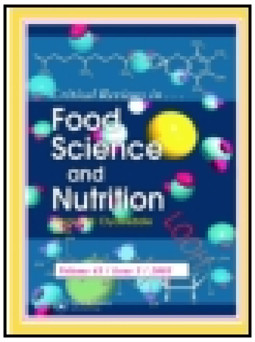

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REVIEW

A critical review of the composition and history of safe use of guayusa: a stimulant and antioxidant novel food

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ABSTRACT
Due to rapid international market development, there is a strong and urgent need to assess the safety of the novel food, *Ilex guayusa*. Guayusa has been consumed for centuries in the western Amazon as an herbal tea, and novel food regulation provide a detailed framework for safety assessment of novel foods with such a history of use. This study reviews guayusa's taxonomy, chemical composition, toxicology, ethnobotany, and history of safe use as key elements of a robust novel food safety assessment. Guayusa is a product of traditional agricultural systems with a continuous history of consumption in Ecuador. Its known chemical composition appears to present no greater risk to human health than existing teas such as green tea or yerba mate, although our understanding of guayusa's chemical profile is still nascent, requiring further investigation. Broad consumption of guayusa is not associated with a history of adverse effects or product safety notifications. Biochemical and phytochemical studies have profiled its nutritional content, metabolite composition, and bioactivity, validating guayusa's antioxidant and stimulant properties. In conclusion, guayusa leaves have a well-documented chemical composition and history of safe use, which are key considerations for authorization as a novel food in the EU.

KEYWORDS
caffeine; *Ilex guayusa*; food safety; food science; traditional food; chemical composition

Introduction

There is strong and growing international interest in herbal teas and their health-promoting properties. Commercially, this interest is driving a 4.5% compound annual growth rate in the global herbal tea market, forecast for the period 2018–2024 (Infinium Global Research 2018). In Europe, consumer interest in herbal teas is also reflected in the diversity of plants that are consumed, with 412 plants or plant parts listed as food herbals (Tea and Herbal Infusions Europe 2014). Internationalization of consumer food preferences and lower barriers to international trade have increased our knowledge of, and access to botanicals as foods. As such, there are a growing number of novel botanicals available which have a history of use as herbal teas in previously inaccessible parts of the world. Guayusa (*Ilex guayusa* Loes.) is such a plant that has recently drawn international attention as an herbal tea.

Guayusa leaves are used as a herbal tea that has stimulant and antioxidant properties (Dueñas et al. 2016; Lewis, Elvin-Lewis, and Gnerre 1987; Sequeda-Castañeda et al. 2016). Like black tea and green tea (*Camellia sinensis* L.) and the lesser-known Ilex species, yerba mate (*Ilex paraguariensis* A.St.-Hil.), guayusa is viewed as having great potential as a healthy and energy-giving drink, more so because it lacks the bitter flavor of other teas (García-Ruiz et al. 2017). Originally consumed in the western Amazon, international exports of guayusa have grown to 100 tons per annum since 2007 (Wise and Santander 2018a). Although it is now consumed in many parts of the world, guayusa is considered to be a novel food under EU legislation because it was not consumed in the EU prior to 1997. For the international market development of guayusa to continue in Europe and elsewhere, it is necessary first to critically assess the safety of guayusa as a novel food.

A new EU Regulation (EU) 2015/2283 on Novel Foods (2015) came into effect in 2018, amending and repealing all prior novel food legislation. The regulation articulates new rules for novel foods that have a history of use as traditional foods in other countries. The new regulation requires characterization of the chemical composition of the food and evidence of a history of safe use. Scientific and technical guidance has been developed by the European Food Safety Authority specifically to assist importers to conform with the legislation, when placing traditional foods from third countries in the EU market (Turck et al. 2016). For novel foods of botanic origin, that guidance stipulates the need to describe:

- Taxonomic, botanic and geographic identity;
- Cultivation and post-harvest production process;
- Compositional data for main constituents, characteristic components, nutritionally relevant components, and substances of possible concern to human health;
- Storage and transport stability;

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- A history of continued use;
- Conditions for safe consumption.

The EU novel food assessment framework provides a rigorous basis for the analysis of consumer risk associated with the market entry of novel foods, in Europe and in other countries that regulate novel foods. This study uses that framework to carry out a focused literature review that delivers a critical analysis of the safety of guayusa as a novel food, and as an herbal tea traditionally consumed in the western Amazon. This study contributes to the timely pre-market risk assessment of guayusa, which is rapidly growing across international consumer markets. It is also a necessary review of the scientific literature concerning guayusa, which has rapidly increased in recent years.

Methods

A search of the public literature indexed in Elsevier Scopus and Clarivate Analytics Web of Science was conducted. All databases of the Web of Science index were searched using the 'Topic' fields back to 1950, including the SciELO Citation Index which covers indexed articles from Latin American journals. All years of the Scopus database were searched using the 'Title Abstract Keywords' field. Search terms used were: 'guayusa' or 'Ilex guayusa'. Since guayusa is a product of Ecuador in Latin America, and some useful Spanish language (Latin American) journals can have poor coverage in the aforementioned databases, similar searches were conducted using Google Scholar. The inclusion criteria applied were: (1) studies having a focus on guayusa, (2) peer-reviewed original research papers, (3) reviews, books, book chapters, historical monographs, conference proceedings and scientific opinions of recognized food safety authorities or advisory committees and (4) Spanish or English language documents. Exclusion criteria included: (1) book reviews, news items, patents and non-peer reviewed reports (unless they were historical monographs) (2) studies that did not have a focus on guayusa. Search results were screened based on a full text review and 37 documents were identified from this initial screening process. The initial screen was followed by a review of the references cited in the retrieved papers to identify other relevant studies of guayusa. Experts in the field were contacted individually to source other documents within their sphere of knowledge. A total of 103 documents were identified for this review.

