall host health is noteworthy. While microorganisms that inhabit the gut play a role in the metabolism of NAD+ and its metabolites (43,44), NMN and NR also affect the composition of the gut microbiota, reversing dysbiose and promoting beneficial effects at both intestinal and extraintestinal levels (45–47).

Hence, this review will cover the biochemistry and metabolism of NAD+ precursors NMN and NR, as well as their latest beneficial physiological effects, interplay with gut microbiota, and future research prospects in the fields of nutrition and food science.

2. NAD+, NMN, and NR metabolism

In eukaryotes, NAD+ performs two important functions: energy transduction and cell signaling. NAD+ was first established as a vital redox cofactor for ATP (adenosine triphosphate) synthesis. Subsequently, NAD+ degradation processes were unveiled, using it as a substrate by CD38/CD157/SARM1, ADP-ribosyl transferases (ARTs), poly-ADP polymerases (PARPs), and sirtuins (7,48–50) (**Figure 1**). These NAD-dependent enzymes mediate essential cellular processes for organismal homeostases, such as DNA repair (51), apoptosis (52), cell survival (53), lifespan regulation (10,49), metabolic adjustments (49), inflammation and infection (54).

NAD+ is a pyridine nucleotide made up of two nucleosides that are joined by a pyrophosphate group. Each nucleoside contains a ribose ring, with one nucleoside containing adenine attached to the first carbon atom (adenosine diphosphate ribose) and the other containing nicotinamide at the same position (NMN) (**Figure 1**).

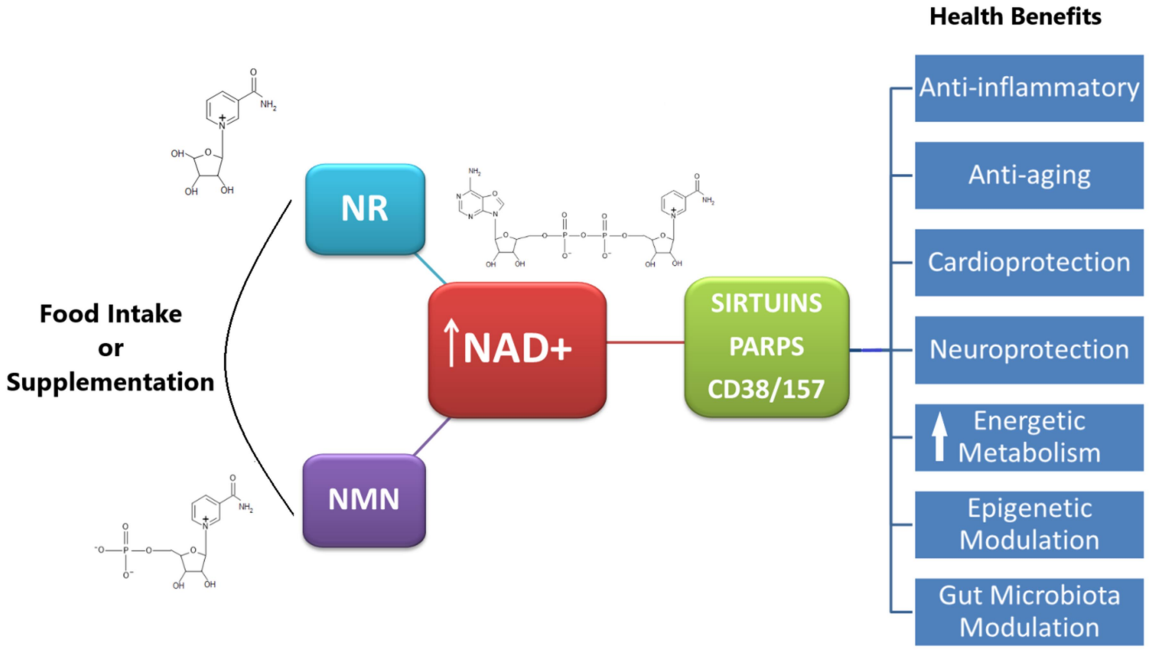
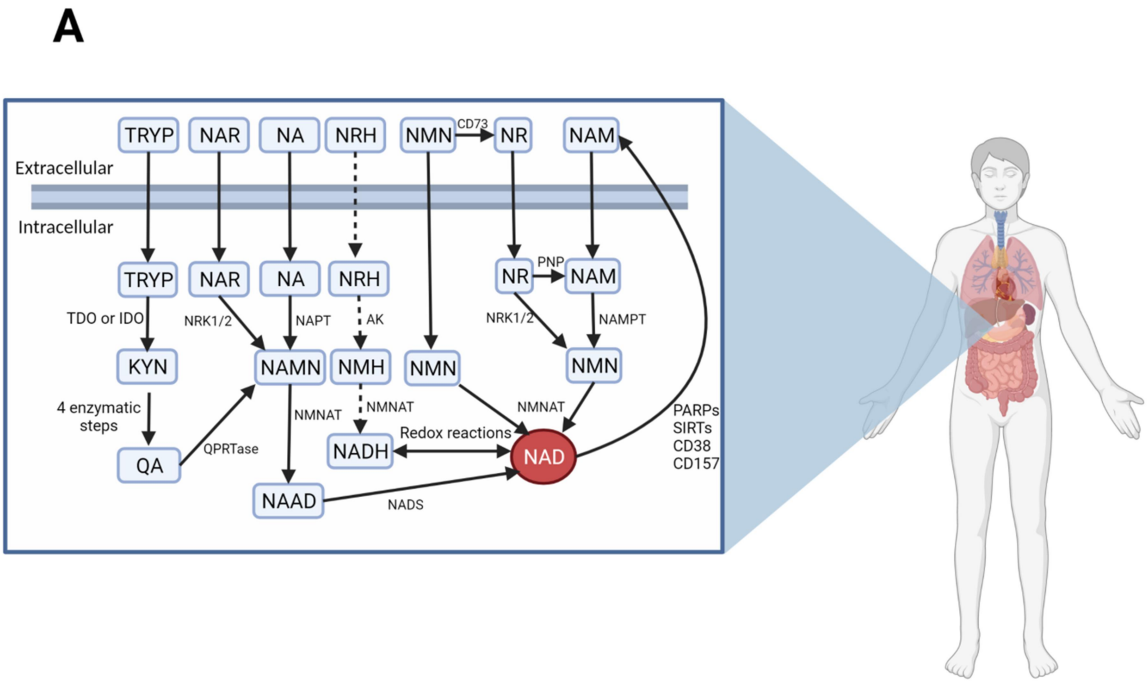
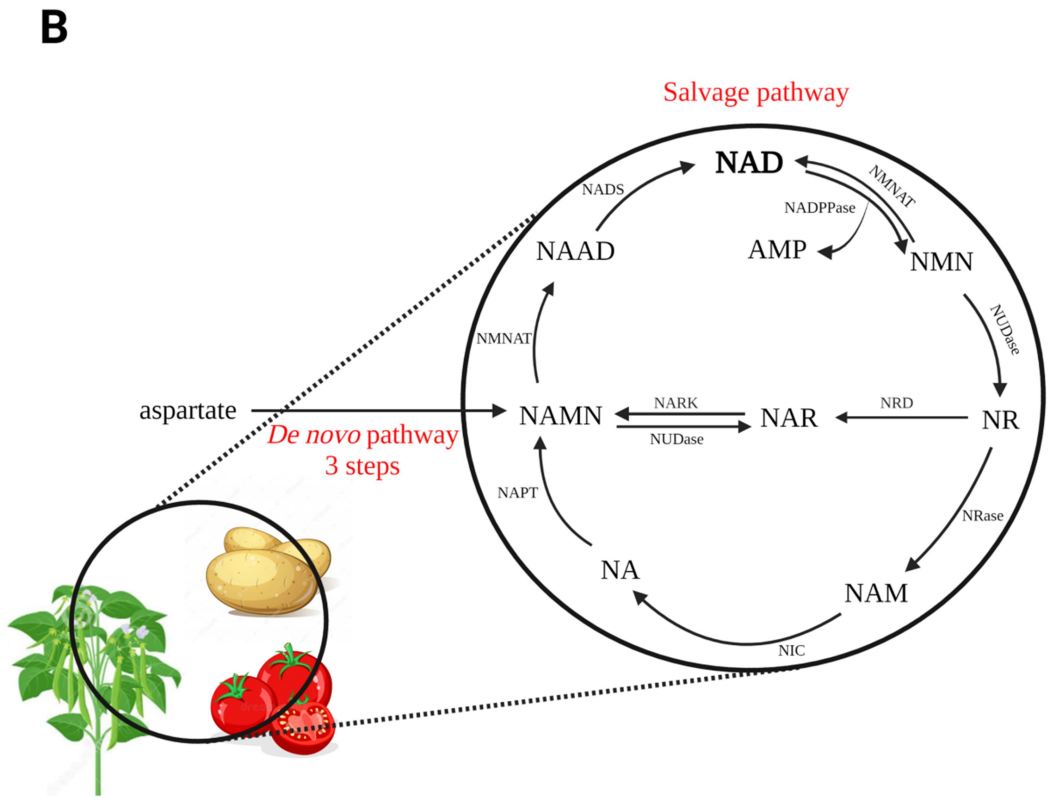


Figure 1. Chemical Structure and Schematic Illustration of the Beneficial Health Effects of NAD+ Precursors NMN and NR.

NAD+ synthesis can occur from tryptophan and vitamin B3 (niacin and nicotinamide). Later, the intermediates NR and NMN have also been identified to promote NAD+ synthesis (13,55). NR is a pyridine nucleoside consisting of an amine (nicotinamide) with a beta-N-glycosidic bond to a ribose. NMN is a pyridine nucleotide which contains a NR molecule bounded to a phosphate group. NMN and NR are involved in the degradation and re-synthesis of NAD+ (**Figure 1**), and this metabolic cycle of NAD+ varies between species and kingdoms (56–58).

In mammals, NAD can be produced in different ways: by *de novo* generation from the amino acid tryptophan; by the nicotinamide (NAM) in the *salvage pathway* which is released from these consuming reactions as a NAD recycling system; or by niacin/nicotinic acid (NA) in the *Preiss-Handler pathway* (**Figure 2A**). The precursors amidated nicotinamide (NAM), NMN and NR are the main substrates in the salvage pathway. Furthermore, more recently the reduced forms of NR and NMN, namely dihydronicotinamide riboside (NRH) (59,60) and dihydronicotinamide mononucleotide (NMNH) (61), respectively, have also been shown to promote synthesis (**Figure 2A**).





Depicted examples: Febaceae family, tomato, potato

Figure 2. NAD biosynthesis pathways in humans (A) and plants (B). NMN could be uptake through CD73-mediated dephosphorylating to NR, or via a transporter encoded by gene SLC12A8. Arrows dotted indicate a putative pathway.
Precursors/Metabolites: KYN: N-formylkynurenine; NMN (nicotinamide mononucleotide); NMNH (dihydronicotinamide mononucleotide); NR (nicotinamide riboside); NRH (dihydronicotinamide riboside); NAR (nicotinic acid riboside); NAM (nicotinamide); NA (nicotinic acid); NAMN (nicotinic acid mononucleotide); NAAD (nicotinic acid adenine dinucleotide), QA (quinolinic acid); TRYP (tryptophan). Enzymes: AK: adenosine kinase; CD73/CD38/CD157: ectoenzimas; IDO: indoleamine 2,3-dioxygenase; NADPPase (NAD pyrophosphatase); NADS (NAD synthase); NAMPT: nicotinamide phosphoribosyltransferase; NAPT (nicotinate phosphoribosyltransferase); NARK (nicotinic acid ribose kinase); NIC (nicotinamidase); NMNAT (nicotinamide/nicotinic acid mononucleotide adenylyltransferase); NRase (nicotinamide riboside nucleosidase); NRD (nicotinamide riboside deaminase); NRK1/2: nicotinamide riboside kinases; NUDase (5′-nucleotidase); PARPs: poli-ADPR polymerases; PNP: purine nucleoside phosphorylase; QPRTase: QA-phosphoribosyltransferase; SIRTs: sirtuins, TDO: tryptophan 2,3-dioxygenase. Image created with Biorender.com.

Moreover, it is worth mentioning the role of the microbiota in NAD+ synthesis. Unlike mammals, the bacteria present in the gut possess enzymes that convert NMN to nicotinic acid mononucleotide (NAMN) through a process of deamidation (62). After that, NAMN follows via the Preiss-Handler pathway to NAD+ synthesis. The isotope-labeled NMN orally administered in rats was partially deamidated into NAMN by the intestinal microbiota before its absorption. Despite to a noticeably lesser extent than the amidated metabolites NMN and NAD+, nicotinic acid adenine dinucleotide (NAAD) levels in the liver increased (43), as was observed for NR supplementation, which resulted in increased NAAD (63). Shats et al. (2020) also reported that NAD+ enhancement by NAM and NR was mostly through microbiota-dependent deamidated pathway (44).

In addition, NR can increase NAD+ levels in two different ways. Through direct absorption following the salvage pathway, or be hydrolyzed to nicotinamide (NAM) by the glycohydrolase bone marrow stromal cell antigen 1 (BST1), and further metabolized by the

gut microbiota to nicotinic acid, following through the Preiss–Handler pathway. Furthermore, was reported BST1 has a base-exchange activity against both NR and niacin riboside (NAR) to generate NAR and NR, respectively, connecting the amidated and deamidated pathways (64).

Thus, several pathways allow alternative precursors to be used to maintain intracellular NAD levels. Whether this redundancy is vital or has tissue-specific importance remains unexplored.

3. Sources of NMN and NR

The biosynthesis NAD+ precursors can be obtained through the diet or by supplementation. The precursors tryptophan, NAM, NA, NMN, and NR are obtained through the diet either in their free form or as metabolites derivatives from the digestion of NAD+.

NR has been named as the third NAD+ vitamin precursor, alongside NAM and NA (65). One of the official methods for measuring NA content in foods is by microbiological assay. In the assessment, the food matrix is submitted to an acid or alkaline treatment at a high temperature (AOAC Method 944.13). It is known that under these conditions, NAM, NAD, and NADP are converted into NA, and the alkaline treatment releases NA eventually bound to polysaccharides, peptides, or glycopeptides. Ummarino et al. (2017) investigated whether NMN and NR would also produce NA when exposed to hot acid or alkaline extraction procedures. And indeed, NR and NMN molecules were hydrolyzed to NA and could account for the total niacin content in the milk samples analyzed (42). Hence, this indicates that the niacin quantified by microbiological assay includes NR and NMN eventually present in the food matrix.

Although NMN and NR can be found in foods of animal origin and mushrooms, this section will focus on their content in plants as a potentially good dietary source, as there are ongoing experiments (results unpublished) supporting this notion.

NAD+ is essential for plants' adaptation to environmental stresses such as UV irradiation, salinity, heat shock, and drought stress (66,67). Additionally, NAD+ is involved in several key factors in plant biology including growth and development, metabolism (energy, reactive oxygen and nitrogen species, harvest), signaling, gene expression, immunity, and biosynthesis (58,68). Gene transcription studies have revealed different expression patterns during fruit development and growth. Among the specific genes related to NAD metabolism,

de novo pathway biosynthetic genes were transcriptionally induced in young tomatoes. Later stages of fruit growth showed an accumulation of genes involved in the salvage pathway, which coincided with increased NAD levels, most likely to sustain the high metabolic activity during the ripening process (69).

In plants, NAD is synthesized by the *de novo* pathway from the amino acid aspartate and by the salvage pathway (66), however, nicotinic acid (NA) is the key metabolite of the pyridine nucleotide cycle (70) (**Figure 2B**). Previously, the pathways in NAD+ biosynthesis consisting of seven metabolites were the most known, however, a cycle that includes nicotinamide riboside deaminase was found in potato tubers (71). The new cycle bypasses nicotinamide and nicotinic acid pathways (**Figure 2B**) indicating that it could occur in some plant species (58).

NMN is generated by breaking the diphosphate link of the NAD+ molecule via the action of NAD diphosphatases (EC 3.6.1.22), such as the nudix hydrolases (66), also named NAD pyrophosphatase (58). Although the salvage pathway deamidated of 6 or 8 steps is most prevalent, NMN can also be converted to NAD in plants by a single step catalyzed by nicotinamide mononucleotide adenyltransferase (NMNAT) (58,66). The NMNAT activity specific in the mitochondrial fraction was demonstrated in Jerusalem artichoke (*Helianthus tuberosus L.*) and plays an important role in NAD metabolism (72). In lentil and prickly pear (opuntia), the enzyme nucleotide pyrophosphatase (EC 3.6.1.9) was identified and exhibited hydrolytic activities on the pyrophosphate bonds of the reduced and oxidized forms of NAD(P), producing NMN and AMP (73,74). In plants such as tea, potatoes, gymnosperms, and Fabaceae, there is the conversion of NMN to NR by activity 5′nucleotidase (EC 3.1.3.5) (58,66). These findings characterizes NMN and NR as metabolites of the plant NAD+ pool (67,69), and vegetal foodstuffs as sources of these compounds.

Accordingly, mammals are exposed to these precursors through the digestive breakdown of dietary NAD+ (39). Bioavailability studies showed that ingested NAD+ was mainly hydrolyzed in the small intestine by pyrophosphatases present in brush border cells or intestinal secretions (75). The expression of a transporter in the gut (76) reinforces that NMN could be made bioavailable through oral delivery.

To date, only a few studies have measured NMN and NR in foods, and as a result, the best sources have not been clearly identified. Mills et al. (2016) published the first evidence of NMN in foods such as edamame beans, avocado, broccoli, cucumber, cabbage, and tomato. Among the investigated sources the highest amount was found in edamame

ranging at 0.47-1.88 (mg/100g) and avocado at 0.36-1.60 (mg/100g), and at smaller amounts in seafood and raw meats (0.06-0.42 mg/100g) (1). Additionally, NR and NMN were also determined in different species of milk and beer (42,77). Since NR was first discovered in milk (55) it had only been measured in milk so far, with bovine milk having the highest NR content (0.5–3.6 μM,) and human milk as the richest NMN source (2.1–9.8 μM) (42). As molarity is usually expressed in units of moles of solute per liter of solution, it is assumed that the concentration is for the volume of 1L of milk, since this information is not stated in the referenced article.

Recently, yeast-mediated NR and NMN production in craft beers was demonstrated, and hop indicated a role in the enhancement of NR levels during fermentation (77). Thus, opens new perspectives for naturally enriched sources of NAD+ precursors through fermented foods and beverages. This would bring new possibilities alongside the potential biotechnological use of microbial species (e.g fructophilic lactic acid bacteria) to produce NMN and NR (78) for functional probiotic products and supplements.

Last, NMN and NR supplements are marketed at high prices in capsule or powder form, with dosages ranging from 100 to 1000 mg, which are doses utilized per day in the studies. However, it's important to note that the optimal dose of NMN and NR supplements for humans is not yet fully understood and further research is needed to determine the most effective and safe dosage. Obtaining NAD+ precursors through the diet may be a more accessible and natural alternative, despite their lower content in food sources compared to supplements. Further research is needed to clarify these issues.

4. Safety and pharmacokinetics

Several studies are ongoing to evaluate the physiological effects of NMN and NR supplementation, including safety and pharmacokinetics in humans (NCT04228640, NCT04910061), aging (NCT04823260, NCT04685096), hypertension (NCT04903210), on heart failure (NCT03565328, NCT04528004), on COVID-19 (NCT04407390, NCT05175768, NCT04818216), on mitochondrial function (NCT03789175, NCT03951285), metabolic and cardiovascular functions (NCT04571008), skeletal muscle and bone metabolism functions (NCT03818802, NCT04691986), on muscle physiology and physical capacity (NCT04691986, NCT04664361), among others (listed in the database *ClinicalTrials.Gov*). Although the results of pre-clinical and clinical trials with NR and NMN

supplementation are promising, remains a need to determine if long-term supplementation and high doses may show side effects.

For example, NAD synthesis inhibition due to NAMPT inhibition decreases cell growth and increases susceptibility to oxidative stress emerging as a therapeutic concept for cancer treatment (79). While the NAD+ precursors, NMN and NR, reversed cell death induced by the NAMPT inhibitor, the CD38 favored cell death, and CD73 allowed cell viability by degrading NAD and NMN into NAM, and NMN into NR, respectively, thus sustaining NAD+ synthesis (80). In an oncogenic mouse model, the supplementation with NMN (500 mg/kg body weight daily for 13 days) significantly increased the secretory phenotype associated with pro-inflammatory senescence, promoting pancreatic ductal adenocarcinoma progression. This raises the hypothesis that dietary supplements can increase NAD+ and may be tumorigenic in vivo under stress conditions, such as premalignant senescent lesions induced by activated oncogenes (81).

Therefore, it is worth considering the extent of complex interactions and regulation between NAD^+-dependent processes.

A toxicity study in rats demonstrated that repeated oral administration of synthetic NMN (NMN-C®) at doses up to 1500 mg/kg/d over a sub-chronic (90-day) treatment period appears to be safe and did not promote toxic effects as seen from body weight change, food and water consumption, feed conversion efficiency, biochemical and blood parameters (82), although differences in several physiological and biochemical parameters were found in animals from mid and high-treatment doses (750 mg/kg/d and 1500 mg/kg/d, respectively). There was an increase in kidney, liver, and adrenal gland weights relative to control, correlating with histopathology findings in the kidney and liver. Correspondingly, elevated levels of hepatic enzymes alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase were found. Thus, the established upper intake level of NMN for a 60 kg individual was 900 mg per day (82).

The first clinical trial reported that a single oral dose of NMN (from 100 to 500 mg) was safely and effectively metabolized in healthy subjects. It showed a significant increase of metabolites in plasma in a dose-dependent manner, without causing adverse effects or any significant clinical symptoms or changes in heart rate, blood pressure, oxygen saturation, and body temperature (40). However, NMN was not detected in plasma, most likely because the sample freezing before extraction may have caused NMN degradation (40). It has been reported that NMN degraded very rapidly in blood at -80°C (83).

In healthy subjects, NMN (250 mg/day for 12 weeks) elevated NAD+ levels in whole blood and no obvious adverse effects, no abnormalities in physiological and clinical laboratory tests were observed (84). Another study involving healthy subjects aged between 40 and 65 years found that oral NMN supplementation (300 mg) for 60 days was also well-tolerated, with no observed deleterious effects. Although the difference between the NMN and placebo groups was not statistically significant, NAD+/NADH levels in the serum increased by 38% (from 6.57 pmol/ml baseline to 9.07 pmol/ml at the end of the study) (85).

Orally administered NMN was rapidly synthesized into NAD+ in mouse tissues. After oral gavage (300 mg/kg body weight), NMN was quickly absorbed from the intestine into the bloodstream and eliminated from circulation within 15 min, with a consistent increase in hepatic NAD+ levels at 15 to 30 min (1). Suggesting plasma NMN levels may also be kept low and constant due to balanced NMN distribution from plasma to tissues.

Oral NR increased NAD+ concentrations >2-fold (NR 47.75 μM *versus* placebo 20.90 μM), NAAD by 4.5-fold, and NMN by 1.4-fold, without causing NAM increases in blood. NAM removal pathways were highly active after NR, with an excess of MeNAM, Me-2py, and Me-4py, and about a 20-fold increase of NAR in urine (29).

Martens et al. (2018) identified around a 5-fold increase in NAAD levels in the blood of healthy middle-aged men and women supplemented with NR (1g/day) for 6 weeks (34). In another study, NAAD was not detected in human blood prior to NR supplementation, while there was a ∼2,900% increase in NAAD baseline in healthy individuals taking single doses of up to 1g NR (100, 300, and 1,000 mg) (63) . Thus, confirming that NAAD is a product of NR utilization in humans and a biomarker of increased NAD+ metabolism. Which corroborates with findings in mice that the metabolic NR deamidated Preiss–Handler pathway is predominant (64).

NMN exerted a pronounced effect on skeletal muscle biology, with a significant increase in differentially-expressed genes of platelet-derived growth factor binding pathway compared placebo group (38). Although muscle NAD+ content did not change after 10 weeks, NMN treatment increased muscle metabolites N1-methylnicotinamide (MeNAM), N1-methyl-2-pyridone-5-carboxamide (Me-2py), and N1-methyl-4-pyridone-5-carboxamide (Me-4py). Irie et al. (2020) also observed the raising of these metabolites after oral NMN supplementation, suggesting increased muscle NAD+ turnover by NMN (40).

5. Preventive and therapeutic effects of NMN and NR

Historically, the NAD biosynthesis precursors tryptophan, NA, and NAM have been used to prevent and treat pellagra, a disease characterized by darkly pigmented skin eruptions causing the so-called three D's (dermatitis, diarrheal, and dementia). Tryptophan alone is not sufficient at sustaining cellular levels of NAD+ (86) as it takes part in other metabolic pathways, such as serotonin, melatonin, and picolinic acid synthesis. NA at pharmacological doses reduced serum cholesterol levels in humans, however, its use is limited due to the side effect causing skin flushing, accompanied by an unpleasant warmth sensation, and often itching (87).

Although NAM has been shown to be effective to increase NAD+ in tissues, it also exerts an inhibitory effect on sirtuin (88) and PARPs activity (89). At high doses, NAM results in increased methylation to generate N1-methylnicotinamide (MeNAM) via nicotinamide N-methyltransferase (NNMT) for elimination in urine, which can lower the cellular methyl pool over time (89). This could lead to a reduction in DNA and protein methylation, and as consequence, alter gene expression patterns and protein activity, once that DNA methylation in gene promoter regions is typically associated with transcriptional repression.

Furthermore, blood and urine pharmacokinetic data performed in rats suggested that NMN administered intraperitoneally appears to be retained in the body longer than NAM (90). It is still unclear why NR administration increases NAD+ levels more than NAM and NA treatment (63), while NMN and NAM administration showed no difference in NAD levels in blood and liver (90). Further studies with direct comparisons of all precursors at the same dose are important to elucidate the superiority purpose of each one.

Proposed as a nutraceutical to prevent age-related physiological decline (1,91), NMN has shown to improve diabetes (13,20,38) and Alzheimer's disease (22,23), enhance aerobic capacity (92) and exhibit cardio- and vasoprotective actions. It inhibits inflammation and decreases oxidative stress, preventing arterial and endothelial dysfunction (32,93), and protecting against heart failure (94), ischemia, and reperfusion (33).

NR demonstrated protective effects on cognitive function, synaptic plasticity, learning, memory, and motor function in Alzheimer’s disease models (24,27). It reduced the beta-amyloid levels, increasing the chance of recognizing a novel object by 20% in NR-treated Tg2576 mice (27). Futhermore, NR significantly reduced neuroinflammation,

apoptosis of hippocampal neurons, phosphorylated Tau and DNA damage, while also increasing neurogenesis by almost 20% (24).

Additionally, NR has been shown to prevent and improve metabolic disorders such as high-fat diet- induced obesity and non-alcoholic fatty liver disease (NAFLD). It promoted metabolic flexibility by enhancing energy expenditure, leading to a significant attenuation of high-fat diet-induced body weight gain and a decrease in fat mass, particularly in the liver, where triglyceride levels decreased by 40% (95). NR increased hepatic β-oxidation and mitochondrial content reversing glucose intolerance, insulin resistance, liver lipid accumulation, and hepatic fibrosis (21).

Although it may have other as yet unraveled mechanisms, the beneficial effects of NMN and NR have been attributed mainly to increase NAD+ levels enabling the sirtuins activities and their underlying targets.

5.1. Anti-aging effect

The classification of aging as a disease or a natural and universal process is seen as controversial, however it is well known the fact that a decline in cellular functions is part of the aging process.

It is recognized the decrease of NAD+ levels in various tissues during aging (11,13,96,97), playing a critical role in the pathophysiology of several diseases, including age-associated metabolic disorders, neurodegenerative diseases, and mental disorders (14). A significant cause for this age-associated NAD+ decline is the decrease in NAMPT-mediated biosynthesis. The NAMPT (nicotinamide phosphoribosyltransferase) protein and NAD+ levels decreased significantly in organs, such as white adipose tissue and skeletal muscle in old mice (13) affecting the activity of NAD+-dependent enzymes such as sirtuins (15,98). As consequence, affect the balance of redox reactions in the cell, leading to functional decline (14).

Another responsible for the low NAD+ levels is the ectoenzyme CD38. The levels and activity of CD38 increase with aging and are necessary for NAD decline and mitochondrial dysfunction (99). It was also found that CD38 degraded NMN altering the pharmacokinetics of both NMN and NR (99). Thus, CD38 regulates the levels of NAD+, NMN, and additionally to SIRT1 activity, playing a critical role in the onset of age-related diseases. And indeed, CD38 is a candidate molecule for regulating neurodegeneration. CD38

knockdown in mice suppressed axon degeneration, demyelination, and immune cell infiltration after facial nerve axotomy compared to wild-type mice. Correspondingly, intraperitoneal injection of NR (400 mg/kg) once daily for one week prior to facial nerve axotomy, delayed axon degeneration, and demyelination, increasing significantly facial nucleus and facial nerve NAD+ levels in wild-type mice (100).

During the aging process, DNA damage accumulates in the cell nucleus causing PARPs activation and reducing both NAD+ levels and SIRT1 activity (15). SIRT1 is one of the sirtuins (SIRT1-7) that use NAD+ as a substrate catalyzing the reaction of deacetylation or mono-ADP-ribosylation of proteins. In addition, accumulated DNA damage in chromosomal regions leads to a progressive and cumulative loss of protective telomere sequences at the chromosome end (101). Short telomeres showed decreased expression and activity of sirtuins and marked mitochondrial dysfunction.

NMN promoted telomere lengthening in aged mice (36), pre-aging mice and humans (37). Treatment with NMN (dissolved in 5 mM drinking water for 8 weeks) promoted telomere lengthening in generation 4 (G4) mice, dampened the DNA damage response and p53, functionally rescued liver fibrosis, and improved the mitochondrial biogenesis and function in a partially SIRT1-dependent manner (36). The increased NAD+ levels in the liver tissue of NMN-treated G4 mice reversed the hyperacetylation of several sirtuin targets indicating the reinforcement of sirtuin activity (36). SIRT1 increases mitochondrial activity and biogenesis by deacetylating and activating PGC1α (peroxisome proliferator-activated receptor-γ co-activator 1α) (102). PGC1α is a transcriptional co-regulator of mitochondrial genes and detoxifies enzymes that eliminate ROS (reactive oxygen species), leading to metabolism improvement and antioxidant protection (103).

Mitochondria are a vital organelle for the body which from food produces most of the energy used by cells. Almost all metabolic processes depend on NAD+, consequently, the maintenance of mitochondrial NAD+ levels is crucial for cell survival (104). As we age, mitochondria become increasingly dysfunctional, with defective mitophagy and bioenergetic capabilities which end up producing excess free radicals inside the cell, damaging the global cellular environment (101). A long-term NMN administration (given in drinking water ad libitum at either 100 or 300 mg/kg/day for 12 months) increased mitochondrial respiratory ability in skeletal muscle and reversed age-associated gene expression changes in a tissue-specific manner (1). In addition, the orally administered NMN was quickly absorbed, transported into blood circulation, and immediately converted to NAD+ in major metabolic

tissues such as the liver and soleus muscle, improving energy expenditure, oxygen consumption, insulin sensitivity, lipid plasma profile, eye function, bone density, and myeloid-lymphoid composition in aged mice (1).

Other animal and human studies of the NR and NMN supplementation, including anti-aging effects, are summarized in Table 1.

Table 1. Effects of NMN and NR using in preclinical and clinical studies.

NAD+ Precursor	Experimental model	Treatment	Outcomes	Reference
NMN	Cerebromicrovascular endothelial cells (CMVECs) isolated from 3- and 24-month-old male F344xBN rats	Cultured primary CMVECs were treated NMN (5×10^{-4} mol/L) for 1 to 5 days	Restoration of angiogenic capacity (formation of capillary-like structures, proliferative and migratory capability) and attenuation of oxidative stress in aged CMVECs.	(140)
	Young (3 months) and aged (24 months) male C57BL/6 mice	Intraperitoneal injections of 500 mg NMN/kg body weight per day for 14 days	Reverse the aging-induced cerebrovascular endothelial dysfunction. Rescued the neurovascular coupling responses associated with an improved cognitive performance by increasing endothelial NO-mediated vasodilation.	(141)
	Male Sprague–Dawley rats (12 weeks old)	Intraperitoneal administration of 100 mg/kg on alternate days for a period of 3 months	Prevented neuronal loss and rescued the memory deficits in diabetic rats. Increased brain NAD+ levels, normalized the diabetes-induced decrease in both SIRT1 and PGC-1α, preserving protein deacetylation, and hippocampal biochemical and mitochondrial respiration.	(26)
	12 and 14-month-old females C57BL6/JAusb mice	2 g/L in drinking water for 4 weeks	Increased NAD(P)H levels and rejuvenated oocyte quality, leading to fertility restoration and reversal of the adverse effect of maternal age on embryo development.	(142)

	Model of isoproterenol-induced cardiac fibrosis in male C57/B6 mice (8–10 weeks old)	Intraperitoneal injections of 500 mg/kg every 3 days before and after isoproterenol injection	Prevention of cardiac dysfunction and attenuation of cardiac hypertrophy. The NAD+ levels and SIRT1 activity were restored, inhibiting oxidative stress and Smad3 acetylation.	(143)
	Model aging mice (male C57BL/6 mice 24-month-old)	Intraperitoneal injections of 500 mg NMN/kg body weight per day for 2 weeks	Anti-aging changes in pro-inflammatory, and pro-atherogenic miRNA expression profile in the aorta. Rescue of vascular function and attenuation of oxidative stress.	(144)
	24-month-old C57BL/6 mice	Intraperitoneal injections of 500 mg/kg body weight per day for 2 weeks	Reversion of age-related changes in neurovascular mRNA expression profile, leading to the rescue of youthful neurovascular phenotype and to the improvement of cerebromicrovascular endothelial function. Induction of genes involved in mitochondrial rejuvenation, anti-inflammatory, and anti-apoptotic effects, such as SIRT1-mediated upregulation of PGC-1α, FOXO3- and FOXO4-	(145)
	Eight healthy men 45–60 years old	Oral NMN (300 mg/day) after 30 min of breakfast for 90 days	Elongating telomere length in peripheral blood mononuclear cells (PBMC)	
NR	Humans 10 twin pairs	Escalating dose of NR supplementation (250 to 1000 mg/day) for 5 months	DNA methylation and modulation epigenetic control of gene expression in muscle and adipose tissue. Reprogramming of tissue NAD+ and mitochondrial	(47)

		metabolism and muscle stem cell identity.	
Alzheimer's disease mouse model	NR treatment (12 mM given in their drinking water for 3 months before the tests)	It normalized reduced cerebral NAD+/NADH ratio, lessened phosphorylated Tau, DNA damage, neuroinflammation, and apoptosis of hippocampal neurons.	(24)
8-week-old specific pathogen-free male C57BL/6J mice	Gavage of NR (400 mg/kg body weight/day) + 50% (v/v) ethanol	NR alleviated the alcohol-induced liver injury. It inhibited the activation of the PP1 pathway, improving serum and liver triglyceride levels and lipid accumulation. Also, NR intervention changed the gut microflora structure and restored the abundance of gut microflora to a level similar to those in normal mice (control). It restored the reduction of bile acid levels in mice feces induced by alcohol exposure, which was correlated with gut microflora.	(8)
C57BL/6J and Fndc5 knockout (*Fndc5*$^{-/-}$) mice with non-alcoholic fatty liver disease (NAFLD) induced by high-fat or methionine/choline-deficient diet	Diet of pellets with NR 400 mg/kg/day for 12-16 weeks. Or intraperitoneal injections 400 mg/kg/day during 2 weeks	Reversion of NAFLD by regulating SIRT2-deppendent Fndc5 deacetylation and deubiquitination, which stimulates the "exerkine" Fndc5/irisin.	(146)
4-week-old male C57BLKS/J *db/db* mice (transgenic diabetic model) and age-matched	NR-supplemented food (approximately 400 mg/kg/day) for 12	Accelerated diabetic wound healing and angiogenesis. Reversion of the reduced NAD concentration in BM-EPCs. It raised	(147)

C57BL/6J mice (control).	weeks	the number of EPCs and elevated the tube formation and adhesion ability of BM-EPCs *in vitro*. NR upregulated Sirt1 expression modulating acetylated PGC-1α expression, and increased p-AMPK/AMPK and VEGF. Furthermore, prevented the accumulation of subcutaneous fat and serum insulin and increasing serum adiponectin levels.	
6-week-old male *Balb/c* mice for C26 Adenocarcinoma–induced cancer cachexia model	Diet supplemented with NR at 200 or 400 mg/kg daily for 3 weeks	Prevention C26 adenocarcinoma–induced muscle atrophy and weight loss. It restored cachexia-induced fat loss, reverting the epididymal lipolysis and inhibiting the adipose triglyceride lipase gene. NR diet decreased the cytokines TNF-α and IL-6. Increased SIRT1 and mitogen-activated protein kinases (ERK1/2 and JNK) were inactivated. Also, it inhibited muscle-specific ubiquitin-proteasome ligases, such as *atrogin-1* and *MuRF-1*. Genes implicated in muscle atrophy and degradation, Pax7 and mitofusin-2 respectively, were attenuated. PCG-1α, a marker for muscle regeneration, was restored.	(148)
15-months-old male C57BL/6J mice	Chow supplemented to provide NR at 300 mg or 600 mg/kg/day for 4 weeks	Enhancement of treadmill endurance and open-field activity in middle-aged mice. NR increased the size of aerobic muscle fibers, enlarging the slow-twitch fibers. In addition, it boosted aerobic and	(149)

	anaerobic, basal and maximal respiration of both mice- and human-derived myogenic progenitors *in vitro.* The differentiation of human myogenic progenitors toward multinucleated skeletal muscle myotubes was improved along with greater myofiber size, fusion index, and expression of differentiation markers.

5.2. Anti-COVID-19 effect

Age is also a risk factor for patients with COVID-19, increasing the chances of severe disease and death (105). The reason is that the aging process leads to several changes, especially immunosenescence and inflammaging that compromise the immune response. The shortening of telomeres and decrease in naïve lymphocytes leading to impaired immune function and a rise in circulating pro-inflammatory cytokines are factors contributing to the worsening of COVID-19 cases (105,106).

Evidence points to the potential relevance of NAD+ in modulating COVID-19 disease outcomes, the pandemic public health outbreak. During SARS-CoV-2 infection the set of genes related to NAD+ synthesis and use is dysregulated (16), potentially due to the increased demand the NAD+ metabolic pathways.

PARPs are known to play a critical antiviral role in inhibiting the translation of transcripts through ADP-Ribosylation in the viral genome, with NAD+ requirement. However, several viral families, including SARS-CoV, encode a macrodomain protein that hydrolyses the ADPR units of proteins and nucleic acids, inhibiting PARPs protective effect and then facilitating replication and virulence. Consequently, excessive activation of PARPs occurs to compensate for ADPR hydrolysis, accompanied by NAD+ consumption, suggesting that increasing NAD+ levels may restore the antiviral functions of PARPs to support immunity to SARS-CoV-2 (16,106).

In addition to the role of PARPS on immune responses, sirtuins can also coordinate the intensity of inflammatory responses, preventing the effects of cytokine storms. SIRT1, SIRT2, and SIRT3 all suppress the activity of NF-κB (factor nuclear kappa B) and the NLRP3 (Nod-like receptor family protein 3) inflammasome via multiple mechanisms (107). SIRT6 attenuates NF-κB signaling via histone H3 lysine 9 (H3K9) deacetylation at chromatin of NF-κB target gene promoters, which are related to apoptosis, and cellular senescence (108).

In a case study of 10 critically-ill patients over 50, the NMN cocktail (83 mL of NMN mixed with 400 mL of water, consumed before breakfast and dinner) was strongly associated with Covid-19 symptom resolution. Before treatment, patients had low oxygen saturation, pulmonary infiltrates, and inflammation. Post-treatment, there was rapid improvement in bilateral pulmonary infiltrates, fever resolution, and lower inflammation biomarkers. However, one patient stopped the treatment after three days due to

miscommunication, and experienced a relapse with fever and pulmonary infiltrates eight days later (17). The rapid and effective clinical improvement suggests NMN may play a role in reversing the potentially fatal cytokine storm triggered by SARS-CoV-2 infection.

A study showed that combined metabolic activators (CMAs), consisting of NR (1g), l-carnitine tartrate (3.73g), N-acetylcysteine (2.55g), and serine (12.35g), improved COVID-19 outcomes. Patients took the mixture orally one dose in the morning after breakfast and one dose in the evening after dinner for 14 days. In both a placebo-controlled, open-label phase 2 study and double-blinded phase 3 clinical trials, patients given CMAs had significantly faster complete recovery times compared to the placebo group (6.6 vs 9.3 days and 5.7 vs 9.2 days, respectively). CMAs were found to reinforce immune response and regulate amino acid and lipid metabolism. Moreover, patients treated with CMAs showed significant improvements in plasma levels of several inflammation and antioxidant metabolism-related biomarkers, such as alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatinine, glucose, and proteins (109).

It was found that alterations in the purinergic metabolism contribute to immune dysregulation during COVID-19, possibly contributing to disease severity. Unvaccinated severe COVID-19 patients presented higher ATP levels and lower levels of adenosine in plasma when compared to healthy controls. Ectonucleotidases CD39 and CD73, responsible for ATP cleavage to adenosine had reduced expression in severe Covid-19 patients' blood. Besides the impaired generation from ATP, the adenosine receptors were also less expressed, in a disease severity-dependent manner (110). Since adenosine and its receptors act on neutrophil and monocyte/macrophage suppressing cytokine production (111), an unbalanced ratio ATP/adenosine disturbs this anti-inflammatory regulation. In addition, in vitro administration of exogenous adenosine prevented inflammatory responses in the leukocytes of patients (110). Whether this alteration in the metabolism of ATP is a cause or effect of the exacerbated inflammatory response to SARS-CoV-2 still needs to be explored. Given the function of NAD+ in ATP generation and the role of both extracellular NAD+ and ATP in the regulation of inflammation and immune response (112), it is worth investigating the possible involvement of NAD+ in this altered ATP metabolism during infection by SARS-CoV-2.

5.3. Anti-inflammatory effect

Boosting NAD+ can reduce inflammation. Consistent with NR's known role in increasing NAD+ levels and consequently SIRT3 activation, the 24-hour administration of NR (amount not described) to PBMCs from healthy subjects replicated the fasting effect of blunting NLRP3 inflammasome activation and enhancing mitochondrial quality control through SIRT3 (30). NR reduced acetylation of SOD2 and isocitrate dehydrogenase 2, while increasing mitochondrial SOD2 activity, thereby reducing mitochondrial ROS levels. Additionally, NR decreased Interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) secretion in monocytes and macrophages from healthy volunteers, with the most significant reductions in monocytes of over 50% for IL-1β (from approximately 22,000 pg/mL to 10,000 pg/mL) and over 75% for TNF-α (from above 2,000 pg/mL to less than 500 pg/mL). In macrophages, the most pronounced reduction was around 20% for IL-1β (from above 300 pg/mL to less than 250 pg/mL) and approximately 45% in TNF-α levels (from around 1,800 pg/mL to 1,000 pg/mL) (30).

In older men, supplementing with 1g of oral NR per day for three weeks increased NAD+ metabolome levels in whole blood and skeletal muscle, and significantly reduced circulating levels of the inflammatory cytokines IL-2, IL-5, IL-6, and TNF-α compared to baseline (from approximately 20 pg/ml to 5 pg/ml for interleukins and from around 250 pg/ml to 200 pg/ml for TNF-α) (29).

In addition to increasing the cellular NAD+ level decreased, treatment with NMN (500 μM) decreased pro-inflammatory cytokines production in LPS-activated mouse and human macrophage cell lines (THP-1, RAW264.7). NMN efficiently alleviated LPS-induced inflammation and oxidative stress via the COX-2-PGE2 axis and inhibition of inflammation-related pathways. Proteomics analysis identified that NMN downregulated the expression of cyclooxygenase-2 (COX-2) and markedly decreased mRNA expressions and extracellular secretion of IL-6 and IL-1β, and the cellular levels of prostaglandin E2 (PGE2). Proteins related to inflammatory response such as RELL1, PTGS2, FGA, FGB, and igkv12-44 were all decreased in LPS/NMN co-treated cells as compared with LPS-treated cells. NMN treatment also suppressed other inflammation-associated pathways such as prostanoid biosynthesis, LPS/IL-1 mediated inhibition of RXR function, IL-6 signaling and NF-κB signaling (31).

5.4. Regulation of energy metabolism

NAD+ functions as a coenzyme in various redox reactions in the major energy production pathways, such as glycolysis, tricarboxylic acid (TCA) cycle, and fatty acid oxidation (113). Declining NAD+ levels is a signature of an imbalance in energy homeostasis (114), which makes it an energy-sensing metabolite. Energy-sensing pathways are important for maintaining an adequate balance between energy production and expenditure. Disturbance of these pathways results in various metabolic disorders, such as insulin resistance and fatty liver (113).

Under disturbed nutrient conditions, such as high-fat and -protein intake, NAD+ levels decreases (12,13). When an excess calorie is consumed, a low AMP/ATP ratio can cause a decrease in NAD+ or NAD+/NADH ratio. The adenosine monophosphate-activated protein kinase (AMPK) is a cellular energy regulator which senses changes in the intracellular AMP/ATP ratio. During energy limitation, low levels of ATP activate AMPK, which acts to maintain cellular energy stores. AMPK switches on catabolic pathways that produce ATP, mostly by enhancing oxidative metabolism and mitochondrial biogenesis, while switching off anabolic pathways that consume ATP, altering the NAD+/NADH ratio. Thus, AMPK enhances the SIRT1 activity by increasing cellular NAD+ levels, resulting in the deacetylation and modulation of the activity of energy metabolism-related downstream SIRT1 targets, which include the PGC1α and the forkhead box O1 (FOXO1) and O3 (FOXO3a) transcription factors (102).

Correspondingly, NR or NMN administration can prevent the reduction in NAD levels. NR (450 mg/kg body weight for 45 days) stabilized myocardial NAD+ levels, increased glycolysis, and citrate and acetyl-coenzyme A metabolism, attenuating the development of heart failure in a mouse model with dilated cardiomyopathy (35). By increasing NAD+ levels in mice's liver and muscle, NR supplementation (400 mg/kg/day) stimulated SIRT1 and SIRT3 activity, enhancing the mitochondrial function, oxidative metabolism, energy expenditure and endurance performance (95). In addition, NR protected against the detrimental metabolic effects of a high-fat diet, including hyperinsulinemia, elevated levels of total and LDL cholesterol, and weight gain, although it had no effect on body weight when given with a normal chow diet (95).

NMN also demonstrated no benefic effect on glucose metabolism in mice consuming a chow diet. Male offspring of both lean and obese progenitress mice, fed either

chow or high-fat diet (HFD) for 30 weeks post-weaning were intraperitoneally injected with NMN (500 mg/kg body weight) for 21 days. These mice had reduced adiposity, and hepatic and plasma triglyceride levels. Additionally, there was a reduction of hepatic genes involved in fat synthesis, transport, and uptake, while the genes involved in fatty acid oxidation were increased by NMN in offspring consuming HFD. However, in relation to glucose tolerance, NMN was beneficial only for the most metabolically compromised mice group (feed HFD and from obese progenitress). In addition, NMN impaired glucose tolerance in mice from lean progenitress consuming chow and had no additional impact in offspring consuming HFD (115). In another study, NMN supplementation (400 mg/kg for 8 weeks) reduced the exercise-induced benefits in obese mice such as hepatic triglyceride accumulation reduction, glucose-stimulated insulin secretion from islets, and glucose tolerance. Although, NMN on its own significantly down-regulated TNF-α and Tlr4 expression and up-regulated expression of PGC-1α in islets supporting its antioxidative effects, the association of NMN and exercise enhanced the ratio of antioxidants to prooxidants, deregulating redox homeostasis (116).

These results suggested that supplementation with NAD+ precursors may be less effective in conditions where the NAD+ levels are balanced.

5.5. Anti-diabetic effect

The hallmark hyperglycemia of diabetes mellitus causes a redox imbalance between NAD+ and NADH ratio, which can lead to oxidative stress and a variety of metabolic syndromes. For cells whose glucose uptake is not dependent on insulin, glucose oversupply can lead to NADH overproduction by both the conventional glucose combustion pathways (glycolysis and Krebs cycle) and the polyol pathway. This NAD+/NADH ratio imbalance, also known as pseudohypoxia, initially causes reductive stress, mitochondrial overload, and dysfunction, which lead to oxidative stress and oxidative damage to macromolecules, including DNA, lipids, and proteins. On the other hand, NAD+ can be diminished or depleted by the overactivation of PARPs that uses NAD+ as their substrate for DNA repair. Accordingly, redox imbalance might be a major factor contributing to the development of diabetic complications (117).

In addition, this redox change characterized by pseudohypoxia causes activation of two pathways: diacylglycerol (DAG)–protein kinase C (PKC) and (DAG)–protein kinase D (PKD) cascade, which impairs AMPK activity decreasing NAD+ synthesis. Another

suggestion could be the insufficient phosphoribosyl pyrophosphate (PPRP) synthesis from the pentose phosphate pathway which is compromised by a decrease in plasma thiamine and protein kinase B activity in diabetes. Protein kinase B is responsible for phosphorylating and activating transketolase to PPRP production, which will be used in NAD+ pathway synthesis (114).

Decreased NAD+ content and imbalanced mitochondrial dynamics occur in the development of diabetic cardiomyopathy. Daily oral gavage of NR 400 mg/kg/d for 4 weeks alleviated diabetes-induced cardiomyopathy improving cardiac function in diabetic mice. NR elevated myocardium NAD+ content and promoted Mitofusin 2-mediated mitochondrial fusion, suppressing oxidative stress and cell apoptosis via the SIRT1-PGC1α-PPARα pathway (19).

Chronic fructose feeding is known to cause suppression of eNAMPT secretion and pro-inflammatory cytokine-mediated islet dysfunction leading to impaired beta cell function and elevated blood glucose. In this case, NMN administration (100 μmol/l) restored insulin secretion in islets cultured with pro-inflammatory cytokines IL1β and TNFα, in a partially SIRT1-dependent manner. In addition, it corrected the inflammation-induced islet dysfunction, reversing the changes in the expression of genes encoding islet markers essential for glucose sensing and beta cell differentiation. Also by improving the glucose-stimulated insulin secretion in mice fed with a fructose-rich diet, NMN (500 mg/kg body weight administrated intraperitoneally 16 h prior to tissue sampling) may indicate an ability to protect against beta cell failure through an anti-inflammatory mechanism (20).

In another study, NMN demonstrated to be an effective intervention to treat the pathophysiology of type 2 diabetes. In both diet- and age-induced diabetic models, the administration of NMN via intraperitoneal (500 mg/kg body weight/day for 11days) ameliorated impaired glucose tolerance substantially and enhanced hepatic insulin sensitivity. The indicated reversing changes in gene expression related to oxidative stress, inflammatory response, and circadian rhythm was in part a result of SIRT1 activation (13). BESTO (beta cell-specific SIRT1-overexpressing) transgenic mice with advanced age had lost their phenotypes of glucose-stimulated insulin secretion and improved glucose tolerance as when they were young, despite maintaining SIRT1 overexpression in pancreatic β-cells. The NMN treatment (500 mg/kg body weight 14 h prior to performing the assay), besides increasing NAD+ levels that had decreased with age, was able to restore the phenotype in aged BESTO mice and also improved glucose-stimulated insulin secretion in aged wild-type mice (98).

Given the multifactorial nature of metabolic diseases, including diabetes, the mechanisms by which NAD+ precursors contribute to improved insulin sensitivity and glycemic control can be attributed to the replacement and/or increase in NAD+ levels and reversal of oxidative stress and inflammation.

In postmenopausal women with overweight or obesity and prediabetes, oral NMN supplementation (250 mg/day for 10 weeks) increased NAD+ content in peripheral blood mononuclear cells (PBMCs), and improved insulin signaling and sensitivity in skeletal muscle (38).

6. **Interplay between gut microbiota and NAD+ in host health**

We reviewed the background and the latest findings about the central function of NAD+ in maintaining cellular homeostasis. It is also already known the important physiological role of gut microbiota in the metabolic homeostasis, hormones secretion, and brain function of the host (118–121). In the same way, link between intestinal microorganisms and NAD+ metabolites suggests an important axis for beneficial physiological effects.

