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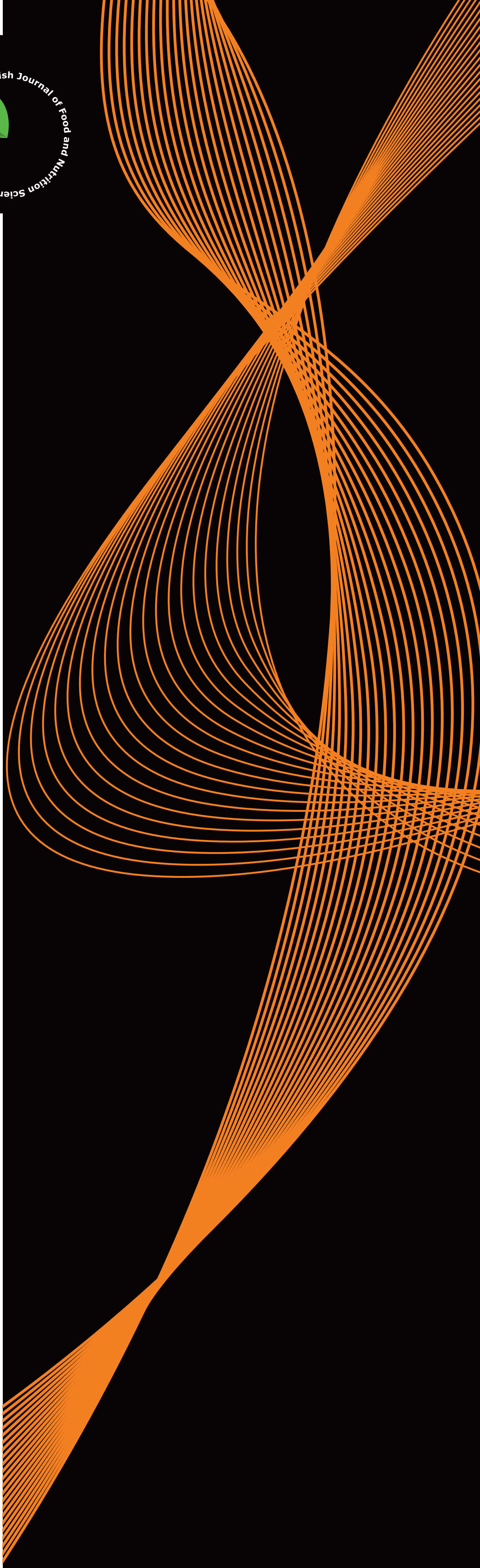

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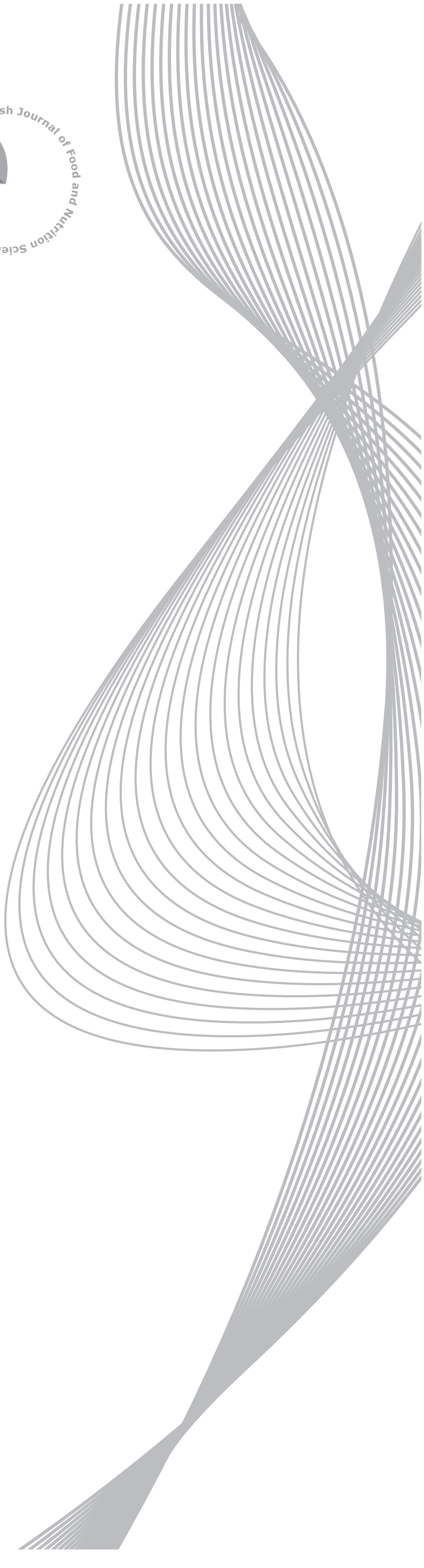

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REVIEW

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Ministerstwo Nauki
i Szkolnictwa Wyższego

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W latach 2019–2020 kwartalnik naukowy ***Polish Journal of Food and Nutrition Sciences*** realizuje zadania z zakresu digitalizacji i upowszechniania publikacji naukowych, które ukazują się na jego łamach oraz utrzymania procedur zapewniających ich oryginalność, w ramach zadania nr 1 pt. ***Digitalizacja artykułów publikowanych w kwartalniku PJFNS w celu zapewnienia otwartego dostępu do nich przez sieć Internet*** oraz zadania nr 2 pt. ***Utrzymanie procedur zabezpieczających oryginalność artykułów naukowych publikowanych w kwartalniku PJFNS***. Oba zadania finansowane są na podstawie umowy nr 607/P-DUN/2019 ze środków Ministra Nauki i Szkolnictwa Wyższego przeznaczonych na działalność upowszechniającą naukę.

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Review article
Nutritional Research Section

Coffee and its Biologically Active Components: Is There a Connection to Breast, Endometrial, and Ovarian Cancer? – a Review

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Key words: coffee, caffeine, breast cancer, ovarian cancer, endometrial cancer

Coffee is an important dietary source of biologically active components, not to mention caffeine, phenolic acids, and diterpenes. It has been suggested that selected coffee secondary metabolites may beneficially modulate several mechanisms of anti-cancer protection. This literature review was intended to present current knowledge related to coffee and its components and hormone-dependent female cancers, such as breast, endometrial and ovarian cancer, and to identify gaps in research that may be exploited in the future. The search for studies was conducted through electronic databases. Publications on coffee composition, coffee preparation and brewing methods, *in vitro* and *in vivo* experiments with the use of substances naturally present in coffee, observational studies, and meta-analyses were collected. In population studies, the greatest attention has been paid to the anticancer effect of caffeinated coffee and/or caffeine. In general, most studies and meta-analyses indicated that there was no clear correlation between coffee and breast cancer or ovarian cancer. Some subgroups of women may benefit from coffee consumption. This is the case for post-menopausal women with regard to the risk of breast cancer and obese women with regard to the risk of endometrial cancer. This paper identifies a number of issues for future research, related to a better understanding of the anti-cancer mechanisms of coffee compounds and further research that would focus on specific target groups, taking into account both the different methods of coffee preparation and lifestyle factors that may influence the results.

INTRODUCTION

Coffee is one of the most popular beverages in the world because of its taste, aroma and stimulating properties, but the perception of coffee as a potentially health-promoting component of the diet is not very high in the global population [Samoggia & Riedel, 2019]. Except some cases, like pregnancy or sensitivity to some coffee compounds, coffee can offer many beneficial health effects [Grosso *et al*., 2016, 2017a; Poole *et al*., 2017; Wierzejska, 2016]. Interestingly, a recent exploratory study has shown that coffee consumption may modulate the expression of 297 genes in healthy women in different ways [Barnung *et al*., 2018], thus affecting metabolic and inflammatory pathways. Several lines of evidence have linked coffee consumption to a reduced risk of cardiovascular diseases [Poole *et al*., 2017; Rodríguez-Artalejo & López-García, 2018], cancer [Gapstur *et al*., 2017; Grosso *et al*., 2017a], neurodegenerative diseases [Liu *et al*., 2016; Qi & Li, 2014], and diabetes [Ding *et al*., 2014], as well as to a lower cancer mortality and all-cause mortality [Grosso *et al*., 2017b; Gunter *et al*., 2017; Poole *et al*., 2017]. On a molecular basis, regular coffee consumption preserves the integrity of DNA by decreasing spontaneous DNA strand breaks [Bakuradze *et al*., 2015].

Cancers in women are a serious global health problem and a leading cause of death. They are responsible for a quarter of the deaths of women in Europe [European Commission, 2019] and a fifth in the USA [Centers for Disease Control and Prevention, 2019]. In sex hormone-dependent cancers, abnormal estrogen and progesterone levels are among the risk factors [Brown & Hankinson, 2015; Diep *et al*., 2015]. With 24.2% incidence, breast cancer cases are the most common in women [Bray *et al*., 2018]. In the global incidence of female cancers, endometrial (4.4%) and ovarian cancers (3.4%) rank sixth and eighth, respectively [Bray *et al*., 2018].

Coffee is a source of biologically active compounds, many of which have anti-cancer properties. However, observational studies with humans, which focus mainly on coffee consumption (caffeinated or decaffeinated) and caffeine intake, show rather conflicting results for breast, uterine, and ovarian cancer. This literature review aims to present current research related to coffee and its components and hormone-dependent female cancers, such as breast, endometrial, and ovarian cancer, and to identify gaps in research that may be exploited in the future.

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SEARCH STRATEGY

From the point of view of current knowledge, the search for studies from the last decade, *i.e.* from 2010 to 2019, was conducted through the electronic databases: Medline and Web of Science. Publications on coffee composition, coffee preparation and brewing methods, *in vitro* and *in vivo* experiments with the use of substances naturally present in coffee, observational studies, and meta-analyses were collected. In some cases, with the exception of case-control and epidemiological studies, studies that were important for this review, but outside the search period, were described as well. The search terms were multiple with “coffee” as a broad search term, and in combination with “breast cancer”, “ovarian cancer”, and “endometrial cancer” as the most critical for this review. The articles published in English that were relevant for this review were selected.

BIOACTIVE COMPOUNDS OF COFFEE

Roasted coffee beans are composed of several substances derived from different chemical groups, not to mention caffeine, phenolic acids, and diterpenes. Their contents in a coffee brew depend, to a large extent, on the type of coffee bean (*Coffea arabica* vs. *Coffea canephora* var. *robusta*), roasting parameters, as well as the methods of brewing. A coffee brew contains substances that are only specific to coffee, such as diterpenes – cafestol and kahweol [Gross *et al*., 1997; Zhang *et al*., 2012]. Others, such as caffeine, can be found in tea, cola-type beverages, and chocolate.

Caffeine

Caffeine is a heat-stable methylxanthine alkaloid (Figure 1) present mainly in coffee, tea, guarana, cola-type soft drinks, cacao, and chocolate. It is very soluble in boiling water. Depending on the type of coffee preparation (ground, instant) or brewing methods (boiling, filtering, French pressing,), coffee may contain 19–803 mg caffeine per serving (Table 1). Decaffeinated coffee, in turn, presents considerably reduced caffeine content <9 mg/serving.