Taxonomic, geographic and botanic identity

Ilex guayusa Loes. is the accepted scientific name for guayusa, which has only one recorded homotypic or nomenclatural synonym, being *I. guayusa* var. *utilis* Moldenke (Galle 1997). Guayusa is variously named by many Ecuadorian Amazon nationalities: Huayusa or wayusa for the Kichwa; wayus, wais or weisa for the Shuar; guayusa for the Secoya; ingurinopari for the Andoa; and wayus for the Achuar (Ministerio de Cultura y Patrimonio 2016). Guayusa is a member of the Aquifoliaceae family, represented by the sole genus, *Ilex*, which is currently circumscribed within the order Aquifoliales (Stevens 2017). Guayusa is a tropical evergreen tree that grows up to 30 m and is native to the western Amazon region (Dueñas et al. 2016; Krause and Ness 2017). Its geographical distribution extends across Ecuador, Peru and Colombia and there is one historical record of guayusa being identified with archeological remains from highland Bolivia (Schultes 1972). However, the full extent of its modern distribution is uncertain. Guayusa is known to be a cultivated plant that is associated with human societies (Schultes 1979; Spruce and Wallace 1908), reputedly having lost its sexual reproduction capacity through generations of vegetative propagation by Amazon indigenous growers (Sequeda-Castañeda et al. 2016). As a member of the *Ilex* genus, guayusa is one of more than 500 species of holly (Galle 1997). It is a relative of yerba mate (Spruce and Wallace 1908), which is the commercially dominant herbal tea in South America (Linck, de Sá, and Elisabetsky 2014). Guayusa is one of few known caffeine-containing holly trees (Alikaridis 1987). Its leaves are ovate, elliptic, oblong or lanceolate; 7-22 cm long, 2.5-7 cm wide; with a serrate or dentate margin (Loesener 1901; Loizeau 1994). There are no formally recognized subspecies, cultivars, or different botanical varieties of guayusa. However, there is one anecdotal report of guayusa plants occasionally exhibiting markedly higher caffeine concentrations that have been confirmed phytochemically (Lewis et al. 1991), which may indicate the need for further examination of intraspecific variation. Guayusa tea is made only from the fresh or dried leaves of *Ilex guayusa*, containing little or no stem material (Krause and Ness 2017). This differs from yerba mate tea that can sometimes include substantial quantities of stem material resulting from a different harvest process (Heck and de Mejia 2007).

Cultivation and processing

Guayusa is predominantly a product of indigenous agroforestry systems. Indigenous farming collectives of the western Amazon region typically employ low-yield agricultural practices, cultivating guayusa without the use of fertilizers, pesticides or heavy machinery (Dueñas et al. 2016; Garrido-Pérez et al. 2018, Jarrett, Cummins, and Logan-Hines 2017; Krause and Ness 2017). Both of Ecuador's largest exporters of guayusa source fresh guayusa leaves predominantly from indigenous farming collectives, and as such, most guayusa exported from Ecuador is grown using organic practices. As a product of polyculture, guayusa is typically grown alongside other commercially useful species. However, if demand for guayusa grows then there will be increasing risk that producers will adopt higher yield forms of agriculture, which are less compatible with biodiversity conservation (Tscharntke et al. 2008) and climate change mitigation (Montagnini and Nair 2004; Ramachandran Nair et al. 2010).

Farmers reproduce guayusa by vegetative propagation beginning with the transplantation of small plant cuttings into preferably loamy soil, ideally having a pH of 4.34 - 5.01 (Sequeda-Castañeda et al. 2016). Ambient light is controlled during early growth and mechanical weed control is used when necessary throughout the life cycle of the plant. Irrigation, artificial light, fertilizers and chemical pest management are not typically used for guayusa agriculture in the western Amazon (Krause and Ness 2017). Guayusa plants reach sufficient maturity to be harvested after three years of growth. Then guayusa leaves can be harvested by manually picking leaves two times per year on an on-going basis (D. Santander, personal communication, September 27, 2018). Leaf regrowth rates are fast due to the high rainfall, high temperature and constant light cycle of the Amazon. This harvest method results in a final product that is almost entirely composed of leaf material, like *Camellia sinensis* tea. The key production steps for guayusa tea processing closely reflect that of *Camellia sinensis* green tea, those steps involve:

- Cleaning to remove potential superficial agricultural contaminants.
- A short resting period to wither the leaves, as a subtle form of fermentation.
- Oven drying to dehydrate the leaves.
- Mechanical maceration to create a course granular material.

The duration of the resting period of botanicals determines their degree of fermentation. The short resting period of guayusa is similar to that of green tea, rather than the partial or complete fermentation that black tea or yerba mate undergo. This brief resting period limits the risk of microbial contamination during processing. Furthermore, traditional guayusa is not roasted or smoked as is sometimes the case for the production of yerba mate (Heck and de Mejia 2007). This simplified processing of dried guayusa leaves is relevant for consumer safety because the intense heat of roasting and smoking in yerba mate is claimed to produce polycyclic aromatic hydrocarbons (PAHs) that could potentially have negative health impacts (Kamangar et al. 2008). Lacking such intense heat during processing, guayusa is not associated with the potential health risks of PAHs. Traditionally in indigenous communities guayusa leaves are sun-dried, however this is not considered to be a viable mainstream commercial production method due to volume and time constraints for processing. Both traditionally, and for commercially-available guayusa tea products, guayusa tea is prepared as a hot water infusion. For commercially-available tea bag products, water is brought to a boil, bags containing 1.5 grams of guayusa are steeped for approximately ten minutes, the bag is removed, and the aqueous extraction is consumed. Since guayusa tea fills a market niche that is already occupied by black tea, green tea and herbal teas, it is logical to project that consumption behavior for guayusa tea in international markets, is most likely to reflect existing consumption patterns for other teas and herbal teas (Wise and Santander 2018b).