Some gut microbial can produce metabolites that exert a protective effect against various diseases (122–124). NMN derived from the gut microbiota ameliorated acute pancreatitis (AP) injury by increasing NAD+ levels and activating the SIRT3-PRDX5 pathway in AP mouse models (125). AP is exacerbated by gut microbiota dysbiosis, which is characterized by an imbalance between beneficial and pathogenic microbes that increases gut permeability, bacterial translocation, and systemic inflammatory responses. Besides reverting gut microbiota dysbiosis, the normobiotic fecal microbiota transplantation (FMT) induced higher levels of NMN in the serum, enhancing NAD+ biosynthesis in the pancreas. Similarly to FMT, pretreatment with NMN (500 mg/kg body weight/day intraperitoneally for 28 consecutive days) increased pancreatic NAD+ levels, mitigating AP-mediated mitochondrial dysfunction, oxidative damage, and inflammation in a partially SIRT3-dependent manner. These findings suggest that NMN is an important mediator of the protective effects of FMT in AP, and reinforces the potential role of gut microbiota on NAD+ metabolism and pathological disorders outcomes of the host.

In addition to gut microbiota activity on B vitamins (118,126) and NAD+ biosynthesis (44), recent evidence demonstrates the role of NAD+ and its precursors NR and NMN on the modulation of gut microbiota, which could be one of the underlying mechanism of beneficial effects observed. NAD+ precursors can modulate gut microbiota by providing an energy source for beneficial bacteria, activating sirtuins, and improving gut barrier function.

NMN and NR can selectively stimulate the growth of beneficial bacteria such as Bifidobacterium and Lactobacillus (46,47). These bacteria have been associated with improved gut health, including prevention of diarrhea, improved gut barrier function, reduced inflammation, and immune system support (127). Additionally, the activation of sirtuins by NAD+ precursors may also contribute to the beneficial effects on gut microbiota and intestinal health. Sirtuins are involved in regulating the gut microbiota (128,129) and in protecting intestinal endothelial cells against oxidative injury (130). Finally, NMN and NR can also improve intestinal barrier function (46,130), which is important for preventing the translocation of harmful bacteria and toxins from the gut into the bloodstream.

Besides changing the composition and functional profiles, the modulation of gut microbiota by NR was demonstrated to be protective against high-fat diet (HFD)-induced weight gain in mice (131). Mice fed with 60% HFD supplemented with 0.4% NR (for 168 days) had almost a 16% reduction in weight gain and a reduction in fasting blood glucose levels compared to the control supplemented with vehicle (water). Interestingly, the fecal transplant (FMT) from NR-treated donors to mice fed a HFD also attenuated the weight gain by decreasing energy efficiency. Both dietary NR supplementation and FMT-NR supplementation caused alteration of the intestinal microorganisms composition with a unique functional metabolic profile enriched of butyrate-producing *Firmicutes*. Even though some Firmicutes species have been associated with obesity (132), there are many species from the Firmicutes phylum that have probiotic and anti-obesity effects (133). For instance, *F. prausnitzii*, *Eubacterium rectale* and *Roseburia* produce short-chain fatty acids, including butyrate which has been shown to reduce adiposity by inhibiting intestinal cholesterol biosynthesis and promoting beneficial metabolic effects, such as insulin secretion, insulin sensitivity, and energy expenditure (133). Additionally, butyrate can increase satiety and gut mobility by upregulating the expression of peptide YY and Glucagon-like Peptide-1 (GLP-1) (133). Additionally, FMT-NR mice also exhibited a significant increase in NADH levels in the cecal contents, indicating altered microbial metabolism in the cecum (131).

And indeed NAD+ can directly affect the metabolism of intestinal microorganisms. Because NAD+ is an important component in the energy metabolism of all living organisms, mainly to be a cofactor for many key enzymes involved in the production of cellular ATP, boosting NAD+ may promote microbial energy production (134). Furthermore, NAD+ can indirectly affect gut microbes by regulating the movement of the colon through the key inhibitory neurotransmitter β-NAD, and by affecting the synthesis of host bile acids (45), which can be antimicrobial when they destroy the bacterial cell membrane (134).

Dietary supplementation of mice with NR for 10 weeks (400mg/kg daily) reduced both pro- and anti-inflammatory cytokine levels, inhibited microglial activation, and provided protection against alcohol-induced depression-like behaviors. This effect was attributed to NR's ability to upregulate brain-derived neurotrophic factor (BDNF) by altering gut microbiota composition (135). The mice model of alcohol-induced depression showed significant differences in intestinal microbiota diversity and composition compared to Control and NR groups. Notably, the model group was enriched with *Akkermansia* and *Clostridium XVIII*, while *Barnesiella* and *Alloprevotella* were dominant in the NR group. Brain inflammatory cytokines were positively correlated with model group-enriched bacteria, whereas brain BDNF levels were positively correlated with model group-deficient bacteria such as *Barnesiella*, *Alloprevotella*, *Prevotella*, *Alistipes*, *Mucispirillum*, and *Odoribacter*. Additionally, fecal microbiota transplantation (FMT) from alcohol-treated mice donors to germ-free recipient mice resulted in similar depression-like behaviors and alterations to microglial activation status, cytokines, and BDNF levels. Conversely, recipient mice of NR-treated donors had similar protective features to their donors (135).

In a clinical trial, NR slightly modulated gut microbiota composition in twins. The long-term NR supplementation (250-1000 mg/day for 5 months) increased the abundance of *Faecalibacterium prausnitzii* (47), which promotes metabolic health and anti-inflammatory responses (136,137). Patients with inflammatory bowel disease (IBD) have been associated with reduced bacterial diversity and a decline in butyrate-producing bacteria such as *Faecalibacterium prausnitzii,* indicating the importance of gut dysbiosis in IBD (138,139). A balanced ratio between the different commensal bacteria species is needed to maintaining the homeostasis intestinal and overall host health (132). Association analysis suggests that the abundance of *F. prausnitzii* may contribute to the regulation of NAD+, inflammation, amino acid, and lipid metabolism (47).

NMN also demonstrated protecting effect on IBD in an experimental mice model of colitis, reversing the intestinal dysbiosis (46). NMN was administered via gavage at a dose of 1 mg/g of body weight either at the same time for 21 days or after colitis induction with dextran sodium sulfate for the last 14 days. In addition to increasing microbial abundance and diversity, NMN improved the mucus secretion and tight-junction proteins' expression, which helped to attenuate intestinal mucosal permeability (46).

The long-term NMN treatment (0.1 to 0.6 mg/mL in drinking water for 12 weeks) modulated gut microbiota diversity and composition, exerting a protective effect on the intestinal tract of mice. Although NMN reduced the diversity of intestinal species, it increased the abundance of butyric acid-producing bacteria (*Ruminococcae*_UCG-014 and *Prevotellaceae*_NK3B31_group) and other probiotics such as *Akkermansia muciniphila*, while the abundance of several harmful bacteria (*Bilophila* and *Oscillibacter*) were decreased (45). In addition, NMN increased the level of bile acid-related metabolites and beneficial metabolites, the number of goblet cells, mucus thickness, and expression of tight junction protein, maintaining the integrity of the intestinal epithelium and reducing mucosal permeability (45). Niu et al. (2021) also found that NMN significantly reduced fecal bacterial diversity. The supplementation of NMN (500 mg/L (w/v) in drinking water for 40 days increased the abundance of *Helicobacter, Mucispirillum*, and *Faecalibacterium*, and lowered Proteobacteria and *Akkermansia* abundance in mice. This changed bacterial was correlated with the composition of serum metabolite constitutes, mainly involved in the purine, nicotinate/nicotinamide, and arginine/proline metabolism pathways (37).

Although NAD+ precursors has a potential modulatory function on gut microbiota, further research is needed to clarify their role in intestinal microorganisms, underlying host physiological effects, and the related mechanisms. Finally, whether boosting host NAD+ levels may promote beneficial intestinal bacteria and inhibit harmful bacteria also remain to be more explored.

7. Conclusion and Future Perspectives

The natural occurrence of NR and NMN in food, along with their potential beneficial effects for animals and humans, is opening up an exciting new area in food science and nutrition. Investigating their content in dietary sources as well as bioaccessibility certainly justifies the research effort.

The search for potential food sources of NMN and NR, from commonly consumed items to waste by-products, could provide insights for improved diets and the development of functional foods. Additionally, this may lead to the identification of new raw materials for the extraction, isolation, and biotechnological production of these compounds. It is also important to evaluate food processing strategies that can preserve and enhance the physiological benefits of NR and NMN.

The metabolism and biodistribution of NAD+ precursors in various tissues and within cells are still little understood. Further research is necessary to investigate the bioavailability, quantitative metabolomics, and pharmacokinetics of NMN and NR. These studies can help identify the optimal doses and contribution of each precursor in maintaining and boosting NAD+ levels, as well as their physiological effects through diet.

Finally, given the crucial role of gut microbiota in human health and the emerging evidence of intestinal microbes modulation by NMN and NR, there is a need to investigate the crosstalk between gut microbiota metabolism and NR and NMN. These studies can help identify the potential impact on health and offer insights into the underlying mechanisms.

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Statement of authors' contributions to manuscript

ALEGRE, GFS: designed research and wrote paper (Conceptualization, writing, review, and editing); PASTORE, GM: designed research (Conceptualization and supervision). All authors have read and approved the final manuscript.

Disclosure statement

The authors declare that they have no conflict of interest.

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In this article, the authors demonstrated the production of NR and NMN in craft beers using yeast-mediated fermentation, and the role of hop in enhancing NR levels during the process. This finding is of great interest to the fields of biotechnology and food science and technology, as it highlights the potential for utilizing fermentation processes to produce NAD+ precursors.

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CAPÍTULO II

The emerging importance of NAD+ metabolome for nutrition and food sciences: a bibliometric analysis

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Highlights

• Initial research of NAD + has focused on biochemistry and cellular biology fields.
• The most cited topic was related with sirtuins and lifespan extension.
• Current focus is on strategies targeting NAD + for reverting diseases.
• There are many unexplored topics related to NAD+ in nutrition and food sciences.
• NAD + metabolome holds promise for nutrition, food quality, and health improvements.

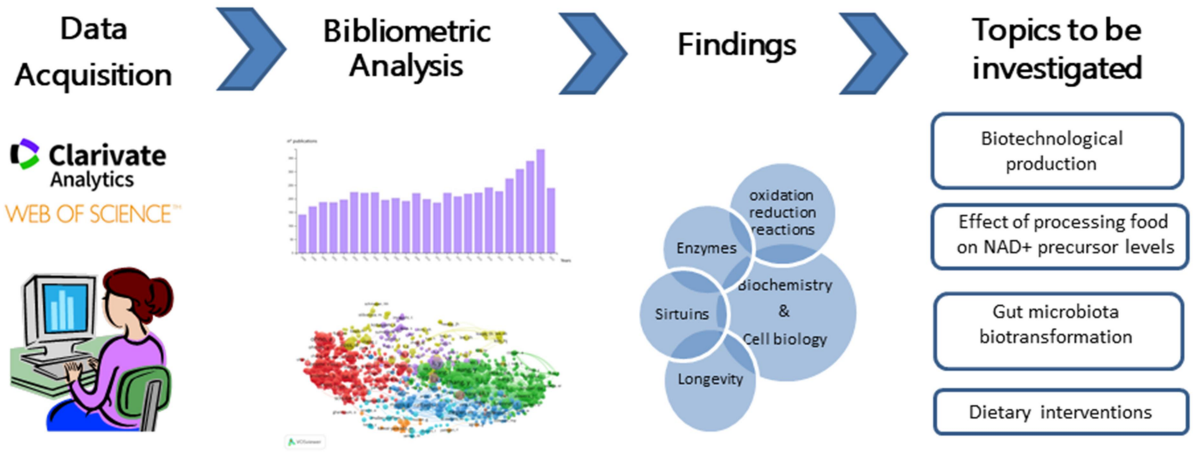
ABSTRACT

NAD+ is crucial for cellular balance in all organisms. Recent interest has focused on its metabolism. Deficiency-related issues can be countered using NMN and NR, potential therapies available through diet or supplementation. These compounds are emerging as nutraceuticals and bioactive food elements. Bibliometric analysis was used to study the scientific output and impact of research on the NAD+ metabolome. For this, the terms "nicotinamide adenine dinucleotide" or "NAD+" or "NAD+ precursors" or "nicotinamide riboside" or "nicotinamide mononucleotide" were searched in the topic title in The Web of Science™ database. Only original articles and articles review were considered. VOSviewer was used for constructing and visualizing the bibliometric networks maps. A total of 10,568 papers were published, with a consistent increase in the number of publications, especially over the last decade. Biochemistry molecular biology, biophysics, cell biology, chemistry multidisciplinary and microbiology were the top 5 field research in the subject. The majority of the publications came from the 'Journal of biological chemistry' and 'Biochemistry' journals. The prominent research topics included longevity, sirtuins, NAD+ precursors, oxidation and reduction reactions, enzymes, microbial biotechnology, cancer therapy, and crop science. Finally, a guide for future investigation in nutrition and food sciences areas was proposed, which holds immense potential for developing innovative strategies to enhance food quality, promote optimal nutrition, and explore new avenues for improving human health. To our knowledge, this is the first bibliometric analysis of NAD+ metabolome, which can provide valuable insights into the research landscape in this multidisciplinary rapidly growing field.

Keywords

NAD+; NAD+ precursors; nicotinamide riboside; nicotinamide mononucleotide; NMN; NR; Bibliometric analysis

Graphical abstract



List of abbreviations

ADPR - adenosine diphosphate ribose

ARTs - mono ADPR transferases

NAD+ - nicotinamide adenine dinucleotide

NADH - reduced form of NAD

NADP - nicotinamide adenine dinucleotide phosphate

NADPH - reduced form of NADP

NMN – nicotinamide mononucleotide

NR - nicotinamide riboside

NAMPT - nicotinamide phosphoribosyltransferase

PARPs - poly ADPR polimerases

SIRT1 – sirtuin 1, an isoform of sirtuins

1. INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) is a pyridine nucleotide found in two oxidation states: oxidized (NAD+) and reduced (NADH), which has a crucial role in maintaining cellular homeostasis across all organisms. It is involved in various vital processes including metabolism, mitochondrial function, inflammation, circadian rhythm, DNA repair, cell division, immune system regulation, signaling and transcriptional events (Chini et al., 2021; Houtkooper et al., 2010; Ummarino et al., 2021; Y. Yang & Sauve, 2016). This multifaceted role has led to a renewed interest in NAD+ metabolism in recent years (Covarrubias et al., 2021; Hosseini et al., 2019; Johnson & Imai, 2018; Xie et al., 2020; Y. Yang & Sauve, 2016).

Discovered more than a century ago as low molecular coferment or “cozymase” in fermentation (Harden & Young, 1906), NAD+ has been receiving abundant attention in researches, which has unraveled their pivotal functions. Since last two decades it changed from being an electron transfer cofactor for redox reactions for energy production, to acting as a rate-limiting substrate for many proteins involved in signaling pathways such as CD38/CD157, ARTs, PARPs, and sirtuins (Berger et al., 2004; Houtkooper et al., 2010), which regulates a large array of cellular functions.

CD38 and CD157 are multifunctional transmembrane glycoproteins which play dual roles as an ectoenzyme and as a regulator of the immune system (Partida-Sánchez et al., 2003; Quarona et al., 2013). They have both NAD glycohydrolase and ADPR cyclase activities to regulate NAD+ availability and to generate second messengers, such as cADPR a cyclic product of ADPR which contributes to calcium mobilization (Houtkooper et al., 2010).

ARTs post-translationally modify proteins by adding one or several ADPR portions to specific amino acids, altering their biological activity (Berger et al., 2004). This modification can inactivate proteins, mainly impacting immune response and inflammation (Fehr et al., 2020). PARPs synthesize ADPR polymers that accept transcription factors (Berger et al., 2004), regulating fundamental cellular events such as transcription, telomere elongation, DNA repair, genomic instability, apoptosis and cell-cycle regulation (Bürkle, 2001; Herceg & Wang, 2001). Although the overactivation of PARPs, caused by constant DNA damage for example, can lead to NAD+ depletion and consequent cell death (Alano et al., 2010).

Another group of NAD-dependent enzymes includes the sirtuins. Sirtuins act as deacetylases for histones and other proteins, either activating or suppressing their functions. They regulate chromatin, transcription factors, and gene expression, as well a variety of biological processes such as metabolism, circadian rhythm, genomic integrity, and cell survival (Carafa et al., 2016; Imai & Guarente, 2014). They remove nicotinamide from NAD and transfer the acetyl group from the lysine residues from the target protein to the ADPR part. This results in the formation of O-acetyl-ADP-ribose, nicotinamide, and a deacetylated substrate.

All these NAD+-dependent signaling pathways require a continuous supply of NAD+, which is maintained by five major precursors and intermediates: tryptophan, nicotinamide, nicotinic acid, nicotinamide riboside (NR), and nicotinamide mononucleotide (NMN) (Johnson & Imai, 2018). Defects in biosynthesis or increased depletion by redox reactions and signaling events contribute to a decline in NAD+ concentration. NAD+ levels decrease with aging (Clement et al., 2019; F. Yang et al., 2022), in the blood of cardiac and neurological patients (Balashova et al., 2022), with high-fat and high-protein intake (Seyedsadjadi et al., 2018; J. Yoshino et al., 2011), and are correlate with mitochondrial dysfunction (Gomes et al., 2013; H. Zhang et al., 2016), metabolic disorders (J. Yoshino et al., 2011), and immune impairments (Minhas et al., 2019). In addition to decreased levels, altered NAD+ metabolism occurs during Covid-19 infection (Brenner, 2022; Fehr et al., 2020; Heer et al., 2020). These findings suggest that NAD+ levels can serve as a clinical indicator of nutritional status, redox state, incidence and progression of age-related diseases (Zhu et al., 2015), and other pathologies.

However, NAD+ deficiency and its associated pathologies can be reverted by boosting NAD+ levels with NMN and NR (Mills et al., 2016; J. Yoshino et al., 2011; H. Zhang et al., 2016), which have been identified as potential therapeutic targets (Jiang et al., 2022; Park et al., 2021; Xie et al., 2020; Y. Yang & Sauve, 2016; J. Yoshino et al., 2018; M. Yoshino et al., 2021). These compounds can be obtained through dietary intake or supplementation, and are emerging as nutraceuticals (Imai, 2010) and new food bioactive compounds (Alegre & Pastore, 2023).

Bibliometric analysis is a quantitative method used to evaluate and measure the impact and relevance of scientific research publications. Studies using bibliometric analysis have grown over the years, for a variety of reasons, such as uncovering emerging trends in article and journal performance, exploring the intellectual structure of a specific domain in the

extant literature, research constituents, and collaboration patterns (Donthu et al., 2021). Therefore, this bibliometric study intends to provide an overview of the current state of scientific production related to NAD+ and their biosynthesis precursors NMN and NR, as well as a better understanding of the main contributors and their interactions in research. In addition, to knowledge of possible gaps, the current and main future areas of interest, suggesting promising novel ideas for investigation and contributions to the nutrition and food sciences field.

2. METHODOLOGY

The methodological approach employed involved bibliometrically tracking scientific articles from Clarivate's Web of Science™ Core Collection (WOS) database. The terms “nicotinamide adenine dinucleotide” or “NAD+” or “NAD+ precursors” or “nicotinamide riboside” or “nicotinamide mononucleotide” were searched in the topic ‘title’. Original articles and articles reviews published since the appearance of the first article in 1912 until the search date (August 30, 2022) were analyzed.

The descriptive analysis, including the total number of articles, the number of publications in open access, time of publication, areas of research, citations, authors, and countries/regions, was conducted using the Web of Science Core Collection website. This database offers numerous options to refine the search results. All data obtained were exported in plain text file (.txt) format, containing full record information and cited references, for the construction of bibliometric network analysis.

To visualize the bibliometric networks of authors, countries, and keywords, maps were created using the VOSviewer software (version 1.6.18, Centre for Science and Technology Studies (CWTS) of Leiden University). Network analysis of authors and international collaborations was conducted using the co-authorship analysis option, selecting the unit of analysis as ‘authors’ and ‘countries’, both without restrictions on the number of authors per document. For keyword mapping, the co-occurrence analysis option was employed with the unit 'all the keywords', and a minimum of 30 occurrences was defined. All the analyses were built using a full counting methodology. For more detailed instructions on how to use the VOSviewer software, a tutorial is provided in (van Eck & Waltman, 2014).

3. RESULTS

The total number of papers found was 10,568, of which 3,695 (~ 35%) were open access, 10,080 (95%) were research papers and 488 (5%) reviews. There was a significant increase in the number of publications over time, which more than doubled in the last 25 years, starting 142 in 1998 rising to 383 in 2021 (Figure 1).

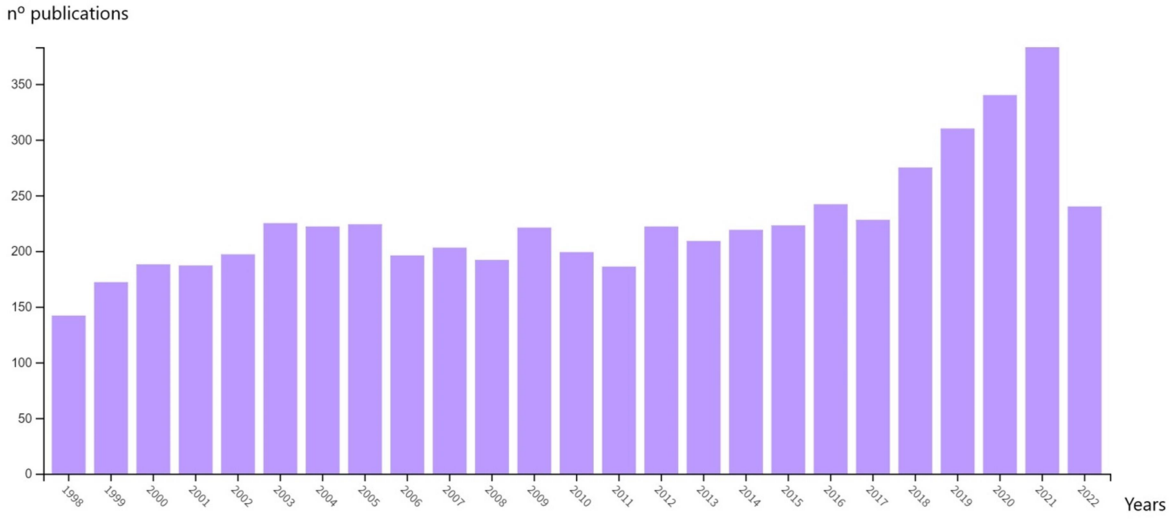


Figure 1. Evolution of scientific publications relating NAD+ subject over the last 25 years, until August 2022.

In relation to area of research, of the total 176 categories the Biochemistry Molecular Biology was the highest number of publications (4,247) representing 40% of total papers. The first 30 categories of journal areas and their publications count are described on Table 1.

Table 1. Ranking main 30 areas/field research of papers published related-NAD+ subject.

Collocation	Subject Area	Publications number	Percentage representative total papers (10,568)

1º	Biochemistry Molecular Biology	4,247	40.19%
2º	Biophysics	1,157	10.95%
3º	Cell Biology	891	8.43%
4º	Chemistry Multidisciplinary	608	5.75%
5º	Microbiology	591	5.59%
6º	Pharmacology Pharmacy	538	5.09%
7º	Multidisciplinary Sciences	524	4.96%
8º	Plant Sciences	511	4.84%
9º	Biotechnology Applied Microbiology	492	4.66%
10º	Chemistry Analytical	391	3.70%
11º	Neurosciences	339	3.21%
12º	Endocrinology Metabolism	326	3.09%
13º	Biochemical Research Methods	306	2.90%
14º	Oncology	306	2.90%
15º	Chemistry Organic	270	2.56%
16º	Medicine Research Experimental	246	2.33%
17º	Physiology	233	2.21%
18º	Chemistry Medicinal	229	2.17%
19º	Peripheral Vascular Disease	172	1.63%
20º	Chemistry Physical	168	1.59%
21º	Electrochemistry	168	1.59%
22º	Genetics Heredity	166	1.57%
23º	Toxicology	162	1.53%
24º	Cardiac Cardiovascular Systems	150	1.42%
25º	Biology	143	1.35%
26º	Immunology	138	1.31%
27º	Food Science Technology	136	1.29%
28º	Hematology	125	1.18%
29º	Nutrition Dietetics	123	1.16%
30º	Geriatrics Gerontology	84	0.795%

Considering the areas of interest in our study, the category of Food Science Technology had 136 published articles, the majority of which covered biochemistry, mainly regarding the enzymes involved in the NAD+ synthesis and metabolic pathways, and biotechnology, including microbial activity in the production and consumption of NAD+. The first article was published in 1968 which studied the substrate specificity and activity of the enzyme alcohol oxidoreductase dependent on NAD from pea (Eriksson, 1968). Among the most recent stands out the yeast-mediated NR production in craft beers and the role of hop in boosting the NR levels during fermentation (Garofalo et al., 2021), and the quantification of NAD+, NR and NMN in milk of different species (Ummarino et al., 2017). Among the most cited stands out the NAD+-dependent xylitol dehydrogenase purification from yeast *Pichia stipitis* (Rizzi et al., 1989) and about the NAD Kinase and NADP Phosphatase enzymes involved in regulation of oxidized and reduced forms of NAD and NADP.

In relation to Nutrition Dietetics area 123 articles were published (Table 1), which the oldest is dated 1965, describing the effect of dietary orotic acid on the hepatic concentrations of adenine, guanine, uracil nucleotides and NAD+ synthesis in rat (Windmueller, 1965). Between the most cited are an article review about the role molecular of NAD+ precursors B3 vitamin and NR in nutrition human (Bogan & Brenner, 2008) and a clinical trial of NR supplementation in obese men (Dollerup et al., 2018).

A total of 1929 journals published papers relating the NAD+, NMN and NR subject. The main 25 journals with the highest number of publications, their number of papers and impact factor are listed in table 2.

Table 2. The 25 top most productive journals in NAD+ metabolome and their impact factor range.

Ranking	Journal	Publications number	Percentage representative total papers (10,568)	Impact factor
1º	Journal of biological chemistry	614	5.810%	5.157
2º	Biochemistry	325	3.08%	3.321

3º	Biochimica et biophysica acta	251	2.38%	4.117
4º	Biochemical journal	229	2.17%	3.766
5º	Archives of biochemistry and biophysics	208	1.97%	4.114
6º	Biochemical and biophysical research communications	206	1.95%	3.322
7º	Journal of bacteriology	183	1.73%	3.476
8º	European journal of biochemistry	151	1.43%	*
9º	Febs letters	151	1.43%	3.864
10º	Plant physiology	123	1.16%	8.005
11º	Analytical biochemistry	97	0.92%	3.191
12º	Journal of the american chemical society	94	0.89%	16.383
13º	Biochemistry moscow	88	0.83%	2.824
14º	Proceedings of the national academy of sciences of the united states of america	88	0.83%	11.205
15º	PLoS ONE	80	0.76%	3.752
16º	Doklady akademii nauk SSSR	79	0.75%	*
17º	Scientific reports	67	0.63%	4.996
18º	Free radical biology and medicine	65	0.62%	8.101
19º	Biochemical pharmacology	63	0.60%	6.100
20º	Cancer research	62	0.59%	13.312
21º	Agricultural and biological chemistry	57	0.54%	*
22º	Journal of biochemistry	50	0.47%	3.241
23º	FEMS microbiology letters	49	0.46%	2.820
24º	Methods in enzymology	45	0.43%	1.682
25º	Plant and cell physiology	43	0.41%	4.937

* The journal no longer exists or the impact factor was not available.

The 25 publications with the highest number of citations are shown on Table 3. The oldest was in 1967 until newest in 2015.

Table 3. The top 25 most cited scientific papers relating to NAD+ subject.

Authors	Title	Type of document	Source	Number Citations
Imai, S; Armstrong, CM; Kaeberlein, M; Guarente, L	Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase	Article	Nature 403, 795–800 .(2000)	2,605
Griendling, KK; Sorescu, D; Ushio-Fukai, M	NAD(P)H oxidase - Role in cardiovascular biology and disease	Review	Circulation research, 86(5), 494-501. (2000)	2,412
Canto, C; Gerhart-Hines, Z; Feige, JN; Lagouge, M; Noriega, L; Milne, JC; Elliott, PJ; Puigserver, P; Auwerx, J	AMPK regulates energy expenditure by modulating NAD(+) metabolism and SIRT1 activity	Article	Nature, 458(7241), 1056-1060. (2009)	2,149
Vaziri, H; Dessain, SK; Eagon, EN; Imai, SI; Frye, RA; Pandita, TK; Guarente, L; Weinberg, RA	hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase	Article	Cell, 107(2), 149-159. (2001)	2,112
Williamson, Dh; Lund, P; Krebs, Ha	Redox state of free nicotinamide-adenine dinucleotide in cytoplasm and mitochondria of rat liver	Article	Biochemical Journal, 103(2), 514. (1967)	1,773
Lin, SJ; Defossez, PA; Guarente, L	Requirement of NAD and SIR2 for life-span extension by calorie	Article	Science, 289(5487), 2126-2128. (2000)	1,372

	restriction in Saccharomyces cerevisiae			
North, BJ; Marshall, BL; Borra, MT; Denu, JM; Verdin, E	The human Sir2 ortholog, SIRT2, is an NAD(+)-dependent tubulin deacetylase	Article	Molecular cell, 11(2), 437-444. (2003)	1,163
Inoguchi, T; Li, P; Umeda, F; Yu, HY; Kakimoto, M; Imamura, M; Aoki, T; Etoh, T; Hashimoto, T; Naruse, M; Sano, H; Utsumi, H; Nawata, H	High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells	Article	Diabetes, 49(11), 1939-1945. (2000)	1,157
Lee, IH; Cao, L; Mostoslavsky, R; Lombard, DB; Liu, J; Bruns, NE; Tsokos, M; Alt, FW; Finkel, T	A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy	Article	Proceedings of the National Academy of Sciences, 105(9), 3374-3379. (2008)	1,028
Nakahata, Y; Kaluzova, M; Grimaldi, B; Sahar, S; Hirayama, J; Chen, D; Guarente, LP; Sassone-Corsi, P	The NAD(+)-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control	Article	Cell, 134(2), 329-340. (2008)	976
Ying, WH	NAD(+)/ NADH and NADP(+)/NADPH in cellular	Review	Antioxidants & redox signaling, 10(2), 179-	929

	functions and cell death: Regulation and biological consequences		206. (2008)	
Venugopal, R; Jaiswal, AK	Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase(1) gene	Article	Proceedings of the National Academy of Sciences, 93(25), 14960-14965. (1996)	882
Du, JT; Zhou, YY; Su, XY; Yu, JJ; Khan, S; Jiang, H; Kim, J; Woo, J; Kim, JH; Choi, BH; He, B; Chen, W; Zhang, S; Cerione, RA; Auwerx, J; Hao, Q; Lin, HN	Sirt5 Is a NAD-Dependent Protein Lysine Demalonylase and Desuccinylase	Article	Science, 334(6057), 806-809. (2011)	862
Araki, T; Sasaki, Y; Milbrandt, J	Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration	Article	Science, 305(5686), 1010-1013. (2004)	850
Lassegue, B; Clempus, RE	Vascular NAD(P)H oxidases: specific features, expression, and regulation	Review	American Journal of Physiology-Regulatory, Integrative and	836

			Comparative Physiology, 285(2), R277-R297. (2003)	
Gomes, AP; Price, NL; Ling, AJY; Moslehi, JJ; Montgomery, MK; Rajman, L; White, JP; Teodoro, JS; Wrann, CD; Hubbard, BP; Mercken, EM; Palmeira, CM; de Cabo, R; Rolo, AP; Turner, N; Bell, EL; Sinclair, DA	Declining NAD(+) Induces a Pseudohypoxic State Disrupting Nuclear-Mitochondrial Communication during Aging	Article	Cell, 155(7), 1624-1638. (2013)	832
Nakahata, Y; Sahar, S; Astarita, G; Kaluzova, M; Sassone-Corsi, P	Circadian Control of the NAD(+) Salvage Pathway by CLOCK-SIRT1	Article	Science, 324(5927), 654-657. (2009)	828
Guzik, TJ; Mussa, S; Gastaldi, D; Sadowski, J; Ratnatunga, C; Pillai, R; Channon, KM	Mechanisms of increased vascular superoxide production in human diabetes mellitus Role of NAD(P)H oxidase and endothelial nitric oxide synthase	Article	Circulation, 105(14), 1656-1662. (2002)	804
Benson, AM; Hunkeler, MJ; Talalay, P	Increase of NAD(P)H-quinone reductase by dietary antioxidants - possible role in protection against	Article	Proceedings of the National Academy of Sciences, 77(9), 5216-	789

	carcinogenesis and toxicity		5220. (1980)	
Ramsey, KM; Yoshino, J; Brace, CS; Abrassart, D; Kobayashi, Y; Marcheva, B; Hong, HK; Chong, JL; Buhr, ED; Lee, C; Takahashi, JS; Imai, SI; Bass, J	Circadian Clock Feedback Cycle Through NAMPT-Mediated NAD(+) Biosynthesis	Article	Science, 324(5927), 651-654. (2009)	770
Canto, C; Menzies, KJ; Auwerx, J	NAD(+) Metabolism and the Control of Energy Homeostasis: A Balancing Act between Mitochondria and the Nucleus	Review	Cell metabolism, 22(1), 31-53. (2015)	761
Yoshino, J; Mills, KF; Yoon, MJ; Imai, SI	Nicotinamide Mononucleotide, a Key NAD(+) Intermediate, Treats the Pathophysiology of Diet- and Age-Induced Diabetes in Mice	Article	Cell metabolism, 14(4), 528-536. (2011)	728
Thompson, EA; Siiteri, PK	Utilization of oxygen and reduced nicotinamide adenine-dinucleotide phosphate by human placental microsomes during aromatization of androstenedione	Article	Journal of Biological Chemistry, 249(17), 5364-5372. (1974)	728
Yang, HY; Yang, T; Baur, JA;	Nutrient-sensitive mitochondrial	Article	Cell, 130(6), 1095-	721

Perez, E; Matsui, T; Carmona, JJ; Lamming, DW; Souza-Pinto, NC; Bohr, VA; Rosenzweig, A; de Cabo, R; Sauve, AA; Sinclair, DA	NAD(+) levels dictate cell survival		1107. (2007)	
Canto, C; Houtkooper, RH; Pirinen, E; Youn, DY; Oosterveer, MH; Cen, Y; Fernandez-Marcos, PJ; Yamamoto, H; Andreux, PA; Cettour-Rose, P; Gademann, K; Rinsch, C; Schoonjans, K; Sauve, AA; Auwerx, J	The NAD(+) Precursor Nicotinamide Riboside Enhances Oxidative Metabolism and Protects against High-Fat Diet-Induced Obesity	Article	Cell metabolism, 15(6), 838-847. (2012)	716

For bibliometric coupling of authors a minimum number of 5 documents per author was defined to construct a map, of which 1410 authors matched this criteria and are shown on figure 2. The 10 most contributors of total 29,514 authors recorded were: Atsuyoshi Ohno (81 papers published), Mathias Ziegler (50 papers published), Seibi Oka (49 papers published), David Ross (48 papers published), Nadia Raffaelli (45 papers published), Charles Brenner (44 papers published), Anil K. Jaiswal (44 papers published), Hsu-Hsin Tai (42 papers published), Francis Schuber F (40 papers published), and David Siegel (38 papers published).

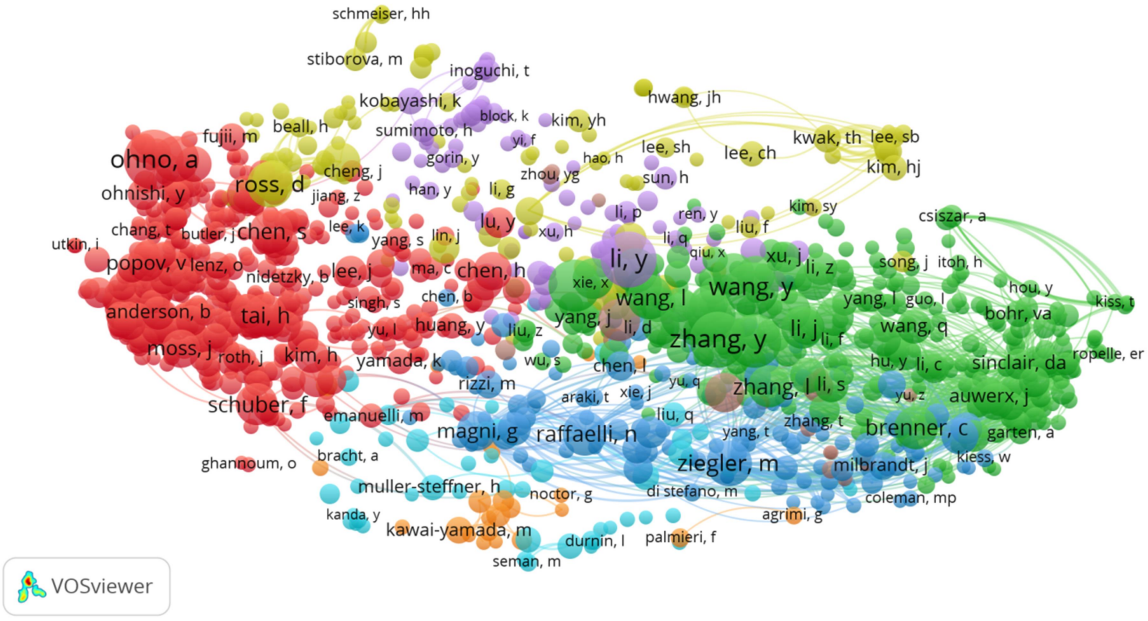


Figure 2. Network visualization map of authors in NAD+-related publications. The circle's size indicates the number of publications for the author and the lines link the network between each other.

In relation to countries contributors to NAD+ subject The United States of America (USA) was the leader in publications with 32.74% of total, adding to Japan and China accounted for 50% of all papers published. The 25 most productive countries on NAD+-related publications are listed in table 4.

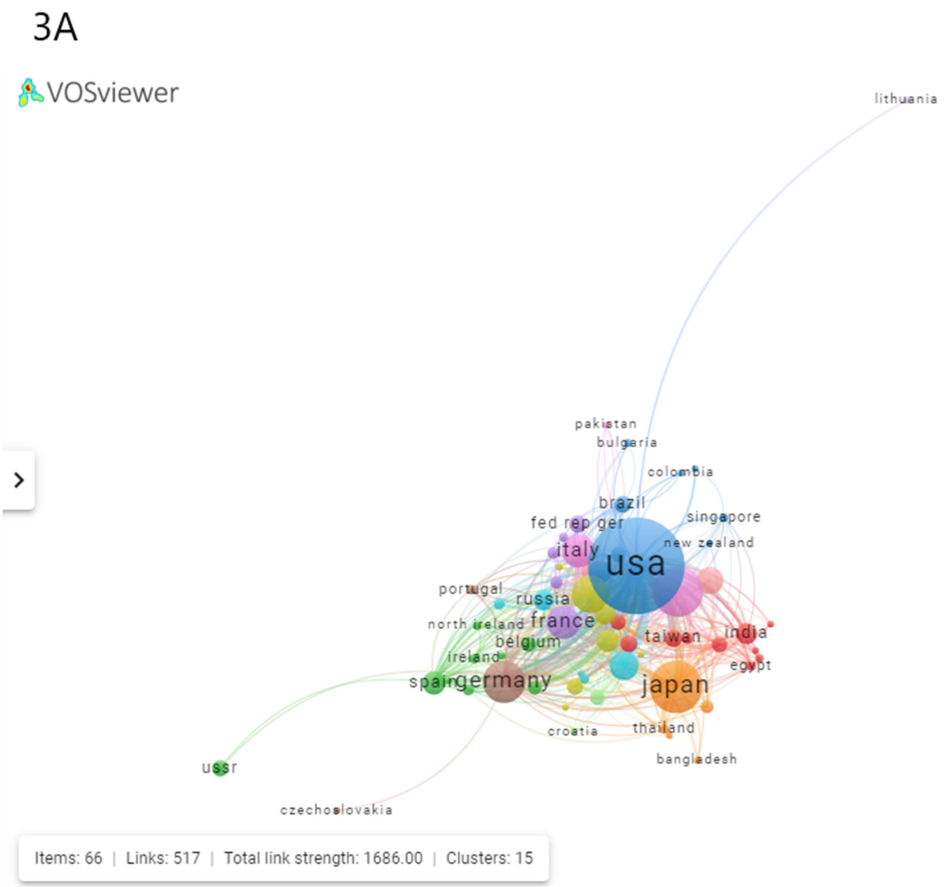
Table 4. Top 25 countries/regions in publications related to NAD+.

Ranking	Country/Region	Publications number	Percentage representative total papers (10, 568)
1º	USA	3,460	32.74%
2º	Japan	1,151	10.89%
3º	Peoples R China	870	8.23%
4º	Germany	654	6.19%
5º	England	534	5.05%
6º	France	432	4.09%
7º	Italy	419	3.97%
8º	Canada	320	3.03%
9º	South Korea	258	2.44%
10º	Netherlands	213	2.02%
11º	USSR	199	1.88%
12º	Sweden	195	1.85%
13º	Spain	194	1.87%
14º	Australia	181	1.71%
15º	India	171	1.62%
16º	Federal Republic of Germany	155	1.47%
17º	Russia	154	1.46%
18º	Switzerland	151	1.43%
19º	Poland	118	1.12%
20º	Brazil	105	0.99%
21º	Czech Republic	104	0.98%
22º	Taiwan	104	0.98%
23º	Scotland	92	0.87%
24º	Belgium	91	0.86%
25º	Denmark	91	0.86%

Of the 105 documents registered in Brazil (Table 4), the first was in 1979 until August 2022 (date when the bibliometric analysis was made). The most cited described the

protection of mice to loss of muscle stem cells and extension life span through NR supplementation-mediated boosting NAD+ levels (H. Zhang et al., 2016).

For co-authorship network analysis was considered a minimum number of 5 papers published per author, counting a total of sixty-six countries according to authorship affiliations, totalizing 517 links (Figure 3A). The United States of America, the leader in publications related to NAD+, was not coincidentally also the country with the highest number of publications in collaborations. Brazil had 17 links, of which USA, Germany, France, Italy and Spain were the main collaborators publishing a total of 95 documents (Figure 3B). The collaboration between Brazil and USA was more recent, from the year 2010, but the main USA collaborations were with Japan, China, Germany, France and Canada (Figure 3C). Besides the USA, China had collaboration mainly with Japan, Germany, France and Italy (Figure 3D). The relation co-authorship of the USA was predominant in the 2000's year indicated for color green (Figure 3D), while relation of China between others countries was in the most in 2010's year indicated by the color yellow (Figure 3D).



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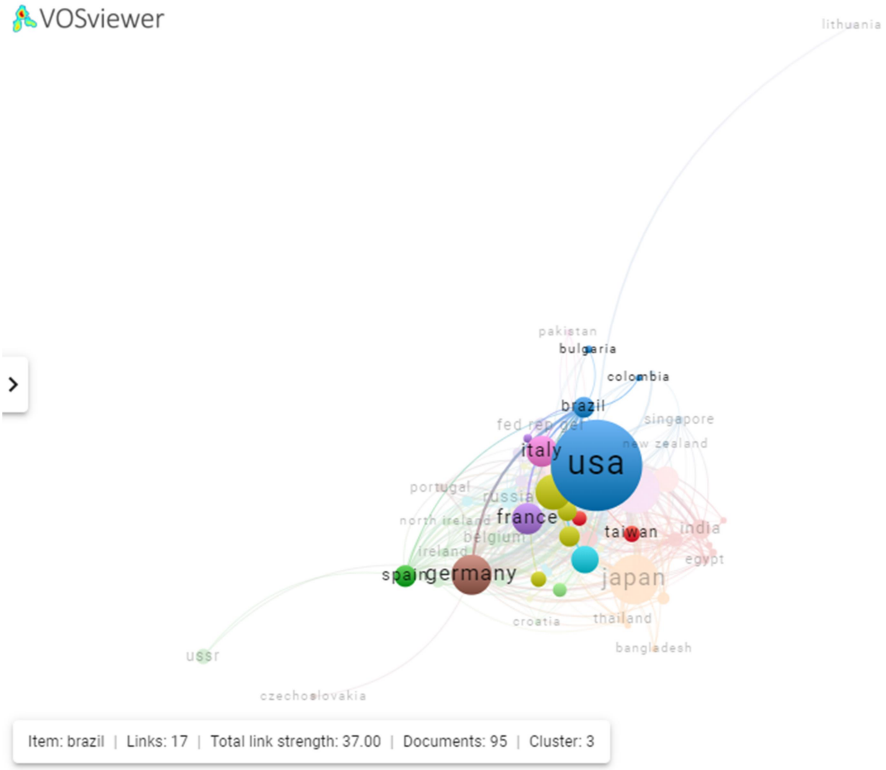
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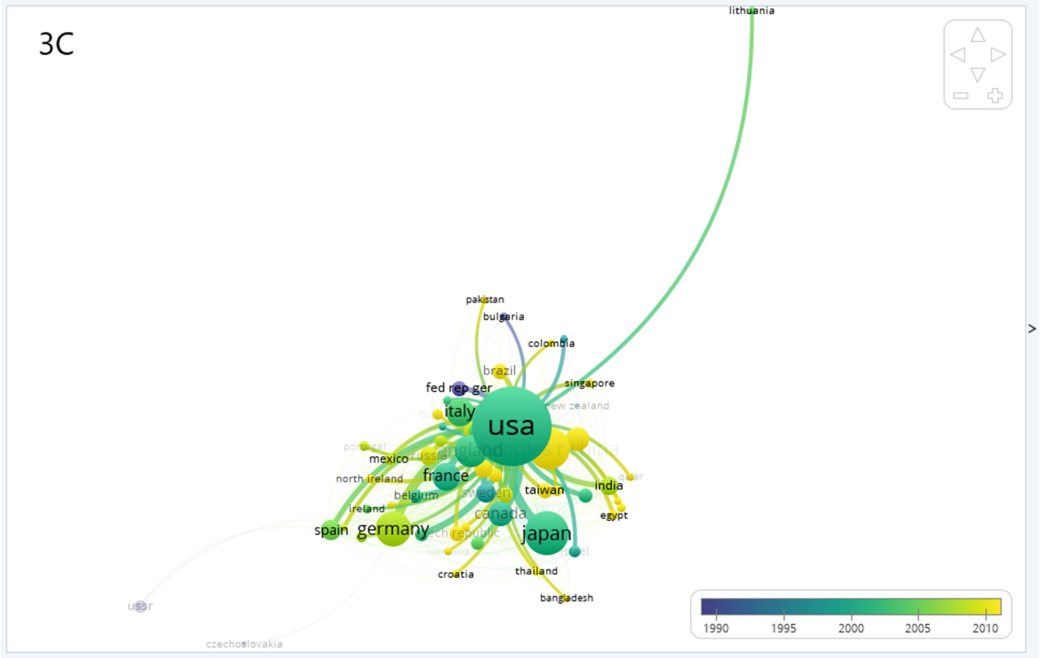
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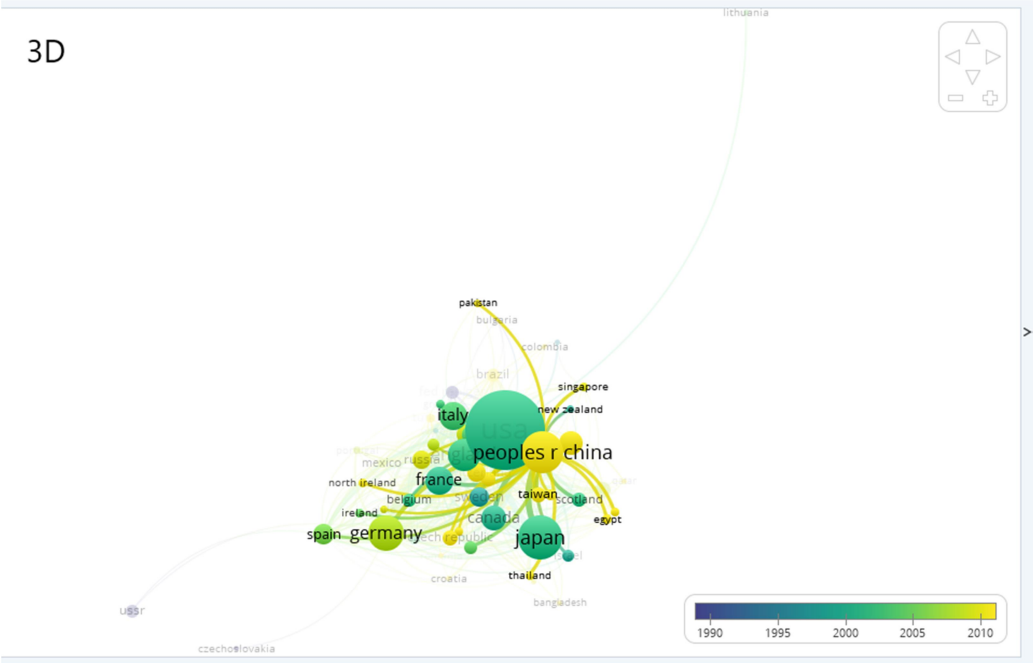

Figure 3. International collaboration in research related to NAD+ (A). Network analysis of Brazil in research related to NAD+ (B). USA collaboration network in research related to NAD+ over time (C). China collaboration network in research related to NAD+ over time (D). The circle's size indicates the number of publications for that country. The lines link the network between countries, while the color of the circle indicates the period of collaborations, according to the year scale on the bottom right.

In relation to keyword analysis, the 346 keywords obtained were colored according to the generated clusters and are schematized on figure 4. A total of 5 clusters were obtained: Cluster 1 (in red) contained 107 keywords with higher occurrence of "NADH, escherichia-coli, protein, purification", cluster 2 (in green) had 102 keywords with higher occurrence "metabolism, NAD+, inhibition, biosynthesis", cluster 3 (in blue) had 69 keywords which main were "oxidative stress, expression, activation, NADPH oxidase", cluster 4 (in yellow) had 35 which "gene, identification, mitochondria, growth" were the most occurred, and cluster 5 (in purple) with 33 keywords which "dt-diaphorase, gene expression, cells, cancer" had higher occurrence.

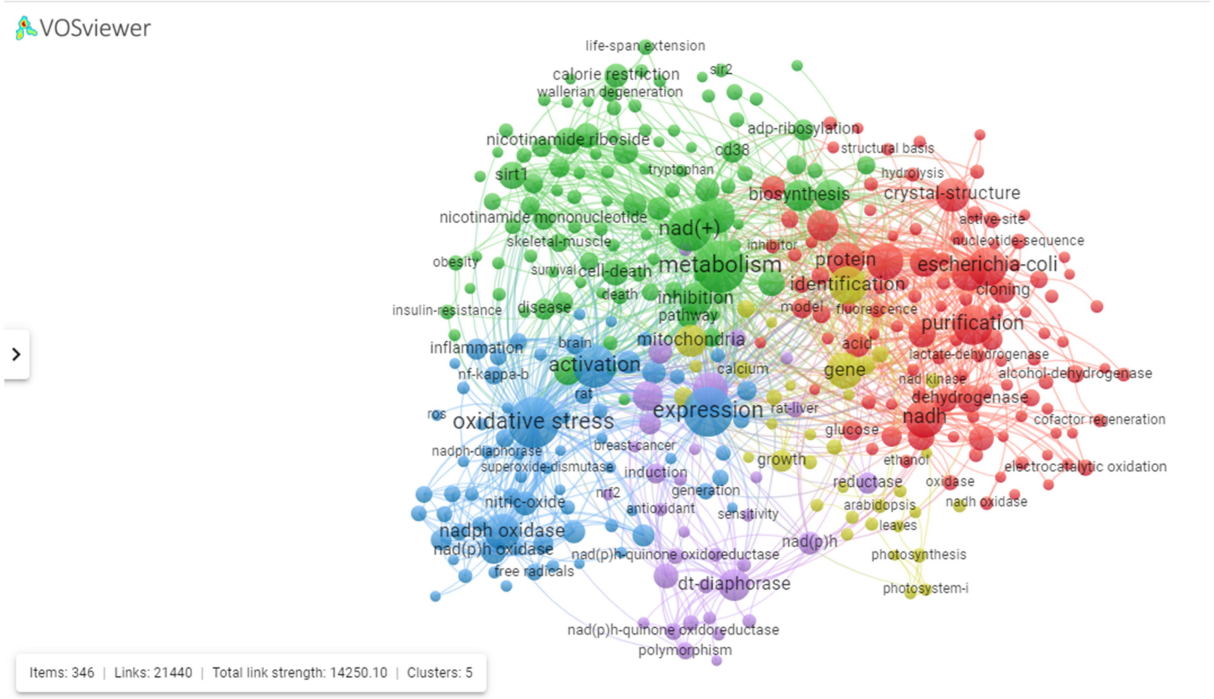

Figure 4. Network visualization map of keywords occurrence in publications related-NAD+. The size of the circle is related to the number of times the keyword occurs. Lines between circles represent the linking, and the color of circles shows its relationship to other circles indicating the cluster to which the keyword belongs.