Several beneficial or adverse biological effects are attributable to caffeine. As an adenosine receptor antagonist, it is a central nervous system stimulant and anti-inflammatory agent [Madeira *et al*., 2017]. *In vitro* studies have demonstrated cell cycle modulating and apoptotic properties of caffeine, mediated through various mechanisms, that can lead to tumor suppression [He *et al*., 2003; Ito *et al*., 2003; Saiki *et al*., 2011]. Caffeine also exhibits antiproliferative activity in ovarian cancer cells, even several times higher than that of chlorogenic acid and caffeic acid [Tai *et al*., 2010]. Besides, it may possibly alter circulating levels of luteal estrogens and sex hormone-binding globulin (SHBG), and thereby affect the development of cancers [Kotsopoulos *et al*., 2009]. Variants in cytochrome P-450 (CYP) genes involved in caffeine metabolism may increase or decrease the risk of ovarian cancer development [Kotsopoulos *et al*., 2009]. It has been

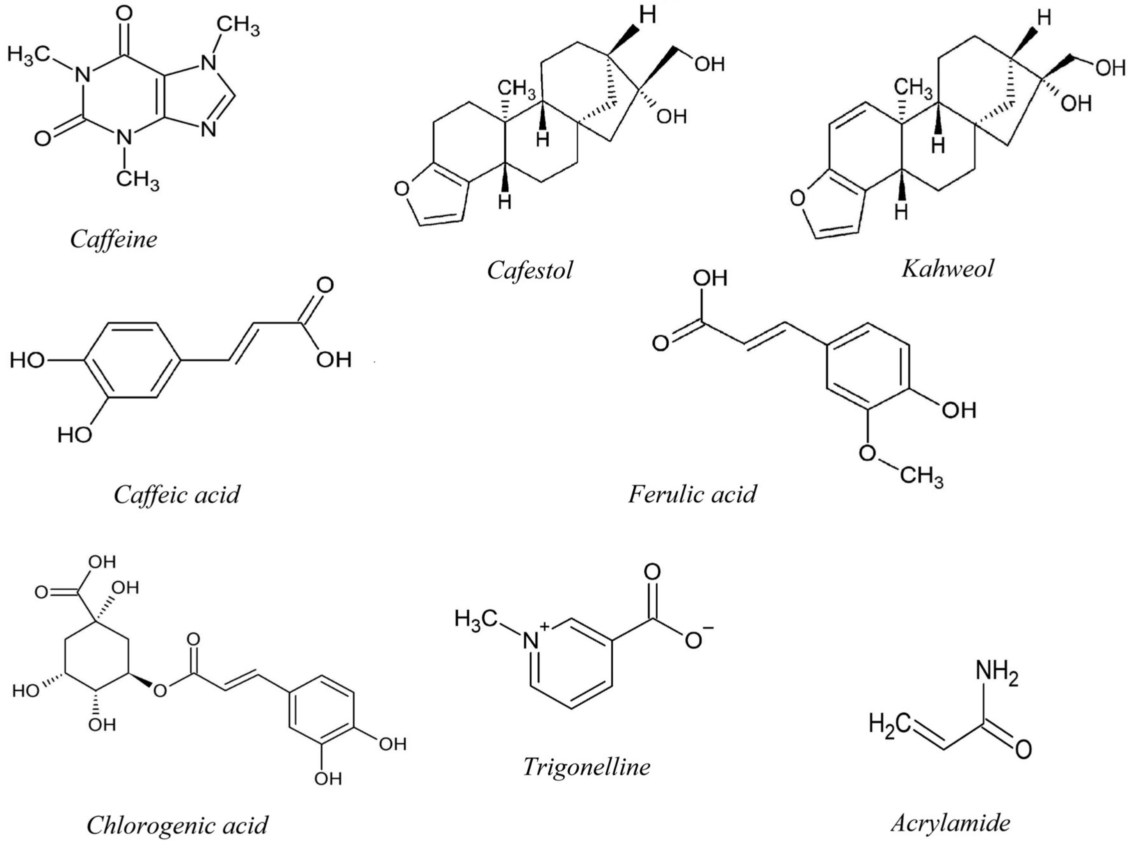
Caffeine
Cafestol
Kahweol
Caffeic acid
Ferulic acid
Chlorogenic acid
Trigonelline
Acrylamide

FIGURE 1. Chemical structure of major coffee compounds.

TABLE 1. Caffeine contents in varied coffee infusions*.

Coffee infusion type	Caffeine content (mg/L)	Caffeine content (mg per serving**)
Boiled/ Turkish	850–2330	201–552
Decaffeinated	1–37	0.24–8.77
Espresso	722–5334	19–144
Filtered	397–2008	81–416
French press	520–1564	123–371
Instant	390–2690	92–638
Mocha	924–2268	327–803

*adopted from: [dePaula & Farah, 2019]; **serving size for coffee: boiled/ Turkish, French press, instant, decaffeinated, 237 mL; espresso 27 mL; filtered 207 mL; mocha 354 mL.

suggested that caffeine from coffee may protect against endometrial cancer [Hashibe *et al*., 2015].

Coffee diterpenes

Diterpenes are organic compounds that consist of four isoprene units. In coffee, their main representatives include cafestol and kahweol (Figure 1), which are specific to the lipid fraction of coffee. In filtered coffees, this lipid fraction is absorbed by the cellulose paper filter [Gross *et al*., 1997]. Therefore, the contents of cafestol and kahweol in filtered coffee are negligible. Higher levels of diterpenes have been found in *Coffea arabica* than in *C. robusta* [Mensink *et al*., 1995]. The content of coffee diterpenes varies between 0.02–9 mg cafestol per serving and 0.02–7.2 mg kahweol per serving, depending on the coffee preparation procedure (Table 2).

Coffee diterpenes cause controversy because they may increase the levels of serum total cholesterol and LDL cholesterol fraction [Mensink *et al*., 1995]. Cafestol and kahweol demonstrate several biologically beneficial properties and are known as inducers of glutathione *S*-transferase, the enzyme which catalyzes detoxication reaction *via* conjugation of xenobiotics with the sulfhydryl group of glutathione [Huber & Parzefall, 2005]. Consequently, conjugates are eliminated. Several lines of evidence connect coffee-specific diterpenes with anticancer properties. In male F344 rats, kahweol and cafestol (1:1) or only cafestol caused an increase in the level of hepatic O(6)-methylguanine-DNA methyltransferase (MGMT), an enzyme which is involved in the reversal of precarcinogenic damage of O(6)-alkylguanine, a DNA adduct formed by alkylating agents in a dose-dependent manner [Huber *et al*., 2003]. Anticancerogenic activity of coffee diterpenes was observed in several cancer cell lines. Most of the papers on female cancers in conjunction with coffee diterpenes have so far been limited to mammary tumors. Antitumor activity of kahweol was tested in a few cancer cell lines. It has been shown to be manifested in both inhibited tumor cell growth and clonogenicity, and a decreased survival of cancer cells [Cárdenas *et al*., 2014]. These effects were mostly pronounced in estrogen-negative human breast cancer cells MDA-MB231, and were accompanied by the activation of caspases 3/7 and 9, and the release of cytochrome c. Kahweol increased the production of reactive oxygen species (ROS) and their cytotoxicity against breast cancer cells, but it did not affect the healthy cells [Cárdenas *et al*., 2014]. Other anticancer properties of kahweol are associated with its anti-angiogenic and anti-inflammatory characteristics [Cárdenas *et al*., 2011].

Cafestol has recently been hypothesized as a preventive compound in the pathology of diabetes [Mellbye *et al*., 2015]. In recent years, the link between diabetes and cancer has become of particular interest. With regard to the results of the research published in the last few years, diabetes can be thought of as a risk factor for certain types of cancer, including endometrial cancer. In an *in vitro* study, cafestol acutely stimulated insulin secretion from β-cells and improved insulin sensitivity in skeletal muscle cells.

Phytoestrogens

Phytoestrogens (plant estrogens) are compounds with a structural similarity to estradiol (E2), the primary female sex hormone. By binding to estrogen receptors (ERs), phytoestrogens can induce different biological activities asso-

TABLE 2. Diterpene contents in various coffee infusions*.

Coffee infusion type	Cafestol		Kahweol	
	(mg/L)	(mg per serving**)	(mg/L)	mg per serving**
Boiled	26–49	4–8	48.0+2.5	7.2
Espresso	22–30	1.0	16.3–17.1	1.0
Filtered	0.12+0.02	0.02	0.14+0.03	0.02
French press	29–53	5–9	–	–
Instant	0.7–1.9	0.1–0.3	0.7–1.9	0.1–0.3
Mocha	19–33	1–2	38.5+0.9	2.3
Turkish	23–41	1.4–2.5	89.9+4.1	5.4

*adopted from: [Zhang *et al*., 2012; Buchmann *et al*., 2011; Gross *et al*., 1997];**serving size for coffee: boiled, 150–160 mL; French press 160 mL; filtered, instant 150 mL; espresso, mocha, Turkish 60 mL.

ciated with estrogenic or anti-estrogenic activity [Rietjens *et al*., 2017].

Coffee contains rather small amounts of phytoestrogens, which belong to different classes of polyphenols, such as lignans and isoflavones. Ground coffee is a source of lignan secoisolariciresinol in average amounts of 5.61 mg/kg but does not contain matairesinol [Mazur *et al*., 1998]. In a recent study, lignans found in espresso coffee prepared from coffee samples from 5 different geographical regions comprised secoisolariciresinol, from 27.9 to 52.0 μg/L, and lariciresinol, from 5.3 to 27.8 μg/L [Angeloni *et al*., 2018]. Again, matairesinol was not detected. Considering the consumption of lignans in the range of 1000–2000 μg/d in European countries [Tetens *et al*., 2013; Witkowska *et al*., 2018], the usual daily coffee intake of 2–3 coffee cups may provide a mere 1.3–2.4% of total lignans. In the intestines, lignans are metabolized by anaerobic bacteria to enterolignans such as enterolactone and enterodiol, which demonstrate estrogenic activity [Zhu *et al*., 2017]. Enterodiol was also found in coffee brews in amounts ranging from 97 to 135 μg/L [Sapozhnikova, 2014].

Isoflavones found in coffee are mainly daidzein, genistein, and formononetin. In ground coffee, they account for 3.2–5.2 mg/kg, 0.9–1.4 mg/kg, and 3–6 μg/kg, respectively [Sapozhnikova, 2014]. Overall, isoflavone consumption in Europe has been described as low, and originated mainly from soybean products [Zamora-Ros *et al*., 2012].

Chlorogenic, caffeic, and ferulic acids

Structurally, chlorogenic, caffeic, and ferulic acids are phenolic compounds that are classified as hydroxycinnamic acids (Figure 1). Ferulic acid (4-hydroxy-3-methoxy cinnamic acid) is synthesized in plants from caffeic acid (3,4-dihydroxycinnamic acid). Chlorogenic acid is an ester of caffeic and quinic acids (3-caffeoylquinic acid). In nature, there is a whole range of different derivatives of hydroxycinnamic acids, which are also typical of green coffee. They are intermediates in lignin biosynthesis. Hydroxycinnamic acids are ubiquitous in plant foods, but their significant amounts were also found in coffee. Therefore, coffee can be the main source of hydroxycinnamic acids in countries with high coffee intake [Witkowska *et al*., 2015]. Caffeoylquinic acids, which are the main hydroxycinnamic acids in coffee, can vary in coffee infusions up to a wide range of 6–188 mg/cup [Jeon *et al*., 2019; Ludwig *et al*., 2014]. They have been ascribed various activities related to carcinogenesis. On the one hand, hydroxycinnamate demonstrates antioxidant, anti-inflammatory, antidiabetic, and antimicrobial potential [Teixeira *et al*., 2013; Vinholes *et al*., 2015]. But on the other hand, chlorogenic acid, caffeic acid, and caffeic acid phenethyl ester (CAPE) are *in vitro* inhibitors of catechol-*O*-methyltransferase (COMT)-mediated *O*-methylation of catechol estrogens to their less estrogenic derivatives [Zhu *et al*., 2009]. This inhibition of COMT may lead to the reduced formation of antiproliferative 2-methoxyestradiol and increased accumulation of reactive catechol estrogen intermediates, which may affect cancer development. Interestingly, caffeic acid has been recently suggested as an enhancer of ovarian cancer cells' resistance to treatment [Sirota *et al*., 2015].

Trigonelline

Trigonelline (*N*-methylnicotinic acid), a product of mammalian metabolism of niacin (vitamin B_3), is an alkaloid that also occurs in various plants. Its chemical structure is presented in Figure 1. After caffeine, it is the second most abundant alkaloid in coffee [Acidri *et al*., 2020], with average concentration approximating 300 mg/L [Lang *et al*., 2008]. During the roasting process, much of the trigonelline is degraded to nicotinic acid [Lang *et al*., 2008].

Trigonelline may have estrogenic properties. In estrogen-dependent human breast cancer cells MCF-7, it has been reported to enhance cell proliferation, induce activation of estrogen response element (ERE), and activate ERs [Allred *et al*., 2009].

Many compounds found in plants exhibit antioxidant properties. However, trigonelline was identified as a potent suppressor of the Nrf2/ARE pathway [Boettler *et al*., 2011], which regulates the expression of genes involved in cellular antioxidant and anti-inflammatory protection. In turn, trigonelline degradation product, the *N*-methylpyridinium ion (NMP) – which is formed during the roasting process, has strong, long-lasting effects that enhance Nrf2/ARE-dependent gene expression [Boettler *et al*., 2011].