Ethnobotany

Historical ethnobotanical studies of guayusa have been published in many forms since the Jesuit priest Juan Lorenzo Lucero observed in 1683 indigenous uses of guayusa in the western Amazon (Patiño 1968). The reviews of Patiño (1968), Lewis et al. (1991), and Dueñas et al. (2016) have each reported how Jesuit priests and other early visitors to the western Amazon perceived the indigenous usage of guayusa and its claimed efficacy against various maladies including: Stomach pain; respiratory congestion; fever; venereal infection; hyperglycemia; female sterility; and dysentery. These early observations of indigenous ethnobotany are subjectively filtered through the lenses of western religious doctrine and 17th - 18th century medical understanding. However, they clearly illustrated the long-standing and broad health value that has been attributed to guayusa by many indigenous nationalities of the western Amazon.

Recent ethnobotanical studies of guayusa have provided more detailed insights into indigenous usage of guayusa, based on its stimulant and health-giving properties. Ethnobotanic studies have reported the use of guayusa in Ecuador and Peru as a blood purifier for the treatment of diabetes with potential as being orally hypoglycemic (Chianese et al. 2019; Dueñas et al. 2016; Marles and Farnsworth 1995; Sequeda-Castañeda et al. 2016). The indigenous use of guayusa as a mouth wash for oral health is widely reported (Burrows and Tyrl 2013; Gill 1940; Karsten 1935; Schultes 1972). Perhaps this usage is associated with guayusa's antibacterial activity on *Staphylococcus aureus* and *Escherichia coli* (Bussmann et al. 2010; Bussmann, Glenn, and Sharon 2010), and on the periodontal pathogens, *Porphyromonas gingivalis*, *P. intermedia*, and *Fusobacterum nucleatum* (Gamboa et al. 2018). An encyclopedia of useful plants of Ecuador lists guayusa as being used for body aches (de la Torre et al. 2008), while Iglesias (1987), Russo (1992) and McClatchey et al. (2009) specifically described its use for headaches. These studies highlight the caffeine content of guayusa as a potential mechanism of action. Fertility is another area of health for which guayusa is broadly cited as being beneficial (Albis 1936; García 1992; Patiño 1968; Serra 1956; Tene et al. 2007). Most recently Contero et al. (2015) have investigated this property, identifying that guayusa extract has estrogenic activity in rats, proposing this as a potential mechanism of action relating to fertility. Other modern studies have cursorily observed uses for guayusa in association with gastritis (Saltos et al. 2016; Tene et al. 2007), repelling insects, skin care (Dueñas et al. 2016), asthma, lactation, weight loss, sex drive, prostate and kidney function, and for good luck relating to snake bites, hunting and fishing (Sequeda-Castañeda et al. 2016). While strong evidence exists for the use of guayusa for many purposes relating to human health, many of these health claims remain largely unsubstantiated or incompletely investigated.

Irrespective of the plethora of health functions that guayusa has been associated with, its ethnobotanic use as a stimulant has gained the most attention in scientific literature. In a key historical botanic study, the renowned botanist and explorer Richard Spruce reported on his western Amazon voyages of 1849-1864, identifying the association of the guayusa plant with human habitation, and detailing its use as a stimulant tea, similar to guaraná or yerba mate (Spruce and Wallace 1908). Since that time, guayusa's use as a stimulant among Ecuadorian and Peruvian nationalities of Jivaroan origin has been reported in studies by Cooper and Steward (1949) and Schultes (1972). A number of studies have since documented the use of guayusa as a stimulant among men of the Ecuadorian Achuar nationality (Descola 1996; Lewis, Elvin-Lewis, and Gnerre 1987; Lewis et al. 1991), the Ecuadorian Shuar nationality (Bennett 1992; Bennett, Baker, and Andrade 2002), the Kichwa nationality (Innerhofer and Bernhardt 2011) and other indigenous nationalities of the western Amazon (de la Torre et al. 2008; Dueñas et al. 2016; McClatchey et al. 2009; Russo 1992; Schultes and Raffauf 1990; Sequeda-Castañeda et al. 2016).

Associated with its ritualistic consumption in the morning as a stimulant, is the often-repeated claim that guayusa is an emetic (Bennett 1992; Bennett Baker, and Andrade 2002; Galle 1997; Patiño 1968). Perhaps this claim stems from historical observations of guayusa as an emetic, see Patiño (1968) for a review. Lewis et al. (1991) have questioned this activity, identifying no emetic compounds, no emetic effect through self-administration, and substantial evidence for emesis as a learned behavior. They further propose that behavioral emesis serves as a mechanism to moderate caffeine intake.

Guayusa has a long history of use as a stimulant and a health tonic, and it is an integral dietary feature of many indigenous nationalities in the western Amazon. Its importance to indigenous cultures is indicated by the work of Saltos et al. (2016), who demonstrated among the Kichwa that guayusa is cited as a medicinal plant more often than any other plant. Innerhofer and Bernhardt (2011) further established that guayusa is planted and harvested by the Kichwa more often than any other medicinal plant. They went on to state that guayusa is "the most significant plant in the daily life of the Kichwa, indicating that it is representing their culture as a national plant" (Innerhofer and Bernhardt 2011, 434). While much work is required to establish the scientific bases for health claims attributed to guayusa, the existing ethnobotanical scientific literature establishes that guayusa has a long and ongoing history of use among many western Amazon cultures, particularly in Ecuador.