4. DISCUSSION

The NAD+ metabolome encompasses a wide range of subjects, from defense responses against plant diseases such as citrus canker (Alferez et al., 2018) to the biotechnological production of NAD+ precursors by microbial species (Garofalo et al., 2021; Sugiyama et al., 2021). After the key role of NAD+ in signaling pathways, including intracellular calcium mobilization and post-translational protein modification, was unveiled (Berger et al., 2004), there was a large emphasis on discovering the enzymes that participate in these reactions starting in 2000's. This emphasis is confirmed by the continuous increase in the number of publications on the subject since that time (Figure 1).

Correspondingly, NAD+ metabolome research was primarily conducted in the fields of Biochemistry, Molecular Biology, Biophysics, and Cell Biology. As a result, the top

journals in this area were 'Journal of Biological Chemistry,' 'Biochemistry,' and 'Biochimica et Biophysica Acta.'.

Through the most cited papers it was possible to see how the relation NAD+ and Sirtuins gained prominence and interest, since the discovery of activity and role of these deacetylases (Imai et al., 2000). These enthusiasm was sparked from the fact that sirtuins regulate healthspan across various organisms (Imai & Guarente, 2014), in accord with the availability of dietary energy and nutrients, proposed to be mediated by increased NAD/NADH ratio (Lin et al., 2004). This highlighted the role of sirtuins activity in mediating the positive effects of caloric restriction, particularly in terms of longevity (Guarente, 2013; Zullo et al., 2018). The systemic regulatory network between SIRT1, one most studied of sirtuins family, and NAMPT-mediated NAD+ biosynthesis covering the metabolism, biological rhythm, aging and longevity in mammals was denominated as ‘NAD+ world’ by Shin-Ichiro Imai (Imai, 2009). Later, was updated to ‘NAD+ world 2.0’ after identifying the role of inter-tissue communication between the hypothalamus, skeletal muscle, and adipose tissue in this control system (Imai, 2016).

Additionally, Imai's group was one of the pioneers in studying the effects of increasing NAD+ levels with the precursor NMN in animal models and clinical trials (de Picciotto et al., 2016; Irie et al., 2020; J. Yoshino et al., 2011; M. Yoshino et al., 2021). Apart from Shin-Ichiro Imai, Atsuyoshi Ohno was another significant contributor to the growth of publications on NAD+. From 1975 to 2001, he unveiled the chemistry of reduction and oxidation reactions in which NAD(P)+/NAD(P)H participates (vide some examples in (Ohno et al., 1981, 1994).

Charles Brenner was also one of authors with the highest number of publications on NAD+. He discovered NR as a novel precursor for the eukaryotic NAD+ biosynthetic pathway. In this study, which was his second most cited, it was also shown that milk is a source of NR (Bieganowski & Brenner, 2004). His work has been primarily focused on the genes and metabolic pathways of NAD+ biosynthesis precursors, as well as their beneficial effects in animal models and clinical trials (Belenky et al., 2007; Elhassan et al., 2019; Trammell et al., 2016).

American, Chinese and Japanese researchers have made a significant contribution to NAD+ subject, mainly unraveling the role and mechanisms in which NAD+ is involved at the molecular and cellular biochemistry level. This prominence supports the significant

impact these countries have on the entire field of science, which may be due to their advantageous economics and superior investment in research.

In 2021 a conference named “NAD+ Metabolism and Signaling” was carried out to provide a platform for junior researchers to present their latest scientific results. Presentations were primarily selected from submitted abstracts, with a limited number of invited keynote lectures (Gorbunova et al., 2021). The conference focused on topics: 'NAD signaling,' 'poly(ADP-ribosyl)ation and PARPs,' 'sirtuins,' and 'metabolism and interventions'. Although it was an online conference, the authors of the presented works were only from countries in the northern hemisphere, including Germany, USA, China, Italy, United Kingdom, Japan, The Netherlands, Sweden, Israel, France, Russia, Croatia, Spain, Norway, Switzerland, and Austria. Similarly, the authors of the meeting report were from continents in the northern hemisphere (North America, Europe, Middle East, and Asia) (Gorbunova et al., 2021). While this event did not encompass all works and papers related to the subject of NAD+, it brought together researchers with the most current and important findings, representing the current leaders in NAD+-related research and highlighting the absence of countries in the southern hemisphere.

The analysis of keyword clusters revealed five predominant themes. The green cluster focused on NAD+ biosynthesis and metabolism, including NMN, SIRT1, calorie restriction, and diseases as keywords. The red cluster was associated with oxidation and reduction reactions, proteins, and purification. The blue cluster was characterized by oxidative stress, activation, expression, and inflammation. The yellow cluster was centered on plants, growth, genes, and mitochondria. Lastly, the purple cluster was related to enzymes, polymorphism, and antioxidants. Accordingly, five clusters of keyword occurrences corresponded to the five most cited topics. The topic of 'longevity', which aligns with SIRT1 of the green cluster, was the most cited. It was followed by 'microbial biotechnology', which is related to proteins and purification of the red cluster. The third most cited topic was 'molecular and cell biology-pharmacology', closely associated with the blue cluster. Next, the topic of 'crop science' correlated with the plant and growth focus of the yellow cluster. Lastly, 'cancer drugs' is related to the activation and expression of the blue cluster, as well as the antioxidants and enzymes found in the purple cluster.

In the last years, a large number of published articles have focused on the physiological effects of increasing NAD+ levels using precursors NMN and NR in animal models and clinical trials (Elhassan et al., 2019; Freeberg et al., 2023; Mehmel et al., 2020; J.

Yoshino et al., 2018; M. Yoshino et al., 2021). Many beneficial effects include, but are not limited to: brain (Campbell, 2022; Long et al., 2015; Vreones et al., 2023), cardiovascular (Diguet et al., 2018; R. Zhang et al., 2017), muscle (Igarashi et al., 2022; Lapatto et al., 2023), and intestinal health (Huang et al., 2021, 2022; Kolba et al., 2022; W. Li et al., 2022), anti-aging (Geng et al., 2023; Kiss et al., 2019; Mills et al., 2016), fertility (Bertoldo et al., 2020; H. Li et al., 2023), and anti-inflammatory action (Elhassan et al., 2019; Tian et al., 2023).

Attention has also been focused on NAD+ pathways related to tumorigenesis, which constitute a complex and evolving area of research. The role of NAD+ in apoptotic cell death reactions remains controversial, as it can exhibit both pro- and anti-tumorigenic effects. NAD+ regulates key cellular processes in tumorigenesis, including inflammation, oxidative stress, and cell survival pathways. Restoring NAD+ levels can counteract malignancy by promoting repair and regulating cell cycle arrest and apoptosis. However, elevated NAD+ during cancer progression may enhance growth, resistance, and cell survival (Poljsak, 2016).

Alterations in the levels or activity of enzymes involved in NAD+ synthesis and degradation have been implicated in cancer development. For example, overexpression and high levels of NAMPT, which is responsible for the conversion of nicotinamide to NAD+, has been observed in many tumor types (reviewed in (Gasparrini & Audrito, 2022; Navas & Carnero, 2021). In addition to NAD-biosynthetic activity, NAMPT can also function as a growth factor, cytokine and adipokine. For acting as a multifunctional protein, NAMPT regulates different processes in tumor cells including metabolic adaptation, DNA repair, signaling pathways, cell growth, invasion, stemness, immune modulation of tumor microenvironment, and resistance to genotoxic stress (Gasparrini & Audrito, 2022).

Although NAD+ depletion can impair DNA repair mechanisms by PARPs and sirtuins contributing to cancer development and progression, can also lower chemoresistance toward DNA targeting therapies. Therefore, inhibition of NAMPT and PARPs has been explored as a therapeutic strategy for cancer treatment (Gasparrini & Audrito, 2022; Mateo et al., 2019).

However, much effort is still needed to fully understand the regulation of NAD+ metabolism in human cancer cells, the resulting metabolic changes from pharmacological inhibitor or NAD+ precursor supplementation, and their precise mechanisms and therapeutic implications.

5. FUTURE DIRECTIONS FOR NUTRITION AND FOOD SCIENCES

Food Science Technology and Nutrition Dietetics ranked 27th and 29th, respectively, among research fields, each accounting for 1% of the total published papers (Table 1). This demonstrates the potential for further exploration of NAD+ subject in these areas.

NAD+ represents a promising area of research within nutrition and food science, encompassing several key topics yet to be unraveled. An example was the profiling of NAD+ precursors in human milk (Redeuil et al., 2019), which can facilitate the development of better infant formulas. Study have also demonstrated the influence of lactation time on NAD+, NR, and NMN levels in human milk, as well as the effect of heat treatment on NR content in bovine milk (Ummarino et al., 2017). These findings suggest that processing techniques can have an impact on the NAD+ precursors content in foods, and the influence of circadian rhythm on NAD+ metabolism.

Other example is investigating individual variations in NAD+ metabolism and understanding how genetic factors and epigenetic modifications influence NAD+ levels and response to nutritional interventions. This research may lead to personalized nutrition approaches tailored to an individual's NAD+ needs and metabolic profile. Additionally, exploring the potential of NAD+ precursors as nutraceuticals or functional food ingredients to enhance NAD+ availability and support overall health. This involves evaluating their safety, bioavailability, and effectiveness in maintaining NAD+ homeostasis.

It is also worth noting the interactions between gut microbiota, including the potential contribution of NAD+ precursors from the diet to gut modulation and overall host health (Alegre & Pastore, 2023; Ren et al., 2022). In addition, efforts should be made to investigate the biotransformation on the gastrointestinal tract and its impact on NAD+ metabolism. This is crucial because the gut microbiome appears to play a critical role on the composition and levels of dietary NAD+ precursors that ultimately enter the bloodstream (Chellappa et al., 2022; Shats et al., 2020). For example investigate whether different types of diets and sources of dietary fiber could affect the NAD+ synthesis by intestinal microbiota, given that the colonic microbiome relies on complex carbohydrates that are fermented in the large intestine to produce NAD (Chellappa et al., 2022).

The exploration of NAD+ in nutrition and food sciences fields offers promising opportunities to revolutionize our understanding of dietary interventions, metabolic pathways,

and personalized nutrition. Understanding the intricate mechanisms of NAD+ metabolome holds immense potential for developing innovative strategies to promote optimal nutrition, enhance food quality, and unlock new avenues for improving human health.

CONCLUSIONS

NAD+ is a comprehensive and multidisciplinary research field. Since the discovery of its role as a substrate for proteins involved in signaling pathways, there has been a continuous increase in publications, particularly in the last 10 years. The investigations have shed light on the intricate pathways, mechanisms, and interactions associated with NAD+ metabolism and pathophysiology. Ongoing research in this field holds great promise for uncovering novel therapeutic strategies and interventions targeting NAD+ for improved health outcomes.

According to citation and keyword analysis, the prominent research topics included longevity, sirtuins, NAD+ precursors, oxidation and reduction reactions, enzymes, microbial biotechnology, cancer therapy, and crop science. The research on NAD+ was primarily conducted in the fields of Biochemistry, Molecular Biology, Biophysics, and Cell Biology.

Several gaps in the field of nutrition and food sciences can be observed, prompting the proposal of future research directions. These gaps include investigating the effects of processing techniques on NAD+ and its precursor content in foods, while evaluating their safety, bioavailability, and effectiveness in maintaining NAD+ homeostasis. Additionally, the contribution of NMN and NR from the diet to gut modulation and overall host health, as well as their biotransformation in the gastrointestinal tract and their impact on NAD+ metabolism. Furthermore, studying possible alterations in NAD+ levels in response to nutritional interventions can pave the way for personalized nutrition approaches tailored to individuals' NAD+ requirements and metabolic profiles.

To our knowledge, this is the first bibliometric analysis of NAD+ metabolome, which can provide valuable insights into the research landscape and aid in the growth of this area.

LIMITATIONS OF STUDY

While facilitating the analysis of extensive and complex bibliographic data by visually representing essential data elements, bibliometric network visualization tools do have limitations. VOSViewer generates distance-based maps in which item proximity indicates relation strength, with smaller distances indicating stronger connections. Uneven distribution aids cluster identification but can complicate labeling. The software defaults to displaying a subset of labels to prevent this overlap, which implies information loss. Even though previously hidden labels may become visible when zooming in, it would require numerous figures to display all zoom angles. Furthermore, it is not possible to identify which articles are within the links of international collaborations, thereby preventing the determination of the most cited ones, the journals, and so on.

Finally, due to the time limitations for completing this work, articles published after August 31, 2022, were not included.

CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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CAPÍTULO III

Determination of NAD+ precursors nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) in vegetal foodstuff: exploring dietary sources

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Highlights

- Screening of NMN and NR in vegetal was conducted by validated HPLC-DAD method.
- For the first time NAD+, NMN and NR were determined in Amazon food plants.
- NMN and NR are potential new bioactive compounds with roles in overall health.
- This study adds to the knowledge of dietary strategies to optimize NAD+ levels.

ABSTRACT

NAD+ is an essential molecule for all living organisms, involved in redox reactions and cellular signaling. Given their importance, studying the levels of its biosynthesis precursors, NMN and NR, in foods is crucial for understanding their potential dietary contributions to human nutrition and health. We conducted a comprehensive screening of NMN, NR, and NAD+ levels in Amazonian non-conventional food plants, as well as various common vegetables, for the first time. This analysis was carried out using a validated HPLC-DAD/UV method, further confirmed by LC-MS/MS. NMN and NR concentrations ranged

from 40 to 13,000 and 62 to 1,600 μg per 100g of fresh-weight vegetables, respectively. Broccoli and green beans were rich sources of NMN, while NR was most abundant in wild chicory, banana, and orange. This study contributes to understanding how to optimize NAD+ levels through diet. Moreover, our work advances NAD+ knowledge in the fields of food science and nutrition.

KEYWORDS

NAD+ precursors; NMN; NR; foods; vegetal; chromatographic analysis

1. INTRODUCTION

Nicotinamide adenine dinucleotide (NAD+) is a multifunctional and crucial molecule for all living organisms, and research on its topic has gained prominence and interest in recent years (Alegre & Pastore, 2023b). Pointed out as a key player in the reversal of physiological decline and longevity (Covarrubias et al., 2021), it acts as a substrate in redox reactions, exchanging electrons for energy production, and plays a role in cellular signaling for DNA repair and response to cellular damage through NAD-dependent enzymes such as sirtuins and PARPs (poly adenosine diphosphate ribose polymerases) (Berger et al., 2004).

The presence and proper availability of NAD+ are crucial for the health and functionality of cells. Advancing age, disturbed nutrient conditions, and many disease states, such as heart failure, metabolic and neurological disorders, are associated with reduced NAD+ levels (Balashova et al., 2022; Clement et al., 2019; Mills et al., 2016; Seyedsadjadi et al., 2018; Yoshino et al., 2011). Boosting the abundance of NAD+ with precursor compounds, such as nicotinamide mononucleotide (NMN) or nicotinamide riboside (NR), has promising effects on physiological function in many animal models of aging and diseases (Diguet et al., 2018; Huang et al., 2022; Mills et al., 2016; Trammell et al., 2016).

Dietary supplementation with NMN and NR has also shown many beneficial effects in humans. NMN improved motor muscle function (Igarashi et al., 2022), physical performance (Yi et al., 2023), and cardiometabolic parameters (Pencina et al., 2023). NR has been shown to improve body composition and sleeping metabolic rate (Remie et al., 2020), increase respiration, and decrease proinflammatory cytokine expression in heart failure patients (Wang et al., 2022). It has also been found to modulate cerebral metabolic function in Parkinson's disease (Brakedal et al., 2022).

It is worth highlighting that one potential mechanism for the observed beneficial outcomes may involve gene expression regulation mediated by NAD-dependent enzymes: the histone deacetylase known as sirtuins and Poly-(ADP) Ribose Polymerase (PARPs), which participate in multiple epigenetic pathways (Ciccarone et al., 2017; Jing & Lin, 2015).

MicroRNAs are small, single-stranded RNA molecules whose main function is to bind to specific messenger RNA (mRNA) molecules, inhibiting their translation or promoting their degradation. They are involved in post-transcriptional regulation and the regulation of gene expression. NMN restored microRNA expression to a youthful profile in the aorta of

aged mice, indicating epigenetic rejuvenation and anti-atherogenic effects (Kiss et al., 2019). These miRNAs targeted genes linked to intracellular signaling, protein homeostasis, and inflammation.

NR is marketed as a supplement in doses ranging from 100 mg to 500 mg, usually at a relatively high price. In the United States, NMN has been prohibited from being sold as a dietary supplement due to its classification as a new drug under investigation (Food and Drug Administration, 2022). Food intake could be a more accessible and natural way to obtain NMN and NR for boosting NAD+ levels (Alegre & Pastore, 2023a).

Fruits and vegetables are low in calories and nutritionally dense, rich in carbohydrates, vitamins, minerals, dietary fiber, and phytochemicals (such as polyphenols, sulforaphanes, and carotenoids). These vegetal foods provide various health benefits and are essential for a balanced diet. According to the World Health Organization (WHO), adults should aim for a daily intake of at least 400 grams of fruits and vegetables and 25 grams of fiber. Consuming fruits and vegetables has been associated to reduced risk of cardiovascular disease, cancer and all-cause mortality (Aune et al., 2017).

Given the significance of NAD+ for organismal homeostasis and proper functioning, the precursors NMN and NR could potentially be essential dietary components, much like vitamin B3, with their recommended daily intakes established in the future. This necessitates knowledge about their quantities in foods and optimized, validated techniques for their determination. In addition, Food Science Technology and Nutrition Dietetics fields represent slightly more than 1% of the papers published on the subject of NAD+ (Alegre & Pastore, 2023b).

Our study aimed to conduct a broad survey of NMN, NR, and NAD+ levels in vegetal foods, utilizing a validated HPLC-UV/DAD method. This research will contribute to understanding how to optimize NAD+ levels through diet and advance our knowledge of the NAD+ metabolome in the fields of food science and nutrition.

2. MATERIAL AND METHODS

2.1. Samples material

The non-conventional food plants, known as PANC, were collected in March or June 2022 at a farm called 'Sítio PANC Ramal do Brasileirinho,' located in the forested area of Manaus, Amazonas, Brazil (latitude 03º01'29"S, longitude 59º52'39"W, and an altitude of

51 m). To assess the impact of seasonality on NAD+ and its precursors, some samples of PANC were collected during both March and June. The collected samples were frozen (-18 ºC) until analysis.

The conventional vegetable samples were obtained on at least three different occasions between January and June 2023 from the retail market in Campinas, São Paulo, Brazil. These samples were freshly analyzed in separate batches to ensure their representativeness.

To evaluate the effect of thermal treatment on NAD+ precursor levels and estimate their content in vegetables that are consumed cooked, some samples were steamed and compared with levels in their raw form.

2.2. Chemicals and Reagents

The standards nicotinamide adenine dinucleotide (CAS: 53-84-9), nicotinamide mononucleotide (CAS: 1094-61-7), nicotinamide riboside chloride (CAS: 23111-00-4), and nicotinamide (CAS: 98-92-0); and potassium phosphate dibasic and potassium phosphate monobasic grade HPLC were purchased from Sigma-Aldrich. Methanol grade HPLC was purchased from J.T.Baker. Methanol and chloroform analytical grade were purchased from Synth (Diadema, São Paulo, Brazil).

2.3. Extraction in food samples

A liquid-liquid extraction of NAD+ and its precursors' was performed according to (Ozaki et al., 2022). Briefly, 1 g of sample was mixed with 2 mL of chloroform/methanol (1/2, mL/mL) and 400 µL of ultrapure water (~18.2 M Ω, 25 ºC) obtained from Millipore Milli-Q system. The samples were centrifuged at 7400g for 10 min. Then, approximately 2 mL of the supernatant was transferred into a microtube and dried by evaporation in SpeedVac Eppendorf® Concentrator Plus 5305 (Eppendorf AG 22331 Hamburg - Germany). Subsequently, the residue was dissolved with 1 mL of Milli-Q ultrapure water, and filtered into glass vials using 0.45 µm nylon membrane.

2.4. In-house HPLC-UV/DAD method validation

For the determination of NAD+ precursors, an HPLC-UV/DAD method was validated using two different columns: a COSMOSIL PBr column (3.0 mm I.D. × 150 mm,

particle size 3 µm, Nacalai Tesque) and a ACCLAIM 120 C18 column (4.6 I.D. × 250 mm, particle size 5 µm, Thermo Fisher).

The method performance requirements were established according to (INMETRO. Instituto Nacional de Metrologia, Normalização e Qualidade Industrial, 2020). The parameters linearity, limit of detection (LOD), limit of quantification (LOQ), precision (repeatability, intermediate precision) and accuracy were determined using both PBr and C18 columns.

2.4.1. Selectivity

Given the impracticality of evaluating potential interferents in all analyzed samples, the method's selectivity was ensured through several measures. These included comparing the UV-visible spectrum of the separated peaks with that of standards and confirming analytes presence using LC-MS/MS.

2.4.2. Linearity

For linearity, calibration curves were prepared in Milli-Q ultrapure water using a mixture of four standards at seven concentration levels. The corresponding determination coefficient (r^2) obtained for each calibration curve was higher than 0.99 (Table 1).

2.4.3. LOD and LOQ

The limits of detection (LOD) and quantification (LOQ) were calculated based on the parameters of the analytical curve, as shown in Equations 1 and 2, respectively.

LOD = 3,3 * s / slope (1)

LOQ = 10 * s / slope (2)

Where:

s = standard deviation of the lowest level of the analytical curve

slope = angular coefficient of the analytical curve

2.4.4. Precision

Precision was evaluated through repeatability (intra-day) and intermediate precision (inter-day). Repeatability assessment included triplicate injections of three independent mixed standard solutions (5, 50, and 100 µg/mL) on the same day (n = 3 per

concentration). Intermediate precision was determined from three replicates of three concentration levels (5, 50, and 100 µg/mL) on three different days (n = 9 per concentration).

It was determined by calculating the standard deviation divided by the mean concentration and expressed as the percentage of relative standard deviation, as shown in Equation 3.

% Relative standard deviation (RSD) = standard deviation/average * 100 (3)

2.4.5. Accuracy (Recovery)

For accuracy evaluation, avocado, Moringa (*Moringa oleifera*) and green bell pepper samples were spiked at three levels concentrations (1, 50 and 100 µg/mL) of each standard. Accuracy was expressed in percentage of recovery, and calculated as:

% recovery = (concentration founded – concentration initial of sample) / concentration added * 100

2.5. Identification and quantification by HPLC-UV/DAD using a validated method

The levels of NAD+ and its precursors' were determined by high-performance liquid chromatography (Dionex Ultimate 3000 Thermo Fisher Scientific) coupled with a photodiode array detector (DAD). The HPLC run was performed according to (Yoshino et al., 2011) with slight modifications. The mobile phases consisted of freshly prepared potassium phosphate buffer (KH_2PO_4/K_2HPO_4) 50 mmol/L pH 7 (A), and methanol (B), eluted as follows: 100% buffer (A) from 0-5 min, a linear gradient to 60% A/40% (B) from 5-10 min, 60% (A)/40% (B) from 10-17 min, a linear gradient to 100% buffer (A) from 17-19 min, and maintained at 100% buffer until 22 min. For performance comparison, two different columns were used with the same gradient: a COSMOSIL PBr column (3.0 mm I.D. × 150 mm, particle size 3 µm, Nacalai Tesque) at a flow rate of 0.4 ml/min and a ACCLAIM 120 C18 column (4.6 I.D. × 250 mm, particle size 5 µm, Thermo Fisher) at a flow rate of 0.9 ml/min, both maintained at 28º C. A volume of 20 µL of sample or mixed standards was injected, and the absorbance was monitored at 220 nm for NMN and NR, and NAM (nicotinamide), and at 260 nm for NAD+. The sample compounds were identified based on their retention time and the UV spectrum of the peak obtained in the separation of the standards, and quantitated based on the peak area compared to a standard curve calibration. For curve calibration, standard working solutions were prepared by diluting stock solutions of

each compound at 1 mg/mL in water to a concentration range from 1 to 50 µg/mL. Data acquisition and analysis was carried out with the Chromeleon™ software.

Samples containing NMN, NR, NAM, and NAD+ were further analyzed and confirmed using LC-MS/MS.

2.6. Confirmation by UHPLC-MS/MS

Liquid chromatography-tandem mass spectrometry was carried out with a UHPLC Waters Acquity™ coupled to a Quattro Micro Triple Quadrupole mass spectrometer. For the separation, a Waters ACQUITY UPLC® BEH Amide column (2.1 mm I.D. × 100 mm, 1.7 µm) was used at a constant flow rate of 0.300 mL/min and temperature of 30 ºC. Injection volume for samples was 10 µL. The optimized gradient conditions are described in Supplementary Table 1. The analysis was performed using positive electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode, and the parameters for each analyte are showed in Supplementary Table 2. The Triple Quadrupole operated at a capillary voltage of 3.60 kV, a source temperature of 150°C, a gas (desolvation) temperature of 350 °C, and a gas flow rate of 900 L/hr. MassLynx™ v4.1 software (Waters) was used for data acquisition and analysis.

2.7. Statistical Analysis

The data are presented as mean values ± standard deviation of at the least three replicates. One-way ANOVA and post hoc Tukey tests were conducted to identify significant differences in the levels of NAD+ and its precursors among the samples. The t-Student test was employed to compare NAD+ and its precursors levels using two different columns. The statistical analysis was conducted with Minitab® version 18.1 software at a 95% confidence level (p-value ≤ 0.05).

3. RESULTS AND DISCUSSION

3.1. HPLC-UV/DAD method validation

The method was linear for both columns (PBr and C18), with determination coefficient (r^2) > 0,99. Except for NAM, the C18 column had a lower limit of detection (LOD) and limit of quantification (LOQ) (Table 1).

Table 1. Retention time (RT), slope (a), intercept (b) of linear regression, linearity (r^2), linear range, limit of detection (LOD), and limit of quantification (LOQ), for the HPLC-UV/DAD method using two different columns.

	PBr column							***C18 column***						
Compounds	**RT (min)**	**Slope (a)**	**Intercept (b)**	**r^2**	**Linear range (µg/mL)**	**LOD (µg/g)**	**LOQ (µg/g)**	**RT (min)**	**Slope (a)**	**Intercept (b)**	**r^2**	**Linear range (µg/mL)**	**LOD (µg/g)**	**LOQ (µg/g)**
NMN	3.34	1.0312	0.187	0.9985	1-50	0.11	0.33	3.40	0.491	0.9014	0.9949	1-50	0.06	0.18
NR	8.68	1.2108	-0.5195	0.9978	1-50	0.26	0.78	6.60	0.5415	-0.1364	0.9976	1-50	0.14	0.42
NAM	12.6	3.204	1.4498	0.9998	1-50	0.11	0.32	11.8	1.4613	0.5338	0.9998	1-50	0.17	0.53
NAD	13.2	1.1989	0.6788	0.9994	1-50	0.30	0.91	10.8	0.5278	0.8239	0.9983	1-50	0.20	0.61

r^2: determination coefficient

With the exception of inter-day precision for NAD+ at the lower level (5 µg/mL) using the PBr column, the relative standard deviation percentages were all below 15% (Table 2), which is generally the value considered acceptable. Our results for precision were close to those reported by (Martino Carpi et al., 2018). They obtained relative standard deviation percentages for intra-day and inter-day precision of 4.9% and 6.1% for NMN, 7.1% and 16.8 % for NR, 4.0% and12.5% for NAM, and 4.0% and 7.9% for NAD+, respectively.

Additionally, with exception of NMN at 5 µg/mL and NR at 50 µg/mL intra-day and inter-day precision, and NAD+ at 50 µg/mL intra-day precision, the C18 column showed the lowest RSD percentages (Table 2).

Table 2. Relative standard deviation percentages (RSD, %) calculated from standards to intra-day and inter-day precision determination, using PBr and C18 columns.

PBr column					***C18 column***				
Intra-day (% RSD)					Intra-day (% RSD)				
Level (µg/mL)	**NMN**	**NR**	**NAM**	**NAD**	**Level (µg/mL)**	**NMN**	**NR**	**NAM**	**NAD**
5	5.55	13.4	3.06	13.8	5	9.90	4.09	1.18	2.64
50	7.36	3.02	5.13	8.33	50	4.96	6.16	3.80	8.75
100	5.65	4.06	2.99	9.79	100	3.40	4.01	0.22	1.09
Inter-day (% RSD)					Inter-day (% RSD)				
	NMN	**NR**	**NAM**	**NAD**		**NMN**	**NR**	**NAM**	**NAD**
5	10.5	7.65	8.98	15.2	5	14.5	2.59	1.09	3.50
50	7.32	3.25	6.65	11.7	50	4.76	4.25	2.95	7.52
100	6.58	2.94	4.63	9.72	100	2.73	2.79	1.02	1.43

The acceptable average recovery for the concentration level of 1 µg/mL (0.1% analyte) should fall within the range of 95-105%. For the 50 µg/mL level, the acceptable range varies between 97-103%, and for 100 µg/mL (10% analyte), the variation is narrower at 98-102% (INMETRO. Instituto Nacional de Metrologia, Normalização e Qualidade Industrial, 2020). Only the recovery of NR at 1 µg/mL in avocado using the C18 column and NR at 50 µg/mL in avocado using the PBr column, as well as NAM at 50 µg/mL in moringa and green bell pepper with the C18 column, fell within the acceptable range (Table 3). NMN and NAD+ showed to be the most challenging compounds to recover, especially at the lower level (1 µg/mL).

Table 3. Recovery percentage (%) of analyzed samples: avocado, moringa, and green bell pepper using C18 and PBr columns.

			% recovery	
Sample	**Level**	**Compounds**	**PBr column**	**C18 column**
Avocado	1 µg/mL	NMN	128	138
		NR	56.3	99.1
		NAD	n.d.	n.d.
		NAM	129	59.8
Avocado	50 µg/mL	NMN	112	6.56
		NR	97.2	196
		NAD	19.1	73.8
		NAM	88.5	92.4
Avocado	100 µg/mL	NMN	95.6	0.91
		NR	72.9	170
		NAD	15.1	28.6
		NAM	79.3	91.9
Moringa (*M. oleifera*)	1 µg/mL	NMN	35.8	n.d.
		NR	n.d.	47.0
		NAD	240	n.d.
		NAM	n.d.	n.d.
Moringa (*M. oleifera*)	50 µg/mL	NMN	115	22.2
		NR	68.6	162
		NAD	87.6	80.7
		NAM	284	104
Moringa (*M. oleifera*)	100 µg/mL	NMN	76.5	11.9
		NR	57.9	121
		NAD	82.8	84.5
		NAM	163	91.3
Green bell pepper	1 µg/mL	NMN	n.d.	n.d.
		NR	77.6	14.3
		NAD	n.d.	n.d.
		NAM	n.d.	n.d.
Green bell pepper	50 µg/mL	NMN	177	199
		NR	52.0	51.6
		NAD	76.0	78.8
		NAM	95.6	102
Green bell pepper	100 µg/mL	NMN	87.3	88.3
		NR	51.6	55.2
		NAD	76.7	86.6
		NAM	81.0	89.0

* n.d. = not detected.

What could justify these results is that the added standards may not necessarily be in the same form as they are present in the sample, as is the case with NR, which contains a

chlorine molecule for greater stabilization (nicotinamide riboside chloride), and NMN which has the nicotinamide in the beta configuration (β-NMN). On the other hand, the presence of the added standards in a more easily detectable form can lead to overly optimistic assessments of recovery.

Another potential reason is that components of the food matrix may affect the determination of NAD+ and its precursors in plant samples. In the developed and validated method using LC–MS for the determination of NMN and related pyridine compounds in several mouse tissues, the matrix effect was 58% for NMN, 61% for NR, 40% for NAM and 80% for NAD. The recovery of NAD+ and its metabolites (NMN, NaMN, NR, NaR, NAM and Na) ranged from 66.3 – 87% (Martino Carpi et al., 2018).

For comparison purposes, we measured the content of NAD+ and its precursors in some samples (collard, cilantro, and spinach) using both PBr and C18 columns. The content of NAD+ precursors measured using the two different columns is showed in Table 4.

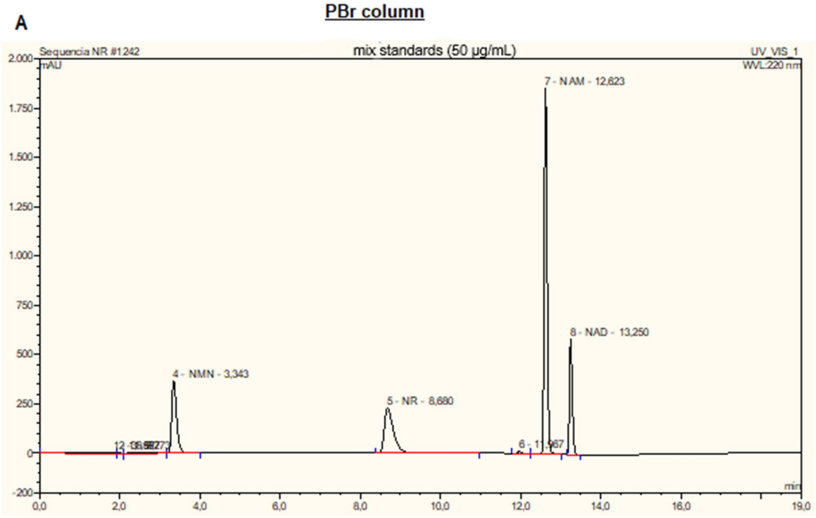
Table 4. NAD+ and its precursors content in collard, cilantro and spinach samples analyzed with different columns: PBr and C18.

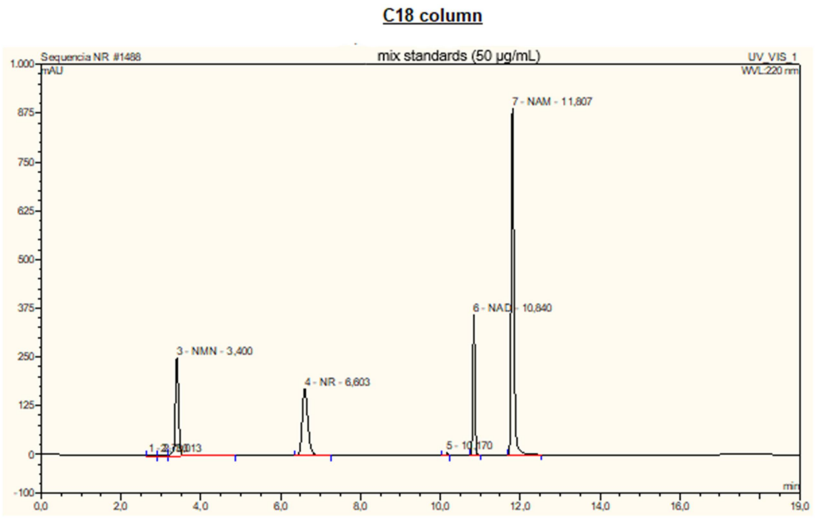
Sample	Compound	PBr column	C18 column
		(µg/100g) FW	
Collard	**NMN**	890 ± 149[a]	308 ± 27.6[b]
	NR	303 ± 50.2[a]	356 ± 59.6[a]
	NAD	3767 ± 128[a]	3816 ± 329[a]
	NAM	n.d.	n.d.
Cilantro	**NMN**	995 ± 163[a]	18.3 ± 11.0[b]
	NR	725 ± 19.0[a]	641 ± 9.45[b]
	NAD	20165 ± 3239[a]	1192 ± 191[b]
	NAM	n.d.	n.d.
Spinach	**NMN**	872 ± 156	n.d.
	NR	343 ± 39.3[a]	337 ± 183[a]
	NAD	1348 ± 799[a]	3048 ± 1252[a]
	NAM	n.d.	n.d.

* Different letters on the same line indicate a significant difference (p-value < 0.05).
n.d. = not detected.

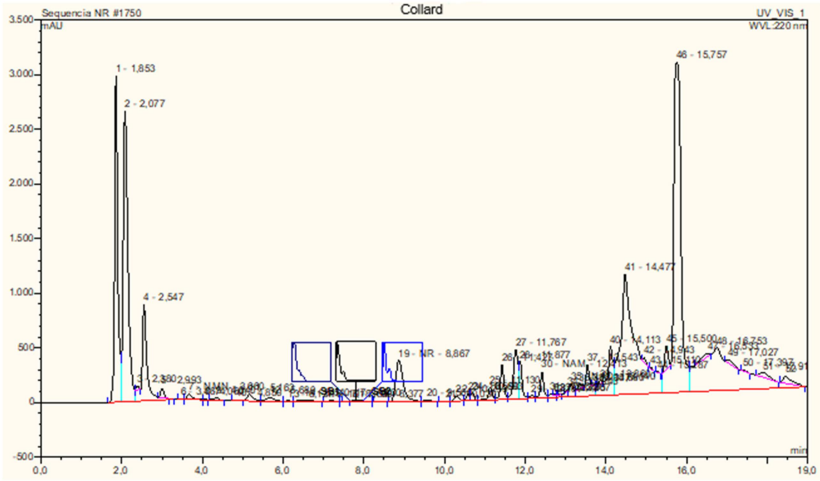
Chromatograms of standards and analyzed samples using C18 and PBr columns are shown in Figure 1.

A


PBr column
Sequencia NR #1242
mix standards (50 µg/mL)
UV_VIS_1
WVL:220 nm
mAU
7 - NAM - 12,623
8 - NAD - 13,250
4 - NMN - 3,343
5 - NR - 8,680
6 - 11,967
min


C18 column
Sequencia NR #1488
mix standards (50 µg/mL)
UV_VIS_1
WVL:220 nm
mAU
7 - NAM - 11,807
6 - NAD - 10,840
3 - NMN - 3,400
4 - NR - 6,603
min

B


PBr column
Sequencia NR #1750
Collard
UV_VIS_1
WVL:220 nm
mAU
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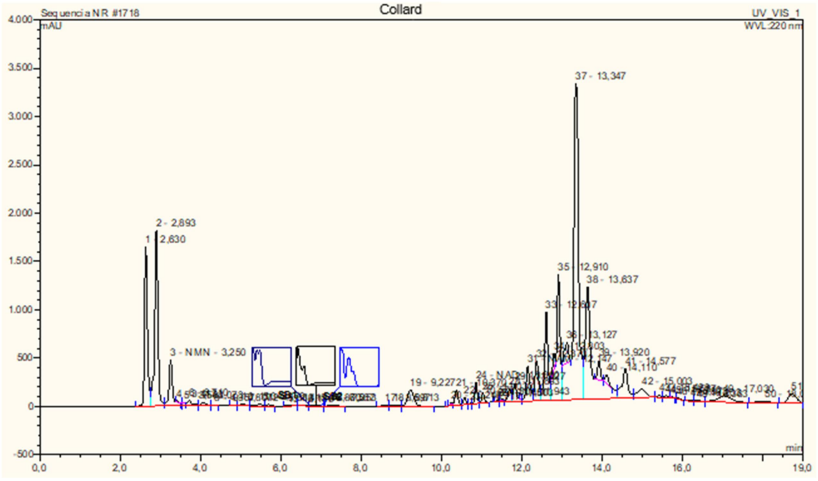
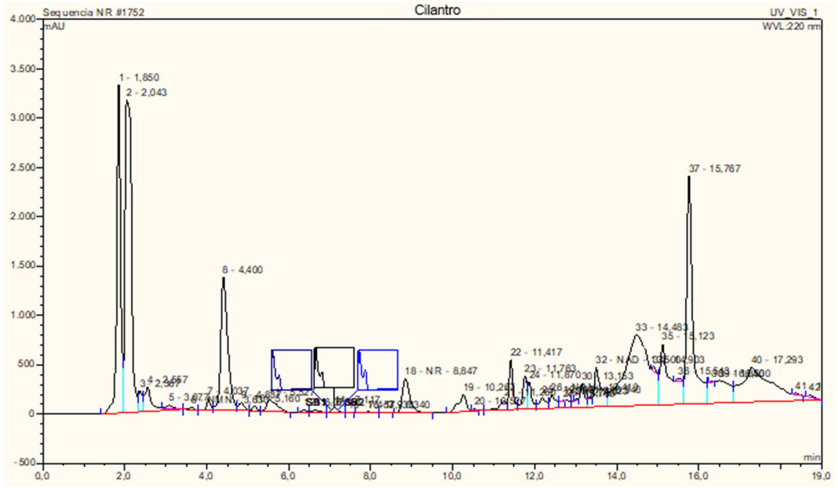
2 - 2,077
4 - 2,547
46 - 15,757
41 - 14,477
19 - NR - 8,867
27 - 11,767
min

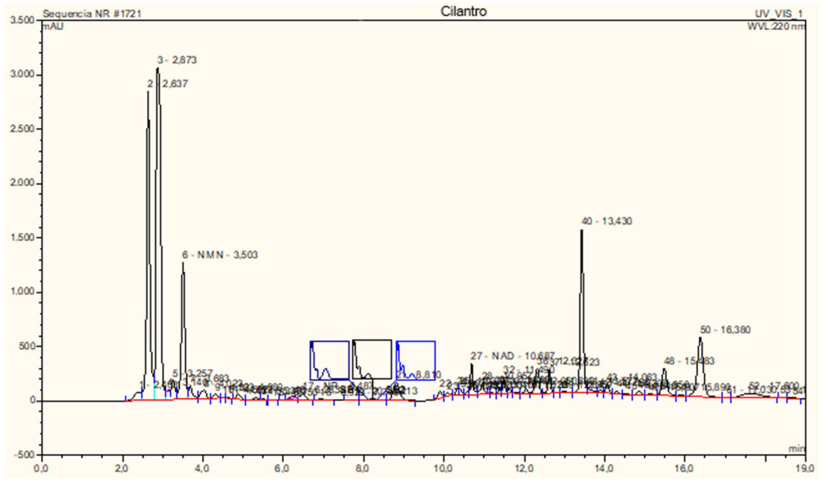

C18 column
Sequencia NR #1718
Collard
UV_VIS_1
WVL:220 nm
mAU
37 - 13,347
2 - 2,893
1
2,630
3 - NMN - 3,250
35
12,910
38 - 13,637
min

C

PBr column

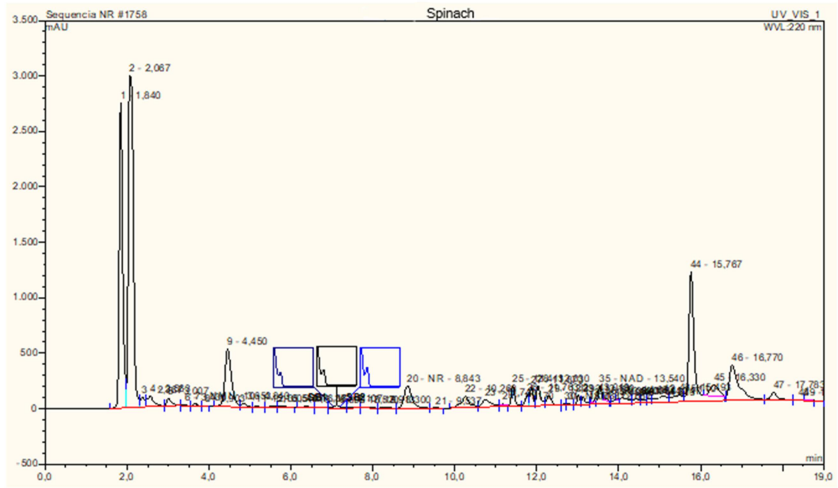


C18 column



D

PBr column



C18 column

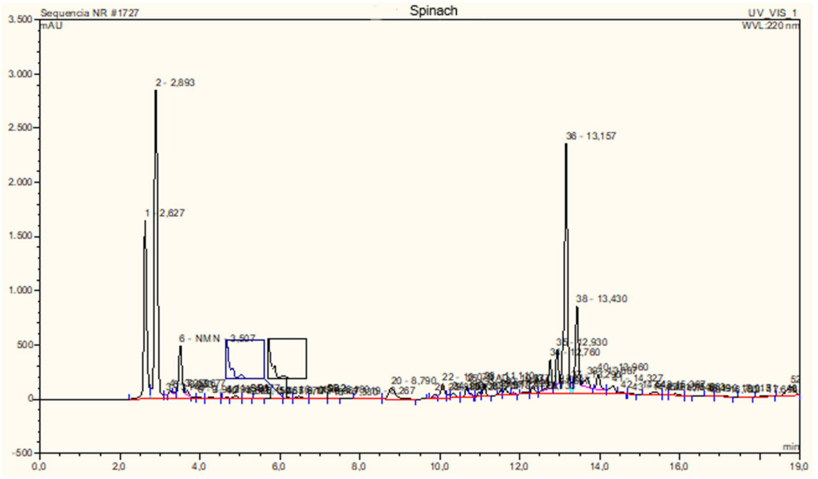


Figure 1. Chromatograms of standards (A) and analyzed samples: collard (B), cilantro (C), and spinach (D), using PBr and C18 columns.

There was a slight difference in the retention times of compounds separated by the two different columns (Table 1). In contrast to what occurred with the PBr column, in the C18 column NAD eluted before NAM, as was also found by (Ozaki et al., 2022).

Among the samples analyzed, cilantro showed significantly higher contents of all compounds when using the PBr column, while collard exhibited significantly higher NMN levels with the same column. In the case of spinach, NMN was exclusively detected with the PBr column (Table 4). Furthermore, the area (mAU*min.) and height (mAU) of the standard peaks were higher with PBr. NAM and NAD+ exhibited nearly double the area and height with PBr compared to C18 (Figure 1A).

And indeed, Ozaki et al., (2022) also found a more sensitive detection with PBr than using a C18 column. The PBr column is packed with a stationary phase of the pentabromobenzyl group, featuring five bromine atoms on the silica gel. It operates based on strong dispersion forces and exhibits good retention ability for various compounds of high polarity (Ozaki et al., 2022).

NAD+ is a pyridine nucleotide, which consists of an amide (NAM), a nitrogenous base (adenine), two ribose sugars, and two phosphate groups. NMN is an immediate intermediate nucleotide in NAD+ synthesis, composed of an amide (NAM), a ribose, and a phosphate group. NR is a nucleoside that serves as a precursor to NMN in the NAD+ synthesis pathway, composed of an amide (NAM) and a sugar molecule (ribose). Finally, NAM is an amidated form of vitamin B3, the smallest precursor present in all subsequent precursors and in NAD+ (Figure 2). Despite their high hydrophilicity, these compounds could also be separated in reverse phase using a C18 column under the same separation conditions as the PBr column.

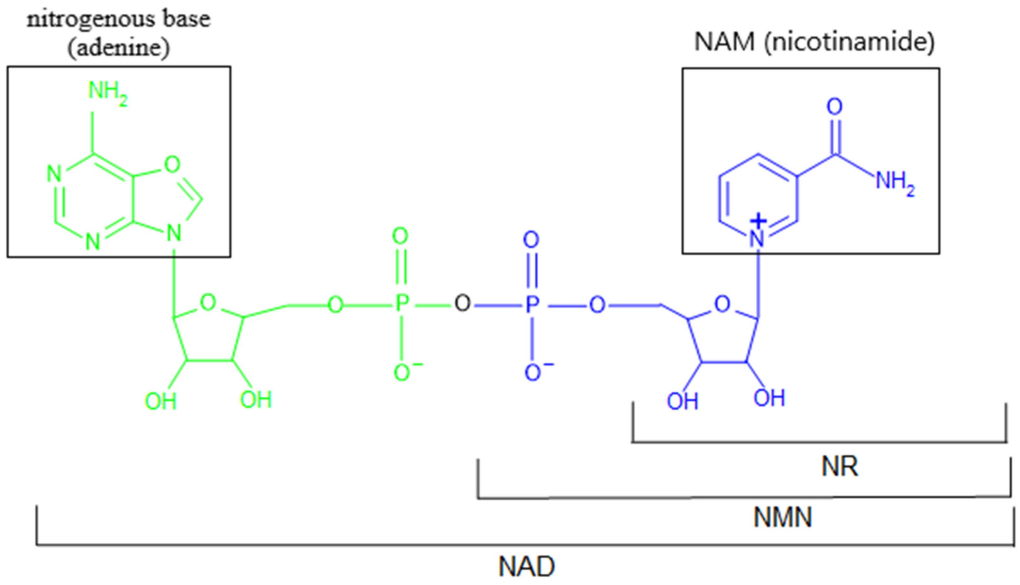


Figure 2. Chemical structure of NAD+ and its precursors (in blue) NAM, NR and NMN.

3.2. NAD+ and its precursors' content in food sources

Due to the superior results obtained using the PBr column (Table 4), we chose to proceed with the screening of NAD+ and its precursors in plant-based foods using this column.

Among the analyzed NAD+ precursors, NMN exhibited the highest levels, with concentration ranging from 40 to 13000 µg/100g of fresh-weight (Table 5). Broccoli and green beans had higher amounts of NMN. Despite raw broccoli having significantly more NMN than cooked, the heat treatment did not decrease NMN in green beans, as cooked green beans had a significantly higher amount than raw ones. The steaming process also had no impact on the NMN content in beets, carrots, and purple cabbage.

For NR, wild chicory had the highest content, followed by banana, orange, and the PANC moringa (*Moringa oleifera*), as well as June's purple malanga (*Xanthosoma violaceum*). By the way, it is worth including the definition of the Portuguese term 'PANC'. PANC is an acronym for 'non-conventional food plants', which includes plants—both subspontaneous or cultivated—with one or more parts suitable for direct human consumption. These plants are also used in the production of beverages, tonics, and infusions, serve as salt substitutes, sweeteners, spices, condiments, and aromatic substances. However, they are not

commonly used in the daily lives of the majority of the population within a region, country, or globally (Kinupp & Lorenzi, 2014).

Despite raw broccoli having a higher NR content than cooked broccoli, this difference was not significant. Eggplant and purple cabbage also did not experience much influence from heat on NR content, as there was no significant difference between cooked and raw samples. Unlike the situation with NMN, the heat treatment degraded NR in green beans, which was not detected in cooked green beans. Previous studies have shown that NR is more heat-sensitive than NMN, as shown in commercially processed UHT bovine milk (Ummarino et al., 2017). While we did not control the time-temperature combination to simulate real culinary practices, our moist heat treatment may have been gentler than ultra-high temperature (UHT), especially considering the different sample types involved.

The varied effects of heat on our samples could probably be attributed to variations in vegetable structure/tissue and the duration/temperature of the treatment. This presents an opportunity to explore the impact of other types of food processing techniques.

Nicotinamide (NAM), one of the forms of vitamin B3, was found only in peanut butter and brown rice, which was already anticipated, as cereals, legumes, and seeds are the primary sources of this vitamin among vegetables (Çatak, 2019). It has been demonstrated that hot acid and base extraction procedures in foods convert NMN and NR to NA (nicotinic acid), thereby contributing to the overall quantified content of vitamin B3 (Ummarino et al., 2017). This explains the disparity observed in the NAM content among the vegetables we also analyzed (Çatak, 2019; Çatak & Yaman, 2019), where NAM was not detected, but rather NMN and NR were found.

This was the first time NR was determined in vegetal samples. NR and NMN content were measured in milk from various species, including human, bovine, ovine, caprine, buffalo, and donkey. The study found that buffalo milk had the highest NR content, ranging from 0.3 to 15.0 μM, while human milk contained NMN in the range of 2.1 to 9.8 μM (Ummarino et al., 2017). NR and NMN were also detected in craft beers (concentration varied from 0.48 to 3.25 μM, and 0.9 μM respectively), and its presence was influenced by the *S. cerevisiae* strain used for fermentation. Furthermore, the addition of hops during the beer fermentation process was found to enhance NR levels (Garofalo et al., 2021).

The NMN content in broccoli and cucumber peel exceeded the only previously reported data for NMN in vegetables, to the best of our knowledge (0.25–1.12 and 0.65 mg/100g, respectively) (Mills et al., 2016). Meanwhile, the NMN content in tomato, cabbage,

and avocado aligned with the values reported by (Mills et al., 2016) (0.26–0.30, 0.0–0.90, and 0.36–1.60 mg/100g, respectively).

NR and NAM were found in smaller quantities compared to NAD+. The levels of precursors varied between 62 to 1600 µg/100g and 63 to 460 µg/100g, respectively. In contrast, the average for NAD+ ranged from 58 to 8800 µg/100g, with purple malanga, malanga, spinach, and collard showing the highest amounts.

Among the PANC, Ora-pro-nobis Amazonian (*Leuenbergeria bleo*) was the only one that did not contain NR. *Leuenbergeria bleo* is an unexplored species of Ora-pro-nobis. While *Pereskia aculeata Mill.* has already had its nutritional composition and functional applications characterized, *Leuenbergeria bleo* lacks scientific data regarding its composition and properties. Our findings on NAD+ and its precursor content highlight the potential of Brazilian Amazon plants, which is supported by recognized local medicinal knowledge.