Acrylamide

The most relevant dietary sources of acrylamide are solid coffee constituents, coffee, and fried potato products [EFSA Panel on Contaminants in the Food Chain (CONTAM), 2015; Freisling *et al*., 2013]. Acrylamide molecules are formed in coffee beans during the process of roasting. The acrylamide content of coffee can vary greatly. In European countries, its average content in ground coffee was estimated at 225–231 μg/kg (depending on the year of sampling), while the maximum value of up to 2223 μg/kg [EFSA, 2011]. For instant coffee, these results were higher with an average value of 357–595 μg/kg and a maximum value of 1470 μg/kg. The latest results of research conducted in Poland demonstrated its contents in the range from 61 to 397 μg/kg in ground coffee and from 152 to 830 μg/kg in instant coffee [Mojska & Gielecińska, 2013]. The average percentage exposure of adults across Europe to acrylamide from roasted coffee, as a proportion of total food intake, was estimated at 0.5–39.9% [EFSA, 2011; Mojska *et al*., 2010]. The chemical structure of acrylamide is presented in Figure 1. In 1994, it was classified by the International Agency for Research on Cancer (IARC) as "probably carcinogenic to humans (group 2A)" [WHO, 1994], and this definition is still valid. In rodent studies, acrylamide administered in drinking water showed a dose-related carcinogenic potential by inducing mammary and ovarian tumors [Beland *et al*., 2013]. In humans, dietary acrylamide exposure is much lower than this in animal studies, ranging from 12 to 39 μg/day in European women [Freisling *et al*., 2013]. Large cohort studies allowed concluding that the usual food intake does not seem to be associated with an increased risk of breast cancer [Kotemori *et al*., 2018; Wilson *et al*., 2009] or ovarian cancer [Larsson *et al*., 2009; Obón-Santacana *et al*., 2016] induced by acrylamide. Also, no associations have been found between the intake of food high in acrylamide, including coffee, and an increased risk of premenopausal breast cancer development [Wilson *et al*., 2009]. Still, associations between acrylamide and ovarian or endometrial cancers cannot be completely excluded among high acrylamide consumers [Hogervorst *et al*., 2007; Pelucchi *et al*., 2015; Wilson *et al*., 2010].

COFFEE AND SEX HORMONES

The main female sex hormones are estrogen and progesterone. Natural estrogenic steroids are estrone (E1), estradiol (E2), and estriol (E3), of which estradiol is the most potent form. Ovarian-produced estradiol is the main estrogen in menstrual years, whereas estrone dominates in the menopause, produced in peripheral tissues from steroid precursor androstenedione. Unlike estriol, estrone can be converted to estradiol (Figure 2).

Estradiol exerts its biological effects through ERs located in the ovary, uterus, and breasts. Estrone and estradiol are hydroxylated by hepatic CYPs to catechol estrogen metabolites of various biological activities such as 2-pathway metabolites (2-hydroxyestradiol, 2-hydroxyestrone) and 4-pathway metabolites (4-hydroxyestradiol, 4-hydroxyestrone) (Figure 2). The 2-hydroxylated catechol estrogens are weak estrogens with potential anti-estrogenic activity [Ziegler *et al*., 2015]. Hydroxylated catechol estrogens are metabolized in the liver by catechol *O*-methyltransferase (COMT) to methoxylated estrogens such as 2-methoxyestradiol, 2-methoxyestrone and 4-methoxyestradiol, 4-methoxyestrone. The 2-methoxyestradiol exhibits anti-angiogenic, pro-apoptotic, and antitumor activities [Aquino-Gálvez *et al*., 2016; Gorska-Ponikowska *et al*., 2017]. Furthermore, a 16-pathway metabolite – 16α-hydroxyestradiol (estriol, E3) being a weak estrogen – is produced in small quantities in non-pregnant women *via* hepatic 16α-hydroxylation of estradiol and estrone by CYPs.

A growing number of studies have shown that plasma estrogens, especially free estradiol, which is unbound to the main estrogen carrier — sex hormone-binding globulin (SHBG) — are strongly associated with the risk of breast cancer development, mainly in the postmenopausal but also in the premenopausal women [Endogenous Hormones and Breast Cancer Collaborative Group, 2013; Folkerd & Dowsett, 2013]. Only the free estradiol fraction, which is just a few per cent of the total plasma estradiol, can penetrate to a cell and stimulate steroid receptors. Conversely, estrogens transported by SHBG are in this bound form inactive, whereas increased SHBG concentrations are linked to the lower breast cancer risk.

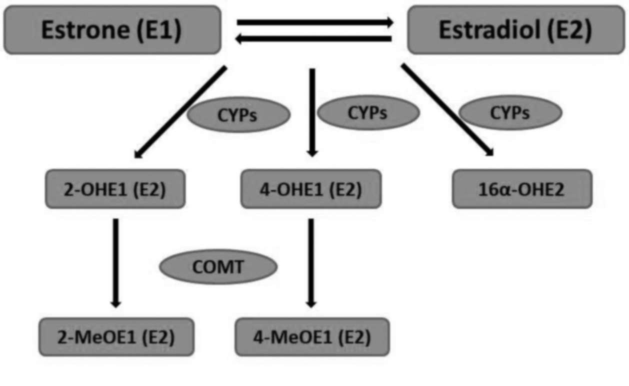

FIGURE 2. Metabolism of estrogen hormones. CYP – cytochrome P450, 2-OHE1 – 2-hydroxyestradiol, 4-OHE2 – 4-hydroxyestradiol, 16αOHE2 – 16α-hydroxyestradiol, 2-MeOE1 – 2-metoxyestrone, and 4-MeOE1 – 4-metoxyestrone.

Several studies provided evidence for associations between coffee or caffeine intake and sex hormone concentrations [Kotsopoulos *et al*., 2009; Nagata *et al*., 1998; Sisti *et al*., 2015]. Kotsopoulos *et al.* [2009] found that the total and free luteal estradiol concentrations in premenopausal women were inversely associated with coffee and caffeine intake. In contrast, the progesterone level was positively associated with caffeine but not with the intake of coffee. In turn, in the postmenopausal women, caffeine and coffee consumption was positively related to SHBG level. No relationship was found between coffee, caffeine or decaffeinated coffee and the concentrations of androgens or prolactin. This result suggests that coffee and caffeine may favorably modulate estrogen metabolism and protect against breast cancer both in pre- and postmenopausal women, but the mechanisms of this protection are entirely different.

A recent study which used data from 587 premenopausal women, participants of the Nurses' Health Study II, demonstrated that the estrogen metabolism could be influenced by coffee intake and by caffeine [Sisti *et al*., 2015]. This study measured mid-luteal urinary concentrations of 15 estrogens and estrogen metabolites and connected them to both caffeine and coffee intake ascertained on the basis of self-reported food frequency questionnaires. It was found that high (>4 cups/day) *versus* low coffee intake (<4 cups/week) was associated with higher excretion of 2-pathway estrogen metabolites of low estrogenic activity, *i.e.* 2-hydroxyestradiol and 2-hydroxyestron. Conversely, the 2-pathway metabolism was not affected by the consumption of decaffeinated coffee. However, the intake of at least 2 cups of decaffeinated coffee per day was associated with a significant reduction of 16-pathway metabolites with estrogenic activity, *i.e.* estriol and 17-epiestriol (17α-epimer of estriol), as compared to occasional coffee drinkers (<1–3 cups/month). In turn, higher caffeine intake was associated with higher urinary concentrations of 16α-hydroxyestrone and 16-epiestriol. This study concluded that the consumption of caffeine and coffee may differentially alter patterns of estrogen metabolism in the premenopausal women.

COFFEE AND BREAST, ENDOMETRIAL, AND OVARIAN CANCERS IN WOMEN

In vitro, coffee brews and some compounds present in coffee demonstrate antiproliferative activities in cancer cells [Tai *et al*., 2010]. Study results have shown that coffee compounds have an additive antiproliferation effect on cancer cell lines [Tai *et al*., 2010]. It has been suggested that selected coffee secondary metabolites may beneficially modulate several mechanisms of anti-cancer protection.

A number of studies have demonstrated an inverse relationship between coffee consumption and cancer risk [Arthur *et al*., 2018; Oh *et al*., 2015; Park *et al*., 2018]. Recently, one study threw light on the nature of associations between coffee consumption and some markers of immune system activation that may have a role in the development of cancer and chronic diseases. It was found that heavy coffee drinkers had lower circulating levels of host response proteins (IFNγ, CX3CL1/fractalkine, CCL4/MIP-1β), cell growth regulators (FGF-2), and proinflammatory cytokine sTNFRII than coffee abstainers [Loftfield *et al*., 2015].

Although breast cancer occurs in both sexes, it is most common in women. The current state of knowledge about breast, endometrial, and ovarian cancers in the context of coffee intake is discussed below.

Breast cancer

Cancers of the mammary glands are the most common invasive female cancers worldwide, and are also the leading cause of all cancer deaths in women [Ferlay *et al*., 2015]. The degree of morbidity increases with age and grows significantly after the age of 40. Breast cancer incidence is associated with both modifiable and genetic factors. The modifiable risk factors include being overweight or obese, physical inactivity, and alcohol use. As estimated, these factors together contribute to 21% risk of developing breast cancer [Danaei *et al*., 2005]. The genetic factors are mostly associated with the presence of abnormal *BRCA-1* (breast cancer 1) and *BRCA-2* (breast cancer-2) genes, which are normally involved in DNA repairing processes [Ford *et al*., 1998]. Mutations of these tumor-suppressor *BRCA* genes may lead to an increased risk of breast cancer development.

In the *BRCA-1* gene mutation carriers, high intake of caffeinated coffee amounting to 6 cups or more daily demonstrated a favorable effect by lowering breast cancer risk as compared to coffee abstainers [Nkondjock *et al*., 2006], an effect not observed in *BRCA-2*. Recent research has indicated that coffee and/or caffeine might have a chemopreventive effect, regardless of whether the women had the *BRCA-1* gene or not [Nikitina *et al*., 2015]. This effect was attributed to a more efficient repair processes of damaged DNA [Nikitina *et al*., 2015]. On the other hand, excessive consumption of coffee could have adverse effects. In female non-carriers of abnormal *BRCA* genes, extremely large coffee consumption of 8 or more cups daily was associated with an increased risk of developing breast cancer [Bissonauth *et al*., 2009].

In women with defective *BRCA-1*, a protective effect seems to be associated with a mechanism of caffeine elimination through cytochrome P450 1A2 (CYP1A2), which is also common for the metabolism of estrogen. The protective effect of coffee in the *BRCA-1* abnormal gene carriers was limited to those females who had at least one C variant of cytochrome CYP1A2 [Kotsopoulos *et al*., 2007].

Until now, several dozen cohort and case-control studies have been devoted to the influence of coffee consumption on the incidence of breast cancer (Table 3). Most of the population studies showed no correlation between coffee drinking and breast cancer [Arthur *et al*., 2018; Bhoo-Pathy *et al*., 2010; Boggs *et al*., 2010; Fagherazzi *et al*., 2011; Gierach *et al*., 2012; Grosso *et al*., 2017a, b; Hashibe *et al*., 2015; Lukic *et al*., 2016; Yaghjyan *et al*., 2018], while some showed reduction [Nilsson *et al*., 2010; Oh *et al*., 2015]. However, a 2018 dose-response meta-analysis of prospective cohort studies, which included more than a million women, showed that daily coffee consumption of 2 cups reduced the risk of breast cancer in postmenopausal women [Lafranconi *et al*., 2018]. Similarly, the earlier meta-analysis of 37 case-control studies showed a negative trend in postmenopausal women [Jiang *et al*., 2013].

Several studies assessed the risk of breast cancer based on the status of ER and progesterone receptor (PR). A Swedish *Women's Lifestyle and Health* cohort study demonstrated that coffee consumption and caffeine intake was negatively associated with the risk of developing ER positive and PR negative (ER+/PR–) breast cancer [Oh *et al*., 2015]. In turn, in a European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study, a linear trend was observed for a lower risk of postmenopausal breast cancer with increasing caffeinated coffee intake for ER–/PR– cancer [Bhoo-Pathy *et al*., 2015]. Another Swedish study reported a lower risk of ER– breast cancer in postmenopausal high coffee consumers [Li *et al*., 2011]. Furthermore, a study undertaken in Sweden, which involved 1090 patients with invasive primary breast cancer, shed light on the effects of coffee on cancer progression [Rosendahl *et al*., 2015]. This study showed that smaller invasive primary tumors and lower prevalence of ER+ tumors were observed in moderate (2–4 cups/day) to high (>5 cups/day) coffee consumers compared to low (<1 cup/day) coffee consumers. Also, this moderate to high coffee intake was associated with a lower risk of breast cancer events in ER+ tumor patients treated with tamoxifen. These findings were translated into molecular and cellular levels [Rosendahl *et al*., 2015]. Coffee contains several biologically active substances which may interact with breast cancer cells to impair their cell-cycle progression and increase cancer cell death. Two of them are caffeine and caffeic acid. Both seem to sensitize breast cancer cells to tamoxifen and suppress growth of ER+ and ER– cells [Rosendahl *et al*., 2015]. In addition, caffeine reduces the number of ERs and cyclin D1 in ER+ cells, and also reduces insulin-like growth factor-1 receptor (IGFIR) expression, which is implicated in carcinogenesis of some tumors, including breast cancer, and phosphorylated Akt (pAkt) levels in both ER+ and ER– cells, the enzyme which contributes to poor prognosis in breast cancer.

Recently, there has been a report from a case-control study showing that instant coffee, unlike brewed coffee, can increase the risk of breast cancer development [Lee *et al*., 2019]. This study concluded that there is a need for research on the effects of different types of coffee on the risk of breast cancer development.