Chemical composition

Recent analytical and phytochemical investigations of guayusa have provided initial qualitative and quantitative evaluations of nutritional content and chemical composition for the species. Published research results have been summarized to review currently available analytical data for guayusa which include proximate analysis, dietary composition, free and total amino acids, caffeine content, elemental analysis, antioxidant activity, and secondary metabolite profiles (Table 1). Data compiled represent values for processed guayusa leaves.

Caffeine composition

The presence of caffeine in guayusa was proposed by Patiño (1968) in response to his ethnobotanical review of guayusa's stimulant effects. Then Holmstedt and Lindgren (1972) identified this alkaloid in both modern and ancient guayusa samples, importantly finding no other "psychotomimetic phenolic amines" that might otherwise account for guayusa's stimulant properties. Later, Lewis et al. (1991) described a caffeine concentration range of 17.3 ± 0.4 mg/g — 75.7 ± 01.2 mg/g in fresh guayusa leaves. Wide in range, these early determinations are only broadly consistent with the modern determinations of Pardau et al (2017) at 29-32 mg/g and Wise and Santander (2018c) at 19.08 ± 0.31 mg/g. Wise and Santander also reported that determination of caffeine in guayusa has become a common focus for undergraduate student projects in Ecuador, again reporting wide concentration ranges (for examples see Barriga Coronel 2017; Cobos Morales 2016; Melo Gallegos 2014). Most previous studies establish that the caffeine concentration of guayusa presents no greater risk to human health than do yerba mate or *Camellia sinensis* tea. However, the broad range of reported values is currently unexplained. If variance is not due to differing analytical methods, then variance between trees, between locations, or throughout the growing period of guayusa could be causative factors for the wide range of determined caffeine values in guayusa. Further studies are needed to elucidate existing phenotypic variation of caffeine content in *Ilex guayusa*, and to determine the rate of caffeine accumulation during the growing period of its leaves.

Proximate and dietary analysis

The proximate composition analysis of dried guayusa leaves, as reported by Wise and Santander (2018c), describes guayusa as being sufficiently dry (5.4 ± 0.7 g/100g) to mitigate the risk of post-manufacture microbial contamination (Scientific Committee for Food 1998). Guayusa is primarily composed of carbohydrate (64.1 ± 1.5 g/100g), its principle component being crude fiber (37.0 ± 1.7 g/100g), and having low energy, salt, fat, and sugar values (Wise and Santander 2018b). The proximate and dietary composition of guayusa generally reflects that reported for tea (Adnan et al. 2013; Ahmad et al. 2014; Mohammed and Sulaiman 2009; Ramdani, Chaudhry and Seal 2013), excepting that the fiber component of guayusa is nearly two times higher. Wise and Santander (2018c) surmise that the relatively lower crude fiber values of black tea and green tea may result from intense industry development activity to maximize tea quality. There is an inverse association between crude fiber content and perceived tea quality (Ozdemir, Gökalp, and Nas 1993; Werkhoven 1974), formalized by ISO 3720-2011, which calls for a crude fiber content below 16% for conformance with good production practice. These crude fiber comparisons indicate the potential for guayusa product development through improvements to guayusa cultivation and harvest practices that might minimize crude fiber content.

Amino acid analysis

The two studies that have determined the amino acid composition of guayusa have identified 17 amino acids

Table 1. Summary of results: Compiled chemical composition studies of guayusa.