Another factor evaluated in our study was seasonality. In the Brazilian Amazon region, with its tropical climate, there are essentially two seasons in a year: the rainy season, referred to as "winter" due to lower temperatures (mid-December to May), and the dry season, similar to summer due to higher temperatures (mid-June to November). We measured the levels of NAD+ and its precursors in PANC malanga (*Xanthosoma sagittifolium*) and purple malanga (*Xanthosoma violaceum*) collected during these two periods: the rainier (March) and the drier month (June). There was a difference between the seasons only in terms of NR levels in purple malanga.

Although no difference was observed in NAD+ and NMN content between PANC samples from the rainier (March) and the drier (June) seasons, it is important to consider how planting conditions (such as soil nutrients, sunlight exposure, etc), and the stage of maturation may interfere with the plant's metabolism, and consequently, with the levels of NAD+, NMN, NR, and NAM, since NAD+ plays a crucial role in various aspects of plant biology and is essential for plants' adaptation to environmental stresses (Hashida et al., 2009; Smith et al., 2021). This could explain the variation we observed in the levels of NAD+ and its precursors in different batches of the vegetal samples (see standard deviation (SD) in Table 5).

Table 5. NMN, NR, NAD+ and NAM content in vegetal foodstuff, determined by HPLC-UV/DAD using PBr column.

	(µg/100g) FW			
Sample	**NMN**	**NR**	**NAD**	**NAM**
PANC (non-conventional food plants)				
Moringa (*Moringa oleifera*)	n.d.	930 ± 130^{bc}	n.d.	n.d.
Moringa Ethiopian (*Moringa stenopetal*)	n.d.	522 ± 176^{de}	847 ± 202^{ef}	n.d.
Ora-pro-nobis Amazonian (*Leuenbergeria bleo*)	125 ± 20.0^{ef}	n.d.	58.7 ± 32.6^{g}*	n.d.
Malanga (*Xanthosoma sagittifolium*) March	91.0 ± 2.65^{ef}	132 ± 1.00^{k}	5089 ± 2642^{bc}	n.d.
Malanga (*Xanthosoma sagittifolium*) June	77.7 ± 7.77^{ef}	239 ± 73.7^{jk}	7251 ± 1848^{ab}	n.d.
Purple malanga (*Xanthosoma violaceum*) March	180 ± 40.7^{def}	561 ± 50.2^{def}	8839 ± 2411^{a}	n.d.
Purple malanga (*Xanthosoma violaceum*) June	93.7 ± 5.69^{ef}	923 ± 58.4^{bc}	6568 ± 1306^{ab}	n.d.
Green leafy vegetables				
Broccoli	13059 ± 3566^{a}	181 ± 28.9^{jk}	n.d.	n.d.
Broccoli steamed	3637 ± 64.7^{c}	100 ± 4.16^{k}	n.d.	n.d.
Cabbage	180 ± 19.8^{def}	77.3 ± 3.21^{k}*	1887 ± 395^{def}	n.d.
Capers	n.d.	509 ± 33.3^{defghij}	n.d.	n.d.
Cilantro	956 ± 140^{def}	531 ± 21.9^{defghi}	828 ± 89.8^{def}	n.d.
Collard (*Brassica oleracea L. var. viridis L.*)	833 ± 67.3^{def}	223 ± 14.0^{ghijk}	3087 ± 448^{cde}	n.d.
Lettuce	n.d.	n.d.	n.d.	n.d.

Leek	n.d.	243 ± 18.8^{efghijk}	n.d.	n.d.
Mint	291 ± 73.1^{def}	62.3 ± 1.15^{k}*	110 ± 14.5^{g}	n.d.
Onion	n.d.	n.d.	n.d.	n.d.
Scallion	872 ± 423^{def}	233 ± 49.0^{fhijk}	321 ± 202^{ef}	n.d.
Spinach	177 ± 8.02^{def}	257 ± 46.3^{ijk}	3845 ± 1844^{cd}	n.d.
Parsley	128 ± 26.8^{ef}	191 ± 37.3^{jk}	1627 ± 304^{def}	n.d.
Purple cabbage	182 ± 12.4^{def}	90.2 ± 4.20^{k}	n.d.	n.d.
Purple cabbage boiled	40.7 ± 6.81^{ef}	153 ± 1.53^{k}	n.d.	n.d.
Wild chicory (*Cichorium intybus* subsp. *Intybus*)	n.d.	1644 ± 63.0^{a}	n.d.	n.d.
Roots and tubers				
Beetroot	2294 ± 211^{cd}	n.d.	n.d.	n.d.
Beetroot steamed	2070 ± 154^{cde}	n.d.	n.d.	n.d.
Carrot	1326 ± 126^{def}	89.3 ± 6.81^{k}	n.d.	n.d.
Carrot steamed	1531 ± 248^{cdef}	n.d.	n.d.	n.d.
Vegetables				
Cucumber peel	1027 ± 186^{def}	n.d.	431 ± 37.1^{ef}	n.d.
Cucumber	n.d.	n.d.	n.d.	n.d.
Eggplant	n.d.	198 ± 2.42^{jk}	n.d.	n.d.
Eggplant steamed	n.d.	170 ± 16.7^{k}	400 ± 65.5^{ef}	n.d.
Green bell pepper	294 ± 104^{def}	64.3 ± 6.66^{k}*	128 ± 53.7^{ef}	n.d.

Tomato	235 ± 45.1[f]	152 ± 14.2[k]	313 ± 86.1[f]	n.d.
Fruits				
Apple	n.d.	n.d.	n.d.	n.d.
Avocado	639 ± 163.6[def]	654 ± 238[cd]	n.d.	n.d.
Banana	n.d.	1209 ± 85.6[b]	569 ± 223[ef]	n.d.
Dragon fruit	645 ± 15.0[def]	n.d.	173 ± 10.0[ef]	n.d.
Dragon fruit peel	458 ± 36.0[def]	133 ± 18.0[k]	n.d.	n.d.
Fig	40.0 ± 8.83[f]	195 ± 16.8[jk]	n.d.	n.d.
Guava	n.d.	n.d.	n.d.	n.d.
Orange	n.d.	1013 ± 421[b]	n.d.	n.d.
Pear	76.7 ± 33.4[ef]	n.d.	n.d.	n.d.
Persimmon	n.d.	n.d.	n.d.	n.d.
Plum	n.d.	146 ± 5.88[k]	244 ± 30.4[ef]	n.d.
Legumes				
Bean (canned)	n.d.	n.d.	n.d.	n.d.
Chickpea (canned)	n.d.	n.d.	n.d.	n.d.
Lentil (braised)	n.d.	n.d.	n.d.	n.d.
Green bean	8765 ± 330[b]	599 ± 155[cd]	304 ± 103[ef]	n.d.
Green bean (steamed)	11769 ± 837[a]	n.i.	1241 ± 264[def]	n.d.
Pea (canned)	n.d.	n.d.	n.d.	n.d.
Peanut butter	n.d.	n.d.	n.d.	467 ± 107[a]

Cereals e pseudocereals				
Brown rice	n.d.	n.d.	n.d.	63.3 ± 6.35^{b}
Quinoa	n.d.	n.d.	n.d.	n.d.
White rice	n.d.	n.d.	n.d.	n.d.

3.3. Confirmation of NAD+ and its precursors' content in food sources by LC-MS/MS

To further confirm the content determined by the HPLC-DAD/UV method, we also analyzed the samples using LC-MS/MS.

Due to the differences found in the time retention of standards and the analytes present in the samples (Supplementary Figure 1 and 2), we decided to spike the samples with a mixed standards solution to confirm the peaks detected by LC-MS/MS. The fortification of the samples with standards allowed us to observe the matrix effect, which mainly interfered with the NAD+ signal in most vegetal (Supplementary Figure 2). Residues of brine salts from caper may have caused ionization suppression, as even in the sample fortified with standards, the ions of interest could not be detected.

While the liquid-liquid extraction technique allowed for the removal of a significant portion of chlorophyll present in the vegetal, depending on the chemical composition of each sample, certain chlorophyll pigments and other water-soluble compounds may have persisted (Figure 3), potentially interfering with the separation and identification of NAD+ and its precursors.

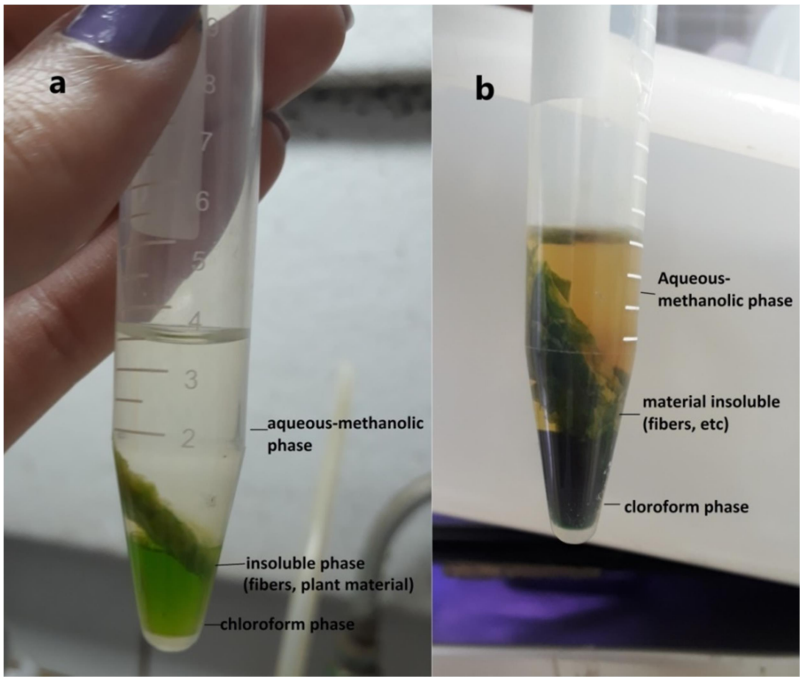


Figure 3. Liquid-liquid extraction of NAD+ and its precursors in cucumber peel (a) and moringa (b) samples.

Several tests for extracting NAD+ and its precursors using techniques employed in animal tissues and cells, as conducted by (Ramsey et al., 2008; Yoshino et al., 2011), and in milk (Ummarino et al., 2017), were conducted but proved ineffective for vegetal samples. The extraction using buffered alcohol (75% ethanol and 25% 10 mmol/L HEPES) at pH 7.1 as described by (Evans et al. (2010) e Trammell & Brenner (2013) removed all chlorophyll from the vegetal, leaving the extracts distinctly green. This caused significant interference in detecting the target compounds. Attempts to use activated charcoal to remove chlorophyll from ethanolic extracts were also unsuccessful, as it retained low to medium molecular weight molecules like NAD+ and its precursors. Activated charcoal is even employed in purifying enzymes to eliminate remnants of NAD+ for use in enzymatic assays determining NAD/NADH (Kanamori et al., 2018).

An alternative extraction method could involve the use of surfactants, which are amphiphilic molecules consisting of a hydrophilic and a hydrophobic moiety. These molecules can diffuse across interfaces with different polarities, reducing surface and interfacial tensions, and aiding in the dissolution of lignin (Melro et al., 2021). An aqueous solution containing the surfactant potassium laurate and acetic acid as the extraction solvent was developed to obtain NMN from broccoli (patent CN110559224A). According to the inventors, potassium laurate was selected through extensive screening to improve the extraction conditions. After adding the surfactant, the yield of nicotinamide mononucleotide was significantly enhanced, and the extraction time shortened to 60-90 minutes (Lu et al., 2019). Another potential, more sustainable alternative could be the use of microbial biosurfactants (Zanotto et al., 2019), which are already employed for the extraction of bioactive compounds in plants (Javed et al., 2022). However, their application in NAD+ precursors extraction remains to be explored.

Despite that, nearly all samples analyzed by HPLC-UV/DAD had their NAD+ and precursors content confirmed through our single run LC-MS/MS method.

4. CONCLUSION

The HPLC-UV/DAD method for the simultaneous determination of NAD+ and its precursors, NMN, NR, and NAM, was found to be linear and precise. Although HPLC-UV/DAD had not sensitivity as LC coupled to mass spectrometry (LC-MS), our method has

proven to be suitable and a more accessible way, as not all laboratories are equipped with LC-MS, to conduct screenings and evaluations of the content of new NAD+ precursors in food samples.

We found that NAD+ and its precursors, NMN and NR, were present in a wide variety of vegetal, including unconventional food plants (PANC) of the Brazilian Amazon region, conventional leafy greens, roots, legumes, and certain fruits. Among PANC, Moringa exhibited the highest NR content, while purple malanga (of March) had the highest NAD+ levels. Among leafy greens, broccoli contained the most NMN, wild chicory had the highest NR levels, and collard had the highest NAD+ content. Beetroot emerged as the richest source of NMN among roots and tubers. Among vegetables, bell peppers and tomatoes contained NMN, NR, and NAD+, with cucumber peel having the highest NMN content. In fruits, NR predominated, with bananas and oranges having the highest levels. Only green beans among legumes contained all three compounds, with cooked green beans having the highest NMN and NAD+ content. Lastly, among cereals and pseudocereals, only NAM was found, exclusively in brown rice. In addition, thermal treatment had varied effects in samples and in NAD+ and its precursor's content, but, in general NR was the most heat-sensitive.

The determination of recently discovered NAD+ biosynthesis precursors, NMN and NR, in natural foodstuffs is crucial for understanding how to optimize NAD+ levels through diet. As well as highlights the importance of evaluate the effects of processing techniques on their content, bioavailability, and effectiveness in maintaining NAD+ homeostasis. Finally, our work adds to the advancement of NAD+ metabolome knowledge in the fields of food science and nutrition.

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AUTHORSHIP CONTRIBUTION STATEMENT

Alegre, GFS: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. Pastore, GM: Conceptualization, Methodology, Supervision, Funding acquisition.

DISCLOSURE STATEMENT

The authors declare that they have no conflict of interest.

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Supplementary Tables

Supplementary Table 1. Gradient conditions for UHPLC-MS/MS separation.

Time	**%A (Water + Formic Acid 0.1%)**	**%B (Methanol + Formic Acid 0.1%)**	**Curve**
Initial	15	85	Initial
1.00	15	85	6
2.00	95	5	6
6.00	95	5	6
6.10	15	85	6
8.00	15	85	6

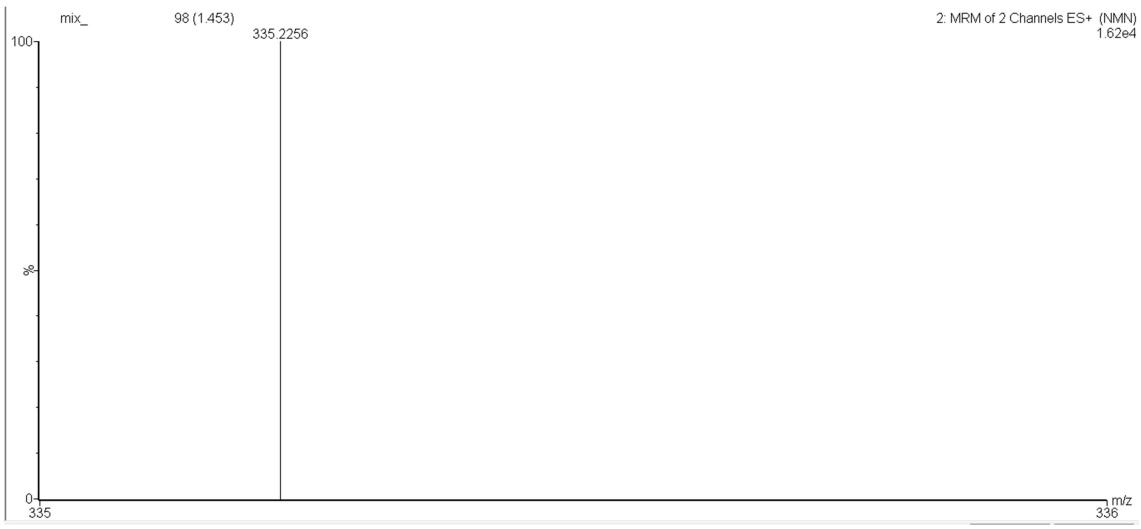
Supplementary Table 2. Multiple Reaction Monitoring (MRM) conditions of NMN, NR, NAM and NAD determined by UHPLC-MS/MS.

Analyte	**Precursor ion (m/z)**	**Product ions (m/z)**	**Collision (V)**	**Cone (V)**	**Dwell (s)**	**RT (min)**
NAM	122.9543	52.7054	27	31	0.100	1.03
		79.8559	19			
NR	255.272	123.0544	7	16	0.100	2.95
		133.0557	9			
NMN	335.2256	96.9507	19	16	0.100	1.38
		123.0479	11			
NAD	664.1552	136.2067	40	21	0.100	2.65
		428.2043	21			

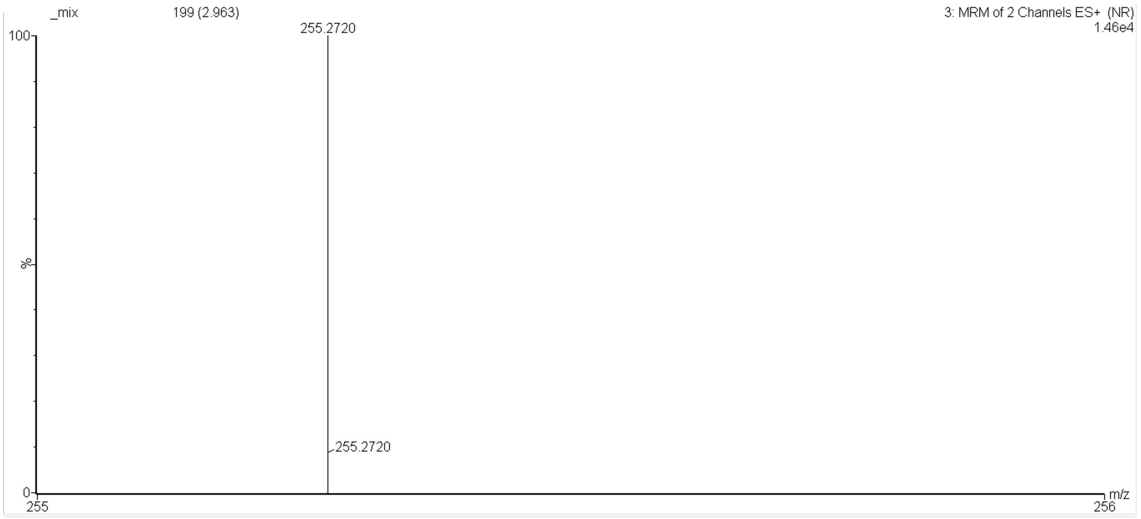
Supplementary Figures

Supplementary Figure 1. Mass spectrum of NMN (A), NR (B), NAM (C) and NAD (D) in aqueous standard solution, using a BEH Amide column (2.1 mm I.D. × 100 mm, 1.7 μm).

A



B



C

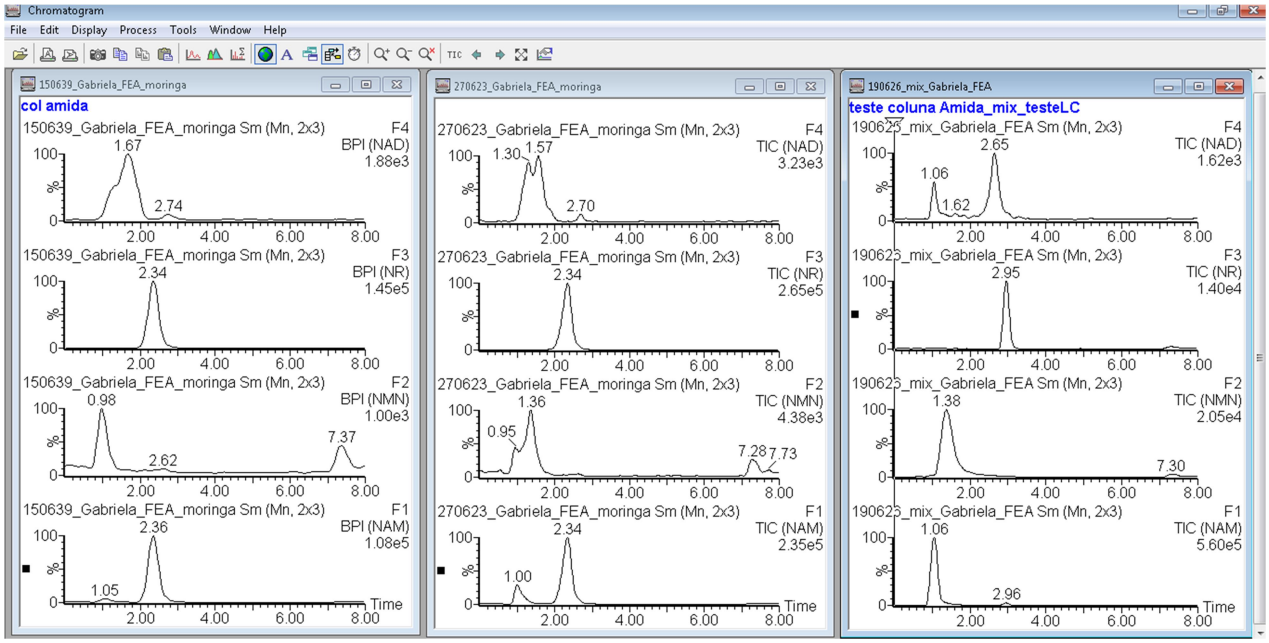

_mix
72 (1.062)
1: MRM of 2 Channels ES+ (NAM)
122.9543 4.56e5
100
%
0
122
123
m/z

D

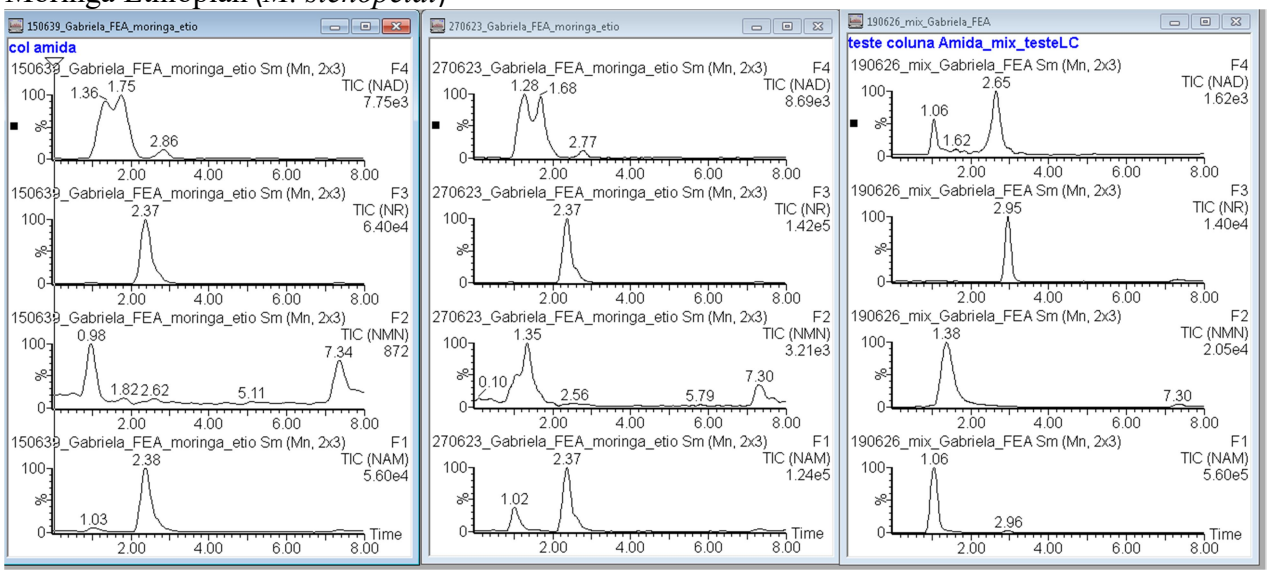

_mix
174 (2.594)
664.1552
4: MRM of 2 Channels ES+ (NAD)
1.17e3
100
%
664.1552
0
664
665
m/z

Supplementary Figure 2. LC/MS/MS chromatograms of NMN, NR, NAM and NAD present in food samples, using a BEH Amide column (2.1 mm I.D. × 100 mm, 1.7 µm). On the left is the sample chromatogram, in the middle is the sample spiked with standards, and on the right is the standard aqueous solution mixture.

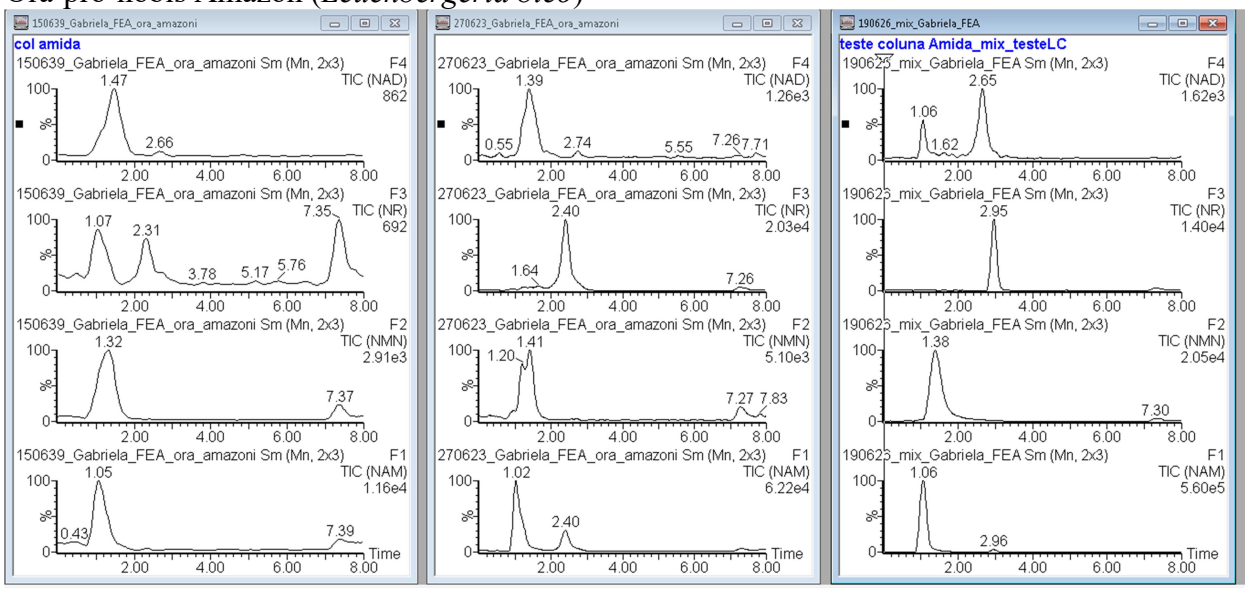
Moringa (*M. oleifera*)



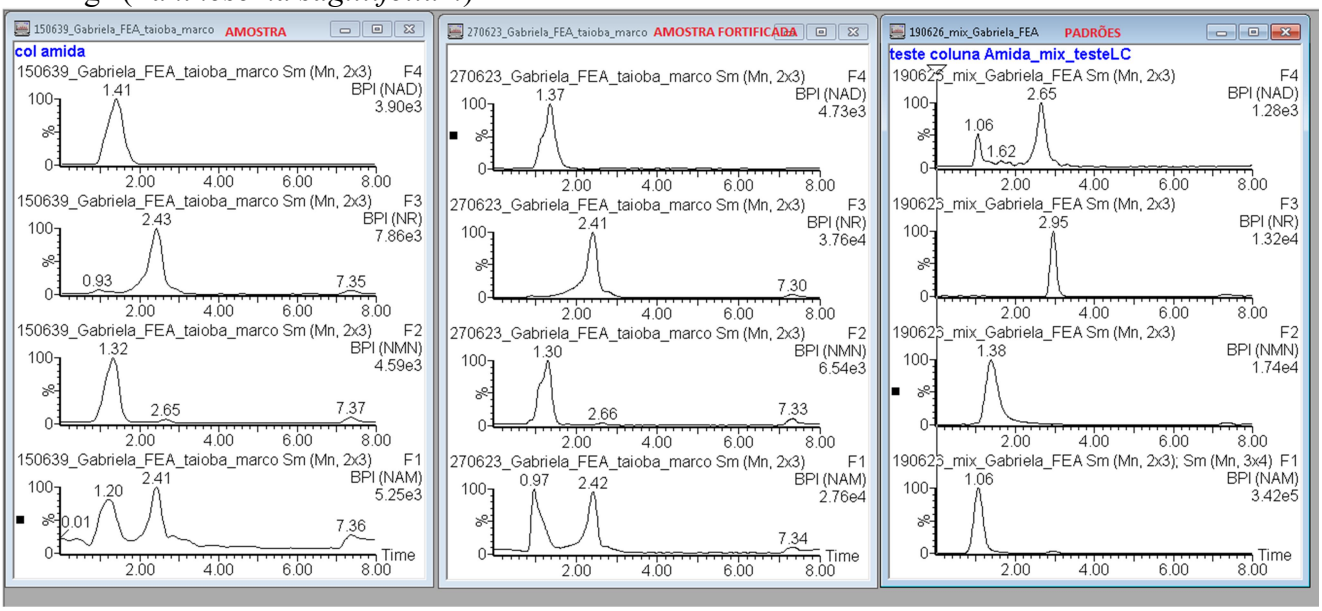
Moringa Ethiopian (*M. stenopetal*)



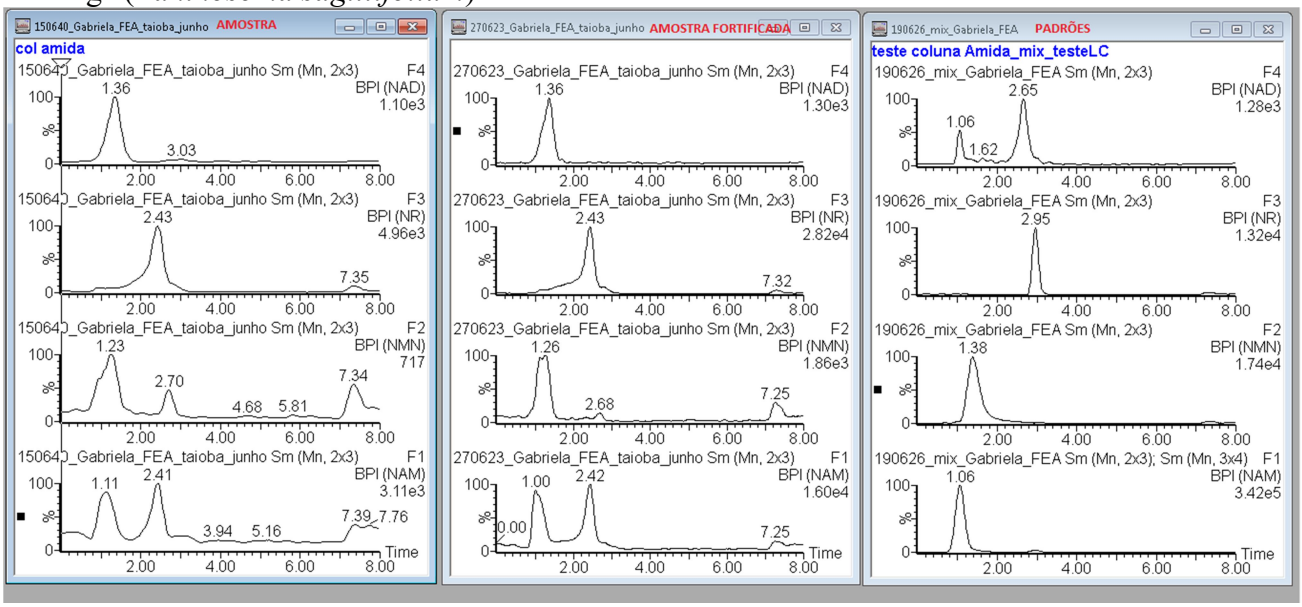
Ora-pro-nobis Amazon (*Leuenbergeria bleo*)



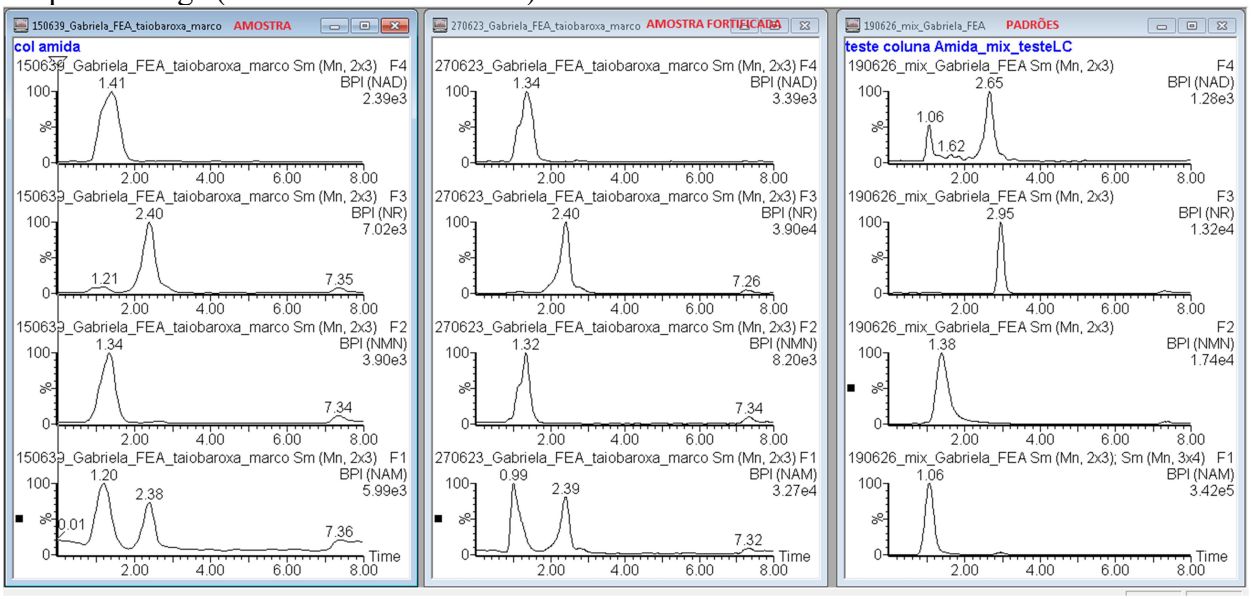
Malanga (*Xanthosoma sagittifolium*) of march



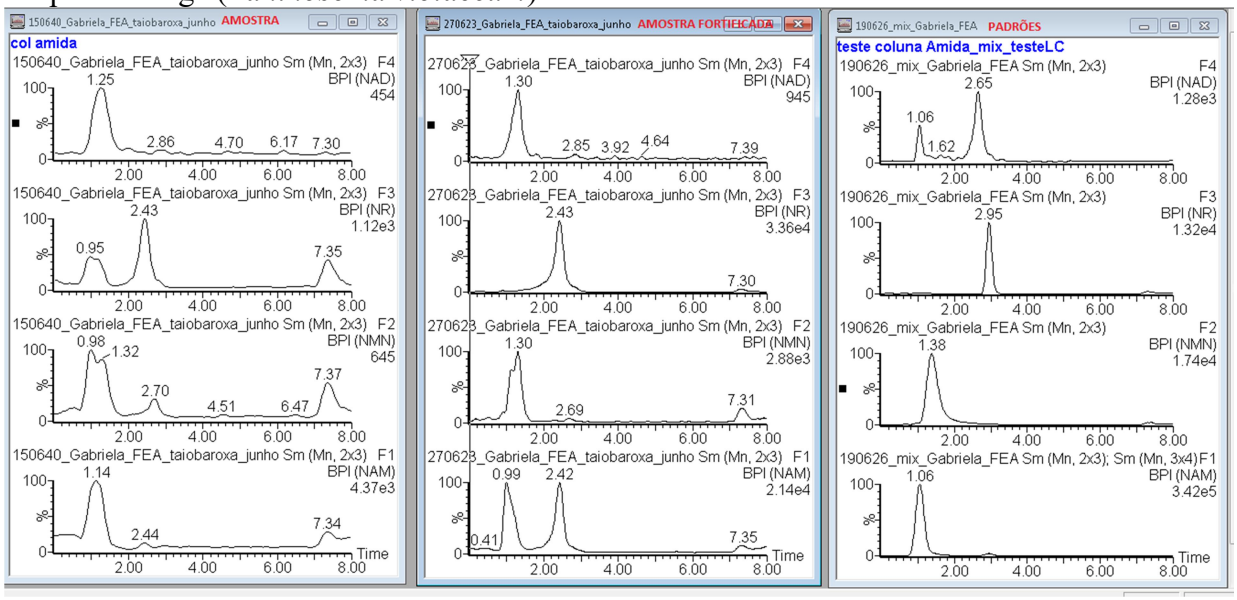
Malanga (*Xanthosoma sagittifolium*) of June



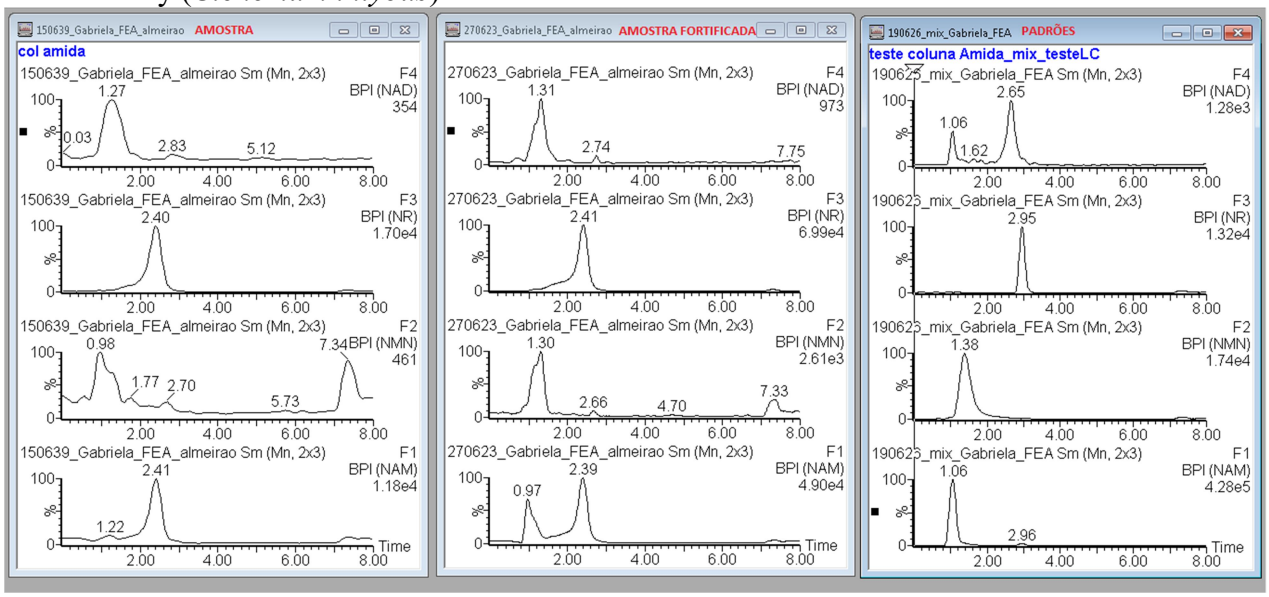
Purple Malanga (*Xanthosoma violaceum*) of March



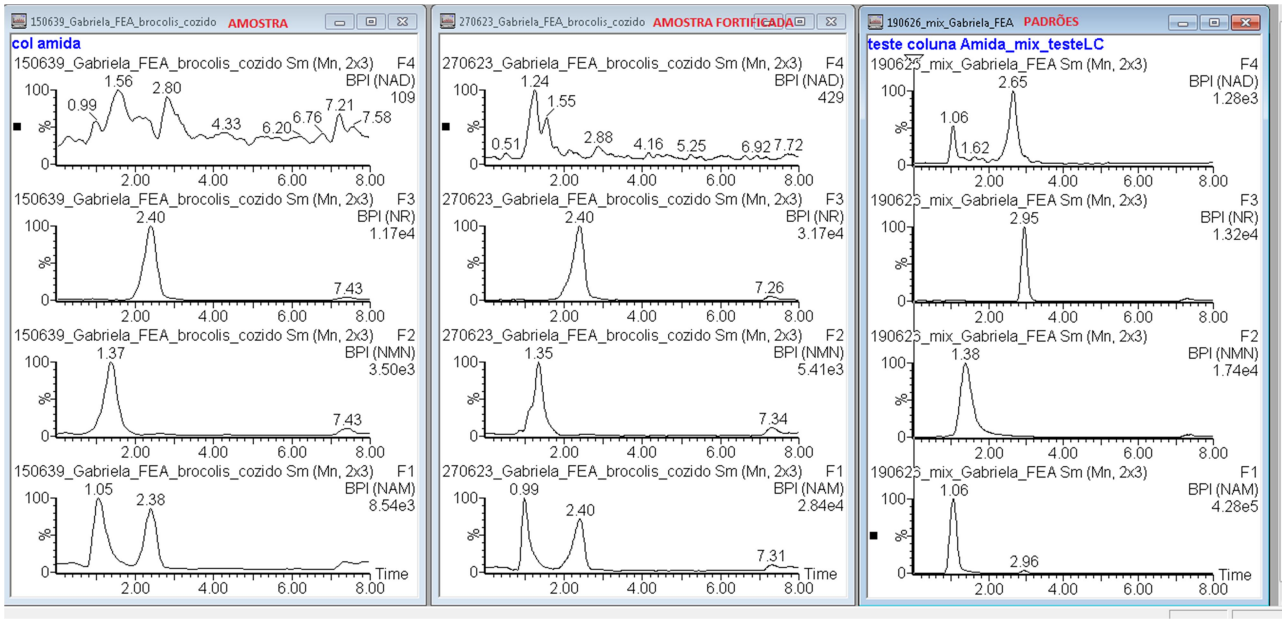
Purple Malanga (*Xanthosoma violaceum*) of June



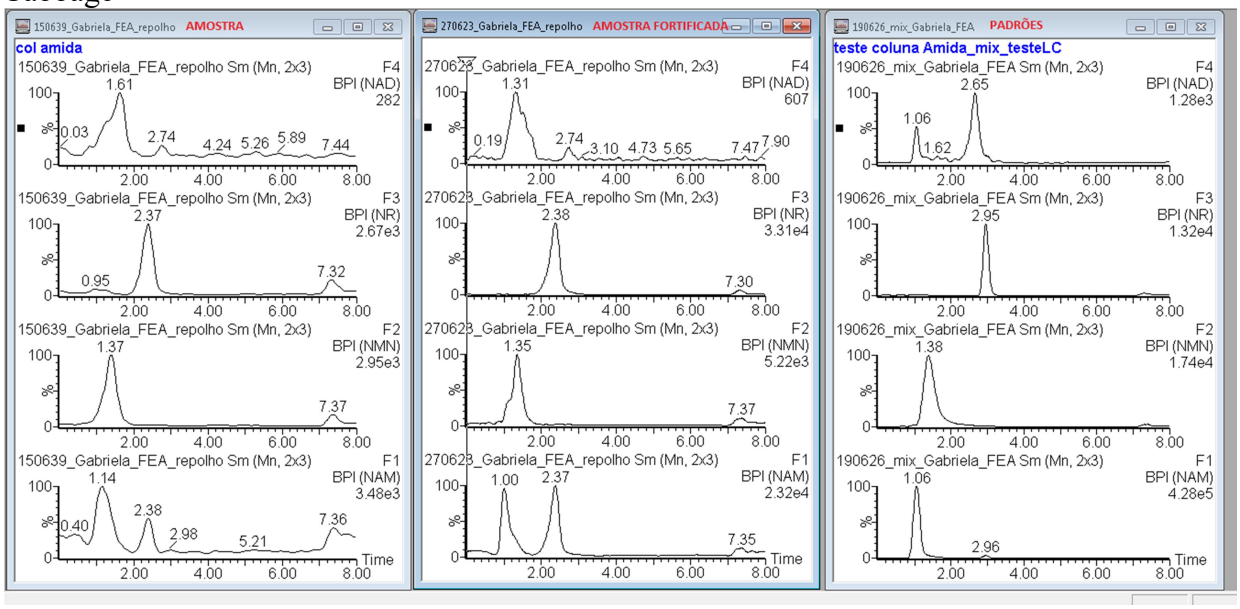
Wild chicory (*Cichorium intybus*)



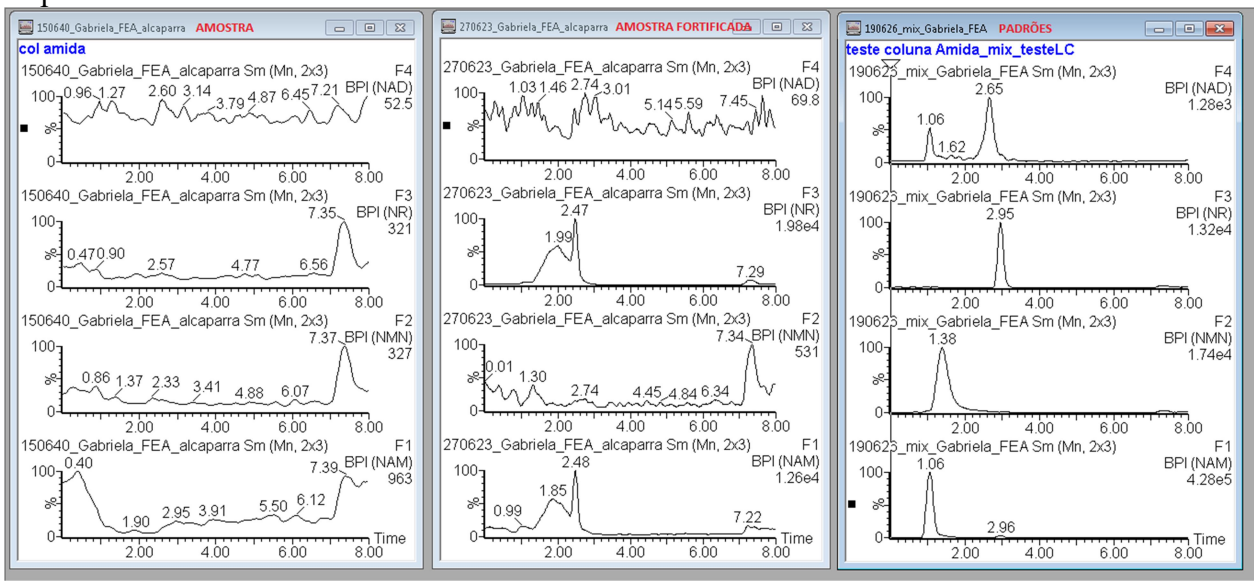
Broccoli steamed



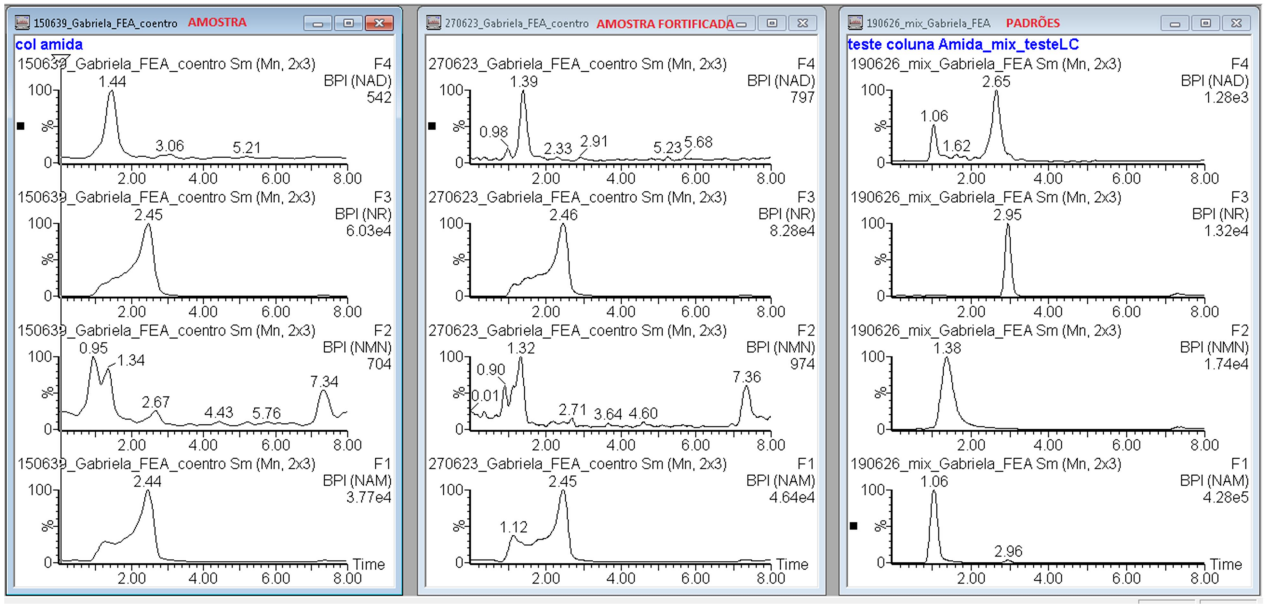
Cabbage



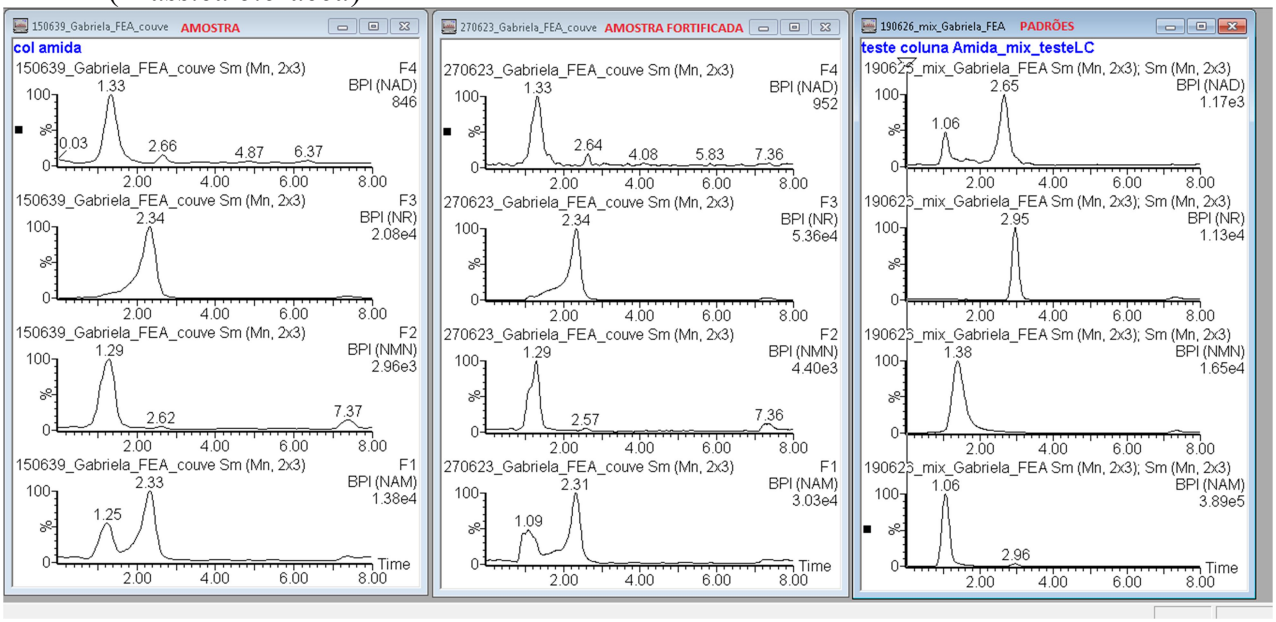
Capers



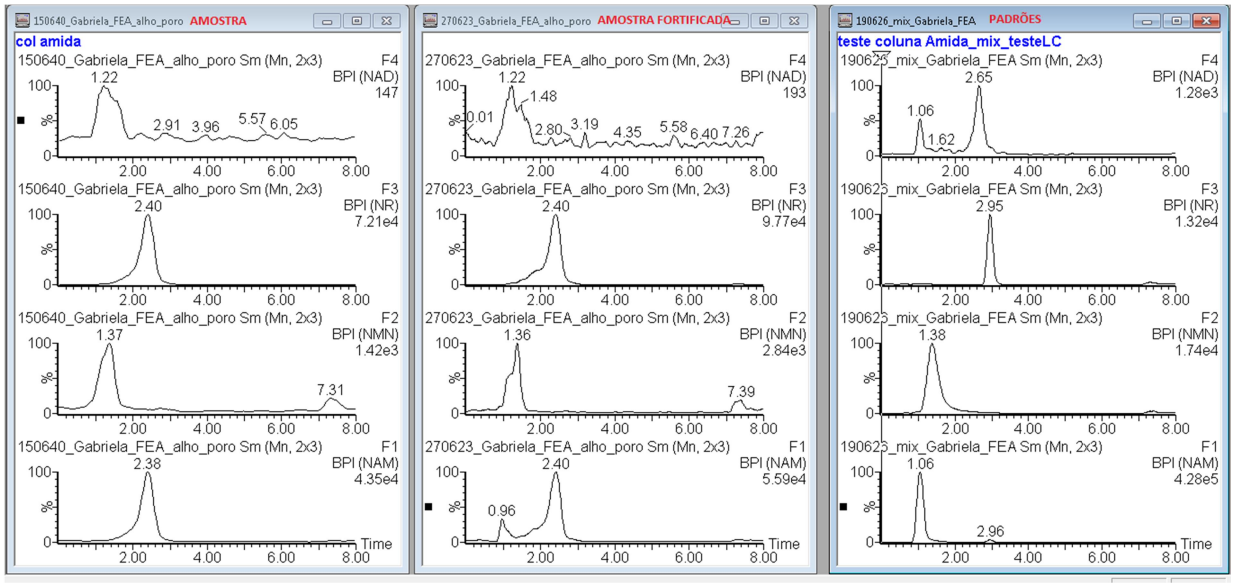
Cilantro



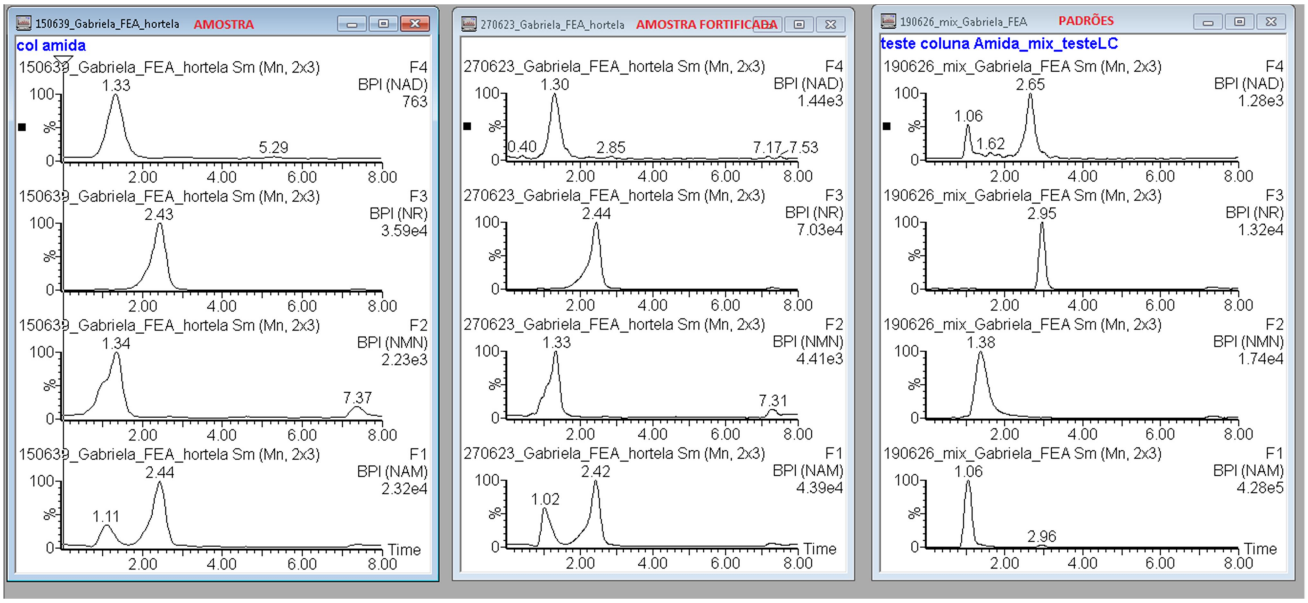
Collard (*Brassica oleracea*)