Ovarian cancer

The number of studies on ovarian cancer and coffee and/or caffeine is steadily increasing. So far, several prospective cohort and case-control studies have been performed. Recently, two meta-analyses have been conducted, one concerning 8 prospective studies and the other – 20 case-control studies [Berretta *et al*., 2018; Shafiei *et al*., 2019].

Epidemiological studies are inconsistent in finding whether coffee, caffeine or decaffeinated coffee could be associated

TABLE 3. Studies between 2010–2019 on coffee/caffeine intake and the risk of breast cancer.

	Name and design of research	Total number of participants	Number of breast cancer (BC) cases	Major study outcomes	Reference
Coffee	EPIC-NL Cohort, Netherlands	27,323	681	No association between coffee consumption and BC risk.	Bhoo-Pathy *et al*. [2010]
Coffee, caffeine	Black Women's Health Study, USA, cohort study	52,062	1268	No association between coffee consumption or caffeine intake and BC risk among African-American women.	Boggs *et al*. [2010]
Coffee	Vasterbotten Intervention Project, Sweden, prospective cohort study	64,603	3034	Boiled coffee ≥4 *versus* <1 intakes/day associated with a reduced risk of BC (HR = 0.52, CI 0.30–0.88, p (trend) = 0.247). Increased risk of premenopausal BC for total coffee (HR=1.69, CI 0.96–2.98, p (trend) = 0.015) and filtered coffee (HR= 1.76, CI 1.04–3.00, p (trend) = 0.045). Reduced risk of postmenopausal BC for total coffee (HR= 0.60, CI 0.39–0.93, p (trend) = 0.006) and filtered coffee (HR= 0.52, CI = 0.30–0.88, p (trend) = 0.045).	Nilsson *et al*. [2010]
Coffee, caffeine	E3N cohort, France	67,703	2868	No association between coffee consumption or caffeine intake and BC risk.	Fagherazzi *et al*. [2011]
Coffee, caffeinated, decaffeinated	Health-AARP Diet and Health Study cohort, USA	198,404	9915 (2051 ER+/PR+ and 453 ER-/PR- cancers)	No association between coffee, caffeinated and decaffeinated coffee and BC risk. No association with ER+/PR+ and ER-/PR- BC.	Gierach *et al*. [2012]
Coffee, decaffeinated coffee, caffeine	Metaanalysis of 37 case-control and cohort studies	966,263	59,018	No association between coffee consumption, decaffeinated coffee, caffeine and BC risk. An inverse weak association of coffee and caffeine with BC risk for postmenopausal women. Strong inverse association of coffee with BC risk for BRCA1 mutation carriers (RR=0.69, P<0.01).	Jiang *et al*. [2013]
Coffee, caffeinated coffee, decaffeinated coffee	European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study	335,060	1064 premenopausal, 9134 postmenopausal	Caffeinated and decaffeinated coffee were not associated with premenopausal breast cancer. Caffeinated coffee associated with lower risk of postmenopausal breast cancer: adjusted HR=0.90, 95% CI, 0.82–0.98, for high *vs.* low consumption. Linear trend for lower BC risk with increasing caffeinated coffee intake most visible for estrogen and progesterone receptor negative (ER-PR-) postmenopausal breast cancer (P=0.008).	Bhoo-Pathy *et al.* [2015]
Coffee	Swedish Women's Lifestyle and Health prospective cohort study	42,099	1395	Decreased RR of BC for > 3–4 cups /day compared to 1–2 cups of coffee in pre- and postmenopausal women.	Oh *et al*. [2015]
Coffee, caffeine	Prostate, Lung, Colorectal, and Ovarian (PLCO) cancer screening trial, USA	50 563	1698	No association between coffee consumption, caffeine and BC risk.	Hashibe *et al.* [2015]
Coffee	Norwegian Women and Cancer (NOWAC) Study, population-based prospective study	91,767	3277	No association between coffee consumption and BC risk.	Lukic *et al*. [2016]
Coffee	Cancer Prevention Study-II	922,896	6113	In nonsmokers, a 2 cup/day increase in coffee consumption inversely associated with death from BC (HR=0.97; 95% CI, 0.94–0.99).	Gapstur *et al*. [2017]
Coffee, caffeinated coffee, caffeine	Canadian Study of Diet, Lifestyle and Health (CSDLH), prospective case-cohort study	3120	922	Coffee and caffeine intake not associated with overall BC risk. Tendency towards increased BC risk with increasing levels of total coffee, caffeinated coffee and/or caffeine in premenopausal and normal weight women.	Arthur *et al*. [2018]
Coffee	UK Biobank	126,182 postmenopausal women	2,636	Coffee consumption not associated with BC risk (HR=1.00; 95% CI, 0.91–1.11 for 2–3 cups/day, and HR=0.98, 95% CI 0.87–1.10 for ≥4 cups/day. No significant interaction between postmenopausal hormone therapy and coffee consumption.	Yaghjyan *et al*. [2018]
Coffee	Dose-response metaanalysis of prospective cohort studies	1,068,098	36,597	2 cups coffee/day associated with BC reduction in postmenopausal women (RR 0.90, 95% CI 0.82–0.99).	Lafranconi *et al*. [2018]
Coffee instant, coffee brewed	Case-control study in Hong Kong Chinese Women	2169	238	Instant coffee associated with increased BC risk (AOR = 1.50, 95% CI 1.10–2.03); brewed coffee negatively associated with BC risk (AOR = 0.48, 95% CI 0.28–0.82).	Lee *et al*. [2019]

BC – breast cancer; ER – estrogen receptor; PR – progesterone receptor; RR – relative risk; HR – hazard ratio; CI – confidence interval; AOR – adjusted odds ratio.

with the risk of ovarian cancer development. Research focuses on both, finding an increased as well as a decreased risk of developing ovarian cancer (Table 4). Some recent findings from the multinational EPIC cohort study, which included over 300,000 female participants, have shown increased mortality due to ovarian cancer in coffee drinkers [Gunter *et al*., 2017]. The remaining studies point rather to a decrease [Gosvig *et al*., 2015; Park *et al*., 2018] or lack of associations with ovarian cancer risk [Arthur *et al*., 2018; Berretta *et al*., 2018; Braem *et al*., 2012; Hashibe *et al*., 2015; Leung *et al*., 2016; Lukic *et al*., 2016; Ong *et al*., 2018; Shafiei *et al*., 2019].

Ong *et al.* [2018] studied genetic predisposition towards higher coffee intake and its implications associated with the risk of epithelial ovarian cancer development. However, they did not confirm this relationship.

Interesting results concerning coffee and ovarian cancer risk are presented in a meta-analysis of 20 case-control studies [Shafiei *et al*., 2019]. Although they did not establish any correlation between total coffee and caffeine intake, the authors found that decaffeinated coffee reduced the risk of ovarian cancer development. This may indicate an anticancer effect from other coffee ingredients than caffeine.

TABLE 4. Studies between 2010–2019 on coffee/caffeine intake and the risk of ovarian cancer.

	Name and type of research	Total number of participants	Number of OC cases	Major study outcomes	Reference
Coffee	European Prospective Investigation into Cancer and Nutrition (EPIC) prospective cohort study and metaanalysis	330,849	1244 EOC cases	No associations with EOC risk HR=1.05 (95% CI, 0.75–1.46) for the top quintile compared with no intake.	Braem *et al.* [2012]
Coffee, caffeine	Danish case-control study	1293	267 OC, 115 with borderline OC	Modest decrease in OC risk for coffee (OR = 0.90, 95% CI 0.84–0.97 per cup/day) and caffeine (from coffee and tea combined) (OR = 0.93, 95% CI 0.88–0.98 per 100 mg/day).	Gosvig *et al.* [2015]
Coffee, caffeine	Prostate, Lung, Colorectal, and Ovarian cancer screening trial, cohort study	97,334	162	No evidence of OC risk associated with coffee consumption. Suggested risk for caffeine intake for some specific OC quartiles.	Hashibe *et al.* [2015]
Coffee, caffeinated soft drinks	population-based Alberta and British Columbia, Canada case-control study	2111	524 EOC	No evidence for risk of EOC associated with coffee or caffeinated soft drinks.	Leung *et al.* [2016]
Coffee	Norwegian Women and Cancer (NOWAC) Study, population-based prospective study	91,767	446	No association with OC risk.	Lukic *et al.* [2016]
Coffee	EPIC (European Prospective Investigation into Cancer and Nutrition), prospective cohort study	321,081	848 OC deaths	Positive association between coffee and OC mortality (HR=1.12, 95% CI 1.02–1.23, P-trend 0.001).	Gunter *et al.* [2017]
Coffee, caffeinated coffee, caffeine	Canadian Study of Diet, Lifestyle and Health (CSDLH), prospective case-cohort study	2826	104	Coffee and caffeine intake not associated with OC overall risk.	Arthur *et al.* [2018]
Coffee, caffeine	Ovarian Cancer Association Consortium (OCAC), Europe	–	44,062 EOC	Single nucleotide polymorphisms (SNP) rs6968865 from the *AHR* gene and rs2472297 from the *CYP1A2* gene, associated with coffee consumption. No evidence on strong association between EOC risk and genetically predicted coffee or caffeine levels.	Ong *et al.* [2018]
Coffee	Multiethnic Cohort Study in Hawaii and Los Angeles, USA	167,720	1795	Reduced OC risk with HR = 0.33 (95% CI 0.17–0.65); Ptrend = 0.007.	Park *et al.* [2018]
Coffee	Metaanalysis of 8 prospective cohort studies	787,076	3541	Coffee intake not associated with OC risk.	Berretta *et al.* [2018]
Coffee, caffeine, caffeinated coffee, decaffeinated coffee	Metaanalysis of 20 case-control studies	40,140	8568	No significant association between total coffee intake or caffeine and OC risk. Inverse association between decaffeinated coffee intake and OC risk (OR=0.72, 95% CI 0.58–0.90). No association with caffeinated coffee.	Shafiei *et al.* [2019]

OC – ovarian cancer, EOC – epithelial ovarian cancer, RR – relative risk, HR – hazard ratio, OR – odds ratio, CI – confidence interval.

Endometrial cancer

Endometrial cancer (EC) is the most common uterine cancer, which is derived from the endometrium, the inner lining of the uterus. The risk factors for endometrial cancer include obesity, long-term stimulation by increased levels of estrogen, high blood pressure, diabetes, early age of menarche, nulliparity, and late menopause [Ali, 2014; Raglan *et al*., 2019]. Postmenopausal women aged 60–70 years with excessive body weight are more predisposed to this cancer. After the menopause, gonadal production of estrogen and progesterone decreases, and androstenedione – another hormone produced by ovaries, is converted by adipose tissue to estrone in obese women. Continuous stimulation of endometrium by estrone without progesterone secretion leads to uncontrolled proliferation of endometrial tissue [Michels *et al*., 2019]. Most cases of endometrial cancer fell into this cause-and-effect pattern (Type I EC); however, there are EC cases (10–20%) which are non-estrogen dependent (Type II EC) [Doll *et al*., 2008]. Whereas molecular characteristics describe Type EC I as endometrial endometrioid adenocarcinomas (EEAs) with or without squamous differentiation and in most cases well differentiated, whereas Type II tumors are high-grade serous papillary or clear cell carcinomas [Doll *et al*., 2008].

Relatively many studies concern the consumption of total coffee, caffeinated, and decaffeinated coffee, and caffeine in relation to the risk of EC (Table 5). At least five meta-analyses of cohort and case-control studies were carried out

TABLE 5. Studies between 2010–2019 on coffee/caffeine intake and the risk of endometrial cancer.