Proximate composition	
Moisture content	5.4 ± 0.7 g/100 g[a]
Crude fiber	37.0 ± 1.7 g/100 g[a]
Crude lipid	8.0 ± 0.2 g/100 g[a]
Ash value	7.7 ± 0.9 g/100 g[a]
Total carbohydrate	64.1 ± 1.5 g/100 g[a]
Total protein	14.8 ± 0.3 g/100 g;[a]
Dietary composition	
Energy kcal / 100g	313.6 ± 7.8 g/100 g[a]
Energy kJ / 100g	1303.4 ± 34.2 g/100 g[a]
Saturated fatty acids	3.362 ± 0.192 g/100 g[a]
Monounsaturated fatty acids	0.806 ± 0.083 g/100 g[a]
Polyunsaturated fatty acids	3.442 ± 0.123 g/100 g[a]
Sodium	<3 mg/100 g[a]
Total sugar	4.84 ± 0.72 g/100 g[a]
Available carbohydrate	27.12 ± 2.77 g/100 g[a]
Phenolic compounds	
Total phenolic content (GAE)	106.62 ± 4.41 mg/g dw;[c] 33.44 ± 0.48 mg/g dw;[d] 54.0 ± 3.8 mg/g;[e] Determined in extract[f]
Caffeoyl derivatives	
Caffeoylquinic acid	Detected[g]
Neochlorogenic acid	11.30 ± 1.06 mg/g dw;[c] 4.10 ± 0.29 mg/g dw[d]
Chlorogenic acid	26.53 ± 0.84 mg/g dw;[c] 7.63 ± 0.54 mg/g dw[d]
Caffeic acid glucoside	Detected[g]
Coumaroylquinic acid	Detected[g]
Caffeoyl-hexose	5.17 ± 0.17 mg/g dw;[c] 0.13 ± 0.01 mg/g dw[d]
Isochlorogenic acid	23.34 ± 0.07 mg/g dw;[c] 7.12 ± 0.48 mg/g dw[d]
Dicaffeoylquinic acid	Detected[g]
3,4-Dicaffeoylquinic acid	4.74 ± 0.32 mg/g dw;[c] 1.12 ± 0.09 mg/g dw[d]
Hydroxycinnamoylquinic isomers	
Feruloylquinic acid	Detected[g]
3-Feruloylquinic acid	0.58 ± 0.02 mg/g dw;[c] 0.08 ± 0.00 mg/g dw[d]
Flavonoids	
Quercetin rutinoside	Detected[g]
Rutin	0.96 ± 0.04 mg/g dw;[c] 0.15 ± 0.00 mg/g dw[d]
Quercetin glycoside	Detected[g]
Quercetin-3-*O*-hexose	5.96 ± 0.18 mg/g dw;[c] 1.45 ± 0.08 mg/g dw[d]
Kaempferol rhamnoside	Detected[g]
Kaempferol glycoside	Detected[g]
Kaempferol-3-*O*-glucoside	1.56 ± 0.12 mg/g dw[c]
Kaempferol-3-*O*-hexose	0.29 ± 0.01 mg/g dw[d]
Terpenoid compounds	
Triterpenoids	
Oleanolic acid	1.18 mg/g dw[h]
Ursolic acid	18.22 mg/g dw;[h] ca. 1% yield[l]
Amyrin esters	ca. 0.1% yield[l]
Uvaol	ca. 0.03% yield[l]
Uvaol aldehyde	ca. 0.01% yield[l]
Carotenoids	
Total carotenoids	468.71 ± 110.49 μg/g dw;[c] 44.00 ± 8.88 μg/g dw[d]
Xanthophylls	
Lutein	266.33 ± 65.38 μg/g dw;[c] 25.72 ± 6.46 μg/g dw[d]
Neoxanthin	1.79 ± 0.59 μg/g dw[d]
Violaxanthin	3.92 ± 0.78 μg/g dw[d]
Violaxanthin + neoxanthin	24.27 ± 2.06 μg/g dw[c]
Carotenes	
α-Carotene	62.52 ± 15.59 μg/g dw;[c] 7.76 ± 1.34 μg/g dw[d]
cis-α-Carotene	2.01 ± 0.76 μg/g dw[d]
β-Carotene	115.59 ± 27.46 μg/g dw;[c] 4.06 ± 1.06 μg/g dw[d]
cis-β-Carotene	0.55 ± 0.13 μg/g dw[d]
Methylxanthines	
Caffeine	19.08 ± 0.31 mg/g;[a] 2.9 − 3.2%;[g] 1.73% ± 0.04 − 7.57% ± 0.12;[i] 0.1% −1.8%[j]
Theobromine	0.02 ± 0.004 − 0.12 ± 0 %;[i]
Carbohydrates	
Fructose	14033.8 μg/g[k]
Glucose	13206.6 μg/g[k]
Mannose	0.0 μg/g[k]
Sucrose	0.6 μg/g[k]
Maltose	39.3 μg/g[k]
Xylose	0.0 μg/g[k]
Sorbitol	248.2 μg/g[k]
Myo-inositol	9833.6 μg/g[k]
Chiro-inositol	0.0 μg/g[k]
Scyllo-inositol	0.0 μg/g[k]

(*continued*)

Free amino acids	
Aspartic acid	83.8 ± 8.3 μg/g;[a] 53.3 μg/g[b]
Serine	31.4 ± 20.4 μg/g;[a] 10.7 μg/g[b]
Glutamic acid	105.3 ± 46.7 μg/g;[a] 50.1 μg/g[b]
Glycine	<LOD;[a] 10.0 μg/g[b]
Histidine	6.2 ± 1.2 μg/g;[a] 12.9 μg/g[b]
Arginine	<LOD;[a] 42.9 μg/g[b]
Threonine	5.5 ± 0.5 μg/g;[a] 13.6 μg/g[b]
Alanine	20.8 ± 1.5 μg/g;[a] 106.9 μg/g[b]
Proline	10.2 ± 2.1 μg/g;[a] 25.3 μg/g[b]
Cystine	<LOD[a]
Tyrosine	<LOD;[a] 12.9 μg/g[b]
Valine	<LOD;[a] 17.4 μg/g[b]
Methionine	<LOD;[a] 5.2 μg/g[b]
Lysine	<LOD;[a] 9.2 μg/g[b]
Isoleucine	<LOD;[a] 13.2 μg/g[b]
Leucine	<LOD;[a] 12.5 μg/g[b]
Phenylalanine	<LOD;[a] 11.0 μg/g[b]
Asparagine	279.5 μg/g[b]
Glutamine	50.2 μg/g[b]
Tryptophan	79.4 μg/g[b]
Total amino acids	
Aspartic acid	1.09 ± 0.01 mg/g[a]
Serine	0.54 ± 0.01 mg/g[a]
Glutamic acid	1.21 ± 0.03 mg/g[a]
Glycine	0.53 ± 0.02 mg/g[a]
Histidine	0.22 ± 0.01 mg/g[a]
Arginine	0.56 ± 0.02 mg/g[a]
Threonine	0.51 ± 0.02 mg/g[a]
Alanine	0.60 ± 0.02 mg/g[a]
Proline	0.53 ± 0.01 mg/g[a]
Cystine	0.10 ± 0.01 mg/g[a]
Tyrosine	0.38 ± 0.01 mg/g[a]
Valine	0.60 ± 0.02 mg/g[a]
Methionine	0.21 ± 0.01 mg/g[a]
Lysine	0.64 ± 0.01 mg/g[a]
Isoleucine	0.48 ± 0.02 mg/g[a]
Leucine	0.86 ± 0.03 mg/g[a]
Phenylalanine	0.55 ± 0.02 mg/g[a]
Other compounds not detected[f]	
Apigenin, β-sitosterol, campesterol, cholesterol, cyanidins, delphinidins, genistein, hesperidin, kuromanin, l-theanine, luteolin, malvidins, naringenin, ononin, peonidins, petunidins, pterostilbene, puerarin, resveratrol, sissotrin, stigmastanol	

[a]Wise and Santander (2018b).
[b]Moldoveanu, Zhu and Qian (2015).
[c]García-Ruiz et al. (2017).
[d]Villacís-Chiriboga et al. (2018).
[e]Jara et al. (2013).
[f]Kapp et al. (2016).
[g]Pardau et al. (2017).
[h]Moldoveanu and Scott (2016).
[i]Lewis et al. (1991).
[j]Holmstedt and Lindgren (1972).
[k]Moldoveanu, Scott and Zhu (2015).
[l]Chianese et al. (2019).
GAE: mg gallic acid equivalents g^{-1} dry weight; LOD: Limit of detection; dw: dry weight.