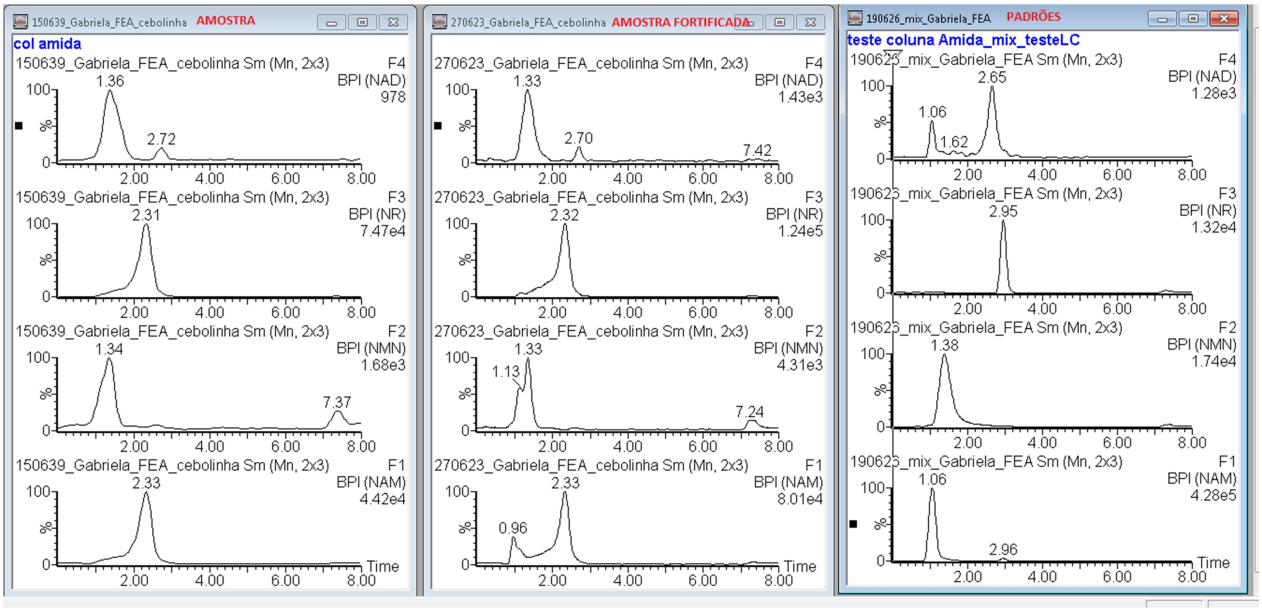
Leek



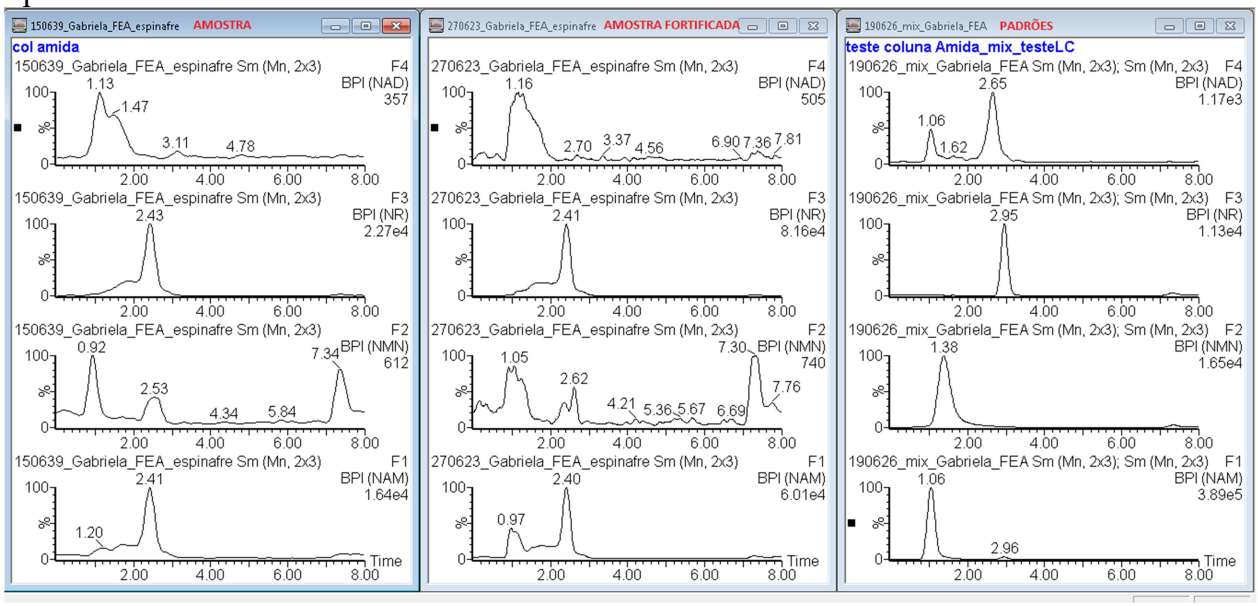
Mint



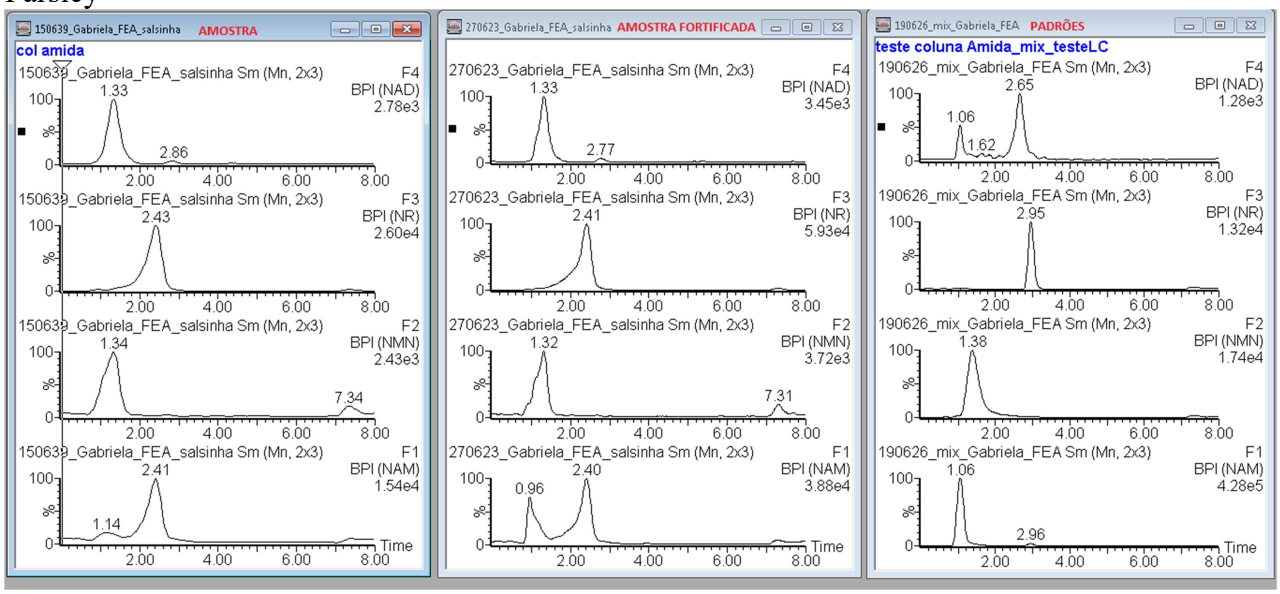
Scallion



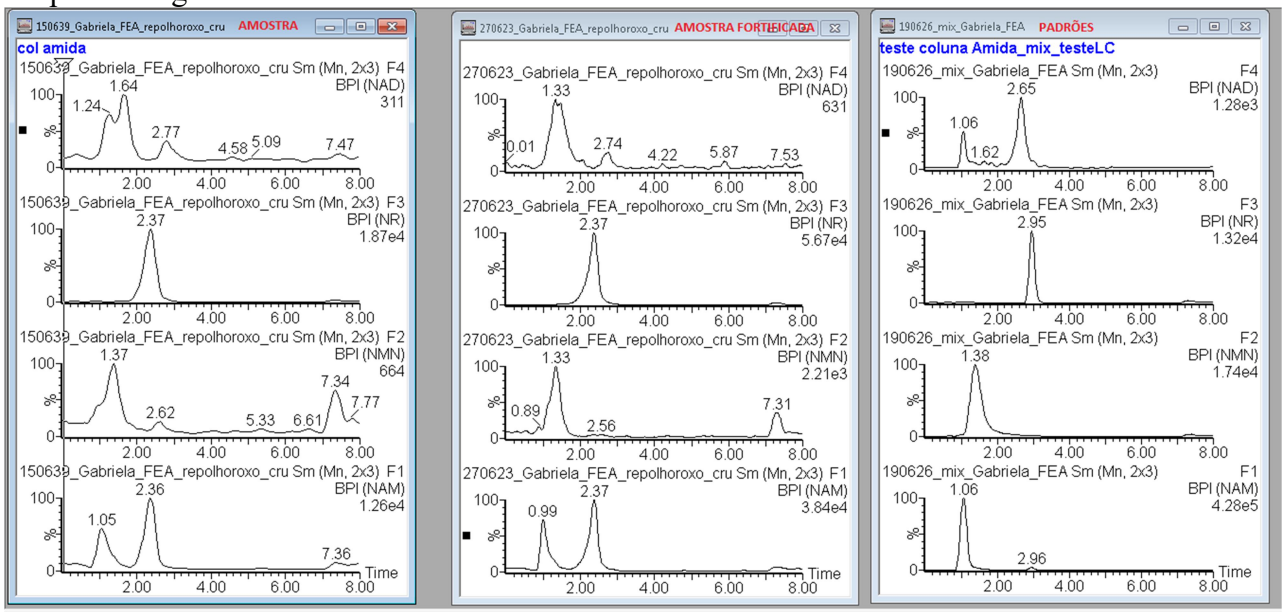
Spinach



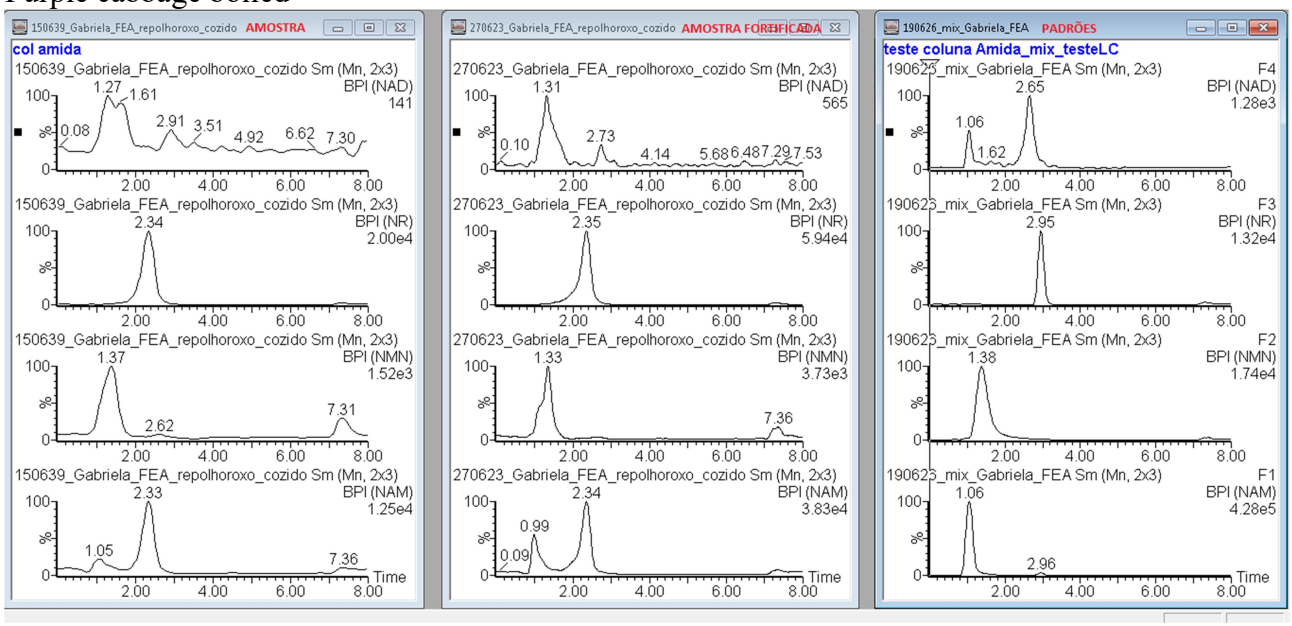
Parsley



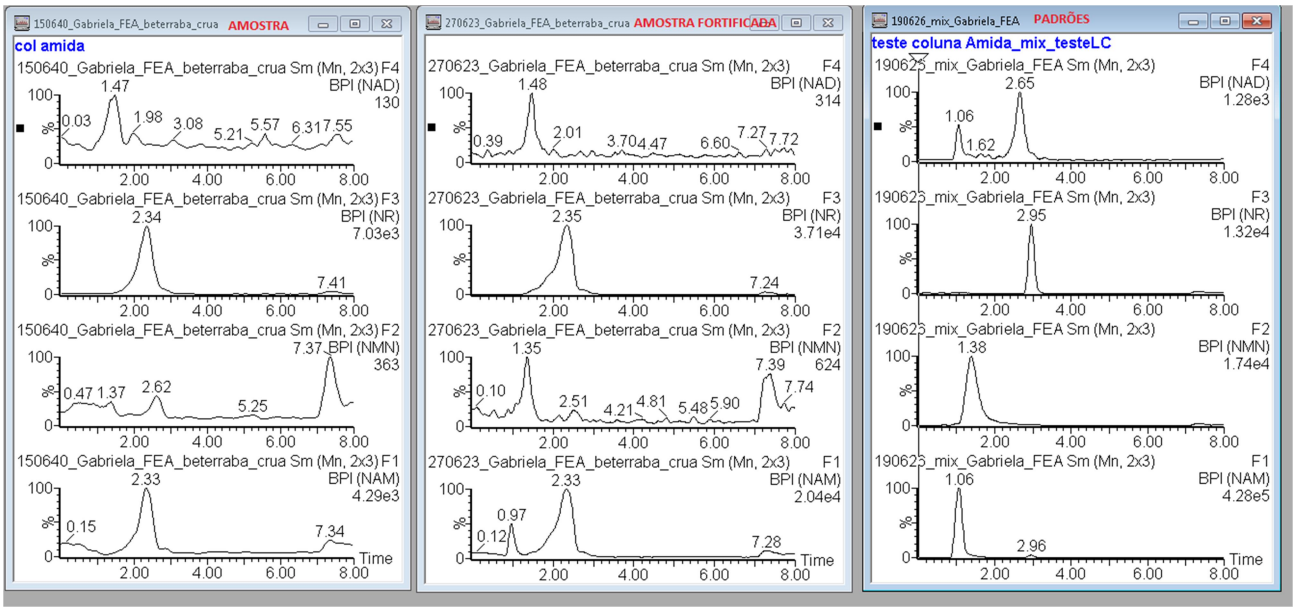
Purple cabbage



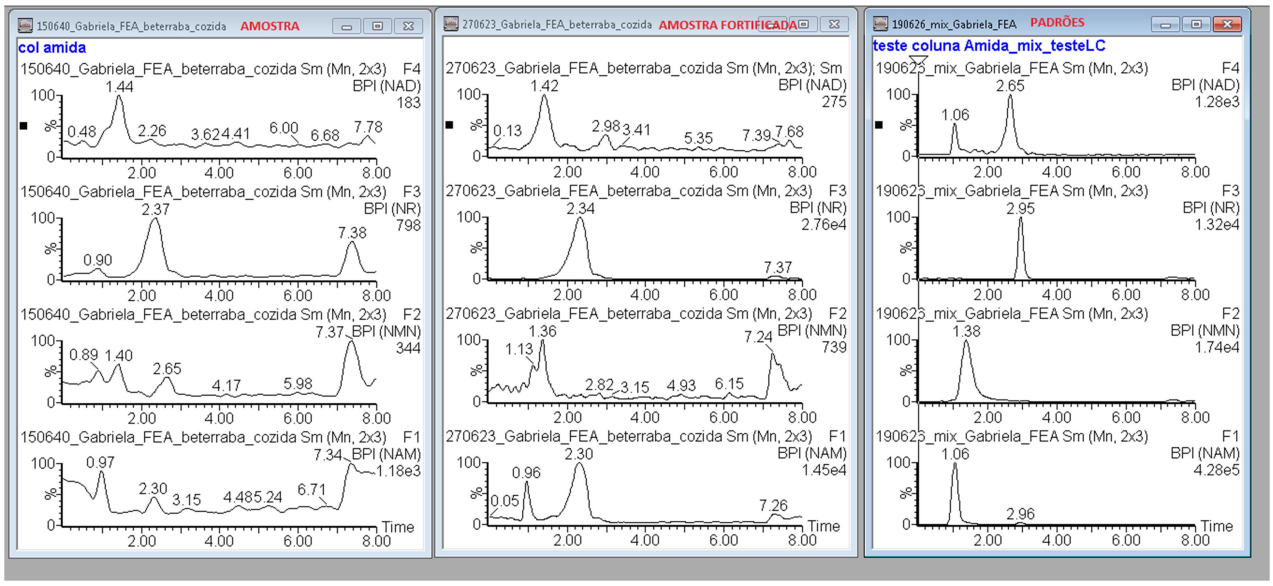
Purple cabbage boiled



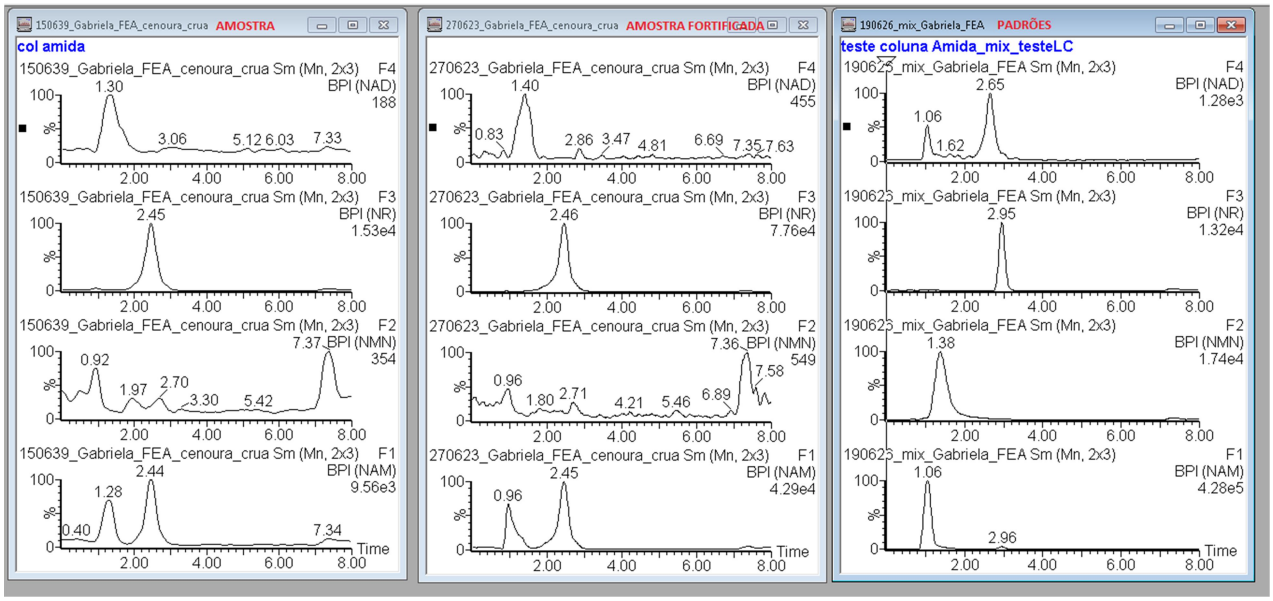
Beet



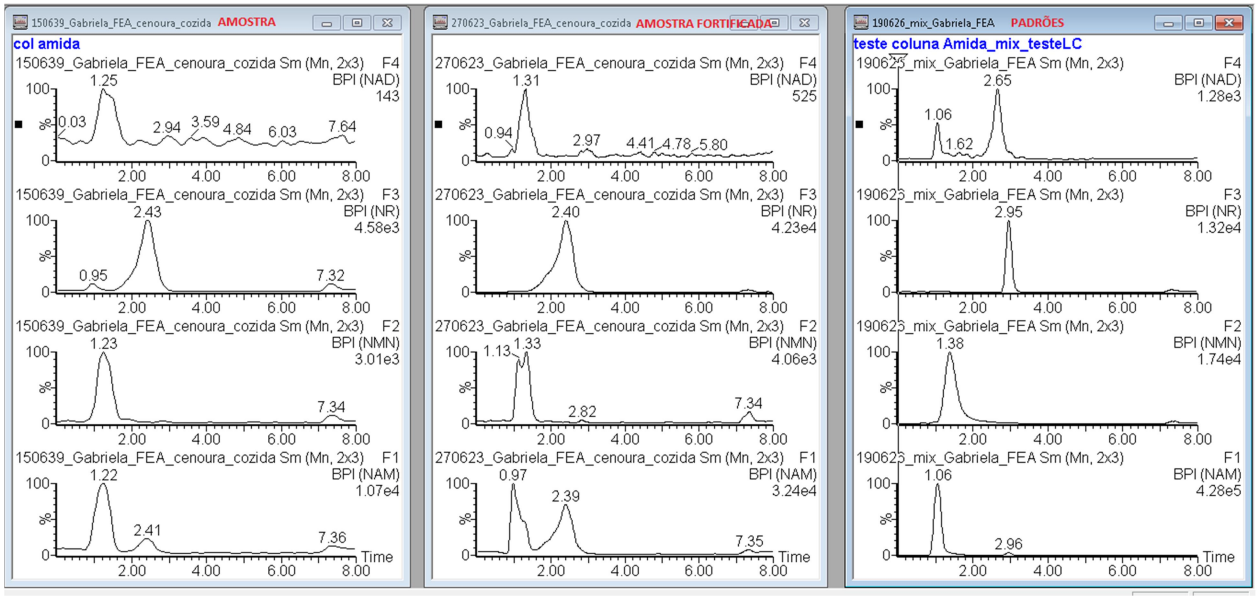
Beet steamed



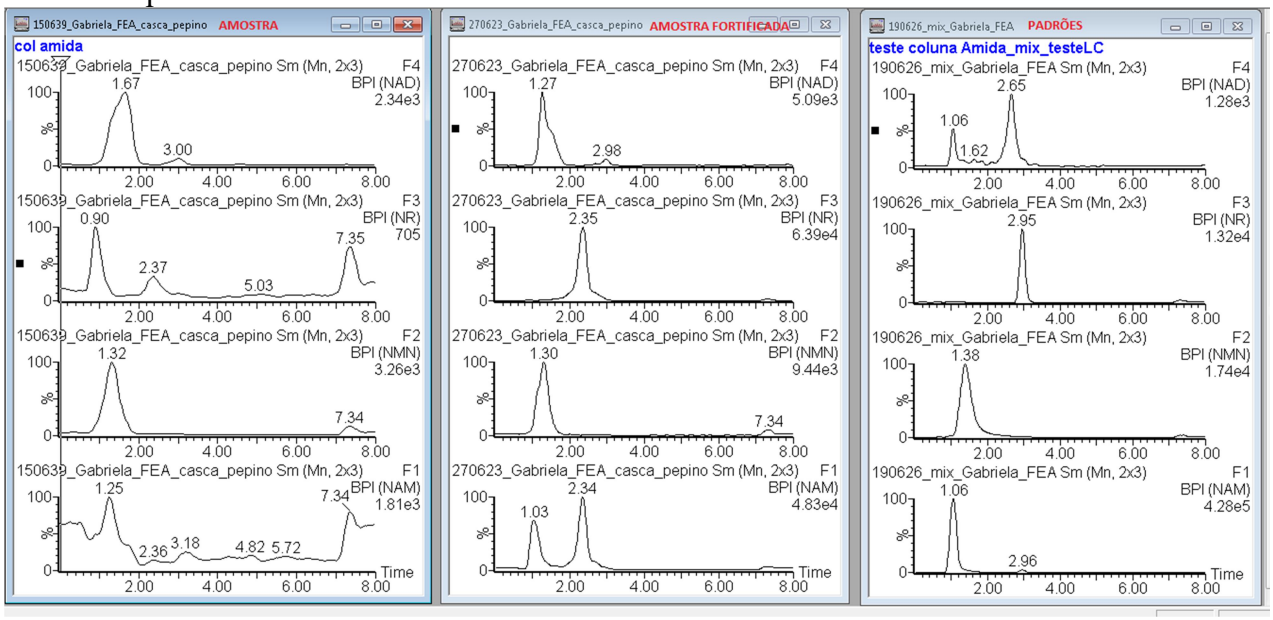
Carrot



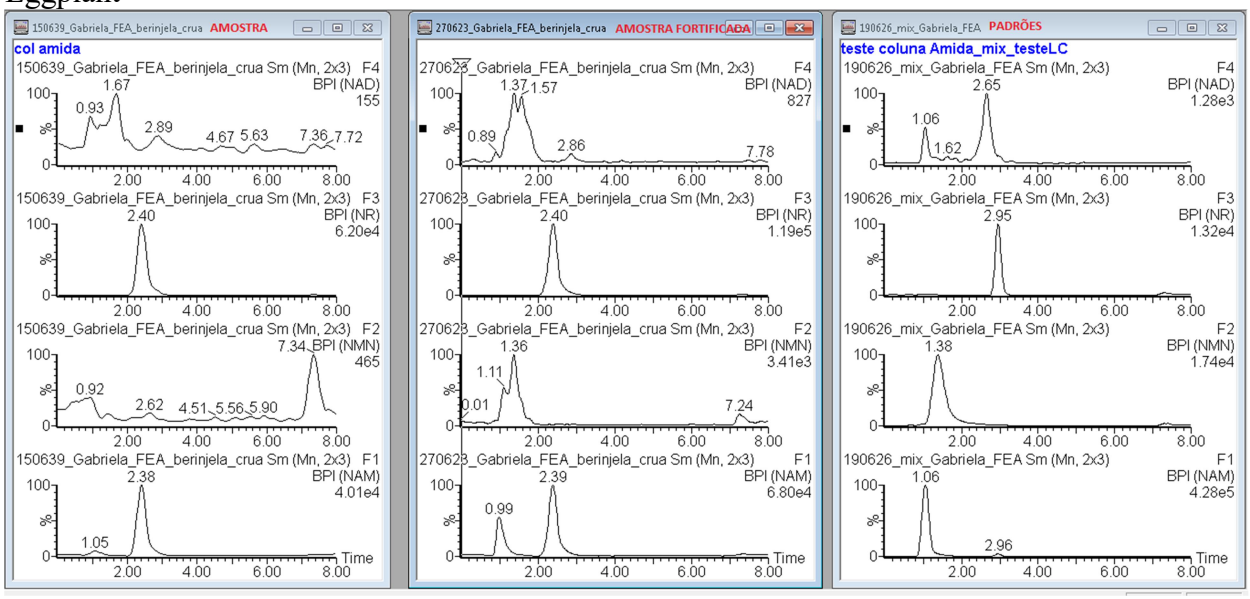
Carrot steamed



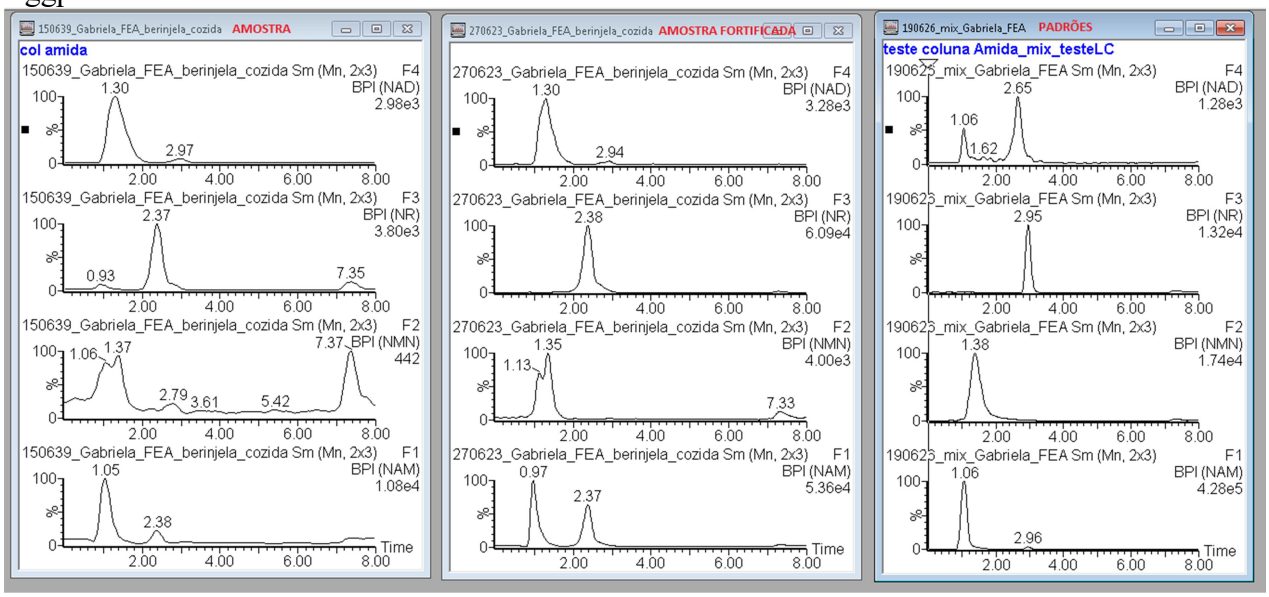
Cucumber peel



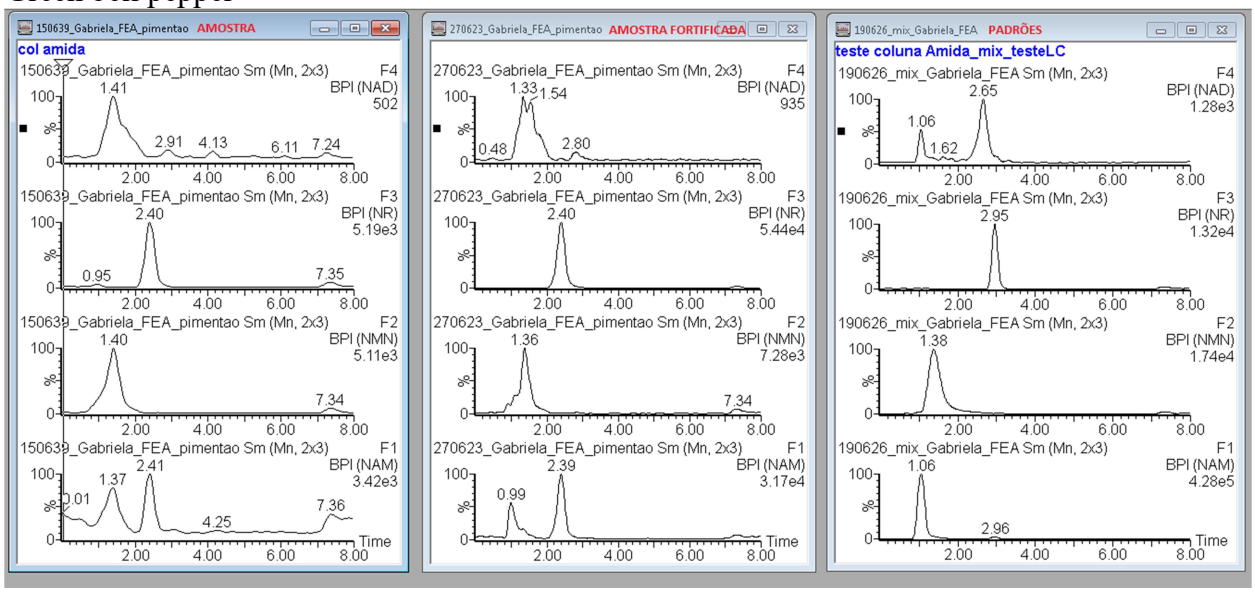
Eggplant



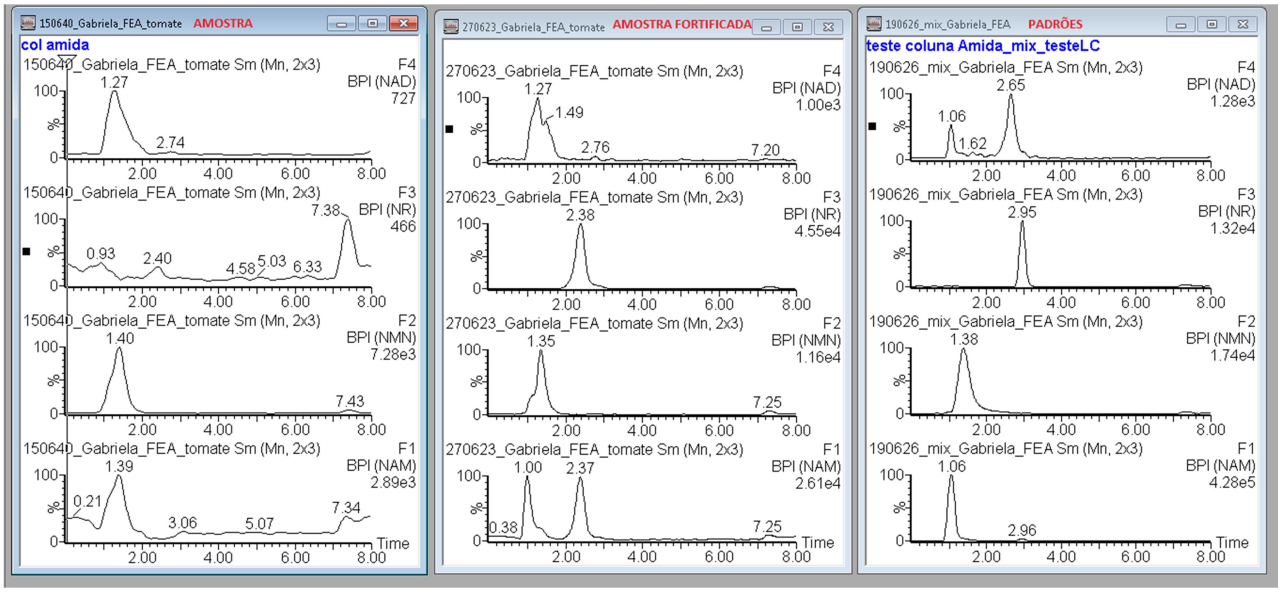
Eggplant steamed



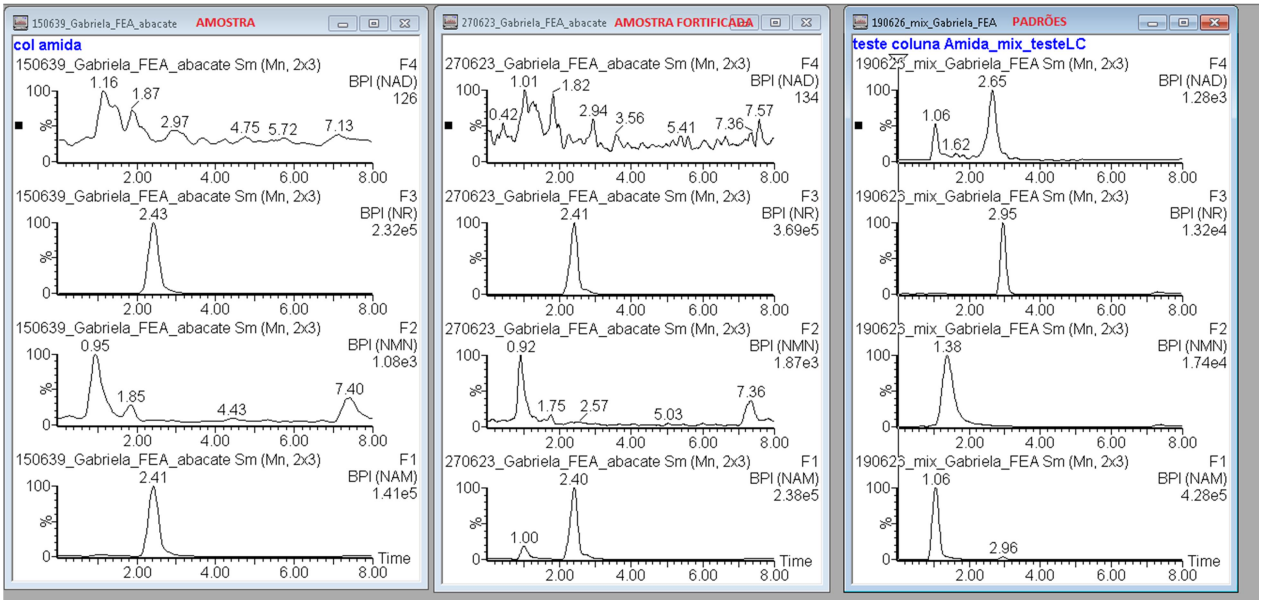
Green bell pepper



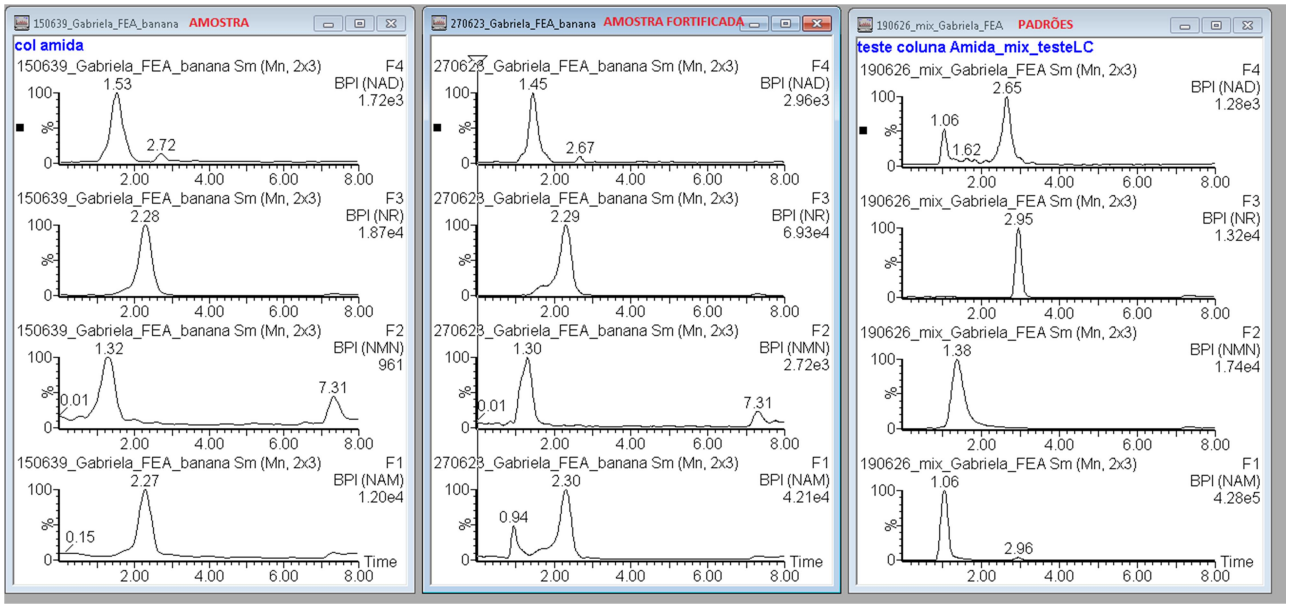
Tomato



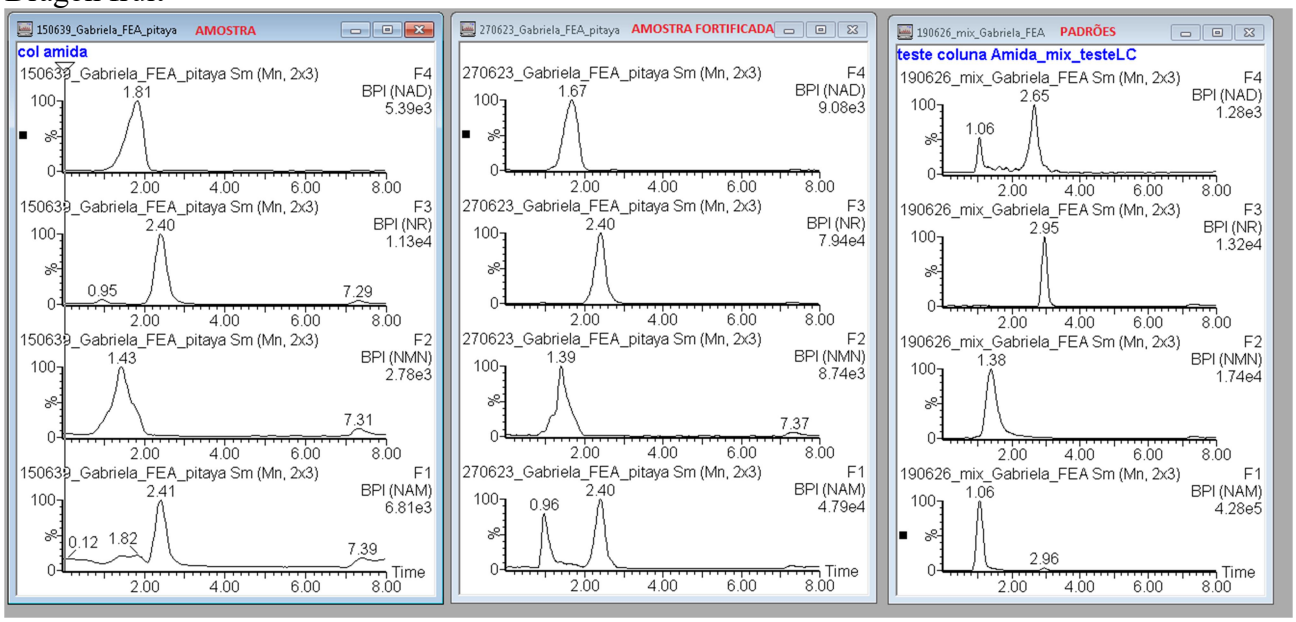
Avocado



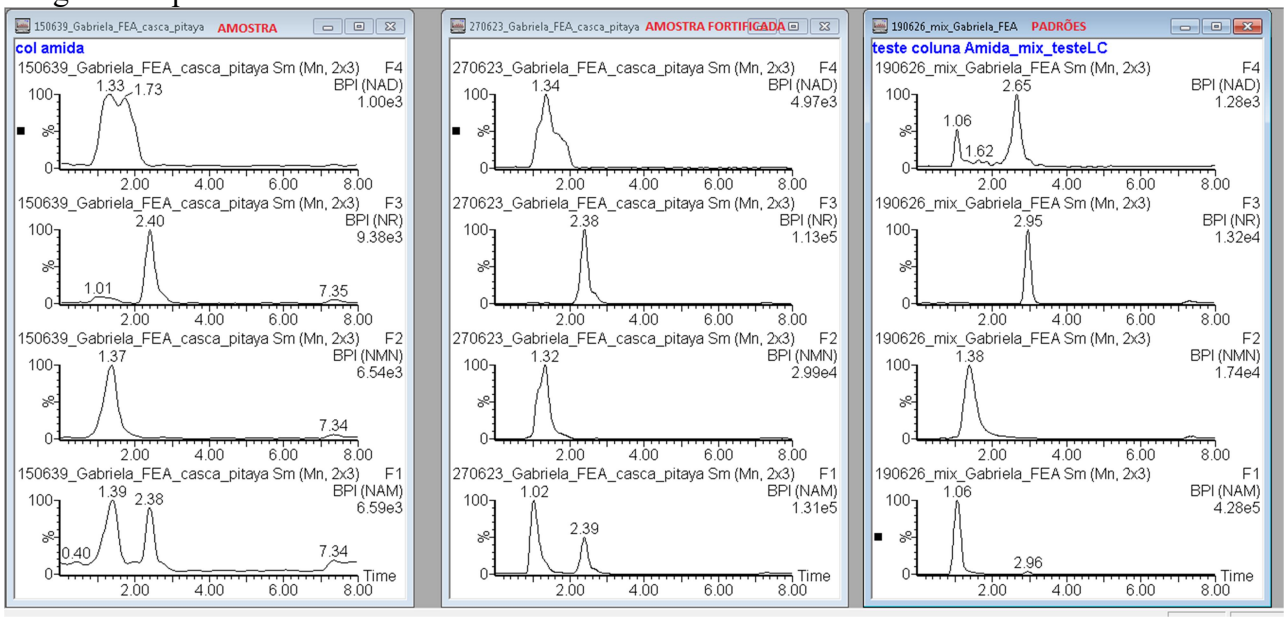
Banana



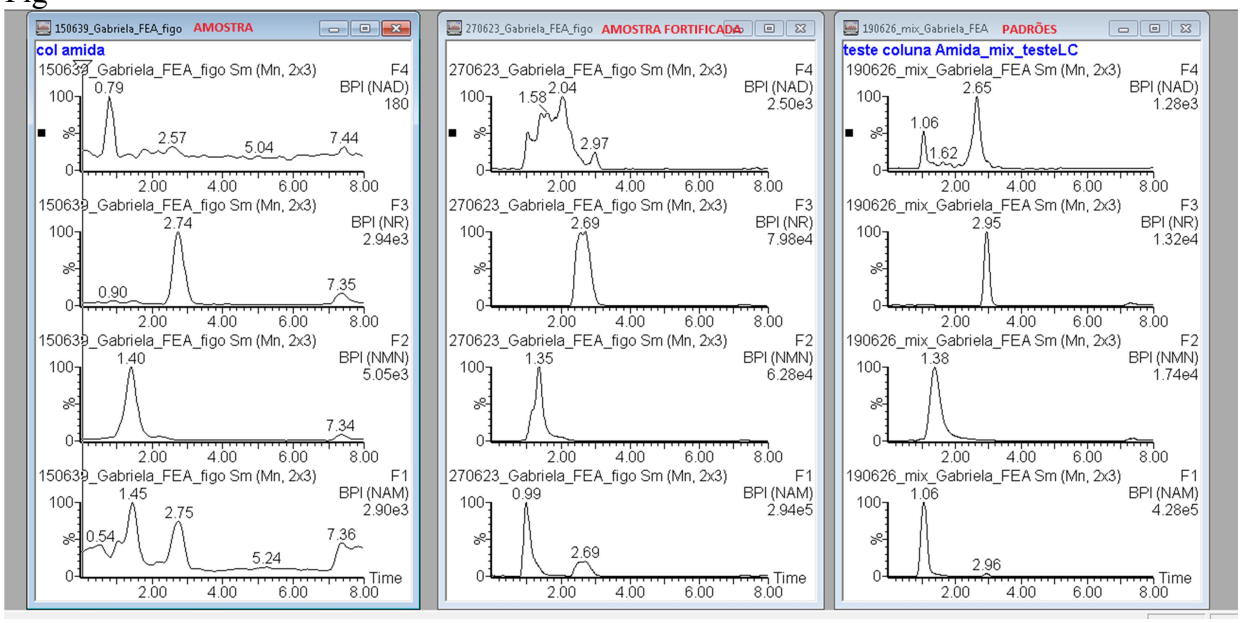
Dragon fruit



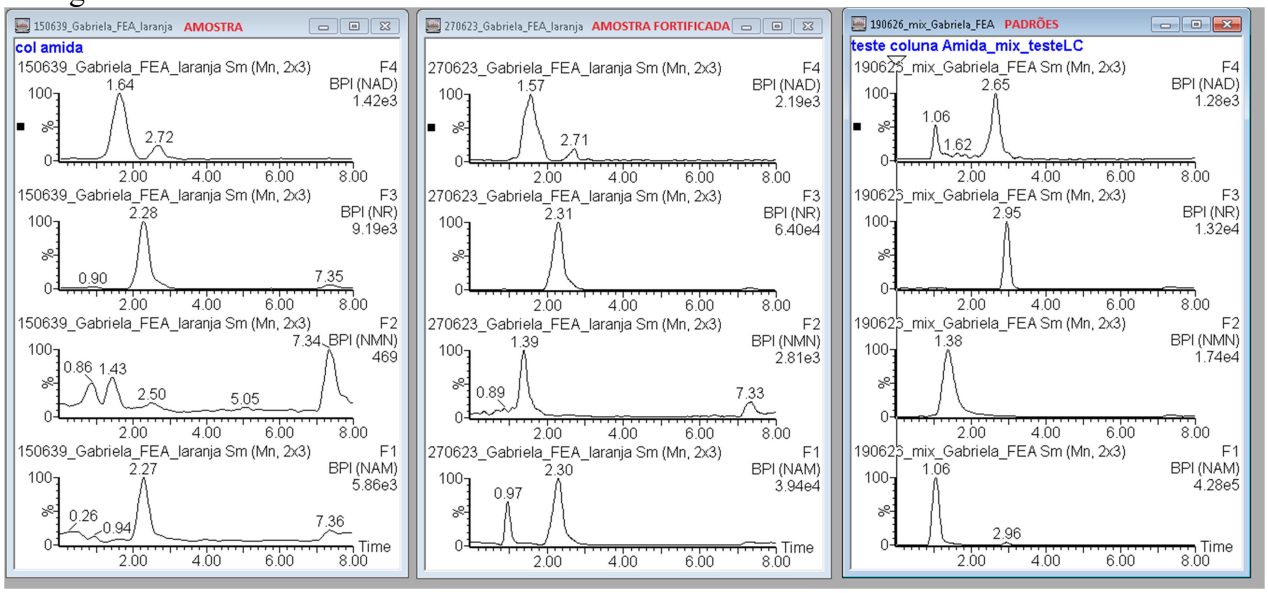
Dragon fruit peel



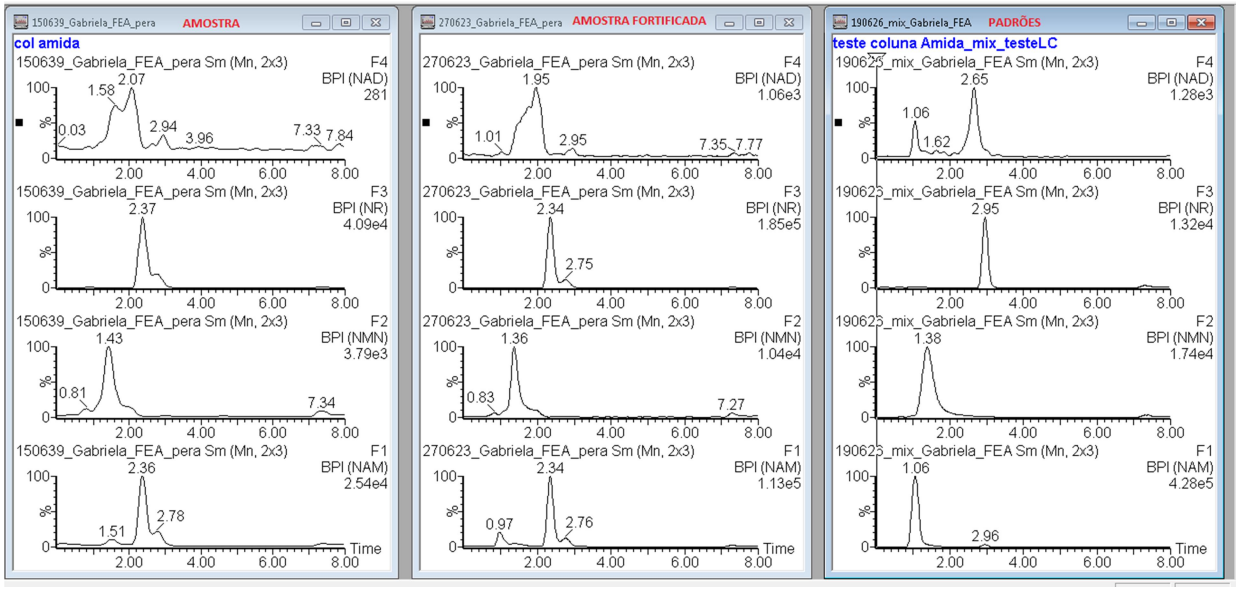
Fig



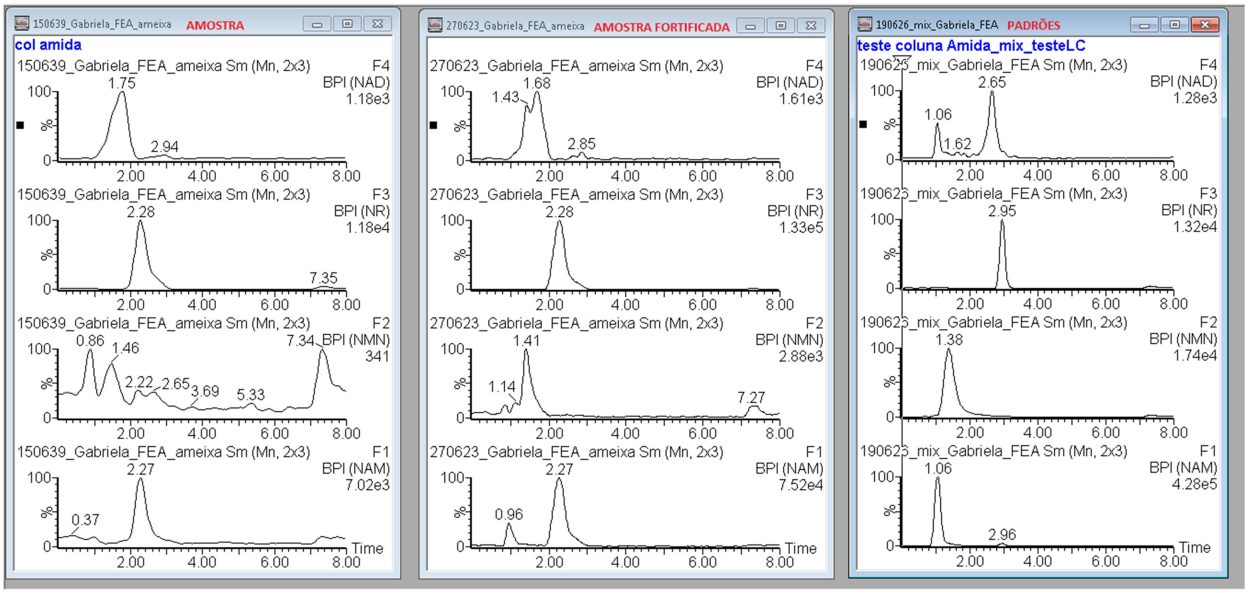
Orange


150639_Gabriela_FEA_laranja AMOSTRA
col amida
150639_Gabriela_FEA_laranja Sm (Mn, 2x3) F4 BPI (NAD) 1.42e3
1.64
2.72
150639_Gabriela_FEA_laranja Sm (Mn, 2x3) F3 BPI (NR) 9.19e3
2.28
0.90
7.35
150639_Gabriela_FEA_laranja Sm (Mn, 2x3) F2 BPI (NMN) 469
7.34
0.86
1.43
2.50
5.05
150639_Gabriela_FEA_laranja Sm (Mn, 2x3) F1 BPI (NAM) 5.86e3
2.27
0.26
0.94
7.36
Time
270623_Gabriela_FEA_laranja AMOSTRA FORTIFICADA
270623_Gabriela_FEA_laranja Sm (Mn, 2x3) F4 BPI (NAD) 2.19e3
1.57
2.71
270623_Gabriela_FEA_laranja Sm (Mn, 2x3) F3 BPI (NR) 6.40e4
2.31
270623_Gabriela_FEA_laranja Sm (Mn, 2x3) F2 BPI (NMN) 2.81e3
1.39
0.89
7.33
270623_Gabriela_FEA_laranja Sm (Mn, 2x3) F1 BPI (NAM) 3.94e4
2.30
0.97
Time
190626_mix_Gabriela_FEA PADRÕES
teste coluna Amida_mix_testeLC
190625_mix_Gabriela_FEA Sm (Mn, 2x3) F4 BPI (NAD) 1.28e3
2.65
1.06
1.62
190625_mix_Gabriela_FEA Sm (Mn, 2x3) F3 BPI (NR) 1.32e4
2.95
190625_mix_Gabriela_FEA Sm (Mn, 2x3) F2 BPI (NMN) 1.74e4
1.38
190625_mix_Gabriela_FEA Sm (Mn, 2x3) F1 BPI (NAM) 4.28e5
1.06
2.96
Time