	Name and type of research	Total number of participants	Number of EC cases	Major study outcomes	Reference
Coffee, coffee caffeinated, coffee decaffeinated	Women's Health Initiative Observational Study, USA	45,696	427 postmenopausal women	No association between coffee consumption and EC risk. No associations among normal-weight and overweight women for total coffee and caffeinated coffee.	Giri *et al*. [2011]
Coffee, coffee caffeinated, coffee decaffeinated	Nurses' Health Study (NHS), USA	67,470	672	< 4 cups of coffee/day not associated with EC risk. Multivariable RR=0.75, 95% CI 0.57–0.97; P(trend) = 0.02) > 4 cups/day *vs.* > 1 cup/day. For ≥4 vs. <1 cup/d RR= 0.70, 95% CI = 0.51–0.95 for caffeinated coffee. For decaffeinated coffee consumption, a suggestive inverse association for 2 or more cups/day *vs.* <1 cup/month.	Je *et al*. [2011]
Coffee	Meta-analysis of 10 case-control and 6 cohort studies	–	6,628	For the highest *versus* lowest categories of coffee intake pooled RR=0.71 (95% CI 0.62–0.81; p for heterogeneity = 0.13) for EC. Pooled RR=0.69 (95% CI 0.55–0.87) for case-control studies and RR=0.70 (95% CI 0.61–0.80) for cohort studies. By geographic region, the inverse association was stronger for three Japanese studies (pooled RR=0.40, 95% CI 0.25–0.63) than five studies from USA/Canada (pooled RR=0.69, 95% CI 0.60–0.79) or eight studies from Europe (pooled RR=0.79, 95% CI 0.63–0.99). An increment of one cup per day of coffee intake conferred a pooled RR=0.92 (95% CI 0.90–0.95).	Je & Giovanucci, [2012]
Coffee caffeinated, decaffeinated, caffeine	Prospective cohort Iowa Women's Health Study (IWHS), USA	23,356	471 Type I, 71 Type II EC postmenopausal women	Type I EC (age range at diagnosis 57.2–89.5 years) significantly associated with caffeinated (RR=0.65 for 4+ cups per day *vs.* ≤ 1 cup per month, 95% CI 0.47–0.89), but not for decaffeinated coffee intake. No associations with tea, cola or chocolate, or for Type II EC. Inverse association with caffeinated coffee in women with BMI >30 kg/ m^2 (RR=0.56; 95% CI: 0.36–0.89).	Uccella *et al*. [2013]
Coffee, caffeine	Swedish Women's Lifestyle and Health cohort study	42,270	144	Similar mean daily coffee consumption in women with and without EC (549 g *vs*. 547 g) and caffeine intake (405 *vs*. 406 mg). No association between coffee consumption or caffeine intake and EC risk among middle-aged women.	Weiderpass *et al*. [2014]
Coffee	Norwegian Women and Cancer (NOWAC) Study, population-based prospective study	97,926	462	Significant risk reduction found for ≥8 cups/day of coffee HR= 0.52 (95% CI 0.34–0.79) in multivariate adjustment model. No significant dose-response relationship. No significant heterogeneity in risk found for filtered and boiled coffee. Reduced EC risk in subgroup analyses among participants who drank ≥8 cups/day and had BMI ≥25 kg/m^2, and in current smokers.	Gavrilyuk *et al*. [2014]
Coffee, caffeine	Prostate, Lung, Colorectal, and Ovarian (PLCO) cancer screening trial, USA	32,293	257	RR=0.69 (95% CI 0.52–0.91) for coffee intake ≥ 2 cups per day. Caffeine not associated with cancer risk in a dose-response manner.	Hashibe *et al*. [2015]
Coffee	UK Million Women prospective study and meta-analysis	560,356	4067	No significant association between EC risk and consumption of coffee. Weak association for coffee consumption with EC in prospective studies.	Yang *et al*. [2015]

TABLE 5. Continued

	Name and type of research	Total number of participants	Number of EC cases	Major study outcomes	Reference
Coffee	European Prospective Investigation into Cancer and Nutrition (EPIC) and Nurses Health Studies (NHS/NHSII)	539,237 (301,107 (EPIC; 238,130 NHS/NHSII)	2834 (1303 EPIC; 1531 NHS/NHSII)	Multivariate-adjusted comparisons of extreme categories of intake: EPIC, median intake 750 g/day *vs*. 8.6 g/day; HR= 0.81 (95% CI 0.68–0.97), Ptrend = 0.09; NHS/NHSII, median intake 1067 g/day *vs*. none; HR= 0.82 (95% CI 0.70–0.96), Ptrend = 0.04.	Merritt *et al*. [2015]
Coffee, caffeinated coffee, decaffeinated coffee, caffeine	Dose-response meta-analysis of 13 prospective cohort studies	1,534,039	10,100	RR=0.80 (95% CI 0.74–0.86) for total coffee consumption (RR=0.60 (95% CI 0.50–0.72) in EC women never treated with hormones, R=0.57 (95% CI 0.46–0.71) in overweight/obese. Overall RR= 0.66 (95% CI 0.52–0.84) for caffeinated and RR=0.77 (95% CI 0.63–0.94) for decaffeinated coffee. Linear dose-response relationship for coffee, caffeinated coffee, decaffeinated coffee and caffeine intake. EC risk decreased by 5% for every 1 cup/day of coffee, 7% for every 1 cup/day of caffeinated coffee, 4% for every 1 cup/day of decaffeinated coffee, and 4% for every 100 mg of caffeine intake/day.	Zhou *et al*. [2015]
Coffee	Dose-response meta-analysis of 12 prospective cohort studies	1,404,541	10,548	4 cups coffee /day associated with RR=0.80, 95% CI 0.72–0.89) for EC risk and with RR= 0.76, 95% CI 0.69- 0.83) for postmenopausal EC risk.	Lafranconi *et al*. [2017]
Coffee, caffeinated coffee, caffeine	Canadian Study of Diet, Lifestyle and Health (CSDLH), prospective case-cohort study	2608	180	For coffee cup increase HR=0.88 (95% CI 0.79–0.95), for caffeinated coffee cup increase HR=0.88 (95% CI 0.80–0.96), for 100mg caffeine increase HR=0.93 (95% CI 0.87–0.99).	Arthur *et al*. [2018]
Coffee	Meta-analysis of 12 cohort studies and 8 case-control studies	–	11,663 cases in cohort studies and 2,746 cases in case-control studies	For highest *vs*. lowest coffee intake summary RR= 0.74 (95% CI 0.68–0.81; p heterogeneity = 0.09, I2 = 32%), for cohort studies RR=0.78 (95% CI 0.71–0.85; p heterogeneity = 0.14, I2 = 31.9%), for case-control studies RR=0.63 (95% CI 0.53–0.76; p heterogeneity = 0.57, I2 = 0%). One-cup increment/day associated with 3% risk reduction (95% CI 2–4%) in cohort studies and 12% (95% CI 5–18%) in case-control studies. After pooling the results from 5 cohort studies that included BMI, the association remained significant only in women with BMI>30 (RR = 0.71, 95% CI 0.61–0.81).	Lukic *et al.* [2018]

EC – endometrial cancer, RR – relative risk, HR – hazard ratio, CI – confidence interval, BMI – body mass index.

between 2010 and 2019 [Je & Giovannucci, 2012; Lafranconi *et al*., 2017; Lukic *et al*., 2018; Yang *et al*., 2015; Zhou *et al*., 2015]. In cohort studies, high coffee intake compared to no intake was associated with a lower EC risk [Merritt *et al*., 2015]. Similar findings were reported for coffee, caffeinated coffee, and caffeine [Arthur *et al*., 2018]. Intake of four or more cups of coffee a day [Je *et al*., 2011], or more recently consumption of two or more cups a day [Hashibe *et al*., 2015], was associated with lower EC risk, but some research showed no conclusive association [Weiderpass *et al*., 2014]. Coffee intake was found to be particularly beneficial in overweight or obese women [Friberg *et al*., 2009; Gavrilyuk *et al*., 2014; Lukic *et al*., 2018; Uccella *et al*., 2013; Zhou *et al*., 2015]. Several studies have indicated that coffee intake might be associated with a decreased cancer risk in obese postmenopausal women [Giri *et al*., 2011, Uccella *et al*., 2013]. Caffeinated coffee consumption seems to have an advantage over decaffeinated coffee [Zhou *et al*., 2015].

In one study, coffee was examined as a contributor to the dietary antioxidant activity (DAA) of EC patients. Coffee-related DAA was found to be inversely associated with the overall EC risk (OR=0.87, CI 0.77–0.99) [Rossi *et al*., 2016]. Such association, however, was weak for DAA with no coffee intake included. The above research points to the role of coffee as a dietary factor with the strongest impact on DAA, whereas the highest DAA quartile is associated with the lowest risk of EC (OR=0.75, CI 0.52–1.08).

The evidence supports the association between coffee and decreased EC risk in postmenopausal women. The mechanism of this association is not clearly understood. One line of evidence points to diabetes, which is a well-established factor in EC development. Most recently, the risk of developing EC has been linked to high fasting insulin, C-peptide and HOMA-IR values, which are the parameters used to assess β-cell function and insulin resistance [Hernandez *et al*., 2015]. Coffee intake combined with hormone-replacement therapy (HRT) seems to sensitize cell receptors to insulin and decrease insulin resistance in postmenopausal women [Catalano *et al*., 2008]. Insulin resistance correlates directly with body mass index (BMI), and coffee intake increases insulin sensitivity in overweight women [Catalano *et al*., 2008].

CONCLUSIONS

As shown in publications cited in this paper, coffee is composed of multiple chemical compounds, that vary according to such factors as: type of beans (Arabica, Robusta), roasting process, preparation (instant coffee) and brewing method. Therefore, their content in a coffee cup can vary greatly. Most long-term observational studies were planned from the viewpoint of consumption of total, caffeinated, decaffeinated coffee, and caffeine, but they took no account of the size of coffee portions and the content of biologically active substances, which may vary several times between cups. Such studies generally do not distinguish between ground roasted coffee and instant coffee. A recent case-control study has raised this issue and showed a positive relationship with breast cancer [Lee *et al*., 2019]. For all these reasons, it is difficult to determine the appropriate dose and type of coffee that would be most effective in preventing cancer. An issue that has not yet been studied is the question of the additives with which coffee is consumed, such as milk or sugar, and how they can affect bioavailability of coffee compounds, as has been shown for tea [Korir *et al*., 2014]. Clinical trials offer more standardized conditions of research. However, in the case of cancer, long-term clinical trials are not feasible. In turn, long-term observational studies are in some cases ambiguous as regards the effect of coffee consumption, showing a positive, negative or no relationship between coffee consumption and breast, ovarian or endometrial cancer. In general, most studies and meta-analyses indicate that there is no clear correlation between coffee and breast cancer or ovarian cancer. However, some subgroups of women can benefit from coffee consumption. This is the case for post-menopausal women with regard to the risk of breast cancer and obese women with regard to the risk of endometrial cancer [Lafranconi *et al*., 2018; Lukic *et al*, 2018; Zhou *et al*., 2015]. The situation is unclear in the case of ovarian cancer, where the effect of coffee is generally not observed, but in the case of decaffeinated coffee the opposite relationship may exist [Shafiei *et al*., 2019]. Further research should therefore be targeted at them with clearly defined portion sizes and preparation methods. In addition, more attention should be paid to whether or not cancer is hormone-dependent and whether there is a genetic predisposition to breast, ovarian or endometrial cancer. Many lifestyle factors such as smoking, improper diet, excessive consumption of alcohol, contribute to the risk of developing cancer. Future research should pay attention to selecting participants in order to minimize the possible influence of confounding factors [Grosso *et al*., 2016].

Coffee is an important dietary source of biologically active compounds with the caffeine being the most abundant. *In vitro* and *in vivo* studies suggest that various classes of secondary metabolites of coffee may beneficially modulate several mechanisms of anti-cancer protection. It would be interesting to examine whether the compounds present in coffee have a synergistic effect and whether caffeine is indeed one of the most important substances that have an anti-cancer effect. Recent studies show that decaffeinated coffee can also exhibit these properties [Shafiei *et al.*, 2019], indicating that other coffee compounds are important as well. The available literature on human studies related to single coffee components is limited to caffeine and therefore this research topic is needed to be developed. Furthermore, the metabolism of many coffee compounds in the body requires better characterization. This applies especially to phenolics, which undergo microbiological transformation in the intestine. And yet, some coffee compounds, such as lignans and trigonelline, have an estrogenic activity, which can be important when studying hormone-dependent cancers.

There are some strengths and limitations of this review. Its strength and at the same time its limitation is the time-period in which literature was sought, which allowed for the collection of the latest publications, but omitted most of the publications before that period. However, in the case of observational studies, most of the previous case-control and epidemiological studies have been analyzed in recent meta-analyses. On the other hand, the search was limited to two electronic databases, thus some studies may not have been included in this review.