(Moldoveanu, Zhu, and Qian 2015; Wise and Santander 2018c). Comparison of the amino acid profiles of yerba mate and green tea reveal broad similarity between the two species, having relatively high asparagine, aspartic acid, glutamic acid and arginine values. Guayusa differs from that profile in having relatively higher values for alanine, glutamine, and tryptophan, but not for glutamic acid and aspartic acid.

We are unaware of any published scientific studies that have identified the amino acid L-theanine in guayusa. That is noteworthy because producers, vendors, and other proponents of guayusa widely report that it contains theanine, imparting a mellow and long-lasting caffeine stimulant effect, rather than the more acute effect of caffeine in coffee. L-theanine, or γ-ethylamino-L-glutamic acid is an amino acid that is reported in scientific literature as being only produced in some species of the *Camellia* genus and the mushroom *Xerocomus badius* (Casimir, Jadot, and Renard 1960; Juneja et al. 1999). It is actively transported across the blood-brain barrier, influencing the levels of other amino acids (Kitaoka et al. 1996; Yokogoshi et al. 1998), decreasing dopamine release, and decreasing synthesis and release of norepinephrine and serotonin (Kimura and Murata 1986; Yokogoshi, Mochizuki, and Saitoh 1998). It is these biochemical mechanisms that are said to underpin the action of L-theanine as a relaxant and a physiological counterpoint to the stimulant effect of caffeine (Bryan 2008).

We are aware of four recently published studies or reviews of L-theanine in tea or herbal teas that repeat claims that L-theanine is present in guayusa (Adhikary and Mandal, 2017; Chatterjee, Chatterjee, and Bandyopadhyay

2016a; Chatterjee, Chatterjee, and Bandyopadhyay 2016b; Kapp et al. 2016; Liang et al. 2015). However, their claims are either uncited or they incorrectly cite unrelated studies. Additionally, Sequeda-Castañeda et al. (2016) cite the results of a 2010 laboratory report of the analytical laboratory, ABC Testing. However, the study that produced those results appear to be unpublished in the scientific literature. The only peer-reviewed published study that has directly addressed the question of L-theanine content in guayusa reported no detectable level (Kapp et al. 2016). The quantification of L-theanine in guayusa is an important question that remains unclear. While this lack of clarity is unimportant for consumer safety, to understand the nutritional value of guayusa it is important to establish a scientific basis for the broad health claims regarding L-theanine that are currently advertised by guayusa producers as fact.

Elemental analysis

Only one elemental analysis of guayusa has previously been published (Wise and Santander 2018c). That study identified 16 elements including the macroelements K, P, Mg and Na, and the microelements Al, Ba, B, Cd, Cu, Fe, Mn, Mo, Ni, Sr, Ti and Zn, as has been reported for yerba mate and black tea (Giulian et al. 2007; Marcelo et al. 2014). Notable differences between guayusa and yerba mate include the additional identification of As, Be, Cr, Co, Pb, Li and V in yerba mate, whereas none of these were detectable in guayusa. Also, Fe, Mn, and Ti were found in yerba mate at levels 1.5 – 30 times higher than in guayusa. Only Mo and Sr are reported in guayusa with markedly higher values than in yerba mate (Wise and Santander 2018c). The amount of Mo in guayusa is $2.33 \pm 0.65\,\mu g/g$, which is two orders of magnitude below estimated daily intake in the USA (Tsongas et al. 1980). Also, Sr is not considered a health risk to humans and at $117 \pm 17\,\mu g/g$ in guayusa, does not exceed the estimated daily intake (Nielsen 2004; Tsongas et al. 1980). As a preliminary analysis, Wise and Santander (2018c) have established that the presently known elemental profile of guayusa appears to present no greater risk to human health than does yerba mate. However, much work remains to understand heavy metal accumulation and speciation in guayusa leaves especially with regards to aluminum, which is a major agricultural challenge in acidic western Amazon andisols (Kochian, Hoekenga, and Piñeros 2004). It is also important to address the elemental composition of herbal tea infusions made from guayusa, since many elements are poorly extractable in aqueous solution (Szymczycha-Madeja, Welna, and Pohl 2012).

The determined level of $252\,\mu g/g$ for aluminum in guayusa leaves is of particular interest since aluminum is toxic for many plants and therefore is rarely present in leaves at high levels (Jansen, Watanabe, and Smets 2002). *Camellia sinensis* is known to be one of a few aluminum-accumulating plants, having a tolerance to aluminum toxicity and possessing up to 300-1500 $\mu g/g$ of aluminum in its leaves (Flaten, 2002). The amount of aluminum reported in guayusa leaves is lower than in tea and in yerba mate (da Costa et al. 2009; Giulian et al. 2007; Marcelo et al. 2014) and therefore presents no greater risk to human health than do tea or yerba mate. However, questions regarding guayusa's aluminum tolerance, accumulation and speciation remain an important focus for future research in order to understand the physiology and potential toxicology of aluminum in guayusa under varying agricultural conditions, harvest methods, and post-harvest processing.