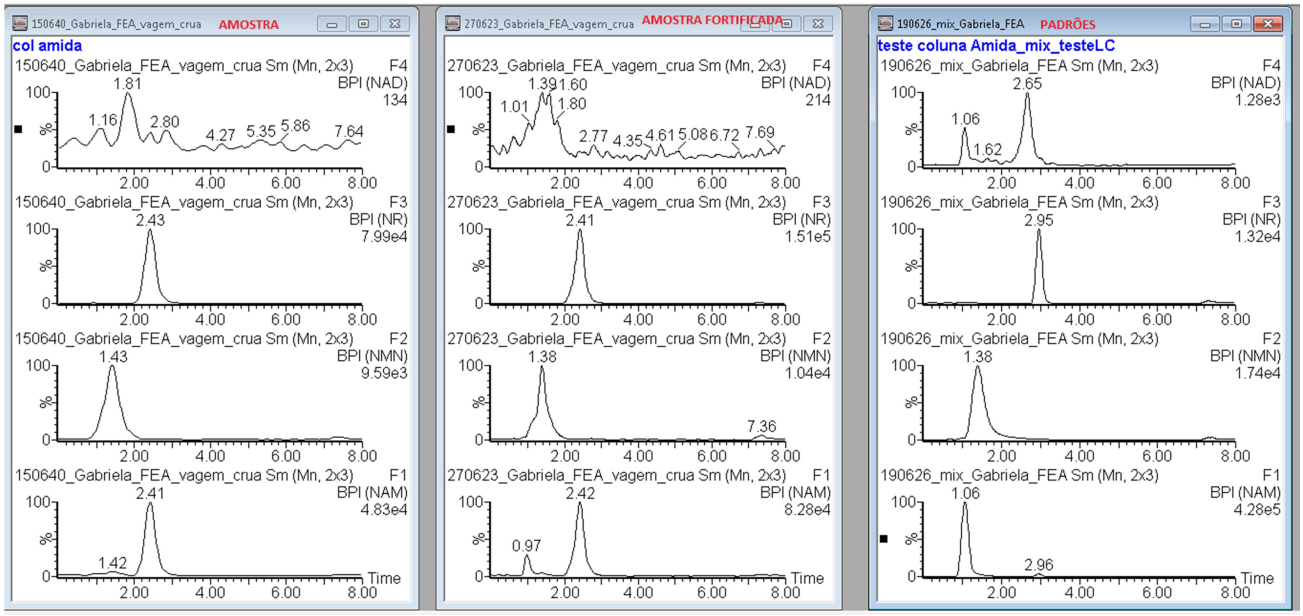
Pear


150639_Gabriela_FEA_pera AMOSTRA
col amida
150639_Gabriela_FEA_pera Sm (Mn, 2x3) F4 BPI (NAD) 281
2.07
1.58
0.03
2.94
3.96
7.33
7.84
150639_Gabriela_FEA_pera Sm (Mn, 2x3) F3 BPI (NR) 4.09e4
2.37
150639_Gabriela_FEA_pera Sm (Mn, 2x3) F2 BPI (NMN) 3.79e3
1.43
0.81
7.34
150639_Gabriela_FEA_pera Sm (Mn, 2x3) F1 BPI (NAM) 2.54e4
2.36
1.51
2.78
Time
270623_Gabriela_FEA_pera AMOSTRA FORTIFICADA
270623_Gabriela_FEA_pera Sm (Mn, 2x3) F4 BPI (NAD) 1.06e3
1.95
1.01
2.95
7.35
7.77
270623_Gabriela_FEA_pera Sm (Mn, 2x3) F3 BPI (NR) 1.85e5
2.34
2.75
270623_Gabriela_FEA_pera Sm (Mn, 2x3) F2 BPI (NMN) 1.04e4
1.36
0.83
7.27
270623_Gabriela_FEA_pera Sm (Mn, 2x3) F1 BPI (NAM) 1.13e5
2.34
0.97
2.76
Time
190626_mix_Gabriela_FEA PADRÕES
teste coluna Amida_mix_testeLC
190625_mix_Gabriela_FEA Sm (Mn, 2x3) F4 BPI (NAD) 1.28e3
2.65
1.06
1.62
190625_mix_Gabriela_FEA Sm (Mn, 2x3) F3 BPI (NR) 1.32e4
2.95
190625_mix_Gabriela_FEA Sm (Mn, 2x3) F2 BPI (NMN) 1.74e4
1.38
190625_mix_Gabriela_FEA Sm (Mn, 2x3) F1 BPI (NAM) 4.28e5
1.06
2.96
Time

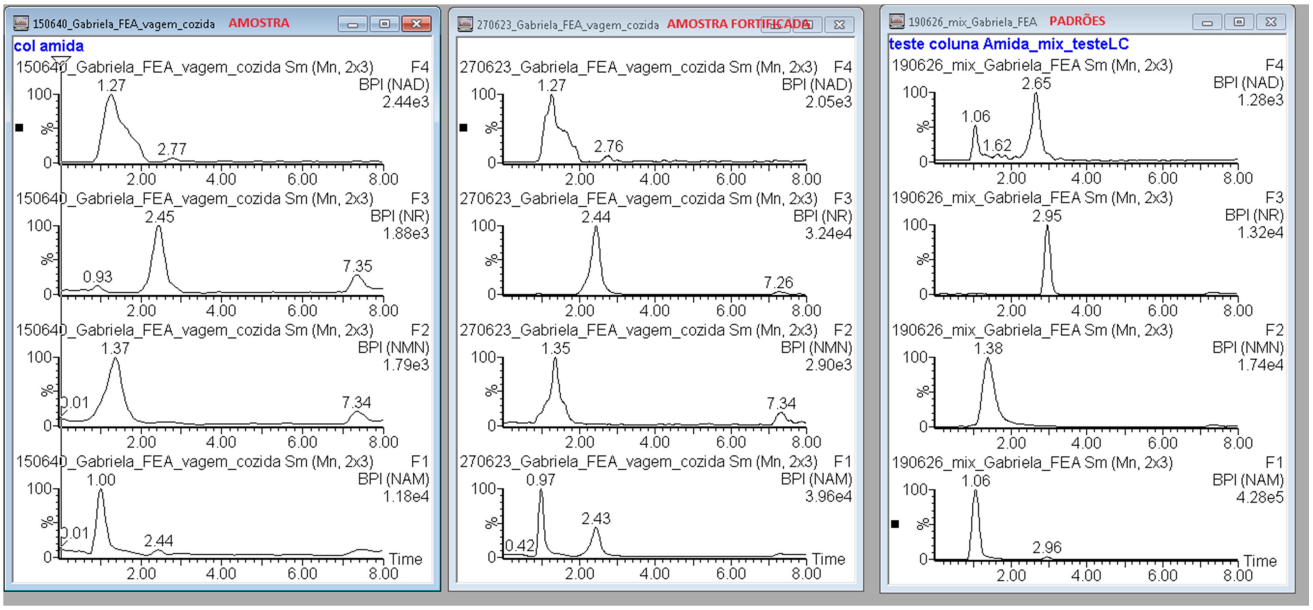
Plum



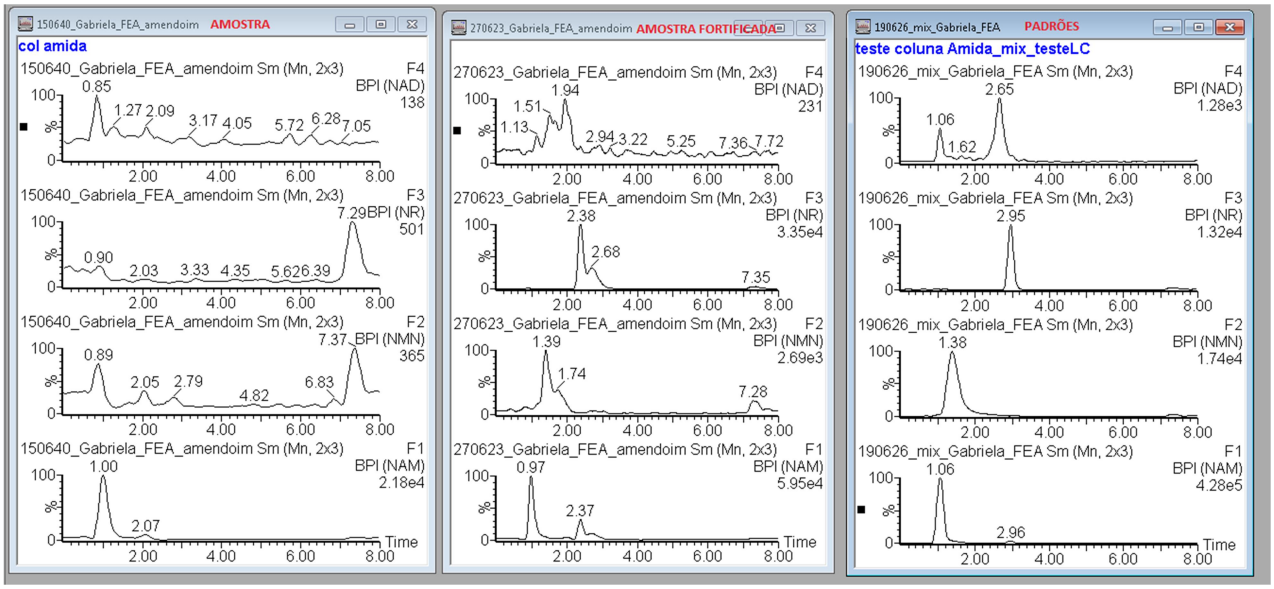
Green bean



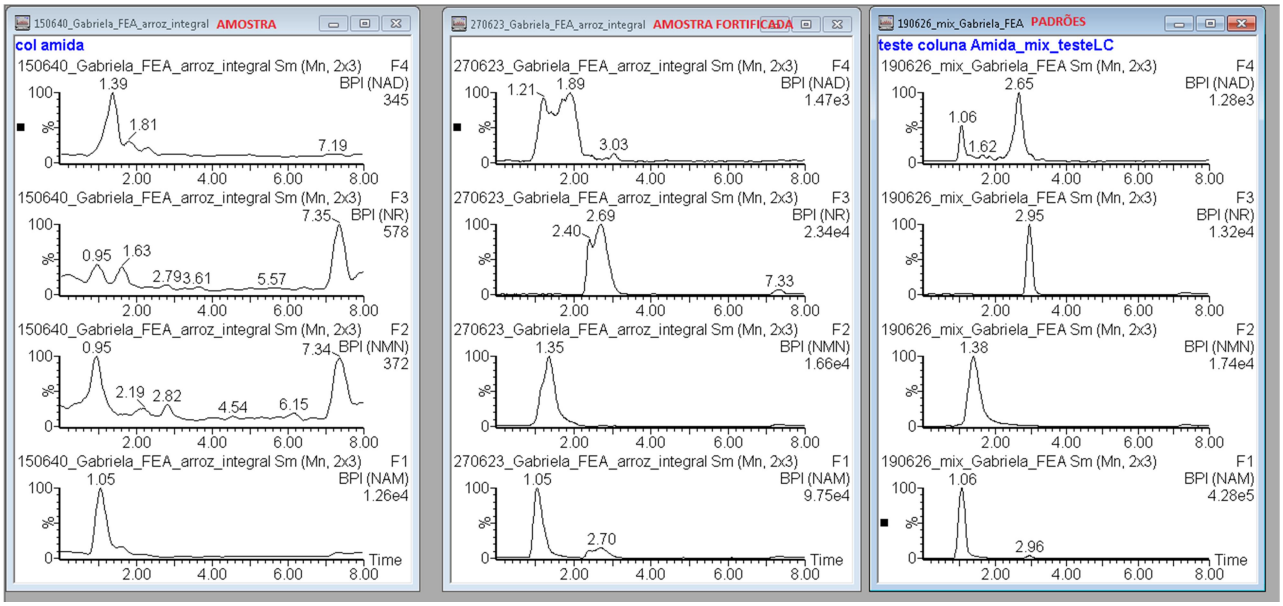
Green bean steamed



Peanut butter



Brown rice


150640_Gabriela_FEA_arroz_integral AMOSTRA
col amida
150640_Gabriela_FEA_arroz_integral Sm (Mn, 2x3) F4
BPI (NAD)
345
1.39
1.81
7.19
150640_Gabriela_FEA_arroz_integral Sm (Mn, 2x3) F3
BPI (NR)
578
7.35
0.95
1.63
2.79
3.61
5.57
150640_Gabriela_FEA_arroz_integral Sm (Mn, 2x3) F2
BPI (NMN)
372
7.34
0.95
2.19
2.82
4.54
6.15
150640_Gabriela_FEA_arroz_integral Sm (Mn, 2x3) F1
BPI (NAM)
1.26e4
1.05
Time
270623_Gabriela_FEA_arroz_integral AMOSTRA FORTIFICADA
270623_Gabriela_FEA_arroz_integral Sm (Mn, 2x3) F4
BPI (NAD)
1.47e3
1.21
1.89
3.03
270623_Gabriela_FEA_arroz_integral Sm (Mn, 2x3) F3
BPI (NR)
2.34e4
2.69
2.40
7.33
270623_Gabriela_FEA_arroz_integral Sm (Mn, 2x3) F2
BPI (NMN)
1.66e4
1.35
270623_Gabriela_FEA_arroz_integral Sm (Mn, 2x3) F1
BPI (NAM)
9.75e4
1.05
2.70
Time
190626_mix_Gabriela_FEA PADRÕES
teste coluna Amida_mix_testeLC
190626_mix_Gabriela_FEA Sm (Mn, 2x3) F4
BPI (NAD)
1.28e3
2.65
1.06
1.62
190626_mix_Gabriela_FEA Sm (Mn, 2x3) F3
BPI (NR)
1.32e4
2.95
190626_mix_Gabriela_FEA Sm (Mn, 2x3) F2
BPI (NMN)
1.74e4
1.38
190626_mix_Gabriela_FEA Sm (Mn, 2x3) F1
BPI (NAM)
4.28e5
1.06
2.96
Time

CAPÍTULO IV

Chemical composition of *Pereskia Violacea*, a new Brazilian Ora-Pro-nobis species from the Amazon, and effects of season

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ABSTRACT

Pereskia violacea, a novel species of Ora-pro-nobis, is a PANC native and endemic to the Brazilian Atlantic Forest, with no prior published reports. Recognizing the importance of fruits and vegetables in a healthy diet, this study aimed to conduct the nutritional and antioxidant characterization of Ora-pro-nobis violacea leaves cultivated in the Amazon region (Brazil) and assesses the impact of seasonality on their composition. For that purpose, proximate composition, mineral content determination by Flame Atomic Absorption Spectrophotometry, saccharides chromatographic profile, total phenolic compounds, DPPH and ORAC antioxidant capacity assays, as well as NAD+ precursors determination by HPLC were performed. Samples from the lower precipitation period (November) exhibited significantly higher results, consistent with its classification as a species belonging to the Cactaceae family. However, samples from both the rainier season (March) and the drier season (November) showed high content of protein, fiber, potassium, calcium, and iron. Total phenolic compounds content was 769.3 and 1043.6 µg GAE/g (in dry weight), respectively, which was significantly correlated with antioxidant capacity. Furthermore, the NAD+ precursor NMN was detected at levels ranging from 6.99 to 7.24 µg/g in fresh samples and 29.50 to 45.97 µg/g in freeze-dried samples. This study reveals new frontiers of *Pereskia violacea* as a nutritious Brazilian native PANC, emphasizing the importance of valuing, cultivating, and consuming these non-conventional food plants. Furthermore, it highlights their potential as a sustainable source of nutraceuticals and promising food ingredients, suitable for enriching various food products, including the plant-based options, which have an increasingly growing demand.

Keywords: Brazilian Amazon food plant; PANC; non-conventional food plant; ora-pro-nobis; NAD+ precursors; NMN

1. Introduction

Pereskia violacea, also known as *Pereskia grandifolia* subsp. *violacea* (Leuenberger) N.P.Taylor & Zappi, is a species of the Cactaceae family, native and endemic to Brazil. It originates from the Atlantic Forest and thrives in a variety of soils and climates (Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, n.d.).

Despite closely resembling *Pereskia grandifolia* Haw., *Pereskia violacea* differs due to its size and purple color on the underside of the leaves, and in its strikingly magenta-coloured floral bracts and much paler-coloured pollen (Taylor & Zappi, 2018) (Figure 1).

Figure 1. Flowers of Ora-pro-nobis violacea (*Pereskia Violacea*), from São Lourenço, Rio Grande do Sul, Brazil. Photo source: Valdely Ferreira Kinupp.

An example of a non-succulent primitive cactus, *Pereskia violacea* is a small tree or shrub, evergreen to deciduous, reaching approximately 2 to 5 m in height. Its main stem is vertical to arched, resembling more a woody plant than a typical cactus (Figure 2). In older stems and trunks, the thorns are more robust and numerous. However, it exhibits considerable variability, with some plants having thorns while others do not.

Figure 2. Image of Ora-pro-nobis violacea (*Pereskia Violacea*) tree and leaves, from Manaus, Amazonas, Brazil. Photo source: Valdely Ferreira Kinupp.

Ora-pro-nobis is a non-conventional food plant, known as PANC (acronym in Portuguese) (Kinupp & Lorenzi, 2014), that contains a high amount of protein, minerals, vitamins, and fiber. Beyond its nutritional richness, research on Ora-pro-nobis (*Pereskia aculeata* Miller), including studies on leaves, mucilage, and ripe and green fruit, highlights its significant importance as a nutraceutical agent. This is attributed to its antioxidant, anti-inflammatory, antimicrobial, neuroprotective, and antiproliferative activities (Garcia et al., 2019; Pinto et al., 2012, 2015; Torres et al., 2022).

Regarding Ora-pro-nobis violacea, there are no published reports available. Data on its chemical and nutritional composition are of particular importance, given the role of

fruits and vegetables in a healthy diet (Ministério da Saúde (BR). Secretaria de Atenção Básica. Departamento de Atenção Básica., 2014; U.S. Department of Agriculture and U.S. Department of Health and Human Services, 2020).

The ability of Ora-pro-nobis to thrive under limited water supply is well-known. It is also recognized that soil conditions, climate (temperature, precipitation, solar incidence), water availability, and other abiotic and biotic factors significantly impact the development and chemical composition of plants (Li et al., 2020; Prinsloo & Nogemane, 2018). Considering the scarcity of data on this specific species of Ora-pro-nobis, this study aimed to conduct the chemical and nutritional characterization of *Pereskia violacea* leaves cultivated in the Amazon region (Brazil) and assess the impact of seasonality on their composition. Additionally, we determined the content of NAD+ precursors, which are postulated as novel bioactive dietary molecules (Alegre & Pastore, 2023).

2. Materials and Methods

2.1. Samples material

The ora-pro-nobis violacea (*Pereskia Violacea*) leaves were collected from the same tree (Figure 2) during two different seasons: rainy in March and dry in November 2022. The collection took place at a farm called 'Sítio PANC Ramal do Brasileirinho,' located in the forested area of Manaus, Amazonas, Brazil (latitude 03º01'29"S, longitude 59º52'39"W, and an altitude of 51 m).

The botanical identification and the exsicate (access number 18804) were deposited at the Herbarium-EAFM of the Federal Institute of Education, Science and Technology of Amazonas (Brazil).

With the exception of the determination of NAD+ precursors, which was carried out with fresh frozen samples (kept at -18ºC), all other samples were concurrently freeze-dried under the same conditions. They were subsequently ground into a fine powder and stored at -18ºC until analysis.

2.2. Proximate composition

The moisture content, ash, protein (Kjeldahl method) and lipids (Soxhlet method) were determined following the methods of (Instituto Adolfo Lutz, 2008). The total fibers were determined according to (AOAC, 1995) (method 991.43). Total carbohydrates were calculated as the difference to 100%.

2.3. Saccharides chromatographic analysis

The samples were extracted with ultrapure water (1:20, w/v) with the aid of an ultrasound bath (UNIQUE, model UCS-2850, 25 kHz, 120 W, Brazil) for 10 min at room temperature. After the centrifugation (4000g, 25 min, 5°C) the supernatants were filtered through 0.22 μm PVDF filter membranes.

The analysis of sugars and oligosaccharides was performed by High Performance Anion Exchange Chromatography coupled to Pulsed Amperometric Detection (HPAEC-PAD) system model DIONEX ICS-5000 (Thermo Fisher Scientific, Waltham, USA) according to (Pereira et al., 2018) with some modifications. Sugars quantification (xylitol, mannitol, sorbitol, arabinose, rhamnose, glucose, fructose, and sucrose) were separated on a CarboPac PA1 column (250 × 4 mm i.d., 10 μm particle size, Thermo Fisher Scientific, Waltham, MA, USA) using an isocratic mobile phase (0.12 mol/L NaOH). The fructooligosaccharides (GF2, GF3, and GF4), and maltooligosaccharides (G2, G3, G4, G5, G6, and G7) were separated on a CarboPac PA100 column (250 × 4 mm i.d., 8.5 μm particle size, Thermo Fisher Scientific, Waltham, MA, USA) using three mobile phases: 0.2 mol/L NaOH (eluent A), ultrapure water (eluent B), and 0.5 mol/L sodium acetate containing 0.2 mol/L NaOH (eluent C). The elution gradient was performed as follows: 0-2 min 47% A, 50% B, and 3% C; 2-18 min, 47-10% A, 50% B, and 3-40% C; 18-23 min, 100% C; 23-28 min 47% A, 50% B, and 3% C. In both analyses, the flow rate was 1.0 mL/min, the column temperature was kept at 30 °C and the injection volume was 25 μL.

The saccharides were identified by comparing the retention times of the standards and the samples. Calibration curves were constructed with commercial standards (0.25–12.50 μg/mL) to quantify the sugars in the samples.

2.4. Minerals

The mineral content was determined according to Silva et al. (2017). Briefly, 0.5 g of the samples was mineralized in a digester block (Marconi, model 4025) and left at room

temperature overnight. Subsequently, the tubes were kept at 110 °C for 2 hours. Afterward, 2 mL of nitric acid and 30% hydrogen peroxide were added, and the tubes were heated again at 130 °C for 2 hours. Following digestion and cooling, the samples were filtered through ash-free filter paper, and the volume was adjusted to 25 mL with ultrapure water. Samples were further diluted to fit within the linear range for each mineral.

Flame atomic absorption spectrophotometry (FAAS, Analyst 200, PerkinElmer, Waltham, USA) was employed in absorption mode for the determination of iron (248.33 nm), calcium (422.67 nm), magnesium (285.21 nm), manganese (279.48 nm), copper (324.75 nm), and zinc (213.86 nm). Sodium and potassium were determined in FAAS emission mode at 589.00 nm and 766.49 nm, respectively. Quantification was performed using external standard calibration curves with five concentration points.

2.5. Total phenolic compounds content

For total phenolic compounds and antioxidant capacity assays, the samples were extracted as described by (Chu et al., 2002) with some adaptations. In the exhaustive extraction process, a volume of 5 mL of a mixture of solvents (1:1) of 80% ethanol and 80% acetone, both in water, was added to 0.5g of lyophilized samples. The mixture was vortexed for 10 seconds, left in an ultrasound bath for 5 minutes in ice water. After that, it was centrifuged at 7400 RCF for 5 minutes at 5 ºC. The supernatant was collected, and the residue extraction process was repeated two more times. The supernatants were combined and evaporated until approximately 80% volume reduction. The resulting evaporated extract was resuspended in 10 mL of deionized water, stored in microtubes, and frozen (-18 ºC) until the analysis.

The phenolic content of the samples was determined using the Folin-Ciocalteu reagent based to the method proposed by (Singleton et al., 1999). Briefly, 30 µL of the extract was added to 150 µL of a 10% Folin-Ciocalteau solution and 120 µL of 7.5% NaHCO3 in a 96-well plate, then incubated for 6 minutes at 45 °C. Absorbances were measured at 760 nm using a microplate reader (Spectrostar Nano, BMG Labtech, Germany). Quantification of total phenolic compounds was performed using a standard curve prepared with gallic acid dissolved in 50% ethanol (10–100 µg/mL). The analysis was conducted in triplicate, and the results were expressed as micrograms of gallic acid equivalent (GAE) per gram of dry weight (dw).

2.6. Antioxidant capacity *in vitro* assays

Various techniques are used to assess the *in vitro* antioxidant capacity of bioactive compounds present in food matrices and natural products. These techniques can be classified based on the reaction mechanism involved. Therefore, it is recommended to use two or more techniques to obtain results that more accurately reflect the true antioxidant potential of the sample. For the measurement of antioxidant capacity in Ora-pro-nobis violacea leaves, DPPH, and ORAC methods were employed.

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2.6.1. DPPH• Scavenging

The assay was performed according to the method proposed by Roesler et al. (2007) with some adaptations. Briefly, a freshly prepared ethanolic solution of DPPH (0.004% w/v) was ultrasonified for 5 minutes and filtered through qualitative paper. For the reaction system, 50 µL of extracts and 250 µL of DPPH were added to a 96-well plate and incubated for 30 minutes at ambient temperature. The absorbance reading was taken at 517 nm on a microplate reader. Trolox, dissolved in ethanol p.a., was used as the standard for the elaboration of the analytical curve (5–250 µM).

The ability to scavenge free radicals is calculated based on the decrease in absorbance observed and expressed as a percentage of radical oxidation inhibition, calculated according to the formula:

% inhibition = ((Abs DPPH – Abs sample/Trolox)/Abs DPPH)*100 (1)

Where,

Abs DPPH = absorbance DPPH solution

Abs sample/Trolox = absorbance of sample or standard solution

A standard curve needs to be constructed using the values of the percentage of Trolox inhibition versus Trolox concentration. The results were expressed as µM Trolox equivalent (TE) g−1 dw and % inhibition DPPH.

2.6.2. Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was conducted according to the procedure described by (Leite et al., 2011). Samples, standards and reagents were prepared in 75 mM potassium phosphate buffer (pH 7.4).

For the reaction, 20 µL of previously diluted extracts or trolox standard, 120 µL fluorescein (3.87 mg/mL) and 60 µL AAPH [2,2-Azobis- (2-methylamidinopropane) - dihydrochloride] (108 mg/mL) were added to a 96 wells black polystyrene plate. Each plate reading included a blank reaction with 20 µL of potassium phosphate buffer instead of a sample, and a trolox standard curve (25-600 µM). Fluorescence intensity was monitored at 37 °C immediately after the addition of AAPH, every 60-second cycle for 80 cycles using the NOVOstar Microplate Reader (BMG Labtech®, Offenburg, Germany). This was done with fluorescence filters set to excitation and emission wavelengths of 485 and 520 nm, respectively, and accompanied by Data Analysis Software MARS Data Analysis version 1.3 (BMG Labtech®, Offenburg, Germany).

The result was calculated using the differences of areas under fluorescein decay curves (AUC) between the blank (net AUC) and sample/standard. AUC and net AUC were calculated as follows:

$$AUC = 1 + \Sigma f_i/f_0 \qquad (2)$$

In which f_0 is the initial fluorescence (t = 0) and f_i is the fluorescence obtained at t = i (minutes).

$$\text{Net AUC} = AUC_{sample/standard} - AUC_{blank} \qquad (3)$$

The net AUC for each point on the standard curve was plotted against the standard concentration. The results were then calculated using the equation of the straight line obtained and expressed as the equivalence of Trolox (µM/g).

2.7. NAD+ precursors content

The content of NAD+ and its precursors nicotinamide mononucleotide (NMN), nicotinamide riboside (NR) and nicotinamide NAM were evaluated as described by (artigo capítulo III). Briefly, fresh frozen samples (1g) or freeze-dried samples (0.1g) were subjected to liquid-liquid extraction with a mixture of chloroform, methanol, and water. The samples were centrifuged at 7400 RCF for 10 min. The aqueous-methanolic phase of the supernatant was aliquoted, transferred into a microtube and dried by evaporation in SpeedVac Eppendorf® Concentrator Plus 5305 (Eppendorf AG 22331 Hamburg - Germany). Subsequently, the residue was dissolved with 1 mL of Milli-Q ultrapure water (~18.2 M Ω, 25 ºC), filtered into glass vials using 0.45 µm nylon membrane and injected into HPLC-UV/DAD (Dionex Ultimate 3000 Thermo Fisher Scientific).

The absorbance was monitored at 220 nm for NMN and NR, and NAM, and at 260 nm for NAD+. Compounds were identified based on retention time and UV spectrum and quantitated by peak area. Standard curve calibration included diluting stock solutions to concentrations ranging from 1 to 50 µg/mL.

Samples were further analyzed and NAD+ precursors content confirmed using LC-MS/MS, as described by (artigo capítulo III).

2.8. Statistical Analysis

The data were expressed as mean values ± standard deviation from three replicates. Statistical analysis involved a one-way ANOVA test, and significant variances among means were further assessed using a post hoc Tukey test at a 95% confidence level, conducted through Minitab® version 18.1 software. The Pearson correlation analysis between the total phenolic content and antioxidant capacity was performed with Excel (Microsoft Office Professional Plus 2010).

3. Results and Discussion

3.1. Chemical composition

The fresh leaves of Ora-pro-nobis violacea (*Pereskia Violacea*) from two different seasons, rainier (March) and drier (November), showed very similar proximate composition, with only a slight difference in lipid and ash content (Table 1). Despite the varying rainfall between the samples, the fresh leaves had moisture content very close to each other (86.37% for March and 86.89% for November samples) (Table 1). However, after freeze-drying, the leaves from November had significantly lower moisture content, resulting in a significantly higher protein content (23.86%) (Table 2).

Our data align with findings from studies on other Ora-pro-nobis species. Silva et al. (2018) reported higher protein content in *Pereskia aculeata* leaves collected in December and June, with lower levels in February and March (Silva et al., 2018). In another study, *Pereskia aculeata*, exhibited slightly higher levels of moisture (89.5%), protein (28.4% dw), lipids (4.1% dw), and ash (16.1% dw), but lower total dietary fiber (39.1% dw) (Takeiti et al., 2009). Almeida et al., (2014) found a higher total carbohydrate content (29% in dry weight), also calculated by the difference, and lower fiber content (18-21% dw) in both *Pereskia*

aculeata and *Pereskia grandifolia* (de Almeida et al., 2014), compared to what we found for *P. violacea*. This demonstrates that *P. violacea* excels in dietary fiber content compared to other Ora-pro-nobis species.

Table 1. Percentage mean of centesimal composition in dry and wet weight of Amazon Ora-pro-nobis violacea (*Pereskia Violacea*) leaves from rainier (March) and drier (November) seasons.

	March		**November**	
	% dry weight	% wet weight	% dry weight	% wet weight
Moisture	6.73	86.37	5.98	86.89
Protein	21.32	2.91	23.86	3.13
Lipids	1.81	0.26	2.78	0.34
Ash	15.64	2.09	12.96	1.65
Total Fiber	45.55	6.21	45.15	5.92
Total Carbohydrates*	7.14	1.91	6.97	1.79
Total Sugar (Mono, Di and Oligossacharides)	1.81	0.25	2.3	0.28

*Calculated by the difference between 100 and the sum of lipids, ash, proteins, and total dietary fiber.

In terms of mineral content, although the Ora-pro-nobis violacea leaves from March have a higher percentage of ash, the November leaves had higher levels of all measured minerals, except for iron, where the March leaves had more than double the content of this mineral (13.14 mg/100g dry weight) (Table 2). This iron content is considered high when compared to that reported for other vegetable leaves such as kale (*Brassica oleraceae L. var. acpehala DC.*) (7.26 mg/100g dw) (Ayaz et al., 2006), and spinach (7.43 mg/100g dw) (Qin et al., 2017).

Iron deficiency is one of the most prevalent mineral deficiencies worldwide (Ramakrishnan, 2002). In 100g of freeze-dried leaves from March, it contains 162.5% of the Adequate Intake (AI) for adult men (19 to > 70 years) and 72% of the AI for women (19 to > 70 years) (Padovani et al., 2006).

In general, 100g of both freeze-dried Ora-pro-nobis violacea leaves contain magnesium, zinc, and manganese, exceeding the Recommended Dietary Allowances (RDA) and Adequate Intake (AI). Additionally, they surpass the RDA or AI for calcium in adults aged 19 years (1000 mg) to those older than 70 years (1200 mg). This is of paramount importance given the scarcity of plant foods rich in calcium, the high requirement for this element, and the difficulty faced by the general population, with particular attention to the elderly, in meeting the AI or RDA.

The samples exhibited very low sodium content and a high level of potassium. A desirable low sodium-to-potassium ratio is indicated, as evidence suggests that a high sodium intake can elevate blood pressure and is associated with an increased risk of hypertension and cardiovascular disease (WHO, 2012b). Conversely, increased potassium intake coupled with controlled or decreased sodium intake was beneficial for hypertensive patients (Binia et al., 2015). The World Health Organization (WHO) recommends a potassium intake of at least 3510 mg/day (90 mmol/day) for adults to reduce blood pressure and the risk of cardiovascular disease, stroke, and coronary heart disease through increased consumption of potassium-rich foods (WHO, 2012a). Fruits and vegetables are sources of potassium, aligning with international health recommendations to increase their consumption (Ministério da Saúde (BR). Secretaria de Atenção Básica. Departamento de Atenção Básica., 2014; U.S. Department of Agriculture and U.S. Department of Health and Human Services, 2020).

These findings highlight the potential of this underutilized plant as a valuable source of minerals. While studies on bioaccessibility and bioavailability are needed to determine the extent to which minerals would be absorbed and available after ingestion, the inclusion of Ora-pro-nobis violacea leaves, whether fresh or in powder form, in the diet would undoubtedly contribute to meeting the AI or RDA for these essential elements.

Table 2. Mean and standard deviation of proximate composition, mineral, and sugar content of Amazon Ora-pro-nobis violacea (*Pereskia Violacea*) leaves from rainier (March) and drier (November) seasons.

	March	November
Proximate composition		
% dry weight (dw)		
Moisture	6.73 ± 0.12a	5.98 ± 0.06b
Protein	21.32 ± 0.71b	23.86 ± 0.31a
Lipid	1.81 ± 0.05b	2.78 ± 0.07a
Total fiber	45.55 ± 2.01a	45.15 ± 1.95a
Ash	15.64 ± 0.09a	12.96 ± 0.06b
Minerals (mg/100g dw)		
Calcium	1458.92 ± 16.01a	1468.40 ± 39.00a
Magnesium	681.84 ± 6.38b	995.04 ± 4.55a
Iron	13.14 ± 0.18a	6.41 ± 0.12b
Zinc	11.79 ± 0.12b	18.92 ± 0.11a
Potassium	4151.80 ± 54.30b	4787.30 ± 60.40a
Sodium	3.87 ± 1.08a	2.63 ± 0.90a
Copper	0.34 ± 0.00b	0.56 ± 0.00a
Manganese	4.93 ± 0.18b	12.05 ± 0.26a
Saccharides (mg/100g dw)		
Mono- and disaccharides		
Xylitol	3.67 ± 0.27b	15.15 ± 3.09a
Mannitol	45.96 ± 0.51a	44.92 ± 0.50a
Sorbitol	102.55 ± 12.50a	90.75 ± 9.48a
Rhamnose	n.d.	n.d.
Arabinose	n.d.	n.d.
Glucose	1111.80 ± 19.50b	1489.80 ± 45.30a
Fructose	504.71 ± 7.07b	573.41 ± 14.50a
Sucrose	n.d.	n.d.
Maltose (G2)	n.d.	n.d.
Total Monossacharides and dissacharides	1768.69 ± 39.85	2214.03 ± 72.87
Oligosaccharides		
1-kestose (GF2)	t.r.	t.r.
Nistose (GF3)	n.d.	n.d.
1-frutofuranosil nistose (GF4)	n.d.	n.d.
Maltotriose (G3)	n.d.	n.d.
Maltotetraose (G4)	31.35 ± 1.81b	50.96 ± 2.40a
Maltopentose (G5)	n.d.	n.d.
Maltohexaose (G6)	7.07 ± 0.65b	38.33 ± 1.27a
Maltoheptaose (G7)	n.d.	n.d.
Total Oligossacharides	38.42 ± 2.46	89.29 ± 3.67
Total sugars	1807.11 ± 42.31	2303.32 ± 76.54

Different letters on the same row indicate a significant difference (p-value < 0.05).

t.r.: Traces; n.d: not detected.

With the exception of mannitol and sorbitol, leaves collected in November (dry season) exhibited a significantly higher content of saccharides, resulting in higher total sugar content (Table 2). Glucose was identified as the major saccharide, and the finding of a glucose/fructose ratio exceeding 2:1 is noteworthy. The presence of luminal glucose significantly increases fructose absorption, preventing symptoms such as abdominal distension, discomfort, or pain, as well as altered intestinal habits due to the production of gases (H2, CO2, and CH4) by colonic bacteria fermentation of excess unabsorbed fructose (Barrett, 2013). Other vegetables analyzed, such as artichoke, endive, spinach, radish, beetroot, and eggplant, also exhibited higher levels of glucose than fructose (Hernández-Hernández et al., 2011); however, these levels were lower than those found in *Pereskia violacea* leaves.

In addition to being used as artificial sweeteners, the sugar polyols xylitol, sorbitol, and mannitol, as well as fructose, are also termed as FODMAPs (fermentable oligo-, di-, and monosaccharides and polyols). These compounds can be poorly absorbed by the small intestine and may cause a wide range of effects on the gastrointestinal tract, whether undesirable or not (Barrett, 2013). In addition to occurring naturally in fruits and vegetables, these compounds can also be used as artificial sweeteners. Many vegetables evaluated showed a higher content of polyols in comparison with ora-pro-nobis violacea leaves, ranging from 0.11 to 0.45 and 0.09 to 2.96g per 100g of fresh weight of the sample, for sorbitol and mannitol, respectively (Muir et al., 2009).

Among the oligosaccharides analyzed, only maltotetraose and maltohexaose were detected, with maltotetraose being the major one in *Pereskia violacea* leaves. This finding is intriguing, considering the lack of data on the presence of these oligosaccharides in vegetable leaves. Typically, they are found in roots and tubers, with sweet potatoes having the highest content (Sancho et al., 2017). Additionally, maltotetraose (G4) was identified as the major oligosaccharide (163 mg/100g dw) in *Eugenia stipitata* fruit (de Araújo et al., 2021), and was detected in low levels in germinated rice (2-5 mg/100g) (Moongngarm et al., 2011). Both maltotetraose and maltohexaose are carbohydrates composed of four and six glucose molecules, respectively, linked by α 1 - 4 glycosidic bonds, derived from maltose or partial hydrolysis of starch.

In fresh leaves (ww), approximately 10% of the total carbohydrates content comprises mono-, di-, and oligosaccharides, while for freeze-dried leaves (dw), this percentage increases to approximately 20% (Table 1).

Although there is no available data on the sugar profile in Ora-pro-nobis leaves, it is anticipated that the remaining sugars originate from polysaccharides, constituting the mucilage present in these leaves. The chemical composition analysis of *Pereskia aculeata* mucilage revealed the presence of polysaccharides, including galactose, arabinose, rhamnose, fucose, and partially esterified galacturonic acid. The mucilage was composed of 48% (w/w) total sugar, 26% (w/w) uronic acid, and 19% (w/w) protein (Martin et al., 2017). Plants belonging to the Cactaceae family produce significant quantities of mucilage, a complex carbohydrate with a high water absorption capacity, which makes it an excellent candidate for use as a hydrocolloid in processed foods. Additionally, a mild potential use as an emulsion and stabilizing agent, due to its interfacial adsorption properties and pseudoplasticity, was demonstrated (Martin et al., 2017), as well as the possible development of films for functional and/or edible packaging (Oliveira et al., 2019). The mucilage present in *Pereskia violacea* certainly warrants further investigation to explore its potential applications.

3.2. Total phenolic compounds content and antioxidant capacity

Ora-pro-nobis violacea leaves from the drier season (November) exhibited significantly higher total phenolic compound content, as well as increased antioxidant capacity measured by DPPH and ORAC assays (Table 3). Additionally, there was a higher percentage of DPPH radical oxidation inhibition, with the November sample showing 49% inhibition, compared to 12.41% in the March sample (Figure 3).

Table 3. Total phenolic compounds content and antioxidant capacity measured by DPPH and ORAC assays of Amazon Ora-pro-nobis violacea (*Pereskia Violacea*) leaves from rainier (March) and drier (November) seasons. Results are expressed per gram of dry weight (dw).

	March	**November**
Total phenolic (µg GAE /g)	769.3 ± 55.10b	1043.6 ± 139.90a
DPPH (µM TE/g)	52.01 ± 4.60b	186.52 ± 2.85a
ORAC (µM TE/g)	13.79 ± 3.42b	41.42 ± 0.21a

GAE: Gallic acid equivalent

TE: Trolox equivalent

Different letters on the same row indicate a significant difference (p-value < 0.05).

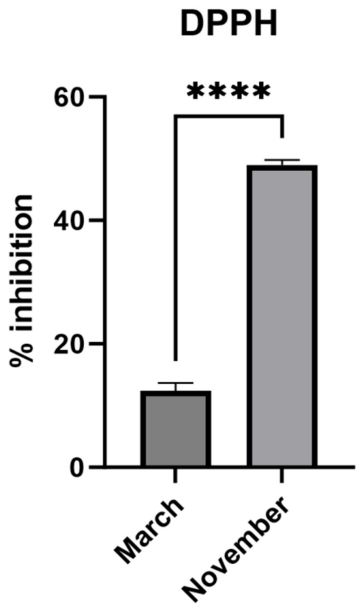

Figure 3. Percentage of DPPH radical oxidation inhibition by Amazon Ora-pro-nobis violacea (*Pereskia Violacea*) leaves from rainier (March) and drier (November) seasons. **** P-value = <0.0001

In addition, a stronger positive and statistically significant correlation between total phenolic content and antioxidant assays was observed through Pearson correlation coefficient ($r = 0.84975$ for phenolic and DPPH and $r = 0.82067$ for phenolic and ORAC; $p < 0.0322$ and $p < 0.0454$, respectively). This suggests the possible reason for the higher antioxidant capacity observed in the November sample, as it also had a higher total phenolic content.

The optimal antioxidant activity is linked to multiple phenolic groups (Leopoldini et al., 2004). The effectiveness of phenolic compounds as antioxidants correlates with the number of –OH groups in the aromatic ring(s) and their arrangements. Phenolic compounds can chelate prooxidative metals, activate antioxidative enzymes, and form adducts with antioxidant properties based on the arrangement of –OH groups. The key factors influencing the antioxidant activity of polyphenolic compounds include chemical structure, hydrogen bond-forming ability, metal ion chelation and reduction capability, adduct formation, kinetic solvent effects, antioxidant reaction mechanisms, activation of antioxidant enzymes, and

reduction potential (Olszowy, 2019). In a study assessing the inactivation of reactive radical species mechanisms by food molecules, gallic acid, caffeic acid, and epicatechin proved highly efficient as hydrogen donors, while kaempferol and resveratrol emerged as prime candidates for an electron-transfer mechanism (Leopoldini et al., 2004).

DPPH is an assay based on an electron transfer mechanism, where antioxidants reduce an oxidant, leading to a color change in the radical chromophore. On the other hand, the ORAC assay involves a hydrogen atom transfer mechanism, wherein antioxidants and substrates compete for thermally generated peroxyl radicals (Dudonné et al., 2009). The higher antioxidant capacity of *Pereskia violacea* measured by DPPH (Table 3) suggests a greater presence of compounds efficient in electron transfer. Another influencing factor is the reaction medium, as antioxidant activities are influenced by the solvent employed. The choice of solvent can impact the reaction mechanism and the availability of antioxidant groups. Additionally, factors such as the presence of metal ions (type and concentration) and pH can affect the antioxidant properties of phenolic compounds (Olszowy, 2019).

The main phenolic compounds identified in the aqueous and ethanolic extracts of *P. grandifolia* leaves were caffeic acid, chlorogenic acid, and catechin (Teixeira et al., 2023). In the hydroethanolic extract of *P. aculeata* leaves, a total of ten phenolic compounds were identified, comprising two phenolic acids (caftaric acid and caffeic acid derivatives) and eight flavonoids (quercetin, kaempferol and isorhamnetin glycoside derivatives) (Garcia et al., 2019). Notably, the total phenolic content in the *P. aculeata* leaf extract, as determined by Garcia et al. (2019) at 23.75 mg/g, surpassed the quantity we found in *Pereskia violacea*.

To our knowledge, there is no available data on the total phenolic compounds or the characterization of individual phenolic compounds and antioxidant capacity of *Pereskia violacea*. This absence makes it challenging to compare with other Ora-pro-nobis species, particularly due to variations in result expression for antioxidant properties. In a study by Garcia et al. (2019), higher activities were observed in the *P. aculeata* leaf extract compared to Trolox in both the DPPH and ABTS trials, with IC50 values of 72.9 µg/ml and 40.5 µg/ml, respectively, for each assay (Garcia et al., 2019). Silva et al. (2018) evaluated the total phenolic compounds and antioxidant capacity of *P. aculeata* leaves at various points throughout the cultivation period. The samples exhibited higher levels of phenolic compounds and antioxidant capacity, as determined by the DPPH assay, during the autumn month of April, with values of 44.99 g of Trolox/kg of fresh plant and 2.66 g of gallic acid equivalent (GAE)/kg of fresh plant, respectively (Silva et al., 2018).

The climate seasons also affected directly the concentration of bioactive components (total phenolics, total flavonoids and L-ascorbic acid), and the antioxidant activity of Brassica vegetables (*Brassica oleracea* L. and *Brassica rapa* L.) (Aires et al., 2011).