This literature review shows that, despite extensive knowledge of coffee and its effects on human health, there are still many open questions to explore in relation to breast, ovarian, and endometrial cancer. Important issues for future research are to better understand the anti-cancer mechanisms of coffee compounds, especially of diterpenes, trigonelline and phenolics, and to undertake research that would focus on specific target groups, taking into account both the different methods of coffee preparation and lifestyle factors that may interfere with the results.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Original research article
Food Technology Section

Physical, Physicochemical, Mechanical, and Sensory Properties of Bioplastics from Phosphate Acetylated Arenga Starches

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Key words: native arenga starch, acetylation, cross-linking, sodium trimetaphosphate, sodium tripolyphosphate, biofilms

Bioplastics are alternative to plastic packaging made from renewable natural materials. They have a great potential for wider application due to their environmental-friendliness and ease of degradation. This research, therefore, aimed to evaluate the physical, physicochemical, mechanical, and sensory characteristics of bioplastics made from native arenga starch (NAS) and phosphate acetylated arenga starch (PAAS). The PAAS was obtained by dual modification of NAS through acetylation using 5% acetic anhydride and crosslinking using a mixture of sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP) at 99:1 (*w/w*). The concentrations of the mixture were varied at 2, 4, 6, 8, 10, and 12% (*w/w*) of the starch. The thickness, water holding capacity (WHC), oil holding capacity (OHC), water vapor transmission rate (WVTR), water content, biodegradation, Fourier transform infrared (FT-IR) spectroscopy, tensile strength, elongation at break, Young's modulus, and sensory properties of the NAS and PAAS bioplastics were investigated. The results showed the thickness of the NAS and PAAS was generally uniform. The WHC of the NAS bioplastic was higher than that of PAAS. The OHC and WVTR of the PAAS bioplastics increased with the increment in the concentration of the STMP/STPP mixture. Furthermore, the water content of the PAAS bioplastics was lower than that of NAS, while the weight loss due to biodegradation of the NAS was higher compared to PAAS. The PAAS bioplastics were characterized by FTIR, which confirmed the acetylation and crosslinking between the arenga starch molecules. Generally, the elongation at break of the PAAS bioplastics was higher than that of the NAS bioplastic, color of the PAAS bioplastics was more transparent and texture of the PAAS bioplastics surface was smoother than of the NAS bioplastic.

INTRODUCTION

Packaging is the final part of the processes involved in the production of food and non-food products purposely to increase shelf life, improve consumer acceptance, and as a means of providing support for the distribution and expansion of product marketing [Prasteen *et al*., 2018]. The major material used for this purpose is plastic and this has led to a continuous increase in its waste thereby causing environmental pollution because it is non-biodegradable, its recycling process is quite expensive, and it has the ability to contaminate foods [Ogunrinola & Akpan, 2018]. There is, however, the urgent need to find alternative materials for packaging and one of these is the bioplastics due to its safe and biodegradable nature.

Bioplastics are produced from renewable natural materials and have been observed to have the potentials of being an alternative to plastic packaging due to their environmental friendliness and easy degradation. Therefore, there is an urgent need for bioplastics due to the rapid increase in the pace of plastic production and air pollution causing several health risks because of their toxic nature [Jain & Tiwari, 2015]. Bioplastics are, however, very suitable alternatives to improve the quality of life and maintain a pollution-free planet [Keziah *et al*., 2018].

It has been discovered that bioplastics made from native starch have poor physicochemical, mechanical, functional and sensory characteristics compared to those modified chemically. According to Shindu & Khatkar [2018], those produced from modified wheat starch are transparent, have greater tensile strength, and lower solubility compared to the native wheat starch. Moreover, edible films of acetylated rice starch were also found to be stable to heat, have high elongation at break, and a rapid process of degradation [Colusi *et al.*, 2017]. Meanwhile, the mechanical properties of bioplastics from tapioca starch modified using acetic anhydride were observed to be superior over these of the native starch

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[Tawakaltu *et al*., 2015]. The elongation at break, solubility, and oxygen permeability characteristics of biodegradable films from hydroxypropyl sago starch were higher and the water vapor transmission rate was lower compared to the native material [Polnaya *et al*., 2013].

One of the potential starches to be used as a bioplastic polymer was native arenga starch (NAS), but it is limited by fast retrogradation, inability to withstand acidic conditions, low viscosity, and paste resistance [Rahim *et al*., 2019]. Therefore, there is a need for its modification in order to obtain its suitable properties required by bioplastic polymer materials. The novelty of this research was the use of dual modification of the main raw material through acetylation and crosslinking. This involved the development of a phosphate acetylated arenga starch (PAAS) through acetylation and crosslinking. These were intended to produce a modified material that is difficult to retrograde, stable to heat and acids, and resistant to water due to the presence of acetyl groups and crosslinking in the starch molecules. This was possible because previous studies have reported the incorporation of acetyl and phosphate in starch molecules was able to inhibit the retrogradation process and aid resistance to heat, acid-base, and water [Rahim *et al*., 2017]. Therefore, the objective of this research was to evaluate the physical, physicochemical, mechanical, and sensory characteristics of the NAS and PAAS bioplastics obtained through acetylation using 5% acetic anhydride and crosslinked using a mixture of sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP) at 99:1 (*w/w*) with different concentrations.

MATERIALS AND METHODS

Materials

The materials used in the research consisted of NAS extracted from the pith of sugar palm trees (*Arenga pinnata*). Distilled water and 98% acetic anhydride were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid (HCl), sodium hydroxide (NaOH), STMP, STPP, 96% ethanol, glycerol, and acetic acid were purchased from Merck (Darmstadt, Germany). All the chemicals used for the bioplastic analysis were of analytical grade.

Arenga starch extraction

The NAS was prepared according to the method described by Sahari *et al*. [2014] with some alterations. *Arenga pinnata* tree was cut down and the stem was then split to release the woody fiber together with the starch from the inward delicate center of its stem. Step by step, water was brought inside the fiber and starch blend got from the stem and was completely massaged by hand. The blend was then separated to permit the water to move through the sifter with starch granules in the mixture. The starch was conceded sufficient opportunity to settle at the base of the compartment, and water was tapped. After that, the starch was kept in an outdoors for momentarily and then dried in the oven for 72 h at temperature 70°C.

Preparation of dual modification arenga starch

Acetylation of NAS was conducted according to the method of Rahim *et al*. [2015] while crosslinking followed the Koo *et al*. [2010] approach with a slight modification. The suspension consisting of 100 g arenga starch and 225 mL distilled water was stirred using a magnetic stirrer for 1 h at room temperature. This was followed by the dropwise of acetic anhydride in such a quality as to give 5% of weight of arenga starch (*v/w*). The pH of the suspension was maintained at 8.0 to 8.5 by adding 3% NaOH. After 60 min, the pH was increased to 10.5 through the addition of 3% NaOH. Next, a mixture of STMP/STPP at 99:1 (*w/w*) was added in such an amount to obtain concentration of 2, 4, 6, 8, 10, and 12% by weight of arenga starch (*w/w*). The suspension was again stirred for 30 min at room temperature and next 0.5 N HCl was added until the pH decreased to 4.5 to end the reaction. After reaction, the suspension was left to sediment for 30 min and the sediment was washed with distilled water three times and 96% ethanol once. Next, the sediment was dried in an oven at 60°C for 15 h, mashed, and filtered with 100 mesh sieve. The modified arenga starches were called 2, 4, 6, 8, 10, and 12% PAAS, respectively, and were used as bioplastic polymer materials.

Preparation of bioplastics

Bioplastic was prepared according to the method developed by Chung *et al*. [2010] with a slight modification. The NAS and PAAS (2, 4, 6, 8, 10, and 12%) were weighed to be 10 g, and 150 mL of distilled water was added. The solutions were heated on a hot plate to a temperature of 100°C with stirring after which 10 mL of acetic acid and 3 mL of glycerol were added. The heating process continued until the gel started forming. The solution was further stirred for 10 min and was poured into stainless steel strips and dried at room temperature for 4 days. The bioplastics obtained were analyzed for physical, physicochemical, mechanical, and sensory properties.

Thickness determination

Bioplastic thickness was measured according to the method proposed by Turhan & Sabhaz [2004] through the use of calipers. The samples were placed between the jaws of the caliper and the thickness was measured at five different places and the average was calculated.

Water and oil holding capacity determination

The water and oil holding capacity (WHC and OHC, respectively) were determined using a method developed by Larrauri *et al*. [1996] with some modification. Briefly, 25 mL of distilled water or olive oil were added to 250 mg of bioplastic samples, stirred, and the mixture was left at room temperature for 1 h. After centrifugation at 3,500×g for 30 min (GS 150 Centrifuge, Clements, Sydney, Australia), the residue was weighed and the holding capacity of water and oil was calculated as g of water or oil, respectively per g of dry bioplastic sample.

Water vapor transmission rate determination

Water vapor transmission rate (WVTR) was determined using the method proposed by Xu *et al*. [2004]. This involved the production of a saturated salt solution in a chamber using a jar with a diameter of 12 cm and a height of 15 cm as well as the adjustment of the relative humidity (RH) in the chamber to 75% by adding a solution of 40% NaCl (*w/v*) at room temperature. Furthermore, an acrylic cup with diameter of 5 cm and height of 1.8 cm was filled with 10 g silica gel, covered with bioplastic according to its size, and placed in the 75% RH chamber. The water vapor diffusing through the bioplastics and silica gel added to the weight and the whole setup was weighed every hour for 8 h to determine the weight gain. The data was used to plot a graph of the time *vs*. weight, and the slope was recorded to calculate WVTR using the following equation:

$$\text{WVTR (g/h/m}^2\text{)} = \frac{\text{Change of bioplastics sample weight (g/h)}}{\text{Surface area of the bioplastics sample (m}^2\text{)}}$$

Moisture content determination

Moisture content was measured following the method applied by AOAC [2005]. The portions of bioplastics (0.5 g) were dried in an oven at 105°C for 2 h.

Biodegradability analysis

The biodegradability of the bioplastic samples was investigated according to the method developed by Ashok *et al*. [2018] with slight modification. The samples were cut into 1 × 4 cm, weighed (W_1), and buried in the soil at a depth of 8 cm for a curing duration of 9 days after which they were weighed again (W_2). The weight loss due to the biodegradation process was determined through the use of the following formula:

$$\text{Weight loss (\%)} = \frac{W_1 - W_2}{W_1} \times 100\%$$

FTIR spectra analysis

FTIR spectra of NAS and PAAS bioplastics were analyzed according to Diop *et al*. [2011]. The bioplastics were mixed with KBr at a ratio of 1:100 (sample: KBr, *w/w*). The infrared spectra were obtained using a Fourier Transform Spectrometer (IR Prestige-21, Shimadzu Corporation, Tokyo, Japan) at a wavenumber range from 4000 cm^{-1} to 500 cm^{-1} regions.

Tensile strength, elongation at break, and Young's modulus determination

Tensile strength and elongation at break were measured using a Mechanical Universal Testing Machine (Model LR30K, LLOYD Instruments Ltd., Hampshire, England). This involved cutting the bioplastics into the required standard and the two ends were clamped to the testing machine at a distance of 50 mm and a speed of 10 mm/min. The start knob was turned on and the machine pulled the sample until it broke and the tensile strength and elongation at break values were recorded afterward. The tensile strength was calculated based on the maximum force (N) applied for the material to break divided by the area of the bioplastic (mm^2):

$$\text{Tensile strength (MPa)} = \frac{F_{\text{Max}}\text{ (N)}}{\text{Bioplastics surface area (m}^2\text{)}}$$

The elongation at break was calculated using the equation:

$$\text{Elongation at break (\%)} = \frac{\text{Maximum length} - \text{Initial length}}{\text{Initial length}} \times 100\%$$

Young's modulus was calculated based on the values of tensile strength and elongation at break according to the formula:

$$\text{Young's modulus (MPa)} = \frac{\text{Tensile strength}}{\text{Elogation at break/100}}$$

Sensory evaluation

The sensory analysis was conducted using a 15-member panel consisting of students from the Faculty of Agriculture, Tadulako University, Central Sulawesi Indonesia. The bioplastic samples were cut into small pieces and the color, texture, aroma, and overall acceptability were tested using a 7-point hedonic scale were 7 indicates highly very like, 6 – very like, 5 – like, 4 – neither like nor dislike, 3 – somewhat like, 2 – dislike, and 1 – very dislike. The panelists were instructed to rate the attributes indicating their degree of likeness using the numbers provided in the hedonic scale according to their preference.

Statistical analysis

All parameters were determined in triplicate except for thickness and sensory analysis, which were carried out four times. The results were analyzed through the application of one-way ANOVA using SPSS version 22 (SPSS Inc., Chicago, IL, USA). The means were compared with Duncan's multiple tests and the statistical significance was defined at $p \leq 0.05$.

RESULTS AND DISCUSSION

Thickness

The thickness of bioplastics of NAS and PAAS obtained using different concentrations of the STMP/STPP mixture are shown in Figure 1. There were no significant differences ($p > 0.05$) in the thickness of both NAS and PAAS bioplastics and this was associated with the equal amount of raw material used. The values (0.36–0.45 mm) were in line with the findings of Marichelvam *et al*. [2019] that the average thickness of bioplastics made from corn and rice starch was 0.25 mm and this means they can be used as biodegradable plastic bags. Moreover, Ghasemlou *et al*. [2013] reported the thickness of the edible film of corn starch was around 0.15 mm while Fakhouri *et al*. [2013] obtained 0.053 to 0.063 mm for those made from potato, rice, wheat, gelatin, and sorghum starch.