Antioxidant composition and bioactivity

Ethnobotanical observations of guayusa as a daily health tonic have stimulated many studies that have aimed to characterize the biochemical basis for its antioxidant properties. Jara et al. (2013) assessed the antioxidant capacity of guayusa using a β-carotene bleaching assay and a 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, also determining polyphenolic and flavonol composition. They concluded that the antioxidant capacity of guayusa sits within range of other traditional medicinal plants used in Ecuador. Pardau et al. (2017) determined the antioxidant capacity of guayusa tea using various bioassays, reporting total polyphenolic content, and determining oxygen radical absorption capacity (ORAC) between 1728 — 2019 µmolTE/g of dry mass. Generally supporting the results of Jara et al., the study of Pardau et al. concluded that guayusa was a good source of phenolic compounds having antioxidant properties. However, their measured values for antioxidant capacity indicated a lower capacity than that of *Camellia sinensis* tea. García-Ruiz et al. (2016; 2017) determined values for 14 phenolic and five carotenoid compounds in guayusa using DPPH and ORAC assays. Their results compared well with previous studies and they further concluded that the phenolic content and antioxidant capacity of guayusa are positively and directly associated. Villacís-Chiriboga et al. (2018) extended on the work of García-Ruiz et al. identifying that the phenolic properties of guayusa leaves are dependent on leaf age and therefore related to harvest time.

The state of knowledge regarding the antioxidant properties of guayusa includes the chemical compositional and functional biochemical evidence to support the empirical discovery of guayusa's antioxidant properties by indigenous peoples. However, the state of knowledge of guayusa's bioactivity lags well behind other studied species such as *Camellia sinensis* and *Ilex paraguariensis*. Studies of the physiological actions of yerba mate reviewed by Riachi and de Maria (2017) may serve as signposts for the future characterization of guayusa's antioxidant properties. Scientific attention in the future is also necessary to validate and fully characterize initial biochemical studies relating to the efficacy of guayusa as an antimicrobial (Bussmann et al. 2010; Bussmann, Glenn, and Sharon 2010; Moldoveanu and Scott 2016), an anti-inflammatory (Moldoveanu and Scott 2016; Pardau et al. 2017; Villacís-Chiriboga et al. 2018), an anti-diabetic (Bailey and Day 1989; Chianese et al. 2019; Marles and Farnsworth 1995; Swanston-Flatt et al.1989) and as an aid for fertility (Contero et al. 2015).

Secondary metabolite composition

Phytochemical analyses of guayusa have identified and quantified secondary metabolites across several broad chemical classes (Table 1). Compounds identified in guayusa samples have been assessed using various analytical methodologies typically employing liquid chromatography. Quantitation of metabolites has been reported for guayusa leaves harvested at various stages of growth (García-Ruiz et al. 2017), for leaves processed using different manufacturing methods (Villacís-Chiriboga et al. 2018), and in tea preparations. Concentrations of carotenoids and phenolics have been reported for green leaves, blanched guayusa, and fermented guayusa (García-Ruiz et al. 2017), and these metabolites have also been compared between young (2 months) and mature (6 months) leaves (Villacís-Chiriboga et al. 2018). Both García-Ruiz et al. (2017) and Villacís-Chiriboga et al. (2018) identified and quantified carotenoids by high performance liquid chromatography tandem to photodiode array detection (HPLC-PDA) using available reference standards. Tentative identification of phenolics was achieved using HPLC-PDA tandem to mass spectrometry detection (HPLC-PDA-MS). Both studies quantified hydroxycinnamic acids and flavonols using chlorogenic acid (5-caffeoylquinic acid) and rutin (quercetin 3-rutinoside) equivalents, respectively. Pardau et al. (2017) analyzed fermented and unfermented guayusa tea using UPLC-PDA-MS, reporting tentative identifications of metabolites based on mass spectral fragmentation patterns and a review of research literature. Though the phenolic compounds cited from these studies are tentative identifications for guayusa, these metabolites and their isomers have been previously identified in samples of *I. paraguariensis* and other *Ilex* species (Dugo et al. 2009; Jaiswal et al. 2010). Identification and quantitation of the triterpenoids, oleanolic and ursolic acids, was achieved using LC-MS/MS and commercially-available reference standards (Moldoveanu and Scott 2016). Their study determined that triterpenoid content in guayusa mostly consists of ursolic acid, with significantly lower content of oleanolic acid. Recent isolation studies of guayusa have confirmed the presence of ursolic acid and the amyrin esters, a-amyrin palmitate, palmitoleate, and their b-series isomers using NMR spectroscopy (Chianese et al. 2019). Kapp et al. (2016) reported the quantitation of methylxanthines and various phenolics in concentrated guayusa tea extractions. Their study was not able to detect various cited metabolites profiled across several classes of compounds. Nevertheless, as their analytical methods are not referenced it is difficult to critically evaluate their data. Further studies are needed to assess quantitative and qualitative variation in metabolite composition for wild and cultivated guayusa.

Toxicology

A limited number of *in-vitro* and *in-vivo* toxicological studies of guayusa have been undertaken using invertebrate and mammalian animal models, and humans. Guayusa was found to have no genotoxicity using an Ames test and a chromosome aberration study in human lymphocytes (Kapp et al. 2016). The same authors determined the lethal dose (LD_{50}) of guayusa to be >5,000 mg/kg in female rats. In that study, the toxicity of guayusa was so low that a definitive LD_{50} could not be established, practical limitations for dosing food with a very high concentration of guayusa extract were reached first. In brine shrimp, the lethal concentration (LC_{50}) for an aqueous extract of guayusa was assessed to be >10,000 µg/mL (Bussmann et al. 2011). In a 90-day subchronic study of male and female rats, guayusa concentrate was administered at 1,200, 2,500 and 5,000 mg/kg/d, and compared with a corresponding caffeine-positive control. Effects observed regarding body weight, food efficiency, triglyceride values, fat pad weights and blood chemistry were comparable between groups, with no additional adverse effects reported in the guayusa concentrate group (Kapp et al. 2016). In humans, a double-blind crossover randomized clinical trial of 12 adult males compared the systemic and central nervous system effects of guayusa extract and synthetic caffeine, each dose containing 200 mg of caffeine (Krieger et al. 2016). No adverse effects were reported, with the effect of guayusa extract on heart rate, blood pressure, and epinephrine no greater than the synthetic caffeine control.