Indeed, seasonality profoundly shapes the secondary metabolism, consequently influencing the chemical and bioactive metabolite profile of plants (Prinsloo & Nogemane, 2018), thus interfering in the antioxidant properties of vegetables. The intricate interplay between plants and environmental factors, such as temperature, light and water availability, trigger adjustments in enzymatic reactions and photosynthesis, impacting the synthesis of specific secondary metabolites. Seasonal changes drive shifts in plant growth phases, affecting their chemical profiles during vegetative and reproductive transitions. Biotic interactions and geographic factors contribute to water stress responses and the production of defensive compounds. Additionally, soil characteristics (such as salinity and nutrient availability) influence the types and quantities of metabolites (Li et al., 2020). In summary, the seasonal dynamics of plant secondary metabolism reflect a sophisticated adaptive strategy.

Water plays an important role in regulating the germination, growth, development, and metabolism of plants, and studies have demonstrated that water stress increases the secondary metabolite content (Prinsloo & Nogemane, 2018). The rainfall during March 2022 in Manaus (AM, Brazil) was 402.1 mm, while in November 2022 it was 104.5 mm (Figure 4). Our results demonstrate that a lower level of precipitation was more beneficial to *Pereskia violacea*, which makes sense since it is a species of Cactacea family, biologically adapted to dry conditions and high solar incidence.

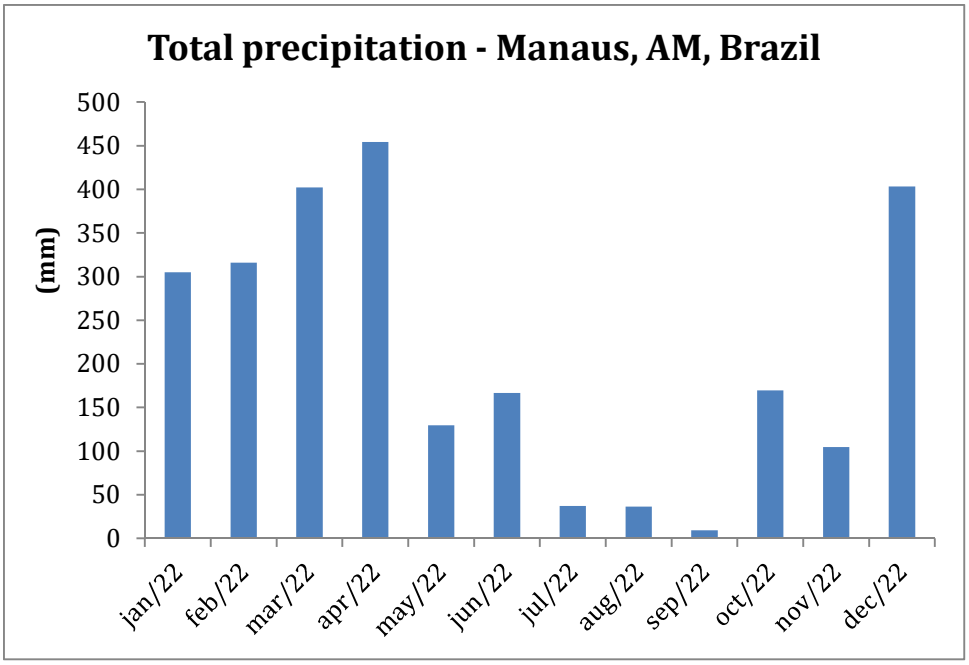

Figure 4. Monthly Accumulated Precipitation from January to December 2022 in Manaus, Amazonas, Brazil. Source: Banco de Dados Meteorológicos (BDMEP) do INMET (Instituto Nacional de Metereologia). Available in: https://bdmep.inmet.gov.br/#

3.3. NAD+ precursors content

NAD+ (nicotinamide adenine dinucleotide) is an important molecule that acts as a redox cofactor in several metabolic reactions and as a substrate in cellular signaling pathways regulating responses to energetic, genotoxic, and infectious stress. Dietary precursors, such as NMN (nicotinamide mononucleotide) and NR (nicotinamide riboside) , can promote NAD+ biosynthesis, boosting intracellular levels, and offering a promising strategy for reversing physiological decline and mitigating the risk of diseases (Xie et al., 2020). These NAD+ precursors have demonstrated numerous beneficial physiological effects, including anti-aging, anti-inflammatory, epigenetic and gut microbiota modulations, as well as enhancing energetic metabolism, neuroprotection, and cardioprotective actions (Alegre & Pastore, 2023).

For the first time, we determined the content of NAD+ precursors in Ora-pro-nobis violacea. The only NAD+ precursor detected in the fresh leaves was NMN, which showed no significant difference between the two seasons, while NR was detected only in the freeze-dried March sample (Table 4). Although the lyophilized samples exhibited a higher

NMN content, it is worth mentioning that fresh leaves have more than 86% water content, while the lyophilized ones had a moisture content of around 6%. Therefore, lyophilization process concentrated dry matter, similar to what happened with the other components (protein, lipids, fiber, etc.) (Table 1).

In comparison with other non-conventional food plants (PANC), Ora-pro-nobis violacea (*Pereskia Violacea*) exhibited a higher NMN content than Ora-pro-nobis Amazonian (*Leuenbergeria bleo*) (1.25 µg/g), malanga (*Xanthosoma sagittifolium*) (0.91 µg/g), and purple malanga (*Xanthosoma violaceum*) (1.80 µg/g) (artigo capítulo III).

Table 4. NMN, NR, NAD+ and NAM content in fresh and lyophilized Ora-pro-nobis violacea (*Pereskia Violacea*) leaves from rainier (March) and drier (November) seasons.

	Fresh (µg/g wet weight)				Lyophilized (µg/g dry weight)			
Sample	**NMN**	**NR**	**NAD**	**NAM**	**NMN**	**NR**	**NAD**	**NAM**
March	7.24 ± 1.39b	n.d.	n.d.	n.d.	29.50 ± 3.02a	14.63 ± 4.01	n.d.	n.d.
November	6.99 ± 3.70ab	n.d.	n.d.	n.d.	45.97 ± 46.51a	n.d.	n.d.	n.d.

Different letters on the same column and row indicate a significant difference (p-value < 0.05).

n.d: not detected

Ultimately, Ora-pro-nobis violacea leaves have the potential to be a functional food. Beyond their possible use in stir-fries, soups, fillings, and various culinary preparations when cooked, similar to other Ora-pro-nobis species like *Pereskia aculeata* and *Leuenbergeria bleo*, as exemplified by (Kinupp & Lorenzi, 2014), the freeze-dried powder or flour form holds promise for application as a nutritious and functional ingredient in various food products, including bread, biscuits, cakes, pie, pasta, sausage, vegan burger formulations, among others, as has already been demonstrated with Ora-pro-nobis (Mota et al., 2021; Teixeira et al., 2023).

There is a growing demand for plant-based products. Whether for health, ethical, and/or environmental reasons, consumers have been seeking alternatives to animal-derived foods. The ora-pro-nobis violacea could also be used as ingredients in these plant-based substitutes, contributing to nutritional value and even technological properties, such as texture, and so on.

It is also important to mention the potential of Ora-pro-nobis violacea as a source for nutrition supplements, particularly through the encapsulation of isolated compounds. This potential is attributed to its rich content of proteins, dietary fiber, minerals, and bioactive compounds such as phenols and NAD+ precursors, along with its antioxidant capacity. Therefore, there is a crucial need for future investigations into the bioactivity and bioavailability, technological applications, and sensory acceptance of products enriched with this valuable plant.

4. Conclusion

Despite being an non-conventional food plant, the leaves of Ora-pro-nobis violacea serve as an important source of nutrients, having a high concentration of proteins, fiber, and essential minerals such as calcium, potassium, and iron, all with low fat content. Furthermore, these leaves contain bioactive compounds, including phenolics and NAD+ precursors, contributing to their antioxidant capacity.

The lower level of precipitation was a determinant factor for the chemical composition of Ora-pro-nobis violacea and, consequently, its antioxidant capacity, since the

sample from the lower level of precipitation season (November) showed significantly higher results (based in dry weight).

We showcase the potential of a novel species of Ora-pro-nobis as a nutritious Brazilian native PANC, opening avenues for future investigations into its vitamin composition, determination of other bioactives such as carotenoids and flavonoids, examination of the presence of anti-nutritional and toxic compounds, and exploration of possible biological effects through *in vitro* and *in vivo* assays.

Our data not only affirm the significance of valuing, cultivating, and consuming non-conventional food plant but also highlight their potential as a sustainable reservoir of nutraceuticals and promising food ingredients, suitable for enriching food products, including the plant-based options, which have an increasingly growing demand.

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Credit authorship contribution statement

Gabriela Fabiana Soares Alegre: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Cynthia Tereza Corrêa da Silva**: Data curation. **Henrique Silvano Arruda:** Data curation. **Felipe Tecchio Borsoi:** Data curation. **Iramaia Angélica Neri-Numa:** Data curation. **Eduardo Adilson Orlando:** Data curation. **Grasieli Beloni de Melo:** Data curation. **Augusto César Costa-Santos:** Data curation. **Juliana Azevedo Lima Pallone:** Data curation. **Valdely Ferreira Kinupp:** Data curation. **Glaucia Maria Pastore:** Supervision, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Disclosure Statement

The authors declare that they have no conflict of interest.

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CAPÍTULO V

NAD+ precursors - NMN and NR - from food plants: effect on human gut microbiota and biotransformation through *in vitro* colonic fermentation

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ABSTRACT

NAD+ is pivotal in energy metabolism and cell signaling. Decreased NAD+ levels are observed during aging and disease. Dietary biosynthesis precursors, NMN and NR, can restore NAD+ levels, reversing physiological decline. These precursors have shown beneficial effects, such as attenuating diabetes and Alzheimer's, and modulating gut bacteria. Despite animal studies, human gut microbiota interaction with NAD+ precursors remains understudied. Our research explores NAD+, NMN, and NR effects on gut microbiota, and their biotransformation by this gut microbiota. We fermented NAD+, NMN, and NR extracted from green leafy vegetables and Amazon food plants with human fecal microbiota in an *in vitro* colonic model. Ammonia and SCFA production were measured, and NAD+ metabolites released post-48h fermentation identified. Microbial structure was analyzed through 16S rRNA gene sequencing. NAD+ and its precursors minimally impacted microbial activity.

Malanga was the only sample that showed a significant difference in ammonia production, with lower levels than the control. Plant extracts increased SCFA levels, potentially from other extract components, as the standards had lower SCFA levels compared to the control. All samples altered microbial composition, increasing Firmicutes over Fusobacteriota. *Escherichia-Shigella*, the prevalent genus, further rose, suggesting stimulation by NAD+ precursors depending on the host's microbiome. Plant extracts enhanced microbial richness, while standards showed low alpha-diversity. Human gut microbiota degraded NAD+ and its amidated precursors, releasing deamidated NA and NAR. Plant extracts, notably spinach, augmented NAD+ biosynthesis precursors pool derived from microbial metabolism. Our study advances understanding of NAD+ precursors and human gut microbiota interactions.

Keywords

NAD+ precursor; nicotinamide mononucleotide; nicotinamide riboside; gut microbiota; human fecal fermentation; Amazon food plants; non-conventional food plants; green leafy vegetables

1. INTRODUCTION

Nicotinamide adenine dinucleotide (NAD+) is essential for all living organisms. As a cofactor for electron transfer in metabolic reactions and a substrate in signaling pathways (Berger et al., 2004; Houtkooper et al., 2010), NAD+ mediates multiple biological processes, including energy metabolism, mitochondrial functions, calcium mobilization, circadian cycle, DNA repair, epigenetic regulation, and cell death (Covarrubias et al., 2021; Ying, 2006). Due to its pivotal role in energetic and signaling processes, NAD+ has been postulated as an important molecule for anti-aging (Johnson & Imai, 2018). During aging and disease conditions decreased NAD+ levels are found (Guest et al., 2014; Massudi et al., 2012; Yoshino et al., 2018).

Tryptophan and nicotinic acid (NA) were the first recognized precursors of NAD+ biosynthesis. When identified as important nutrients for preventing and curing pellagra, tryptophan was initially thought to be a precursor of nicotinic acid (Elvehjem, 1949), but actually, like nicotinic acid, is a precursor of NAD+. Nicotinic acid, in its amide form, nicotinamide (NAM), constitutes a part of NAD, which was initially known as coenzyme I (Elvehjem, 1949). Later, nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) were also established as more direct NAD precursors, bypassing several steps in the synthesis pathway (Bieganowski & Brenner, 2004; Revollo et al., 2004). At equimolar concentrations, NR was even more potent in boosting NAD+ levels than vitamin B3 (NAM and NA) in mice (Trammell, Schmidt, et al., 2016).

By restoring the available NAD +, NMN and NR may mitigate, or even reverse, numerous aging-related dysfunctions and other diseases in pre-clinical studies (for more details see (Yoshino et al., 2018)). Some examples include, but not limited to, Alzheimer's (Wang et al., 2016; Xie et al., 2019; Yao et al., 2017), hearing loss (Brown et al., 2014), retinal degeneration (Lin et al., 2016; X. Zhang et al., 2020), heart failure (Diguet et al., 2018; R. Zhang et al., 2017), vascular dysfunction (de Picciotto et al., 2016), diabetes (Trammell, Weidemann, et al., 2016; Yoshino et al., 2011), among others.

Additionally, it has been shown that NMN and NR supplementation modulates the gut microbiota in mice, increasing the abundance of certain beneficial bacteria while reducing the abundance of certain harmful bacteria (P. Huang et al., 2021, 2022; Peluso et al., 2023). Furthermore, NR altered the gut microbiota of rats and mice, favoring bacterial species capable of synthesizing NAD+ from NR (Peluso et al., 2023). In humans, NR

supplementation for 3 months did not alter the human gut microbiota (Peluso et al., 2023). However in another clinical trial, 5 months of NR supplementation increased the abundance of Firmicutes, specifically of the genus *Faecalibacterium* (Lapatto et al., 2023). To our knowledge, there is no clinical report yet about NMN and gut microbiota.

In addition to supplementation, NMN and NR are potential new food bioactive compounds (Alegre & Pastore, 2023). They are naturally present in vegetables (artigo capítulo III), milk (Ummarino et al., 2017), meat (Mills et al., 2016), and can therefore be obtained through diet. However, it is worth recognizing that the physiological effects of food bioactive compounds and functional foods largely depend on reciprocal interaction with the host gut microbiome (Braune & Blaut, 2016; Rowland et al., 2003, 2018; Russell et al., 2008).

The gut microbiota plays a pivotal role in digesting dietary components and producing metabolites that impact host's health status (Agus et al., 2018; Koeth et al., 2013; Roager & Dragsted, 2019; W. H. W. Tang et al., 2013). Increasing evidence indicates its involvement in diseases, such as irritable bowel syndrome (M.P. Bennet et al., 2015), inflammatory bowel disease (Nishino et al., 2018), metabolic syndrome and obesity (Dabke et al., 2019; Karlsson et al., 2013), autoimmune (Shaheen et al., 2022), neurologic and psychiatric diseases (Gomaa, 2020; Pulikkan et al., 2019; Sherwin et al., 2016).

To evaluate microbiota, animal models, clinical trials, and colonic fermentation models can be used. Among these, the human colonic fermentation model has proven to be an effective method for assessing the potential of probiotics, prebiotics, and food in modulating the microbiota (Singh et al., 2022). Batch *in vitro* colonic fermentations help elucidate microbial metabolism of nutrients and resulting metabolites, informing how diet impacts host health. They are essential for studying diverse foods and their components, enabling initial screenings that would be impractical in continuous systems or constrained by cost and ethical concerns in animal and human studies (Pérez-Burillo et al., 2021)**.**

Given the role of NAD+ in several metabolic processes of the intestinal microbiota, including glycolysis and fermentation pathways, it is of interest to know whether dietary precursors NMN and NR interfere with the activity and composition of the gut microbial community. Additionally, what is the influence of microbial metabolism on the bioactivity of NMN and NR to increase NAD+ biosynthesis and its levels? The activity of the gut microbiota was essential in the NAD+ boosting effect of oral NR across multiple tissues in mice (Shats et al., 2020). However, it remains open whether the same occurs in humans,

since there is a lack of reports about the effect of human gut microbiota on NAD+ and its precursors NMN and NR from food.

To help answer these questions, an *in vitro* batch fermentation protocol was used for the first time to assess the effect of NAD+ and its precursors NMN and NR from plant foods on both human gut microbiota and their biotransformation by this microbiota. We believe that this study will provide insights into this yet unexplored subject in food science and nutrition.

2. MATERIAL AND METHODS

2.1. Samples

We selected some of the green leafy vegetables richest in NMN, NR and NAD+, based on our previous reports (artigo capítulo III), for this human gut microbiota study.

The conventional green leafy vegetables samples, spinach (*Spinacia oleracea*), cilantro (*Coriandrum sativum*) and Brazilian kale, also named collard, (*Brassica oleracea L. var. viridis L.*), were obtained from the retail market in Campinas, São Paulo, Brazil.

The non-conventional food plants, known as PANC (acronym for plantas alimentícias não convencionais, in Portuguese (Kinupp & Lorenzi, 2014)), namely Moringa (*Moringa oleifera*), Malanga (*Xanthosoma sagittifolium*) and Ora-pro-nobis violacea (*Pereskia violacea)* were collected at a farm called 'Sítio PANC Ramal do Brasileirinho' located in the forested area of Manaus, Amazonas, Brazil (latitude 03º01'29"S, longitude 59º52'39"W, and an altitude of 51 m). The ora-pro-nobis violacea samples were collected from the same tree in two different seasons: rainy in March and dry in November 2022, and denominated as Ora March and Ora November in the results, respectively. All the collected samples were kept at -18 ºC until analysis.

The standards NAD+: nicotinamide adenine dinucleotide (CAS: 53-84-9), NMN: nicotinamide mononucleotide (CAS: 1094-61-7), NR: nicotinamide riboside chloride (CAS: 23111-00-4), and NAM: nicotinamide (CAS: 98-92-0) were purchased from Sigma-Aldrich.

2.2. Preparation of samples for fermentation

To evaluate the effect of NAD+, NMN, and NR obtained through food intake on human gut microbiota, while minimizing the background effects of other compounds present in the samples, we conducted a liquid-liquid extraction of these compounds from green leafy

vegetables. The extraction was performed following the procedure described by (Ozaki et al., 2022). Briefly, 7g of fresh leafy bites were mixed with 14 mL of chloroform/methanol (1/2, vol/vol) and 2.8 mL of ultrapure water (~18.2 M Ω, 25 ºC) obtained from Millipore Milli-Q system. The samples were centrifuged at 7400 RCF for 10 minutes at 5 ºC, and then the supernatant was dried by evaporation in a SpeedVac Eppendorf® Concentrator Plus 5305 (Eppendorf AG 22331 Hamburg - Germany). The dried residue was resuspended in 7 mL of Milli-Q ultrapure water, and this aqueous extract was utilized in *in vitro* colonic fermentation. This extraction procediment was performed in triplicate for each sample.

2.3. *In vitro* colonic fermentation

An *in vitro* batch fermentation was performed to simulate the human colon, following the protocol by (Pérez-Burillo et al., 2021) with minor modifications.

Fecal material was obtained from three healthy donors (2 females and 1 male), with an average age of 30.3 years (range: 24–37 years), without intolerances, food allergies, or recent use of probiotics, prebiotics, synbiotics, or antibiotics within the last 3 months. The study protocol was approved by the Ethics Committee of UNICAMP (CAAE nº 72536923.4.0000.5404, approved in 08/29/2023).

The pooled fecal material was diluted in sterile PBS buffer (composition detailed in Supplementary Table 2) to obtain the final fecal inoculum at 20% (w/v) concentration. The mixture was then centrifuged at 5000 rpm for 15 minutes at 4°C, and the supernatant was collected for use in the fermentation.

For each fermentation bottle, 7 mL of each green leafy vegetable extract (at a concentration of 1 g/mL) was added to 56 mL of basal fermentation medium (composition in Supplementary Table 1), along with 7 mL of fecal human inoculum. Then, the bottles were incubated at 37 ºC for 48 hours with gentle shaking, under anaerobic conditions (nitrogen bubbling for 1 minute). Fermentation bottles containing 7 mL of a mixed standard aqueous solution of NAD+, NMN, NR, and NAM (positive control), at a concentration of 100 µg/mL each, were also prepared under the same conditions. Each batch of fermentation included 2 samples in triplicate, with a parallel negative control consisting of 7 mL of Milli-Q ultrapure water instead of the sample, also in triplicate.

At the end of 48 hours, fermentation was stopped by placing the bottles in an ice bath for 15 minutes. Thereafter, the samples were centrifuged at 15000 rpm for 5 minutes, and the supernatant and pellet were stored at -80 ºC until the analysis. The supernatant was used in

the analysis of short-chain fatty acids and NAD+ metabolites, and the pellet was used for 16S rRNA gene sequencing.

2.4. Ammonia measurement

The ammonia produced post fermentation was quantified using a selective ion meter (710A model, Orion) coupled with an ammonia selective-ion electrode (95–12 model, Orion). The apparatus was calibrated using standard solution at 1000 ppm of ammonia. A total of 200 µL ISA solution (Ionic Strength Adjuster, Orion), a pH-adjusting and ionic force solution, was added to every 10 mL of sample.

2.5. SCFA analysis

Short chain fatty acids were determined according to the procedure described in (Pereira et al., 2020). The fermentation supernatants were acidified with a 5M HCl solution, filtered through a nylon membrane (0.45 µm), and then injected into an Agilent 7890A gas chromatograph system equipped with a flame ionization detector (FID). A capillary column (30 m × 0.25 mm i.d. × 0.25 µm Nukol™, Supelco, Bellefonte, PA, US) was used.

The SCFAs were identified on chromatograms by their retention times compared with the standard solution and quantified by peak area compared to a standard curve calibration. For curve calibration, standard solutions of acetic acid (0.04-10.49 mg/mL), propionic acid (0.04-9.93 mg/mL) and butyric acid (0.08-9.64 mg/mL) were prepared in a 5M HCl solution. Ethylbutyric acid (1 mM) was used as an internal standard for runs.

2.6. 16S rRNA Gene Sequencing microbiota analysis

2.6.1. DNA extraction

Bacterial DNA extraction in samples was performed using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research) following the manufacturer's instructions. The DNA samples were then immediately frozen in a freezer at −20°C until molecular analysis.

2.6.2. Sequencing

The library was prepared using primers for the V3V4 region of 16S rRNA (~ 470 bp, amplified with primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'- GGACTACNNGGGTATCTAAT-3'), and bacteria amplicons sequenced by Illumina platform (PE 250, 50-100 mil reads raw data per sample).

2.6.3. Data analysis

The data were processed and analyzed using with QIIME – Quantitative Insights Into Microbial Ecology, version 2022.2.0 (https://qiime2.org/).

The operational taxonomic units (OTUs) were clustered based on sequence similarity at 99%, and taxonomic assignments were made using the SILVA 138 reference database (https://www.arb-silva.de/).

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2, version 2.4.2) was used to predict the metabolic functions of microbial communities. The images and exploration of biological data were done in Python.

2.7. Analysis of NAD+ and Its Precursors Metabolism by Gut Microbiota Using LC-MS/MS

The analysis of the NAD+ metabolome post-fermentation was conducted according to the method outlined by (Peluso et al., 2023).

The fermentation supernatant was filtered using a 0.22 µm nylon membrane. Subsequently, 400 µL of the filtered solution was extracted with 400 µL of a 4:1 methanol:water (vol/vol) mixture and centrifuged again. The final supernatant was dried by evaporation in a SpeedVac Eppendorf® Concentrator Plus 5305 (Eppendorf AG, 22331 Hamburg, Germany), then resuspended in 100 µL of a 4:1 methanol:water (vol/vol) solution. A 10 µg/mL standard mix solution of NAD+, NMN, NR, and NAM was prepared in the same solvent (4:1 methanol:water vol/vol).

Detection and chromatographic separation were performed using an Ultra-high performance liquid chromatography (UHPLC) Ultimate 3000 coupled to a Q-Exactive hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Germany), equipped with a heated electrospray ionization (HESI-II) probe. For hydrophilic interaction liquid chromatography (HILIC) separation of metabolites, an Acquity BEH Amide column (1.7 µm × 2.1 mm, 100 mm, Waters, USA) was employed. Both mobile phases A (water) and B (acetonitrile:water, 9:1; vol/vol) contained 10 mM ammonium acetate. The mobile phase gradient started with 5% of mobile phase A and remained constant for 1 minute, followed by an increase to 55% over 14 minutes. An additional 5 minutes were allocated for gradient re-equilibration to initial conditions. The flow rate was set at 200 µL/min, and the column temperature was maintained at 40°C, with an injection volume of 2 µL. Samples were

detected in positive mode, in the m/z range of 100-1500, with an acquisition rate of 10 Hz, normalized collision energy of 30 eV, and automatic fragmentation (Auto MS/MS) of the 6 most intense ions in each cycle. Metabolite intensities were acquired using Full MS/MS. Data were extracted using Xcalibur software version 3.0.63 (Thermo Fisher Scientific, Germany).

Metabolites were identified through spectral similarity. The fragmentation spectra of experimental samples were compared with spectra of analytical standards injected under the same analysis conditions (level 1 of identification) or spectra from the literature in PubChem (https://pubchem.ncbi.nlm.nih.gov/), Global Natural Products Social Molecular Networking (GNPS) (https://gnps.ucsd.edu/), and Human Metabolome Database (HMDB) (https://hmdb.ca/) databases (level 2 identification).

2.8. Statistical Analysis

The data are presented as mean values ± standard deviation of three replicates.

One-way ANOVA and post hoc Tukey tests were performed to identify significant differences among the samples and control, with a confidence level of 95% (p-value ≤ 0.05). Graphs and statistical analysis were performed using GraphPad Prism software, version 10.1.1.

3. RESULTS AND DISCUSSION

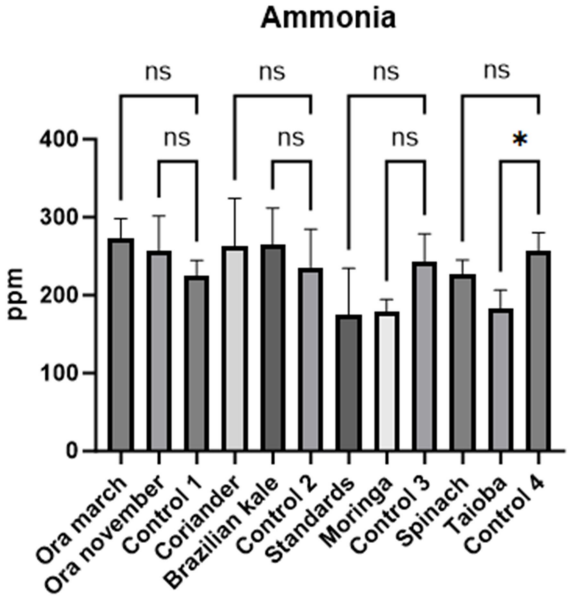
3.1. Influence of NAD+ and their precursors, NMN and NR, from green leafy vegetables on gut microbiota activity and composition

3.1.1. Ammonia production from fecal human fermentation

The media level of ammonia produced varied between 174-272 ppm, what was similar to found in the 7-day treatment with probiotic strains in the gut microbiota of mildly anxious adults, which exhibited significantly lower ammonia content than the control group (De Oliveira et al., 2023).

With exception of Ora-pro-nobis violacea (March and November), cilantro and Brazilian kale extracts, the samples exhibited lower ammonia content compared to their controls (Figure 1). However, only Malanga showed significantly different content from the control (182 ppm and 256 ppm, respectively).

Figure 1. Ammonia production post 48h *in vitro* colonic fermentation in batch of green leafy vegetable extracts and NAD+ and its precursors standards.



* $p < 0.05$. Where it says coriander, read cilantro. Where it says Taioba, read Malanga.

This finding is considered beneficial because excessive ammonia concentrations can affect the energy metabolism of colonic epithelial cells. Studies have shown that ammonia inhibits mitochondrial oxygen consumption in a dose-dependent manner (Andriamihaja et al., 2010) and reduces the oxidation of short-chain fatty acids (SCFAs) propionate, butyrate, and acetate in isolated colonocytes from rats (Cremin et al., 2003). This may negatively impact the availability of energy for colonic epithelial cells and cellular processes, including the regulation of cellular proliferation. In addition, under normal circumstances, ammonia is converted into urea in the liver via the urea cycle and then excreted by the kidneys. However, when the liver is unable to process ammonia efficiently, or if there is excessive production, it accumulates in the bloodstream, leading to hyperammonemia. Elevated ammonia levels can cross the blood-brain barrier, leading to neurotoxic effects that impact brain function. Hyperammonemia is characterized by a range of neurological symptoms, from mild cognitive impairments and mood disturbances to severe encephalopathy, seizures, and coma (Matoori & Leroux, 2015).

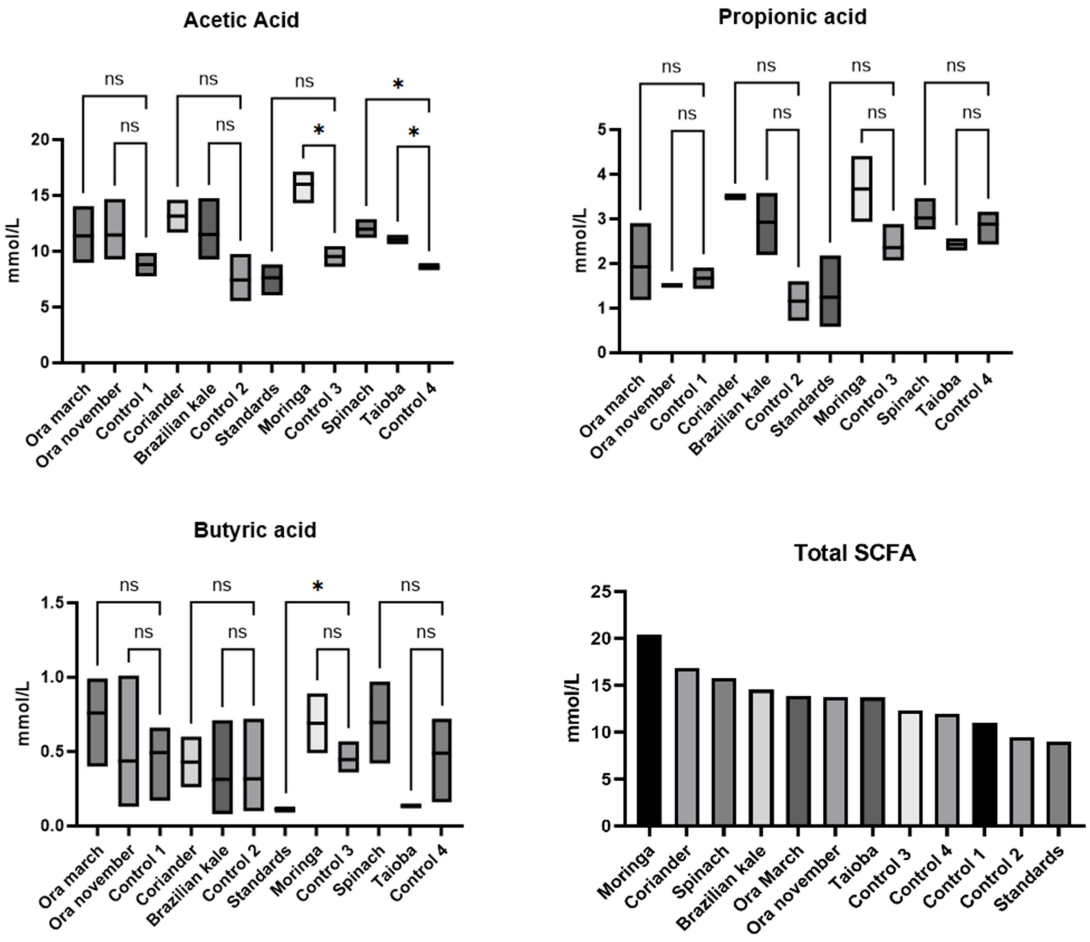
Ammonia is a metabolite resulting from the deamination of amino acids derived of colonic microbiota proteolytic activity, which, in addition to other gases such as H_2, CO_2, and H_2S, also produces short- and branched-chain fatty acids (Blachier et al., 2007; Rowland et al., 2018). Proteins are catabolized by bacteria when the fermentable carbohydrates, the main energy sources, are scarce (Portune et al., 2016). Protein fermentation, resulting from an excessive supply of dietary protein, has been confirmed as harmful to health, increasing the risk of diseases such as colon cancer and inflammatory bowel disease (Ma et al., 2017). This is due to the production of toxic metabolites, including ammonia, indoles, amines, sulfides, and N-nitroso compounds, as well as the proliferation of opportunistic pathogens and pro-inflammatory bacteria (Amaretti et al., 2019).

3.1.2. SCFA production from human fecal fermentation

The levels of short-chain fatty acids (SCFAs) acetate, propionate, and butyrate were quantified as they are primary products of microbial fermentation. They serve as a major energy source for colon cells and microbiota and play crucial roles in intestinal health and function, including protecting the gut epithelial barrier, regulating colonocyte proliferation and inflammation, among others (Liu et al., 2021).

The acetate, propionate, and butyrate levels produced were similar to those normally present in feces, with molar ratios ranging from 3:1:1 to 10:2:1, respectively (Rowland et al., 2018). Although the green leafy vegetable extracts exhibited higher total levels of SCFAs compared to the controls, only Moringa, Spinach, and Malanga demonstrated significantly higher concentrations of acetic acid (11-16 mmol/L) compared to the controls (Figure 2). On the other hand, Malanga, along with the standard solution, showed lower content of butyric acid than their respective controls.

Figure 2. Acetic acid, propionic acid, butyric acid production post 48 hour *in vitro* colonic fermentation in batch of green leafy vegetable extracts and NAD+ and its precursors standards. Total SCFA was determined as the sum of the average concentrations of acetate, propionate and butyrate.



* $p < 0.05$. Where it says coriander, read cilantro. Where it says Taioba, read Malanga.

Besides butyric acid, the positive control, which contained NAD, NMN, NR, and NAM standards, was the only sample that exhibited lower levels of acetic acid, propionic acid, and thus total SCFAs content compared to the control (Figure 2). This suggests that other components present in the green leafy vegetables extracts were preferentially utilized as fuel by the microbiota, contributing to the production of the evaluated metabolites.

Although an extraction of NAD+ and its precursors was performed, it is possible that other polar and water-soluble compounds, such as carbohydrates and dietary soluble fibers, were also extracted and may be present in the plant samples. The content of NAD+ and its precursors, NMN and NR, in plant samples used in *in* vitro colonic fermentation is shown in Table 1.

Table 1. NMN, NR, NAD+ and NAM content of green leafy vegetables extracts used in colonic fermentation, determined by HPLC-UV/DAD.

			(µg/100g) wet weight		Reference
Sample	**NMN**	**NR**	**NAD**	**NAM**	
Cilantro	956 ± 140	531 ± 21.9	828 ± 89.8	n.d.	Artigo capítulo III
Brazilian kale (*Brassica oleracea L. var. viridis L.*)	833 ± 67.3	223 ± 14.0	3087 ± 448	n.d.	
Spinach	177 ± 8.02	257 ± 46.3	3845 ± 1844	n.d.	
Moringa (*Moringa oleifera*)	n.d.	930 ± 130	n.d.	n.d.	
Malanga (*Xanthosoma sagittifolium*)	77.7 ± 7.77	239 ± 73.7	7251 ± 1848	n.d.	
Ora-pro-nobis violacea (*Pereskia Violacea*) March	724.33 ± 138.90	n.d.	n.d.	n.d.	Artigo capítulo IV
Ora-pro-nobis violacea (*Pereskia Violacea*) November	699.38 ± 369.72	n.d.	n.d.	n.d.	

n.d. = not detected

SCFAs originate from bacterial breakdown of complex carbohydrates, mainly polysaccharides and oligosaccharides, although several amino acids released from proteins in the large intestine are also precursors for SCFA synthesis. Studies of *in vitro* fermentation demonstrated that acetate was predominantly produced during the breakdown of pectin and xylan, arabinogalactan resulted in significant propionate production, while of the four substrates tested, butyrate was only formed in substantial amounts from starch (Macfarlane & Macfarlane, 2003). In terms of amino acids, aspartate, alanine, threonine, and methionine are the main sources of propionate, whereas butyrate is predominantly produced through the fermentation of glutamate, lysine, histidine, cysteine, serine, and methionine (Rowland et al., 2018).

Moringa had the highest total SCFAs content (Figure 2). The Moringa leaf powder is reported to contain approximately 33% protein (dry weight), 36.7% carbohydrate content, and 18.1% to 21.1% total dietary fiber, of which 1.94% is soluble (Cuellar-Nuñez et al., 2018). Two polysaccharide fractions, ML-CP 80 (304.7 kDa) and ML-ILP 80 (24.37 kDa), were extracted and identified from Moringa leaf. These fractions exhibited compositions consisting of $_{D}$-xylose (53.14 and 52.55%) and $_{D}$-galactose (33.32 and 38.08%), $_{D}$ -Glucose (6.11 and 3.27%), $_{D}$ -mannose (1.19 and 1.82%), and $_{L}$-arabinose (0.04 and 4.29%), respectively. Galacturonic acid was present exclusively in the ML-CP 80 fraction

(Otu et al., 2020). In another study, a highly branched polysaccharide arabinogalactan (designated as MOP-1) was extracted from Moringa oleifera leaves using a hot aqueous extraction system with ultrasound. Structural characterizations revealed that MOP-1 is composed of rhamnose, arabinose, and galactose in a molar ratio of 1:7.32:12.12 (He et al., 2018).

Water-soluble constituents in cilantro leaves are rarely studied, as research predominantly focuses on essential oil compounds. However, cilantro is found to contain 5.24% fiber and 4.05% protein, which are of interest for SCFAs production (Shahwar et al., 2012).

The composition of spinach is primarily water (91.4%), with smaller proportions of protein (2.9%), carbohydrates (3.6%), fiber (2.2%), and lignans. A 100 g serving of spinach provides significant amounts of several vitamins, including vitamin K, vitamin A, folate, and vitamin C, which meet or exceed their respective Recommended Dietary Allowances (RDAs) (Roberts & Moreau, 2016). Spinach also contains glucuronide derivatives of the flavonoids patuletin and spinacetin (Gil et al., 1999). In addition, a water-soluble natural antioxidant (NAO) mixture, primarily composed of aromatic polyphenols such as p-coumaric acid derivatives, flavonoids, and other hydrophilic molecules, was extracted from spinach and demonstrated considerable antioxidant activity (Lomnitski et al., 2003). Chlorophyll-rich spinach extracts exhibited total SCFAs production similar to our results for spinach extract (ranging from 6 to approximately 16 mM) after 24 hours of *in vitro* colonic fermentation (Li et al., 2021).

Brazilian kale ranked fourth highest in total SCFAs levels. In a study analyzing kale (*Brassica oleracea L. var. acephala*) genotypes grown in South Carolina, USA, it was found that a 100 g serving of fresh kale can provide approximately 0.4–6.7 g of prebiotic carbohydrates, with glucose and fructose being the major soluble sugars (Thavarajah et al., 2016). With approximately 90% water content, 6% carbohydrates, and 2% fiber (Šamec et al., 2019), Brazilian kale, as a cruciferous vegetable, also contains a significant amount of phytochemicals called glucosinolates, including glucobrassicin, glucoraphanin, gluconasturtiin, and glucotropaeolin, all of which have antioxidant properties (Bisht et al., 2023).

Ora-pro-nobis violácea (*Pereskia Violacea*) contains 2.9% protein, 5.9% total fiber, and 2.4% total carbohydrate (wet weight). Approximately 10% of the total carbohydrate content consists of mono-, di-, and oligosaccharides, while the remaining sugars likely

originate from polysaccharides, which constitute the mucilage present in these leaves. In addition to the disaccharides glucose and fructose, monosaccharides such as xylitol, mannitol, and sorbitol were also identified. Among the oligosaccharides, maltotetraose and maltohexaose were found, with maltotetraose being the predominant type (artigo capítulo IV).

Besides being very close to Ora-pro-nobis violácea (Ora march and Ora November), Malanga was the green leaf extract that exhibited the lowest total SCFAs production (average of 13.68 mmol/L). However, a study observed that animals fed diets containing 2.5% lyophilized Malanga leaves had significantly elevated levels of propionic and butyric acids, as well as total short-chain fatty acids (SCFAs), in the colon compared to other diets (containing cellulose and inulin), suggesting the higher fermentability of Malanga by intestinal microbiota (Bernardes Monteiro, 2011). Non-starch polysaccharides were the primary constituents of lyophilized Malanga. Malanga leaves contained 49 g of carbohydrates per 100 g of dry matter, along with 3.4 g of lignin, and 35 g of total dietary fiber, the majority of which was insoluble (28 g). Among the total sugars identified, glucose, fructose, and xylose were the predominant types, totaling 19.3 g (de Almeida Jackix et al., 2013).

The amount and type of SCFA produced are influenced by substrate availability, intestinal transit time, and bacterial composition of the microbiota (Macfarlane & Macfarlane, 2003). While a variety of bacteria are capable of producing acetate, propionate and butyrate are typically generated by specific bacterial species. The main producers of butyrate are of Firmicutes phylum, including *Faecalibacterium prausnitzii* and other *Lachnospiraceae* species. Propionate is produced by species from the Bacteroidetes and Firmicutes phyla, notably from the *Bacteroides* genus, as well as by members of the *Negativicutes* class and certain *Clostridium* species (Rowland et al., 2018).

3.1.3. Gut microbiota composition

According to SCFAs production, the extracts of moringa, cilantro, spinach, and ora-pro-nobis violacea, as well as the standards (positive control), underwent 16S rRNA gene sequencing analysis to evaluate their effect on human fecal microbiota.

Proteobacteria was the predominant phylum representing more than 50% of organisms, which was further increased in all samples compared to the control (Figure 3A). High abundance of Proteobacteria were also observed in others *in vitro* colonic fermentation

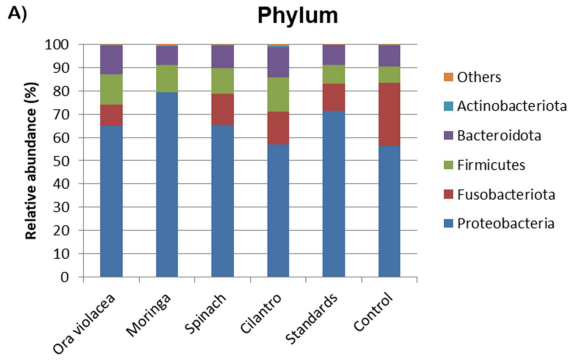
studies with different foodstuffs (Pérez-Burillo et al., 2018), and dietary fibers (Yang et al., 2021).

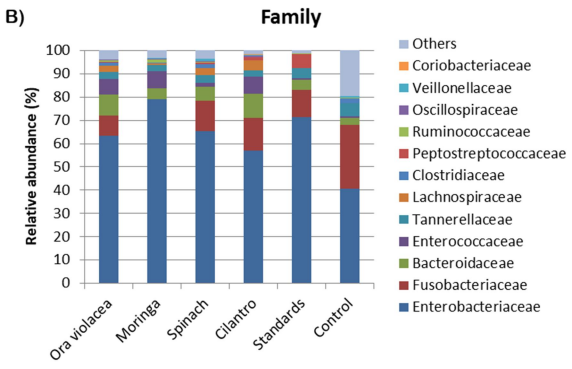
Contrary to what happened with Proteobacteria, Fusobacteriota decreased in all samples and was absent in the moringa extract (Figure 3A), as were *Fusobacterium* genus (Figure 3C, D). This is a beneficial result since, although Fusobacteriota is commonly found in various human mucosal surfaces such as the mouth, gastrointestinal tract, and genital tract, certain species within this phylum, notably *Fusobacterium nucleatum*, are strongly associated with undesirable health effects, including periodontal disease and colorectal cancer (Brennan & Garrett, 2019). Research indicates that *Fusobacterium nucleatum* plays a significant role in the progression of colorectal cancer (Brennan & Garrett, 2019; Rubinstein et al., 2013) and chemotherapy resistance (Yu et al., 2017). Accordingly, moringa has demonstrated antimicrobial (Bancessi et al., 2020; Moyo, 2012) and anticancer activities (Jung, 2014; Khor et al., 2018), including against colorectal cancer cell lines (Al-Asmari et al., 2015).

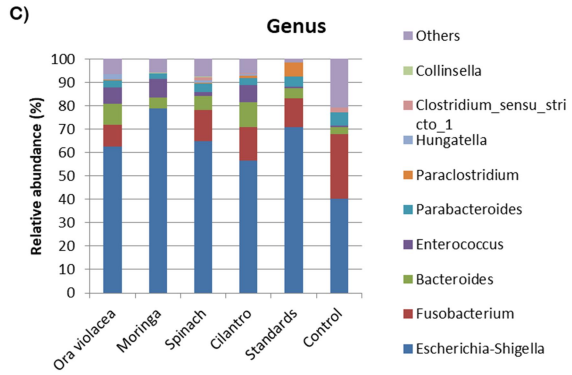
Propionic acid and isovaleric acid SCFAs showed strong inhibitory effects on *Fusobacterium nucleatum* (C. B. Huang et al., 2011). Additionally, moringa exhibited the highest production of propionic acid and total SCFAs among the samples (Figure 2).

Fusobacteriota was also reduced by NMN in *in vitro* fecal fermentation (Z. Tang et al., 2024). Among the NAD+ precursors, moringa contains only NR, but it exhibits one of the highest NR levels among leaves (930 μg/100g wet basis) (Table 1). Whether it is NR and what underlying mechanism promoted this inhibitory effect on Fusobacteriota remain to be further explored.

Figure 3. Effect of green leafy vegetable extracts and NAD+ and its precursors standards on microbial composition, post 48h *in vitro* human fecal fermentation. Panel A, B and C: relative abundances of microbial phylum, family and genus across all samples, respectively. Panel D: percentage of relative abundances of microbial genera across all samples.







D)

	Relative abundance at genus level (%)					
Organism	Ora violacea	Moringa	Spinach	Cilantro	Standards	Control
Escherichia-Shigella	62.7	78.85	65.01	56.59	70.96	40.18
Fusobacterium	9.05	0	13.31	14.37	12.13	27.62
Bacteroides	9.12	4.8	5.9	10.46	4.34	3.15
Enterococcus	6.96	7.78	1.76	7.57	0.79	0.56
Parabacteroides	2.99	2.54	3.57	2.71	4.18	5.7
Paraclostridium	0.36	0	0.4	1.23	5.97	0
Hungatella	2.37	0	0.84	0.61	0	0.02
Clostridium_sensu_stricto_1	0.02	0	1.54	0.19	0.05	1.95
Collinsella	0.05	0.14	0.25	0.26	0.03	0.1
Others	6.38	5.89	7.42	6.01	1.55	20.72

Proteobacteria is a vast phylum composed of approximately 1534 species of facultative anaerobic bacteria, with the majority being pathogenic. Within this large group are bacteria responsible for nitrogen fixation, making Proteobacteria the predominant phylum in various ecosystems, including plant leaves, air, oceans, and soil, with relative abundances of 62%, 77.9%, 57.9%, and 36.5%, respectively (Shin et al., 2015). In humans, chronic enrichment of Proteobacteria in the gut may indicate an imbalanced microbial community structure or disease state, often linked to gut dysbiosis and inflammation (Shin et al., 2015). However, a study of the human microbiome reveals dynamic microbial structures rather than static ones. The relative abundance of Proteobacteria in the human gut transiently varied, with temporal increases and declines possibly influenced by factors like diet, medication, or the adaptive immune system (Caporaso et al., 2011).

Typically, Bacteroidetes and Firmicutes are the two most abundant bacterial phyla in fecal humans, representing the microbiota of the lower gastrointestinal tract (Peluso et al., 2023; Shin et al., 2015; The Human Microbiome Project Consortium, 2012). Firmicutes was the predominant phylum in the human fecal microbiota both before and after 12 weeks of NR

supplementation (1 g twice daily), and no differentially abundant features were detected at any taxonomic levels (Peluso et al., 2023).

Firmicutes was also increased by all samples compared to control. Cilantro, which exhibited the highest abundance of Firmicutes (14.54%), also showed the highest increase in the Bacteroidota phylum abundance (13.08%) (Figure 3A). Cilantro contained NMN, NR and NAD+ (Table 1), and was the one with highest NMN content (956 µg/100g wet basis).

In pre-aging mice, NMN supplementation (in water containing 500 mg/L over 40 days) reduced the abundance of the Proteobacteria and Bacteroidota phyla, while significantly enriching the Campylobacterota and Desulfobacterota phyla (Niu et al., 2021). Twelve weeks of NR supplementation, with a daily dose of 400 mg/kg in drinking water, modulated the mouse gut microbiota by also decreasing the abundances of species from the Proteobacteria and Bacteroidota phyla, while increasing species within the *Lachnospiraceae* family (belonging to the Firmicutes phylum).

While in our results, *Lachnospiraceae* family abundance was increased by leafy vegetables extracts, mainly by cilantro, but negligibly increased (0.1%) by standards. Enterobacteriaceae, belonging to Proteobacteria phylum, was the most abundant family, which was also increased in all samples, mainly in Moringa and standards (Figure 3B).

The nutritional composition of the culture medium showed be the most crucial factor affecting bacterial growth in the *in vitro* fermentation model (Long et al., 2015). In batch fermentations with proteins and peptones as the sole fermentable substrates, *Enterobacteriaceae, Burkholderiaceae, Desulfovibrionaceae, Lachnospiraceae* and *Ruminococcaceae* were the most abundant bacterial families found, while many Firmicutes and Bacteroidota decreased (Amaretti et al., 2019). Considering that we used a basal medium composed of peptone, yeast extract, cysteine, and salts (Supplementary Table 1), it may have contributed to this resulting fecal microbial community.

At the genus level, *Escherichia-Shigella* was the predominant organism, and its abundance increased in all samples, particularly in moringa and the standards (Figure 3C). After *in vitro* fermentation of polysaccharides- cyanidin-3-O-glucoside (C3G) complexes, *Escherichia–Shigella* were also the dominant bacteria detected (Yang et al., 2021).

NMN also increased the abundance of *Escherichia–Shigella* after 24h and 48h of *in vitro* colonic fermentation (Z. Tang et al., 2024). An interesting finding this study was that at 24 hours of fermentation, NMN promoted the abundance of beneficial genera, including *Bifidobacterium, Phascolarctobacterium, Faecalibacterium*, and *Alistipes*, while inhibiting

the proliferation of some harmful bacteria such as *Parasutterella* and *Vibrio* in the human intestinal microbiota. However, this beneficial modulation of microbiota composition observed at 24 hours was reversed by the end of the 48-hour fermentation period. The authors attributed this to depletion of NMN as a substrate for the microbiota (Z. Tang et al., 2024).

One possible explanation could be that this imbalanced gut community originates from donors' microbial composition. At the beginning of the fermentation (0h), *Escherichia-Shigella* was the dominant bacteria genus, with a relative abundance of 50% to 70%, which was reversed with the growth of *Bifidobacterium* and *Lactobacillus* induced by isomalto/malto-polysaccharides at the final 48 hours of *in vitro* human fecal fermentation (Gu et al., 2018).

In our study, despite increasing Firmicutes and decreasing Fusobacteriota abundance, NAD+ and its precursors NMN and NR did not change the presence of Proteobacteria phylum. Instead, they led to a further increase in the abundance of species from the *Escherichia-Shigella* genus, belonging to Proteobacteria phylum, after 48-hour *in vitro* colonic fermentation. This suggests that despite the donors appearing to be healthy, their microbiota profile showed signs of dysbiosis, and that NAD+ precursors may stimulate bacterial species depending on the host's microbiome, or even that longer-term feeding may be necessary to reverse an undesirable gut microbial community.

3.1.4. Richness and diversity of the Gut Microbiota

Alpha diversity refers to the diversity of species within a particular ecosystem. It quantifies the variety and abundance of species within a single sample, or community or habitat. Alpha diversity metrics (or index) typically focus on the number of species (richness) and the number of individuals of different species (distribution or evenness) (Thukral, 2017).

ACE and Chao-1 indices are non-parametric estimators that utilize frequency data of rare species to estimate species richness. The ACE (Abundance-based Coverage Estimator) incorporates the concept of "sample coverage," which represents the proportion of total individuals in an assemblage belonging to the species represented in the sample. To estimate species richness using sample coverage, a cut-off value κ is required to distinguish between "rare" (frequency $\leq \kappa$) and "abundant" (frequency $> \kappa$) species groups (Chao & Chiu, 2016). Chao-1 calculates an estimated lower bound of the total number of species present in a

community, based on the number of unique species observed exactly once (singletons) and species observed exactly twice (doubletons) in a sample (Chao & Chiu, 2016).

Fisher's α index quantifies species diversity within a community based upon the logarithmic distribution of number of individuals of different species (Thukral, 2017), with higher values indicating greater diversity and lower values indicating less diversity. Shannon and Simpson indices incorporate both richness and evenness, what means how evenly species are distributed in terms of abundance, to provide a comprehensive measure of alpha diversity (Thukral, 2017).