Water and oil holding capacity

The WHC and OHC of NAS and PAAS bioplastics are shown in Figure 2. The WHC and OHC of NAS bioplastic were significantly different ($p \leq 0.05$) from those of the PAAS ones. The highest WHC was found for NAS bioplastic. The PAAS bioplastics had lower WHC, which probably was associated with the presence of cross-linking bonds in the PAAS molecules hindering water penetration. However, the concentration of the STMP/STPP mixture used to obtain

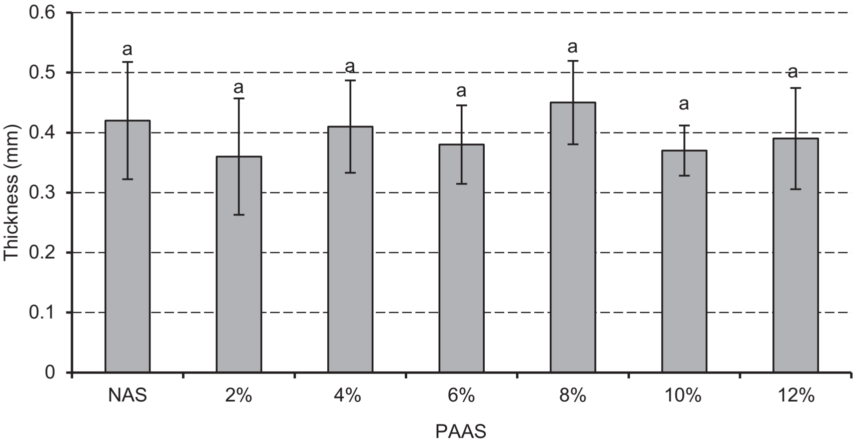

FIGURE 1. Thickness of bioplastics of native arenga starch (NAS) and phosphate acetylated arenga starches (PAAS) obtained using different concentrations of sodium trimetaphosphate/sodium tripolyphosphate (STMP/STPP) mixture (2–12%).

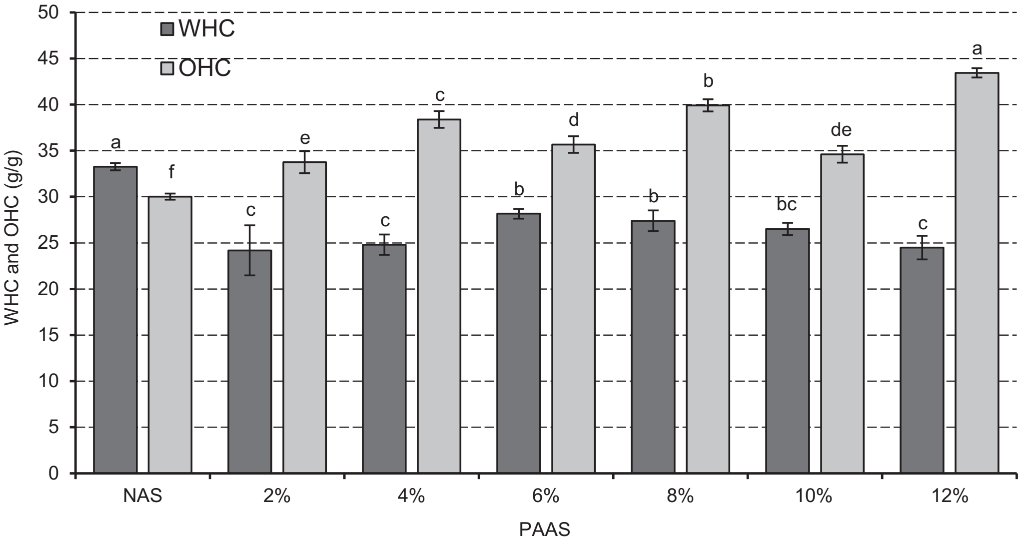

FIGURE 2. The water holding capacity (WHC) and oil holding capacity (OHC) of bioplastics of native arenga starch (NAS) and phosphate acetylated arenga starches (PAAS) obtained using different concentrations of sodium trimetaphosphate/sodium tripolyphosphate (STMP/STPP) mixture (2–12%). Different letters a-f above bars indicate significant differences among values ($p \leq 0.05$).

the modified polymers only slightly differentiated the WHC of PAAS bioplastics. Meanwhile, the highest and lowest OHC were found for 12% PAAS and NAS bioplastics, respectively. Furthermore, the OHC tended to be increasing with the increased concentrations of the STMP/STPP mixture.

The WHC results are in agreement with the study that showed the water uptake of bioplastics from cassava peel starch reinforced with microcrystalline cellulose (Avicel PH101fillers) was lower compared to the original material. This was attributed to the strong hydrogen bonds between microcrystalline cellulose and the molecular structure of starch [Maulida *et al.*, 2016]. The OHC results were in accordance with an earlier report of Sondari & Iltizam [2018] who showed that the OHC of bioplastics from modified cassava starch was lower than that of the original material.

Water vapor transmission rate

WVTR of the NAS and PAAS bioplastics is presented in Figure 3. The highest WVTR (2.40 g/h/m^2) was noted for the bioplastic obtained using NAS. The lowest value (1.05 g/h/m^2) was determined for 4% PAAS sample, however this value did not differ significantly ($p>0.05$) from those determined for 2% and 6% PAAS bioplastics. It is important to note that the values obtained for the PAAS bioplastics were lower than these determined for NAS and this could be associated with the rigidity of PAAS which has the ability to prevent water from entering the starch molecules. Moreover, the incorporation of acetyl groups into the starch molecules has led to a reduction of OH groups and thus to the lower ability to bind water [López et al., 2011]. This is in accordance with an earlier report of Detduangchan *et al.* [2014] that the WVTR of the modified rice starch bioplastic was lower than that of the native material.

According to Fakhoury *et al.* [2012], the water vapor permeability of the native cassava starch film was higher with 4.88 g mm/m^2 d kPa compared to acetylated and crosslinked cassava starch, which was found to be 3.59 g mm/m^2 d kPa. This phenomenon was caused by the strong interaction be-

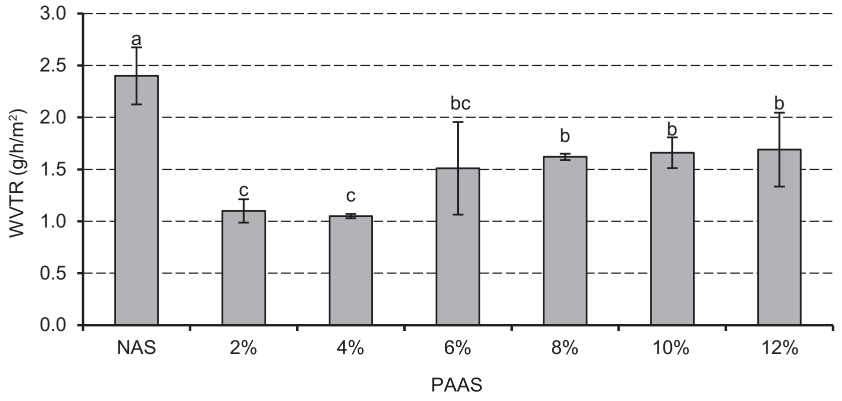

FIGURE 3. The water vapor transmission rate (WVTR) of bioplastics of native arenga starch (NAS) and phosphate acetylated arenga starches (PAAS) obtained using different concentrations of sodium trimetaphosphate/sodium tripolyphosphate (STMP/STPP) mixture (2–12%). Different letters a-c above bars indicate significant differences among values (p≤0.05).

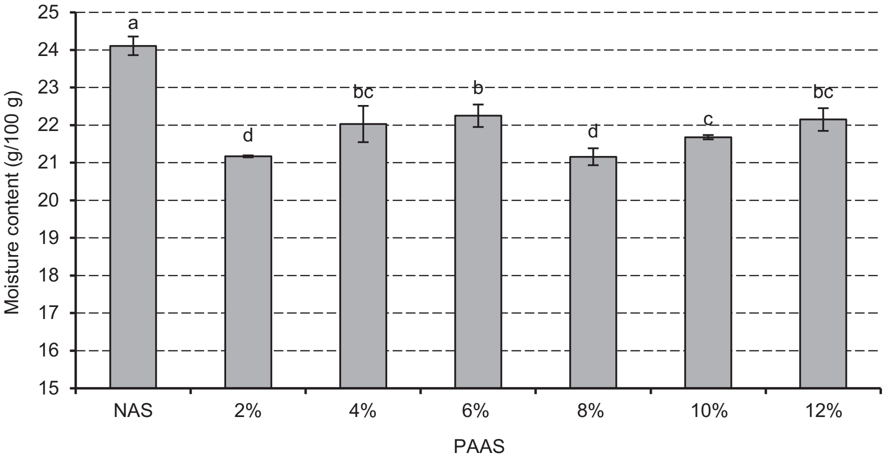

FIGURE 4. Water content of bioplastics of native arenga starch (NAS) and phosphate acetylated arenga starches (PAAS) obtained using different concentrations of sodium trimetaphosphate/sodium tripolyphosphate (STMP/STPP) mixture (2–12%). Different letters a-d above bars indicate significant differences among values (p≤0.05).

tween amylose and amylopectin in the modified starch molecules. However, bioplastics with a low WVTR value are suitable for packaging food products to avoid damage caused by the surrounding environment.

Water content

The water content of the NAS and PAAS bioplastics obtained using different concentrations of the STMP/STPP mixture is shown in Figure 4. The concentration of STMP/STPP mixture significantly ($p \leq 0.05$) affected the water contents in PAAS bioplastics. The highest value (24.11 g/100 g) was obtained for NAS bioplastic, while the lowest (21.16–21.17 g/100 g) was found for 2% and 8% PAAS bioplastics. Moreover, the water content in PAAS bioplastics was found to be generally lower than in NAS. It was supported by the findings of Gutiérrez *et al*. [2015] that the water content in phosphated corn starch edible film was 30%, compared to 44% determined in the native corn starch. However, Atef *et al*. [2015] reported that bioplastics were expected to have a low water content in order to ensure they do not increase the amount of water in the product when used for food packaging.

Biodegradation

The biodegradability of the bioplastic materials was tested using the soil burial method. Biodegradation is defined as a process of decomposition through the activities of microbes in the soil which leads to the transformation of a compound's structure thereby causing changes in molecular integrity [Liu *et al*., 2016]. The average values of weight loss for the NAS and PAAS bioplastics are presented in Figure 5. The differences between the values were significant $p \leq 0.05$).

The weight loss was the highest in NAS bioplastic and lower in PAAS samples. However, for PAAS bioplastics, the values increased with the increasing concentrations of the mixture of STMP/STPP used to obtain the modified polymers. This shows NAS bioplastics are more easily degraded compared to PAAS bioplastics. A previous study showed 71% of potato peel bioplastic degraded in moist soil within four weeks [Arikan & Bilgen, 2019]. Another research reported the biodegradation of bioplastics made from native corn and rice starches and those modified was influenced by temperature, humidity, and microbial activity such that 48.73% was achieved after the materials were placed in the soil at a depth of 3 cm for 15 days [Marchelvam *et al*., 2019].

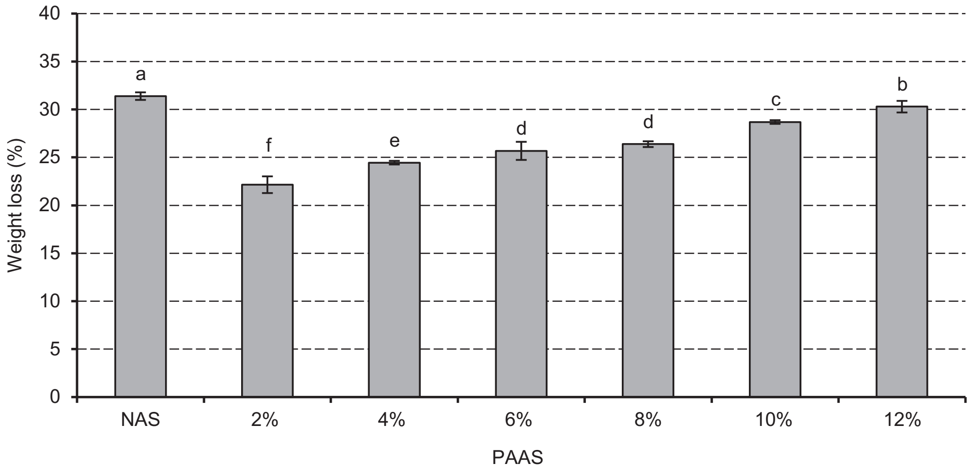

FIGURE 5. Weight loss of bioplastics of native arenga starch (NAS) and phosphate acetylated arenga starches (PAAS) obtained using different concentrations of sodium trimetaphosphate/sodium tripolyphosphate (STMP/STPP) mixture (2–12%). Different letters a-f above bars indicate significant differences among values (p≤0.05).