Agricultural and microbial contaminants

Currently, guayusa is primarily a product of organic agriculture, presenting minimal risk of contamination by agricultural pesticide residues. One study has conducted an LC-MS/MS and GC/MS multi-residue screen covering 389 pesticides, detecting no residues (Wise and Santander 2018b). While that result is reflective of current organic agricultural practices for the production of dried guayusa, if higher-yield agricultural methods using pesticides are adopted in the future, the risk of contamination by pesticide residues will increase. Dried guayusa leaves have been reported not to be associated with *Fusarium*-associated trichothecene mycotoxins, (Wise and Santander 2018b). While testing negative for that grouping of mycotoxins, there is insufficient horticultural knowledge regarding fungal diseases of guayusa and their concomitant mycotoxin threats. Regarding other forms of microbial contamination, dried guayusa leaves processed using standard commercial methods have tested negative to a range of broad bacteriological screens including assays of aerobic colonies; Enterobacteriaceae (presumptive coliforms); coagulase-positive Staphylococci; presumptive *Bacillus cereus*; *Salmonella* spp. serovars; osmophilic yeasts and xerophilic molds; and β-glucuronidase-positive *Escherichia coli* (Wise and Santander 2018b). It is possible that the low moisture content of dried leaves and the inherent antibacterial properties of guayusa provide protection against postharvest bacteriological contamination (Scientific Committee for Food 1998).

History of safe use

Food that has been consumed for many years is often assumed to be safe, owing to a history of use that is not associated with disease or ill-health. Foods that have never been consumed before, such as those derived from genetically modified organisms or those including synthetic food additives come under greater scrutiny since it is necessary to first establish safety in the absence of a history of use. Foods that are considered to be novel within one market but have a history of use outside of that market present a particular challenge for establishing their safety. Pre-market risk assessment is necessary to ensure that novel foods present no risk to the new market, however if a history of safe use can be established in a preexisting market, many scholars recommend that further safety assessment using clinical studies is unnecessary (Bast et al. 2002; Blaauboer et al. 2016; Coppens et al. 2006; Fabiansson 2013; Howlett et al. 2003; Knudsen et al. 2008).

There are a number of studies that anecdotally report on the extensive modern consumption of guayusa in Ecuador and more recently through export to the USA (Dueñas et al. 2016; Jarrett, Cummins, and Logan-Hines 2017; Krause and Ness 2017; Radice and Vidari 2007; Sidali, Morocho, and Garrido-Pérez 2016). This history of use among broad consumer groups, apparently without reports of associated disease is suggestive of guayusa's acceptance as a safe food in its established markets. One study has scientifically addressed the question of guayusa's safe consumption in Ecuador, analyzing provincial hospital admissions data, national disease register data, national toxicology call center data and national food safety authority data (Wise and Santander 2018a). That study revealed no hospital presentations, no product safety notifications, and no disease register records of guayusa-related illness. Furthermore, the study identified only one call center report of a mild self-resolving adverse effect, namely hyperactivity and insomnia after guayusa consumption. These data acquired over a three-year period, considering a national population of 14.5 million people, strongly support the commonly-held belief that guayusa has a history of safe use in its largest preexisting market. The history of safe use of guayusa as a food supplement is less clear, due to the fact that guayusa-fortified foods have only recently appeared in modern consumer markets, and there is little ethnobotanical evidence for the historical use of guayusa as a food supplement.

Conclusions

The current state of scientific knowledge regarding guayusa is now mature enough to draw preliminary conclusions regarding its safety as a novel food. While our understanding of the chemical profile of guayusa still lags behind comparator foods, the currently known chemical composition of guayusa appears to present no greater risk to consumers than do *Camellia sinensis* or yerba mate. The published chemical composition studies of guayusa present compositional data across each of the areas highlighted by an EU guidance study as being necessary for safety assessment (Turck et al., 2016). However, there is still much that remains to be clarified. Further research is needed to develop our understanding of guayusa's secondary metabolite profile and potential bioactivity in humans. Additional work is necessary to further elucidate the elemental composition of guayusa, in particular its accumulation of metals and heavy metals across different soils and growing conditions. Further work would also help to clarify agricultural and post-harvest processing factors affecting the caffeine content of guayusa.

Knowledge regarding the history of safe use of guayusa has now progressed past a reiteration of differing observational accounts of indigenous usage. There is now detailed knowledge about the historical and modern consumption patterns of guayusa tea in Ecuador. Through use of market-based data and the adaptation of novel food post-market surveillance methods, there is now a concomitant understanding that this history of use in Ecuador is not associated with significant adverse effects, nor is there any evidence of association with disease or illness. Such conclusions are important for the market placement of guayusa as a novel food in Europe or elsewhere.

This critical review identifies knowledge gaps surrounding the chemical composition of *Ilex guayusa*, revealing inconsistencies and limitations to our current understanding of this natural alternative to conventional sources of caffeine. In particular, we highlight the wide range of values that have been quantified for the chemical constituents of guayusa using various extraction and separation methodologies. There is a need for additional studies to firmly clarify the relevance of analytical methodologies either for revealing the *in-situ* chemical composition of *Ilex guayusa*, or for characterizing commercial products derived from this plant. In doing so, future studies can develop our scientific understanding of the *Ilex guayusa* plant and further elucidate the safety and nutritional value of guayusa tea, assisting its development as an antioxidant and stimulant novel food with a history of safe use.

Disclosure statement

The authors have no conflicts of interest to declare

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