Gut microbial diversity is proposed as a marker of health since individuals with a low bacterial richness exhibited increased overall adiposity, insulin resistance, dyslipidemia, and inflammation (Le Chatelier et al., 2013).

Moringa exhibited the highest values of ACE, Chao-1, and Fisher metrics, indicating a high total number of species (richness), but had the lowest Shannon and Simpson indices (Table 2). This indicates low diversity, with a predominance of few species such as Escherichia-Shigella and Enterococcus genera, which accounted for almost 90% of the organisms (Figure 3C). The standards (positive control) exhibited low Shannon and Simpson indices similar to Moringa and had the lowest values of ACE, Chao-1, and Fisher indices, indicating that, in addition to low diversity, the standards of NAD+ and its precursors alone also displayed low species richness.

The control exhibited the second-lowest ACE, Chao-1, and Fisher indices, indicating lower species richness compared to the samples. However, it was more diverse, as evidenced by the highest Shannon and Simpson indices.

Among samples, cilantro was the most diverse, with the second-highest Shannon and Simpson indices. Ora-Pro-nobis violacea and Spinach demonstrated close values of all metrics, showing similar intermediate effects on gut microbiota richness and diversity.

Table 2. Alpha-diversity metrics of microbiota post 48h colonic fermentation of green leafy vegetable extracts and NAD+ and its precursors standards.

Samples	ACE	Chao-1	Fisher α	Shannon	Simpson
Control	123.0	123	14.19	3.60	0.82
Cilantro	144.25	144	16.95	3.28	0.71

Ora-Pro-nobis violacea	135.60	135.11	15.57	3.15	0.67
Moringa	188.74	188.43	22.83	2.47	0.53
Spinach	140.19	140	16.22	3.11	0.67
Standards	99.0	99.0	11.00	2.56	0.59

The Moringa leaf aqueous extract with 13.47% nucleotides, given orally for 7 days, had also little effect on the alpha diversity of loperamide-treated mice. ACE and Shannon indices were similar to both control and loperamide-only groups (Gao et al., 2023).

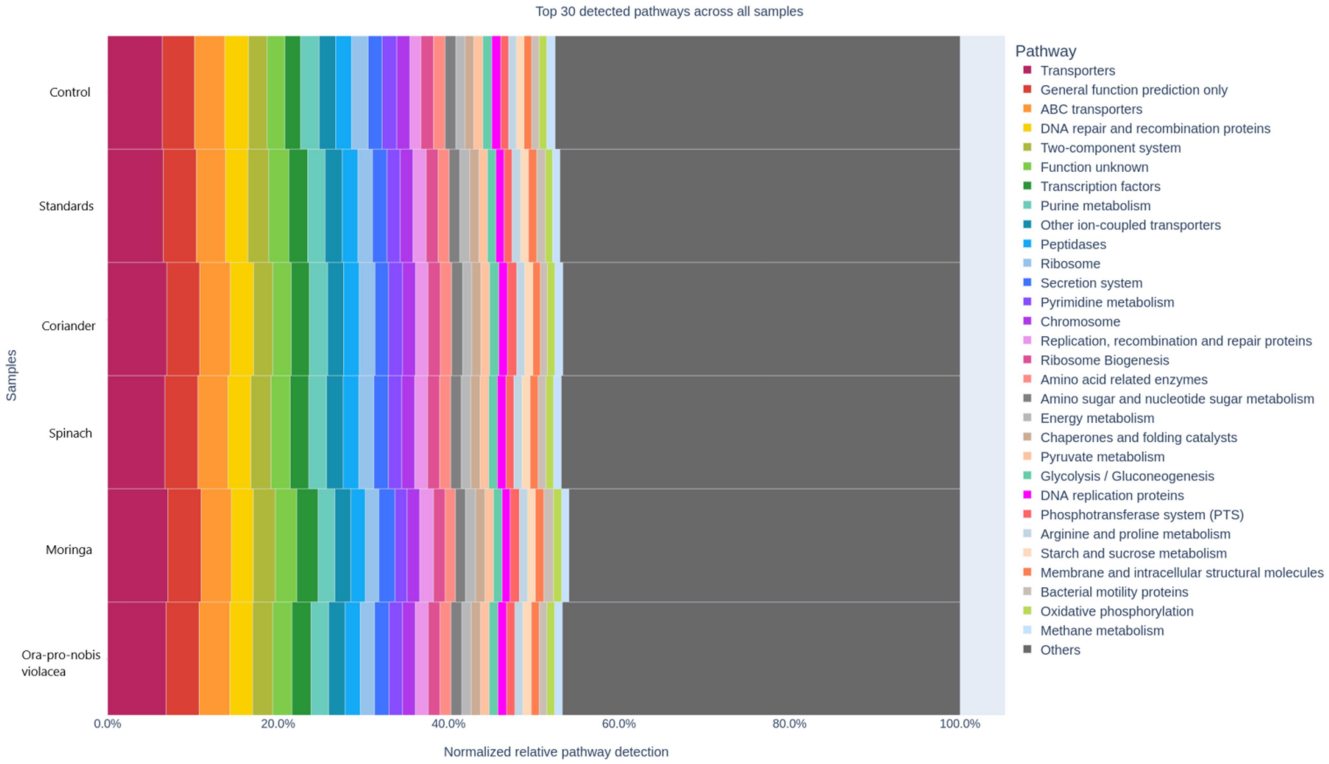
NR supplementation in humans (1 g twice daily) and mice (400 mg/kg/day) for 12 weeks did not significantly affect alpha diversity. Rats supplemented with NR for one to eight weeks showed a slight increase in the Shannon index, but the difference was not significant, and without major changes in overall microbial community structure (Peluso et al., 2023).

In summary, the standards of NAD+ and its precursors exhibited low diversity and richness in the human fecal microbiota, while the green leafy vegetable extracts also showed low diversity but higher richness compared to the control. This suggests that, similar to what occurred with SCFAs production, other components possibly present in the green leafy vegetable extracts contributed to the greater number of species.

3.1.5. Microbial metabolic function analysis

A total of 260 pathways of microbial metabolism were detected, with the 30 most abundant pathways representing more than 50% of those detected. The five main pathways identified were Transporters, ABC transporters, DNA repair and recombination proteins, purine metabolism, and the two-component system (Figure 4).

Figure 4. Top 30 pathways detected across all samples following 48-hour *in vitro* human fecal microbiota fermentation.

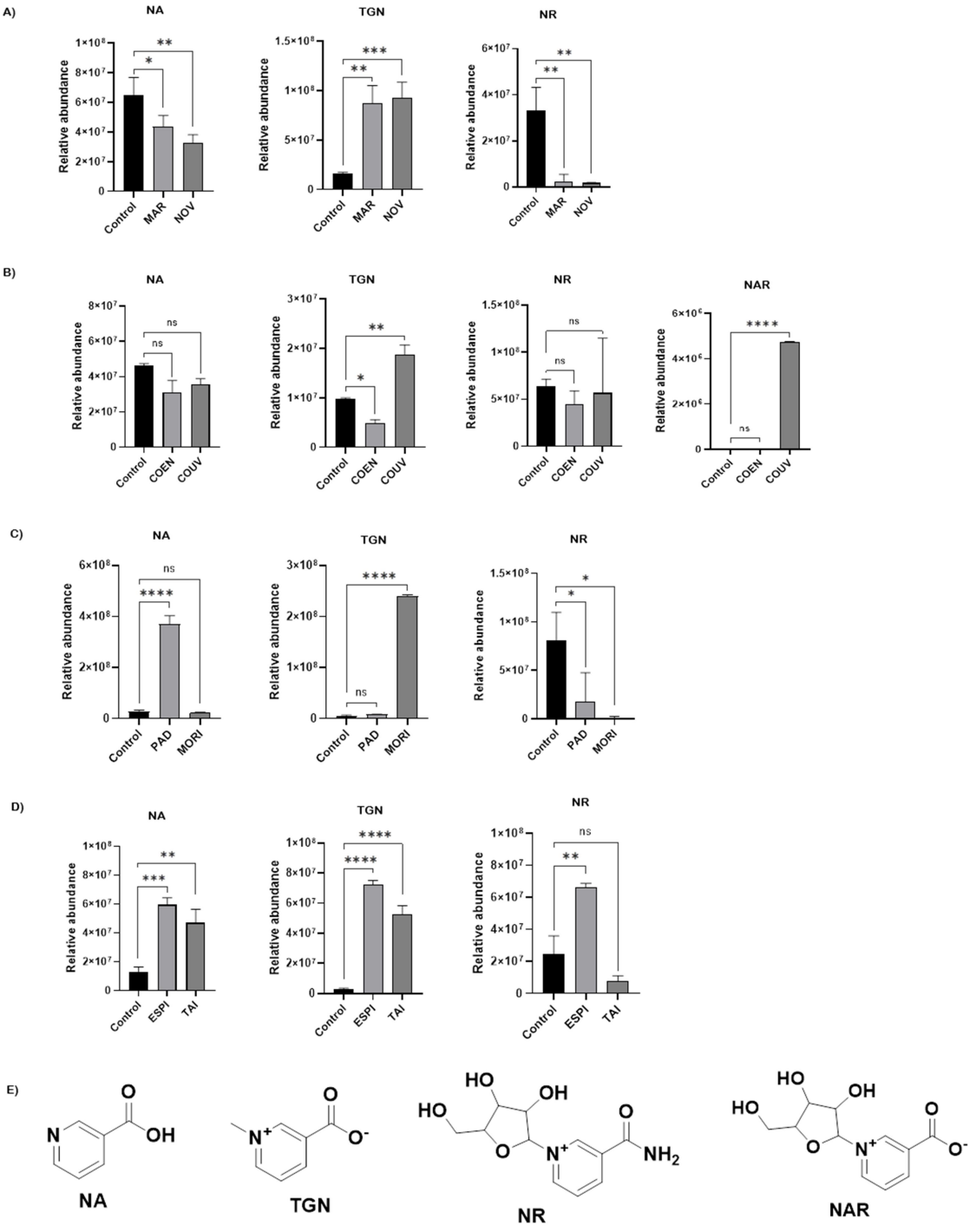


With the exception of moringa, which showed a slightly higher percentage of transporters, the samples exhibited similar percentages among the pathways (Figure 4). This suggests that the fermentation of NAD+ and its precursors NMN and NR had little effect on the functionality of the human fecal microbial community.

3.2. Metabolism of NAD+ and its precursors by gut microbiota

The NAD+-related metabolites derived from gut microbiota found were nicotinic acid, trigonelline, nicotinamide riboside, and nicotinic acid riboside (Figure 5). The LC-MS/MS data for the identified metabolites are presented in Supplementary Table 3.

Figure 5. NAD+ metabolites after 48h *in vitro* colonic fermentation in batch of green leafy vegetable extracts and NAD+ and its precursors standards.



A) Batch 1, where: MAR = Ora-pro-nobis violacea March, NOV = Ora-pro-nobis violacea November. B) Batch 2, where: COEN = cilantro, COUV = Collard/Brazilian kale. C) Batch 3, where: PAD = standards of NAD+, NMN, NR and NAM (positive control), MORI = Moringa. D) Batch 4, where: ESPI = spinach, TAI = Malanga. E) Chemical estructure of

metabolites identified, where: NA = Nicotinic acid, TGN = Trigonelline, NR = Nicotinamide riboside, NAR = Nicotinic acid riboside.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$, ns: not significative.

In general, the profile of metabolites released post-fecal fermentation was unique for each sample and batch. Nicotinic acid (NA) was detected in all samples and controls, with higher levels observed in some samples (spinach and malanga) and lower levels in others (ora-pro-nobis march and November) compared to controls (Figure 5A-D). Considering that yeast extract used in the fermentation medium contains nicotinamide (NAM) and its deamidated form, nicotinic acid (NA) (Ndaw et al., 2002), this may explain the higher NA content observed in some controls (Figure 5A).

In the standards, which had the highest content of NAD+, NMN, NR, and the only sample containing NAM (700 µg each), NA was significantly the major metabolite, while NR was significantly lower compared to the control (Figure 5C). Our findings align with those of (Z. Tang et al., 2024), who found NA to be the primary end product of gut microbiota fermentation. They observed that NMN was initially hydrolyzed into NR, which was subsequently degraded to NAM before deamination to NA. Gut bacteria produce NA from NAM in the colonic lumen through a nicotinamidase enzyme to sustain NAD+ biosynthesis in the epithelial cells (Shats et al., 2020). Indeed, the deamidated NAD+ biosynthetic route is more prevalent in bacteria. PncA genes, responsible for encoding the nicotinamidase enzyme, are more common than nadV genes, which encode the nicotinamide phosphoribosyltransferase enzyme responsible for converting NAM to NMN. These PncA genes were found in the enterobacteria *Escherichia* and *Shigella* (Gazzaniga et al., 2009).

The failure to detect NAD+ post-fecal fermentation could be attributed to a higher rate of catabolism than biosynthesis in the colonic lumen. Another hypothesis is that the majority of NAD+ content found in tissues may be synthesized from NA released by gut microbiota. In fact, the gut microbiota-enabled deamidated biosynthesis pathway was found to be responsible for the majority of NAD synthesis in the colon, liver, and kidney of mice (Shats et al., 2020).

The absence of NMN corroborates previous findings that, after 48 hours of fecal fermentation, NMN was completely consumed by gut microbiota and NA was the primary end product (Z. Tang et al., 2024). It has been demonstrated that the prevalent NAD+ biosynthesis route in gut microbiota is through deamidated *de novo* and *Preiss-Handler*

pathways (Magnúsdóttir et al., 2015; Shats et al., 2020). Additionally, NAD+ can be recycled via the *salvage* pathway, where it is cleaved into NMN. NMN can then undergo hydrolysis to nicotinic acid mononucleotide (NAMN), an intermediate in the *de novo* biosynthetic pathway. Alternatively, NMN can be cleaved into nicotinamide, followed by amide hydrolysis and phosphoribosylation to form NAMN (Begley et al., 2001).

In relation to trigonelline (TGN), except for the standards and cilantro, samples exhibited significantly higher levels than the controls.

Trigonelline is a pyridine alkaloid formed by methylation of the nitrogen atom of NA (Figure 5E). This synthesis is catalyzed by S-adenosyl-L-methionine (SAM)-dependent nicotinate N-methyltransferase (EC 2.1.1.7), also known as trigonelline synthase (Ashihara et al., 2015). Although trigonelline primarily accumulates in seeds of legumes and coffee (with values varying from 1.5 % to 2.9 % in *Coffea Arabica* (Mazzafera, 1991)), it is also present at low levels in other plant organs, including leaves and roots (Ashihara et al., 2015). Considering that trigonelline was detected at low levels in the presence of standards, similar to the control (Figure 5C), and there is no evidence that bacteria metabolize nicotinic acid into trigonelline, it is suggestive that the significantly higher TGN found in leaf extracts compared to controls was derived more from the plant's own composition, which may have been coextracted, than from gut microbial conversion of NA. Indeed, a Moringa leaf aqueous extract contained 0.89% trigonelline in its chemical composition (Gao et al., 2023).

On the other hand, despite having the highest NR content, moringa showed a significantly lower NR level post-fermentation compared to the control (Figure 5C). This suggests that NR and its subsequent metabolite NA were likely consumed by the microbiota, as the NA level was the same as the control.

Intriguingly, NR was identified in all controls, exhibiting higher levels than almost all samples, except for spinach (Figure 5D). One possible reason for this could be the yeast extract used in the medium fermentation, as NR has been found in yeast (Bogan et al., 2009; Holdsworth et al., 1991). Additionally, *Saccharomyces cerevisiae*-dependent NR production has been demonstrated in beers (Garofalo et al., 2021). However, why NR was not converted to NAM, and subsequently to NA as observed in the majority of samples, remains inconclusive.

NAR also participates in human NAD metabolism, contributing to the NAD supply as an alternative precursor (Kulikova et al., 2015). NAR appeared only in collard (Figure 5B), which is an interesting finding since, despite containing NMN, NR, and NAD+

(Table 1), this extract did not have the highest level of any of these compounds. One plausible reason could be the dephosphorylation of nicotinate mononucleotide (NAMN) to NAR by a pyridine 5′-nucleotidase (Bogan et al., 2009; Shats et al., 2020), as the nicotinamidase Pnc1 was inactive in NR, thus failing to produce NAR from NR in yeast (Bogan et al., 2009). While whether this was the case remains to be further explored, as we did not measure NAMN in the collard extract or other samples before colonic fermentation, because our focus was on amidated precursors NMN, NR, and NAM. However, another possibility is that NAMN may have been generated from the deamidation of NMN by a NMN deamidase (EC 3.5.1.42) (Galeazzi et al., 2011).

Finally, spinach was the only sample that had a significantly higher level of all identified metabolites than its batch control (Figure 5D). Similar to collard, spinach also did not have the highest level of NMN, NR, or NAD+. Among the green leafy vegetables assessed, only spinach, collard, and malanga significantly contributed to increasing the pool of NAD+ synthesis precursor metabolites derived from gut bacteria.

Human gut microbiota demonstrated to degrade NAD, NMN, and NR into deamidated precursors NA and NAR. It is worth exploring whether a different gut microbial composition would generate other metabolites.

4. CONCLUSION

Given the growing evidence of the role of gut microbiota in mediating various aspects of health and disease, alongside recent discoveries highlighting the importance of NAD+ in metabolic and signaling processes, efforts to elucidate the effects of NAD+ precursors from plant foods on gut microbiota composition, activity, and biotransformation were initiated in our study for advancing the knowledge of how this interaction may to influence health and disease prevention.

We found that *in vitro* colonic fermentation of NAD+ and its precursors, NMN and NR, extracted from green leafy vegetables changed the microbial composition, increasing bacterial abundance, but had little effect on diversity and activity of human fecal microbiota. While there was an increase in the abundance of the Firmicutes phylum at the expense of the Fusobacteriota phylum, we also observed a further increase in *Escherichia-Shigella*, belonging to the Proteobacteria phyla, as the most abundant genera. This finding

corresponded with the relatively low production of propionic and butyric acid, given that *Escherichia-Shigella* species are not typically known as major SCFA producers.

Human gut microbial metabolism degraded NAD, NMN and NR converting to deamidated NA and NAR precursors. The plants extracts assessed containing NAD, NMN and NR, particularly spinach, contributed to increasing the pool of NAD+ biosynthesis precursors metabolites derived-gut microbial.

Finally, future *in vitro* and clinical studies of long-term feeding of NAD+ and its precursors NMN and NR effects are suggested to simulate chronic intake and compare the results with our 48-hour test on human gut microbiota.

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DISCLOSURE STATEMENT

The authors declare that they have no conflict of interest.

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Supplementary Material

Table 1. Basal medium composition of *in vitro* colonic fermentation.

Compound	Quantity (for 1 L of distilled water)
KH_2PO_4	18.5 g
K_2HPO_4	5.9 g
$NaHCO_3$	2.3 g
L-cysteine	0.6 g
Yeast extract	2.3 g
Peptone	2.3 g
Tween 80	2.3 g
HCl 1M	Enough to adjust the pH to 7.0

Table 2. Composition of PBS buffer for fecal human inoculum preparation.

Compound	Quantity (for 500 mL of 0,1 M PBS buffer)
Sodium thioglycolate	0.5 g
NaH_2PO_4	2.99 g
Na_2HPO_4	3.54 g
NaOH 0,1 M	Enough to adjust the pH to 7.2

Table 3. LC-MS/MS data of metabolites found in green leafy vegetable extracts and NAD+ and its precursors standards post 48h *in vitro* colonic fermentation, in batch.

Compound	**HMDB ID**	**Formula**	**Theoretical *m/z***	**Experimental *m/z***	**Error (ppm)**	**RT (min)**	**MS^2 main fragments**
Nicotinamide riboside (NR)	HMDB00855	$[C_{11}H_{15}N_2O_5]^+$	255.0981	255.0975	-0.07	6.48	255.0975, 133.0498, 123.0554, 106.0288,
Nicotinic acid (NA)	HMDB01488	$[C_6H_5NO_2 + H]^+$	124.0399	124.0393	-0.04	3.68	125.9504, 124.0393, 96.0444, 80.0494
Nicotinic acid riboside (NAR)	HMDB06809	$[C_{11}H_{13}NO_6 + H]^+$	256.0821	256.0815	-0.21	7.55	256.0815, 235.3803, 124.0394, 73.0284
Trigonelline (TGN)	HMDB00875	$[C_7H_7NO_2 + H]^+$	138.0555	138.0549	-0.40	5.29	138.0550, 110.0602, 94.0652, 80.9344

DISCUSSÃO GERAL

Devido ao papel central de NAD+ em várias importantes reações para regulação energética e de sinalização celular, estudos a cerca do seu metabolismo têm ganhado muita atenção nos últimos anos. Isso porque foi identificada uma diminuição dos níveis de NAD+ durante o envelhecimento, estresse oxidativo e infeccioso, e que ao aumentar a síntese de NAD+ através dos mais recentes precursores NMN e NR reverte a queda de seus níveis promovendo benéficos efeitos fisiológicos, principalmente os relacionados ao envelhecimento (J. Yoshino et al., 2018). Atenuação do declínio fisiológico e de patologias como diabetes, Alzheimer, degeneração retinal, esteatose hepática, falha do coração, disfunção endotelial e muscular, entre outras, foram observados após suplementação de NMN e NR em estudos pré-clínicos e clínicos (Chen et al., 2020; Igarashi et al., 2022; Li et al., 2021; Mateuszuk et al., 2020; Tarantini et al., 2019; Trammell et al., 2016; Wang et al., 2016; X. Xie et al., 2019; J. Yoshino et al., 2011; M. Yoshino et al., 2021; R. Zhang et al., 2017; X. Zhang et al., 2020).

Apesar da evidência de NMN e NR em alimentos, e do potencial dietético deles à saúde, pouco enfoque têm sido dado pela comunidade científica da área de alimentos e nutrição. Em análise bibliométrica (capítulo II), constatou-se que as pesquisas relacionadas ao tema de NAD+ e seus precursores NMN e NR, são majoritariamente das áreas de bioquímica e biologia celular (Alegre & Pastore, 2023). Apenas 1% das publicações foram das áreas de ciência e tecnologia alimentos e dietética nutricional, portanto vários importantes tópicos ainda estão inexplorados, incluindo: a) encontrar alimentos fontes desses compostos e avaliar se o processamento dos alimentos interferem no seu conteúdo; b) encontrar novas fontes naturais para extração e isolamento desses compostos, e microrganismos para a produção biotecnológica, suprindo o mercado de suplementos e nutracêuticos; c) intervenções dietéticas visando promover uma otimizada nutrição para sustentar a síntese de NAD+ evitando o declínio de seus níveis; d) estudos de biodisponibilidade e eficácia na manutenção da homeostase do NAD+ através da alimentação; e) interações entre a microbiota intestinal e os precursores NAD+ oriundo da dieta, e os potencias efeitos na saúde do hospedeiro; e f) outras possíveis hipóteses decorrentes dos achados desses anteriores.

Para ajudar a preencher algumas dessas lacunas, um rastreamento do conteúdo de NAD+, NMN, e NR foi feito em vegetais comumente consumidos, e em PANC (plantas alimentícias não convencionais), através de um método que foi desenvolvido e validado para

uso em HPLC/UV-DAD, e os resultados encontrados foram depois confirmados por LC-MS/MS (capítulo III).

As concentrações de NMN e NR variaram de 40 a 13.000 e 62 a 1.600 μg por 100g de vegetais frescos, respectivamente. Brócolis e vagem foram as fontes mais ricas em NMN, enquanto o NR foi mais abundante em almeirão, banana e laranja. O teor médio de NAD+ encontrado variou de 58 a 8.800 μg/100g (peso úmido), tendo taioba roxa (*Xanthosoma violaceum*), taioba (*Xanthosoma sagittifolium*), espinafre e couve as maiores quantidades.

Em relação ao tratamento térmico nos vegetais, o calor úmido teve variados efeitos de acordo com a matriz vegetal e o composto analisado. Enquanto em algumas amostras (como brócolis, berinjela e repolho roxo) o calor não teve efeito significativo no teor de NR, em outras, como a vagem, o calor mostrou o diminuir significativamente. Já para NMN, o tratamento térmico diminuiu significativamente o teor em brócolis, aumentou em vagem, enquanto que não teve efeito para as amostras de beterraba, cenoura e repolho roxo. Esses resultados podem ser atribuídos às diferenças na estrutura/tecido vegetal e às variações na duração/temperatura do tratamento, e representa uma lacuna para ser ainda mais explorada, abrindo insights para investigar o impacto de outros tipos de técnicas de processamento de alimentos no conteúdo de NMN e NR.

Dentre as PANC analisadas, taioba e taioba roxa foram as únicas que tiveram concomitantemente NAD+, NMN e NR. Moringa (*Moringa oleifera*) e taioba roxa tiveram os maiores teores de NR (930 e 923 μg/100g em base úmida, respectivamente), enquanto que a Ora-pro-nobis Amazônica (*Leuenbergeria bleo*) foi a única que não continha NR. Ora-pro-nobis violácea (*Pereskia Violacea*), uma nova espécie de Ora-Pro-nobis, foi a que apresentou o maior teor médio de NMN (724 μg/100g em base úmida). Por ser uma nativa e endêmica PANC da Mata Atlântica brasileira, sem dados publicados ainda sobre sua composição, também foi feita a caracterização nutricional e capacidade antioxidante de Ora-pro-nobis violácea, além da avaliação do efeito da sazonalidade nesses resultados determinados (capítulo IV).

Além de NMN, as folhas de Ora-pro-nobis violácea mostraram alto teor de proteínas, fibras, potássio, cálcio e ferro, chegando até ultrapassar a ingestão diária recomendada (IDR) para cálcio. Também conteve compostos fenólicos (de 769,3 a 1.043,6 μg GAE/g de peso seco), que foi significativamente correlacionado com a capacidade antioxidante. Em relação à sazonalidade, apesar da variação pluviométrica entre as amostras,

as folhas frescas apresentaram teores de umidade muito próximos entre si. Já após a secagem (liofilização), feita no mesmo dia e sob as mesmas condições, as amostras do período de menor precipitação apresentaram resultados significativamente superiores para proteína, lipídeo, para a maioria dos minerais determinados (magnésio, zinco, potássio, cobre, e manganês), maioria dos sacarídeos determinados (xilitol, glicose, frutose, maltotetraose e maltohexaose), fenólicos totais e capacidade antioxidante. Esses achados são consistentes com a sua classificação como uma espécie pertencente à família Cactaceae, indicando a sua adaptação fisiológica em condições de seca. Apesar disso, as folhas da estação mais chuvosa apresentou mais que o dobro de ferro (13.14 mg/100g em peso seco) que as folhas da estação mais seca, uma quantidade maior que a encontrada nas fontes vegetais comumente conhecidas desse mineral (Ayaz et al., 2006; Qin et al., 2017).

E por fim, a possível modulação da composição e atividade da microbiota intestinal humana por NAD+ e seus precursores NMN e NR, extraídos de folhosas, e sua biotransformação e metabólitos gerados por essa microbiota foram avaliados (capítulo V). Para isso extratos de folhas de espinafre, coentro, couve, taioba, moringa e ora-pro-nobis violácea contendo NAD+, NMN e NR foram fermentados pela microbiota de inóculo fecal humano em um modelo colônico *in vitro*. Controles positivos contendo padrões de NAD+, NMN, NR e Nicotinamida (NAM), e controles negativos contendo água também foram avaliados.

Após 48h de fermentação, as amostras aumentaram a riqueza (número total de espécies), e a abundância de espécies de Firmicutes em detrimento de Fusobacteriota. No entanto, NAD+ e seus precursores também aumentaram ainda mais a abundância de *Escherichia-Shigella*, que foi o gênero predominante de todas as amostras e controle. Uma hipótese é que NAD+ e seus precursores podem estimular espécies bacterianas dependendo da microbiota do hospedeiro, ou ainda que uma alimentação por um tempo maior pode ser necessária para reverter uma comunidade microbiana intestinal indesejável.

NAD+ e seus precursores impactaram minimamente na atividade microbiana, sugerindo que não foram um substrato preferível para as bactérias do inóculo usado. A quantidade de amônia produzida variou de 174-272 ppm e não diferiu dos controles, com exceção para taioba que foi a única amostra que apresentou diferença significativa com níveis inferiores ao controle. No entanto, esse resultado é considerado benéfico porque concentrações altas de amônia podem afetar o metabolismo energético das células epiteliais do cólon, e é resultante da indesejável fermentação proteolítica. A produção de ácidos graxos

de cadeia curta (AGCC) também não foi muito significativa, principalmente para o controle positivo, que teve níveis mais baixos de todos os AGCC que o controle. Apenas as amostras de moringa, espinafre e taioba demonstraram concentrações significativamente mais altas de ácido acético (11-16 mmol/L). Para os ácidos propiônico e butírico não houve diferença significativa em comparação aos controles.

Quanto à biotransformação, o metabolismo microbiano intestinal humano degradou NAD, NMN e NR convertendo-os em precursores desamidados ácido nicotínico (NA) e ácido nicotínico ribosídeo (NAR). Os extratos das plantas utilizadas, particularmente espinafre, contribuíram para aumentar o pool de metabólitos precursores da biossíntese de NAD+, possivelmente contribuindo para o aumento da sua síntese nas células colônicas epiteliais, fígado e outros tecidos (Shats et al., 2020).

CONCLUSÃO GERAL

Apesar da relevância, NAD+ e seus precursores NMN e NR ainda são inexplorados nas áreas de pesquisa de ciência de alimentos e nutrição. Nossos esforços em alterar esse cenário permitiu revelar novas fontes alimentícias, incluindo as também pouco exploradas PANC, desses compostos, e o efeito do tratamento térmico nos seus teores.

Ora-pro-nobis violacea revelou ser uma PANC muito nutritiva, o que enfatiza a importância de valorizar, cultivar e consumir plantas alimentícias não convencionais. Além disso, também destaca o seu potencial como uma fonte de nutracêuticos e ingredientes promissores adequados para enriquecer diversos produtos alimentícios, incluindo as opções à base de plantas, que têm tido uma crescente procura.

NAD+ e seu precursores extraídos de alimentos vegetais mostrou alterar a riqueza e a composição da microbiota intestinal humana, indicando o papel dietético de NMN e NR na modulação desse microbioma e os potenciais efeitos decorrentes dessa modulação. Além disso, o metabolismo microbiano mostrou desamidar NAD+, NMN, NR e NAM, liberando outros precursores de NAD+ desamidados, sugerindo sua participação na biodisponibilidade e bioatividade desses compostos.

Todos esses achados reforçam a importância em aumentar a ingestão de folhas, frutas e vegetais, sendo a presença de NAD+ e seus precursores NMN e NR mais uma potencial razão para seus benefícios à saúde, além dos já estabelecidos como baixa caloria, ausência de colesterol, presença de fibras, vitaminas, minerais, polifenóis e outros antioxidantes.

Muito ainda precisa ser investigado e respondido sobre a obtenção dietética de NMN e NR, e suas bioatividades, citando como exemplo às seguintes perguntas: 1) Qual a bioacessibilidade e disponibilidade desses compostos? 2) Quais os efeitos clínicos da ingestão de alimentos fonte de precursores de NAD+ a longo prazo? 3) Quais os potenciais efeitos a nível intestinal e extraintestinal decorrentes da modulação da composição microbiana intestinal por NMN e NR? E várias outras que certamente virão. Mas longe de querer findar o assunto, esse estudo contribuiu para a expansão do conhecimento sobre os precursores de NAD+, NMN e NR, na área de ciência de alimentos, fomentando a abertura para futuras pesquisas.

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ANEXOS

Anexo I - Artigo publicado na revista Current Nutrition Reports

Current Nutrition Reports (2023) 12:445–464
https://doi.org/10.1007/s13668-023-00475-y

REVIEW

NAD+ Precursors Nicotinamide Mononucleotide (NMN) and Nicotinamide Riboside (NR): Potential Dietary Contribution to Health

Gabriela Fabiana Soares Alegre[1,2] · Glaucia Maria Pastore[1,2]

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Abstract
Purpose of Review NAD+ is a vital molecule that takes part as a redox cofactor in several metabolic reactions besides being used as a substrate in important cellular signaling in regulation pathways for energetic, genotoxic, and infectious stress. In stress conditions, NAD+ biosynthesis and levels decrease as well as the activity of consuming enzymes rises. Dietary precursors can promote NAD+ biosynthesis and increase intracellular levels, being a potential strategy for reversing physiological decline and preventing diseases. In this review, we will show the biochemistry and metabolism of NAD+ precursors NR (nicotinamide riboside) and NMN (nicotinamide mononucleotide), the latest findings on their beneficial physiological effects, their interplay with gut microbiota, and the future perspectives for research in nutrition and food science fields.
Recent Findings NMN and NR demonstrated protect against diabetes, Alzheimer disease, endothelial dysfunction, and inflammation. They also reverse gut dysbiosis and promote beneficial effects at intestinal and extraintestinal levels. NR and NMN have been found in vegetables, meat, and milk, and microorganisms in fermented beverages can also produce them.
Summary NMN and NR can be obtained through the diet either in their free form or as metabolites derivate from the digestion of NAD+. The prospection of NR and NMN to find potential food sources and their dietary contribution in increasing NAD+ levels are still an unexplored field of research. Moreover, it could enable the development of new functional foods and processing strategies to maintain and enhance their physiological benefits, besides the studies of new raw materials for extraction and biotechnological development.

Keywords NAD+ precursors · Nicotinamide mononucleotide · Nicotinamide riboside · Pyridine derivatives · Promoting health

Introduction

NAD+ is the oxidized form of nicotinamide adenine dinucleotide, a molecule essential for living organisms for maintaining cellular health. It has been shown to promote several health benefits, including enhancing energy metabolism, cardio and neuroprotection, DNA repair, and anti-inflammatory and anti-aging effects [1••, 2–6].

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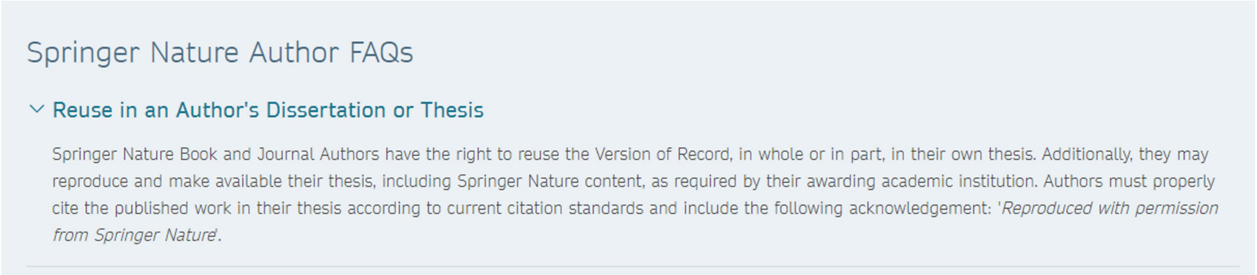
In addition to its key role in energy metabolism as a coenzyme that accepts electrons for catabolic reactions, NAD+ also participates as a co-substrate in signaling pathways of intracellular calcium mobilization and post-translational protein modification [7]. The regulation of these important cellular processes mediated by NAD+ confers protection of mitochondrial function, redox homeostasis control, anti-inflammatory action, attenuation of age-related dysfunctions, cell differentiation, genomic stability, and epigenetic modulation among others [1••, 8–10].

NAD+ is constantly synthesized, catabolized, and recycled in the cell to sustain stable levels. Disturbances such as aging [11] and overnutrition by high-fat and -protein intake [12, 13] affect NAD+ synthesis and are associated with reduced levels of this important molecule. Low NAD+ levels is one of the hallmarks of physiological decline and the onset of age-associated diseases, such as neurodegenerative,

Anexo II - Comprovante de licença para reuso em tese



Anexo III – Artigo publicado na revista Food Bioscience

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The emerging importance of NAD+ metabolome for nutrition and food sciences: A bibliometric analysis

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ABSTRACT

NAD+ is crucial for cellular balance in all organisms. Recent interest has focused on its metabolism. Deficiency-related issues can be countered using NMN and NR, potential therapies available through diet or supplementation. These compounds are emerging as nutraceuticals and bioactive food elements. Bibliometric analysis was used to study the scientific output and impact of research on the NAD+ metabolome. For this, the terms "nicotinamide adenine dinucleotide" or "NAD+" or "NAD+ precursors" or "nicotinamide riboside" or "nicotinamide mononucleotide" were searched in the topic title in The Web of Science™ database. Only original articles and articles review were considered. VOSviewer was used for constructing and visualizing the bibliometric networks maps. A total of 10,568 papers were published, with a consistent increase in the number of publications, especially over the last decade. Biochemistry molecular biology, biophysics, cell biology, chemistry multidisciplinary and microbiology were the top 5 field research in the subject. The majority of the publications came from the 'Journal of biological chemistry' and 'Biochemistry' journals. The prominent research topics included longevity, sirtuins, NAD+ precursors, oxidation and reduction reactions, enzymes, microbial biotechnology, cancer therapy, and crop science. Finally, a guide for future investigation in nutrition and food sciences areas was proposed, which holds immense potential for developing innovative strategies to enhance food quality, promote optimal nutrition, and explore new avenues for improving human health. To our knowledge, this is the first bibliometric analysis of NAD+ metabolome, which can provide valuable insights into the research landscape in this multidisciplinary rapidly growing field.

1. Introduction

Nicotinamide adenine dinucleotide (NAD) is a pyridine nucleotide found in two oxidation states: oxidized (NAD+) and reduced (NADH), which has a crucial role in maintaining cellular homeostasis across all organisms. It is involved in various vital processes including metabolism, mitochondrial function, inflammation, circadian rhythm, DNA repair, cell division, immune system regulation, signaling and transcriptional events (Chini et al., 2021; Houtkooper et al., 2010; Ummarino et al., 2021; Yang & Sauve, 2016). This multifaceted role has led to a renewed interest in NAD + metabolism in recent years (Covarrubias et al., 2021; Hosseini et al., 2019; Johnson & Imai, 2018; Xie et al., 2020; Yang & Sauve, 2016).

Discovered more than a century ago as low molecular coferment or "cozymase" in fermentation (Harden & Young, 1906), NAD+ has been receiving abundant attention in researches, which has unraveled their pivotal functions. Since last two decades it changed from being an electron transfer cofactor for redox reactions for energy production, to acting as a rate-limiting substrate for many proteins involved in signaling pathways such as CD38/CD157, ARTs, PARPs, and sirtuins (Berger et al., 2004; Houtkooper et al., 2010), which regulates a large array of cellular functions.

CD38 and CD157 are multifunctional transmembrane glycoproteins which play dual roles as an ectoenzyme and as a regulator of the immune system (Partida-Sánchez et al., 2003; Quarona et al., 2013). They have both NAD glycohydrolase and ADPR cyclase activities to regulate NAD+ availability and to generate second messengers, such as cADPR a cyclic product of ADPR which contributes to calcium mobilization (Houtkooper et al., 2010).

ARTs post-translationally modify proteins by adding one or several ADPR portions to specific amino acids, altering their biological activity (Berger et al., 2004). This modification can inactivate proteins, mainly impacting immune response and inflammation (Fehr et al., 2020).

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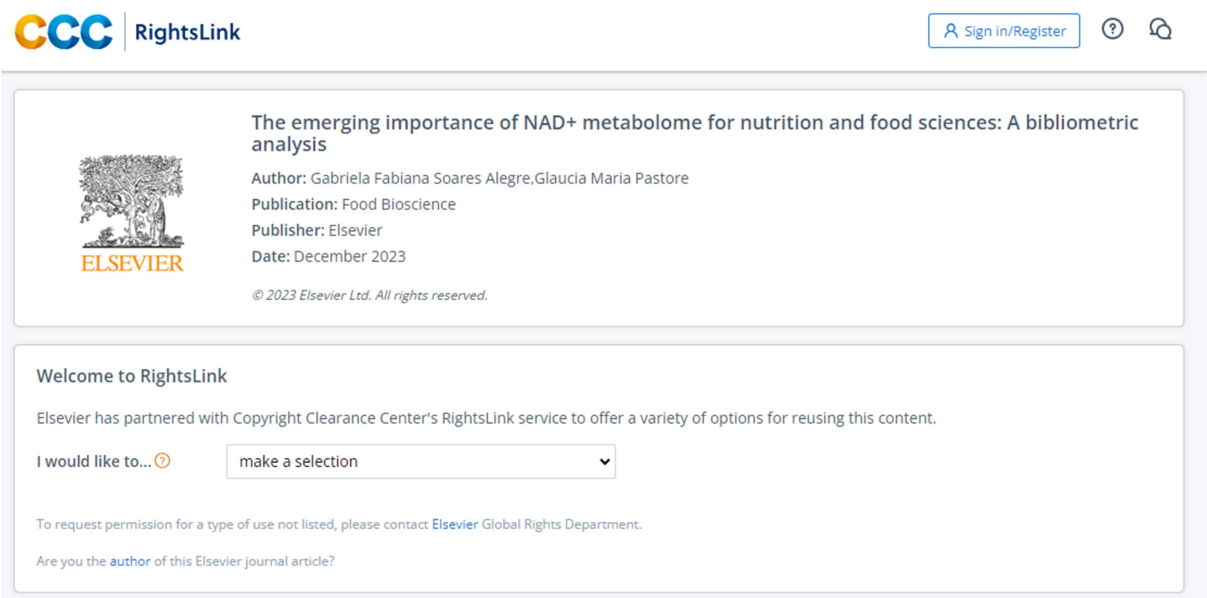
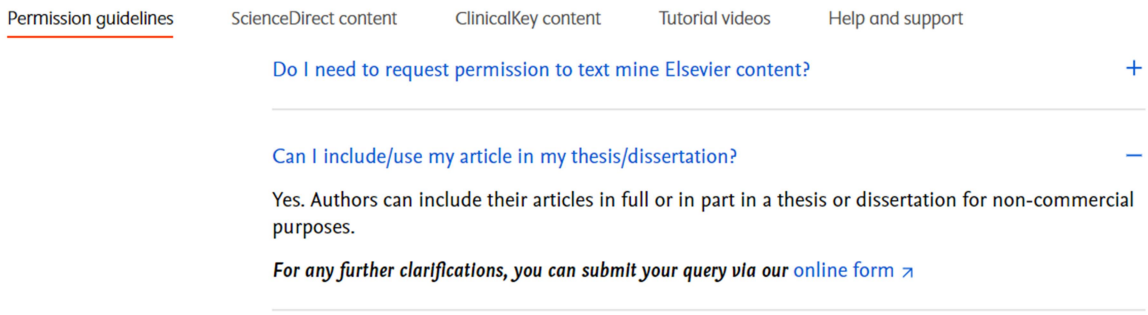
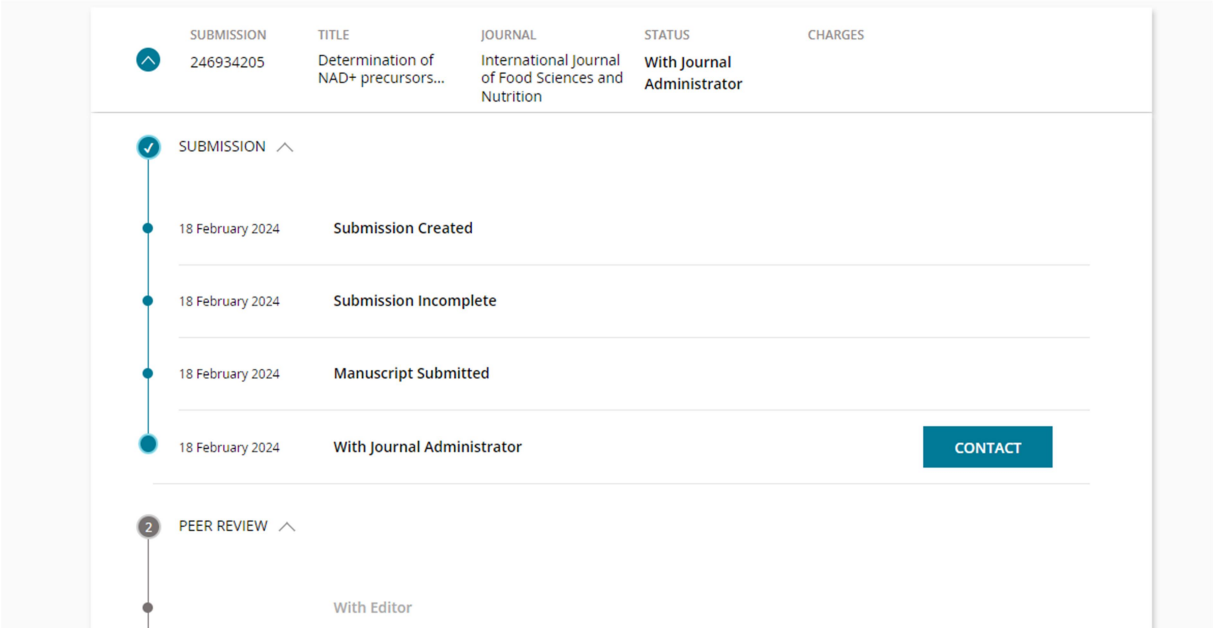
Anexo IV – Comprovante de licença para reuso em tese





Anexo V - Comprovante de submissão de artigo



SUBMISSION	TITLE	JOURNAL	STATUS	CHARGES
246934205	Determination of NAD+ precursors...	International Journal of Food Sciences and Nutrition	With Journal Administrator	

SUBMISSION

18 February 2024	Submission Created
18 February 2024	Submission Incomplete
18 February 2024	Manuscript Submitted
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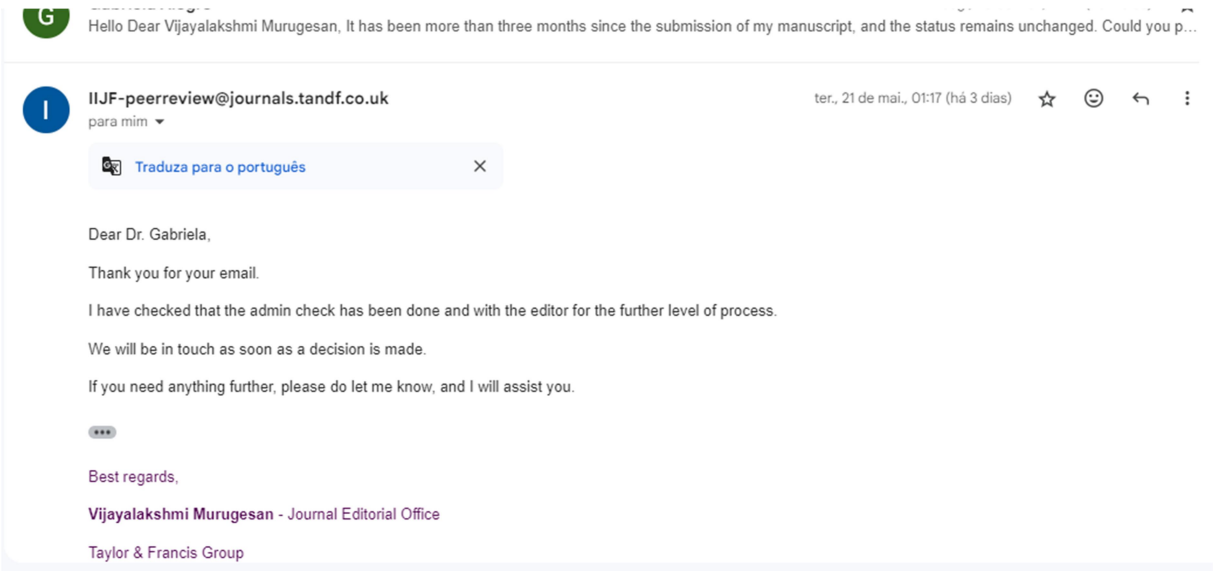
CONTACT

2 PEER REVIEW

With Editor



Hello Dear Vijayalakshmi Murugesan, It has been more than three months since the submission of my manuscript, and the status remains unchanged. Could you p...

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para mim

Traduza para o português

Dear Dr. Gabriela,

Thank you for your email.

I have checked that the admin check has been done and with the editor for the further level of process.

We will be in touch as soon as a decision is made.

If you need anything further, please do let me know, and I will assist you.

Best regards,

Vijayalakshmi Murugesan - Journal Editorial Office

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Anexo VI - Comprovante de aprovação Comitê de Ética

UNIVERSIDADE ESTADUAL DE CAMPINAS - UNICAMP/CAMPUS CAMPINAS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação do teor de NMN e NR - precursores da síntese de NAD - em alimentos, e sua potencial contribuição dietética na promoção da saúde

Pesquisador: GABRIELA FABIANA SOARES ALEGRE

Área Temática:

Versão: 2

CAAE: 72536923.4.0000.5404

Instituição Proponente: Faculdade de Engenharia de Alimentos

Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 6.267.613

Apresentação do Projeto:

As informações contidas nos campos "Apresentação do Projeto", "Objetivo da Pesquisa", "Avaliação dos Riscos e Benefícios" e "Comentários e Considerações sobre a Pesquisa" foram obtidas dos documentos apresentados para apreciação ética pelo CEP e das informações inseridas pelo/a PESQUISADOR/A RESPONSÁVEL pelo estudo, na Plataforma Brasil.

Introdução:

A molécula de nicotinamida adenina dinucleotídeo (NAD+) é essencial para os organismos vivos, pois, participa de centenas de reações regulando os principais processos biológicos, tendo o seu metabolismo um tópico de interesse renovado nos últimos anos (Xie et al. 2020; Yaku, Okabe, and Nakagawa 2018; Yang and Sauve 2016) devido seu papel chave em patofisiologias, principalmente as relacionadas ao envelhecimento. Além de seu papel vital como coenzima no metabolismo energético, transferindo hidrogênio em reações de oxidação-redução, a função de NAD+ se expandiu como um co-substrato para enzimas como CD38, poli adenosina difosfato ribose polimerase (PARPs) e sirtuínas (SIRTs), em várias vias de sinalização que medeiam essenciais processos celulares para homeostase do organismo, tais como reparo do DNA (Herceg and Wang 2001), apoptose (Pittelli et al. 2011), sobrevivência celular (Alano et al. 2010), regulação do tempo de vida (Houtkooper, Pirinen, and Auwerx 2012; Zhang et al. 2016), ajustes metabólicos (Houtkooper et al. 2012), inflamação (Cameron et al. 2019) e infecção (Fehr et al. 2020) como por SARS-COV-2 por exemplo (Heer et al. 2020; Omran and Almaliki 2020).NAD+ é constantemente

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Investigador	Projeto_comite_de_etica.docx	28/08/2023 16:56:53	GABRIELA FABIANA SOARES ALEGRE	Aceito
Outros	Atestado_Matricula.pdf	03/08/2023 12:58:03	GABRIELA FABIANA SOARES ALEGRE	Aceito
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TCLE / Termos de Assentimento / Justificativa de Ausência	Termo_de_consentimento_CEP.docx	03/08/2023 10:42:14	GABRIELA FABIANA SOARES ALEGRE	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CAMPINAS, 29 de Agosto de 2023

Assinado por:
Renata Maria dos Santos Celeghini
(Coordenador(a))

Anexo VII - Comprovante de depósito de material biológico no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado – SISGEN

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº ACB57A4

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**ACB57A4**
Usuário:	**UNICAMP**
CPF/CNPJ:	**46.068.425/0001-33**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie
Pereskia Violacea
Moringa oleifera
Moringa stenopetala
Leuenbergeria Pereskia bleo
Xanthosoma sagittifolium (L.)
Xanthosoma violaceum Schott
Cichorium intybus
Brassica oleracea var. italica
Brassica oleracea var. capitata
Capparis spinosa
Coriandrum sativum
Brassica oleracea L. acephala
Lactuca sativa

Allium ampeloprasum
Mentha crispata Mentha spicata
Allium Allium cepa
Allium schoenoprasum
Spinacia oleracea
Petroselinum crispum
Brassica oleracea var. capitata f. rubra
Beta vulgaris L.
Daucus carota
Cucumis sativus
Solanum melongena
Solanum lycopersicum
Capsicum annuum L.
Malus domestica Borkh.
Persea americana
Musa acuminata 'Dwarf Cavendish'
Hylocereus undatus
Ficus carica L.
Psidium guajava L.
Citrus sinensis
Diospyros kaki 'Fuyu'
Pyrus communis L.
Prunus salicina
Phaseolus vulgaris Cranberry Group
Cicer arietinum
Lens culinaris
Phaseolus vulgaris L.
Pisum sativum
Arachis hypogaea L
Oryza sativa
Chenopodium quinoa

Título da Atividade:	**Avaliação do teor de NMN e NR - precursores da síntese de NAD - em alimentos, e sua potencial contribuição dietética na promoção da saúde**

Equipe

GABRIELA FABIANA SOARES ALEGRE	**UNICAMP**
Glaucia Maria Pastore	**Unicamp**

Data do Cadastro:	**07/10/2023 19:14:15**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **19:17** de **07/10/2023**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - **SISGEN**