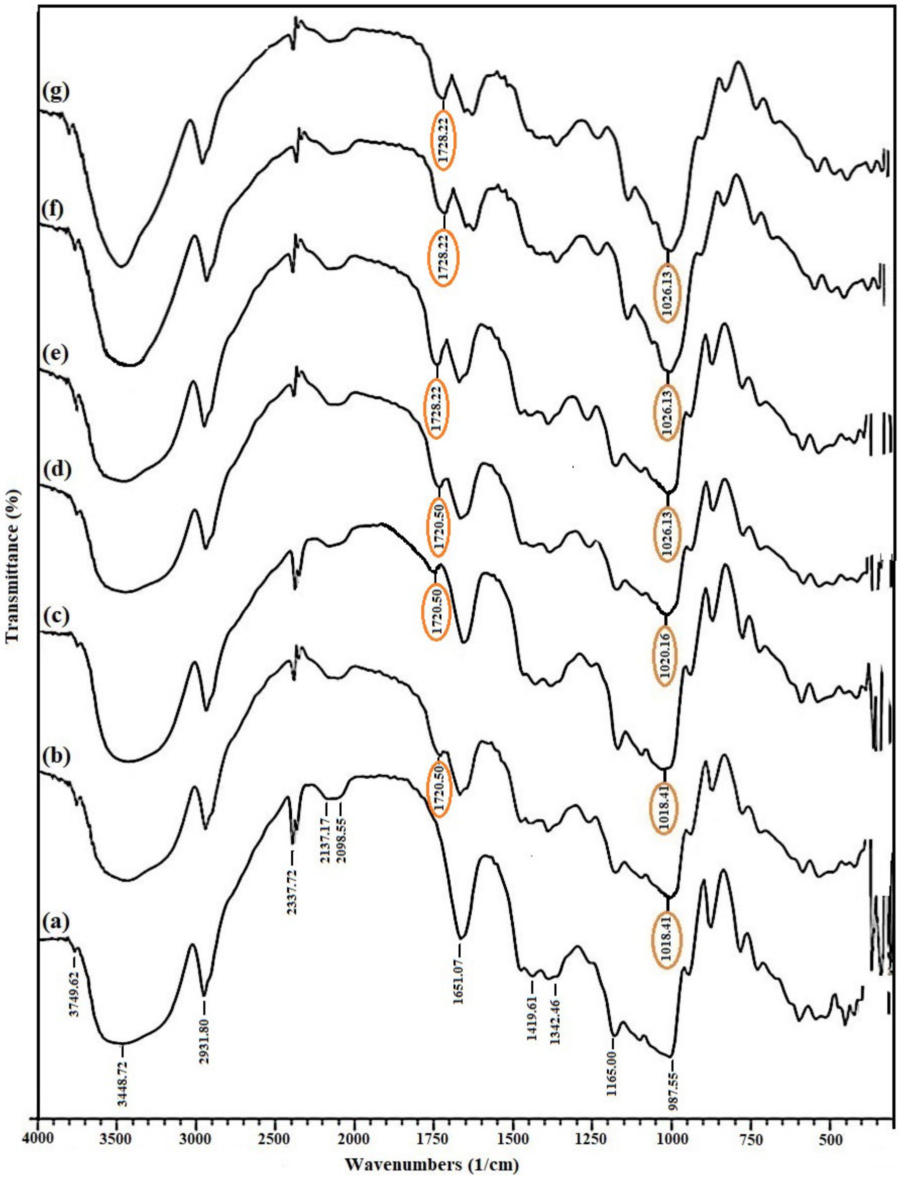

FIGURE 6. The FTIR spectra of bioplastics of native arenga starch (a) and phosphate acetylated arenga starches obtained using different concentrations of sodium trimetaphosphate/sodium tripolyphosphate (STMP/STPP) mixture: 2% (b), 4% (c), 6% (d), 8% (e), 10% (f), and 12% (g).

FTIR spectra

FTIR spectra of NAS and PAAS bioplastics are shown in Figure 6. The characteristic bands were observed at the range of 3700 cm^{-1} to 3000 cm^{-1}, 2931.80 cm^{-1} and 1651.07 cm^{-1} for the hydroxyl groups (O–H), methylene (C–H), and residual bound water (H_2O) stretching the vibration of the glucose unit, respectively. In comparison with the values obtained for NAS bioplastic (Figure 6a), the new absorption bands in the range of 1728.22 cm^{-1} to 1720.50 cm^{-1} and 1026.13 cm^{-1} to 1018.41 cm^{-1} appeared for PAAS bioplastics (Figures 6b–g). These bands corresponded to C=O stretching vibration of an ester group and P-O-C stretching vibration, respectively. This confirmed the occurrence of acetylation and crosslinking between the starch molecules. Previously, acetylation of arenga starch was shown by the presence of the ester carbonyl group bands at 1720 cm^{-1} [Rahim *et al.*, 2017]. In turn, Detduangchan *et al.* [2014] reported a peak at 1035.75 cm^{-1} corresponding to the phosphate stretching (P-O-C) in STMP/STPP cross-linked rice starch films.

Mechanical properties

The tensile strength, elongation at break, and Young's modulus were determined to evaluate the mechanical properties of NAS and PAAS bioplastics. Tensile strength is the magnitude of the force required to achieve the maximum pull in each bioplastic area. Elongation at break is the percentage change in bioplastic length calculated when it was pulled up to break, whereas the Young's modulus represents the division of the tensile strength by the elongation at break [López *et al.*, 2016]. The average values of these parameters for NAS and PAAS bioplastics are shown in Table 1. The ANOVA indicated that tensile strength of the samples did not differ significantly ($p>0.05$), while analysis differentiated bioplastics in terms of elongation at break and Young's modulus ($p \le 0.05$).

Generally, the elongation at break of the PAAS bioplastics was higher than that of NAS bioplastic, although it should be noted that the values determined for NAS and 2%, 4%, 12% PAAS did not differ significantly ($p>0.05$). Zhang *et al.* [2013] reported an increase in elongation at break of oxidized corn starch films due to an increase in the mobility of starch chains resulting from a stronger interaction between oxidized starch and starch chains and a higher degree of oxidation. Moreover, Woggum *et al.* [2014] also found the elongation at break of dual modified rice starch with a mixture of STMP 2% – STPP 5% was higher than that of the native starch.

Furthermore, the elongation at break of the PAAS bioplastics increased with the concentrations of STMP/STPP up to 6% and subsequently remained constant at 12%. Meanwhile, the Young's modulus of the PAAS bioplastics decreased with the concentrations of STMP/STPP up to 8% and afterwards remained constant at 12%. However, the elongation at break and Young's modulus of bioplastics from chitosan cross-linking methylcellulose using STMP 0.1 and 0.3% were found to be higher than these of the chitosan bioplastics [Wang *et al.*, 2019].

Sensory attributes

The degree of likeness of color, texture, aroma, and overall acceptability of the NAS and PAAS bioplastics were evaluated and the results are presented in Table 2. The color and texture were significantly different ($p \le 0.05$), while the differentiations among bioplastics in aroma and overall acceptability were not significant ($p>0.05$).

The color and texture of the PAAS bioplastics were more transparent and their surface was smoother compared to NAS bioplastic, respectively. The transparency and smoother surface of the PAAS bioplastics were achieved through acetylation and crosslinking. In line with the findings of this research, it was reported that the color of the bioplastics from oxidized, crosslinked, and dual oxidation crosslinked lotus rhizome starch was clearer compared to the native material [Sukhija *et al.*, 2019]. This is in agreement with the report by Wang *et al.* [2019] who showed the colors and texture of films made from chitosan methylcellulose crosslinking were more transparent and smoother due to the compactness of the starch molecules.

TABLE 1. The tensile strength, elongation at break, and Young's modulus of the NAS and PAAS bioplastics obtained using different concentrations of STMP/STPP mixture.

Bioplastic	Tensile strength (MPa)	Elongation at break (%)	Young's modulus (MPa)
NAS	1.08±0.09^{a}	7.03±0.83^{b}	0.15±0.01^{a}
PAAS 2%	1.10±0.12^{a}	7.87±1.28^{ab}	0.14±0.01^{ab}
PAAS 4%	1.09±0.29^{a}	7.78±1.45^{ab}	0.14±0.04^{ab}
PAAS 6%	1.16±0.16^{a}	9.14±1.09^{a}	0.13±0.02^{ab}
PAAS 8%	0.94±0.07^{a}	8.09±1.05^{a}	0.12±0.01^{b}
PAAS 10%	0.93±0.03^{a}	8.17±0.54^{a}	0.11±0.01^{b}
PAAS 12%	0.99±0.11^{a}	7.87±1.17^{ab}	0.13±0.01^{ab}

Data are mean ± standard deviation (SD). Values in the same column with different superscript indicate a significant difference ($p \le 0.05$). NAS = native arenga starch, PAAS = phosphate acetylated arenga starches, STMP=sodium trimetaphosphate, STPP= sodium tripolyphosphate.

TABLE 2. Sensory assessment of the NAS and PAAS bioplastics obtained using different concentrations of STMP/STPP mixture.

Bioplastic	Color	Texture	Aroma	Overall acceptability
NAS	4.60±0.74^{b}	4.60±1.12^{b}	4.48±1.13^{a}	5.33±0.72^{a}
PAAS 2%	5.40±0.63^{a}	5.47±0.99^{a}	4.60±0.83^{a}	5.33±0.72^{a}
PAAS 4%	5.27±0.88^{ab}	5.53±0.92^{a}	4.73±0.70^{a}	5.53±0.83^{a}
PAAS 6%	4.93±0.88^{ab}	5.47±0.99^{a}	4.53±0.64^{a}	5.60±0.63^{a}
PAAS 8%	5.67±1.11^{a}	5.27±0.96^{ab}	5.07±0.88^{a}	5.47±0.64^{a}
PAAS 10%	5.13±0.99^{ab}	5.20±0.86^{ab}	5.07±0.96^{a}	5.20±0.68^{a}
PAAS 12%	5.07±1.03^{ab}	5.13±0.74^{ab}	4.73±1.03^{a}	5.20±0.68^{a}

Data are mean ± standard deviation (SD). Values in the same column with different superscript indicate a significant difference ($p \le 0.05$). NAS = native arenga starch, PAAS = phosphate acetylated arenga starches, STMP= sodium trimetaphosphate, STPP= sodium tripolyphosphate.

CONCLUSION

The dual modification of NAS by acetylation and phosphorylation using STMP/STPP mixture with 2–12% concentrations proved to be an advantageous strategy to obtain the PAAS bioplastics with improved physicochemical, mechanical, and sensory properties compared to NAS bioplastic. The WHC, WVTR, moisture content, and Young's modulus of the PAAS bioplastics were generally lower than these of the NAS bioplastic, while the OHC and elongation at break of the PAAS bioplastics were higher compared to NAS bioplastics. The NAS and PAAS bioplastics were decomposed in the soil. Furthermore, the color and texture of the PAAS bioplastics were more transparent and smoother than these of the NAS bioplastic, respectively. This has indicated that the PAAS bioplastics were superior over the NAS bioplastic.

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CONFLICT OF INTERESTS

The authors declare there is no conflict of interest.

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Influence of β-Glucan Structures and Contents on the Functional Properties of Low-Fat Ice Cream During Storage

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Key words: polysaccharides, milk dessert, curdlan, rheology, texture, sensory analysis

The study aimed to determine the effect of the addition level and structure of β-glucans on the functional properties and sensory attributes of low-fat ice cream. The experimental ice cream was produced with the addition of 0.5% and 1% highly purified (1–3)(1–4) and (1–3) β-glucans.

β-Glucans significantly increased overrun and decreased the melting time. The study demonstrated that the β-glucan structure and addition level significantly influenced the consistency and viscosity index, flow behavior, cohesiveness, and firmness of ice cream. The use of (1–3)(1–4) β-glucan led to a significant decrease (~38%), whereas (1–3) β-glucan led to a significant increase (~42%) of ice cream firmness. β-Glucans influenced the sensory properties of ice cream, in particular its texture and mouthfeel but had no significant effect on the taste of ice cream. (1–3)(1–4) β-Glucan isolated from oats is suitable for the production of calorie-reduced ice cream with functional characteristics most similar to the control ice cream (full-fat, with stabilizing substances).

INTRODUCTION

Ice cream is an emulsion (o/w) that contains air bubbles, partially coalesced fat globules and ice crystals. These components are dispersed in a viscous liquid phase composed of proteins, minerals, and polysaccharides that stabilize the emulsion [Soukoulis *et al*., 2010]. The rheological properties of ice cream, including texture and microstructure, are determined by the composition of the ice cream mixture, in particular its fat content that ranges from 8% to 16%. In ice cream, fat is responsible for the production of emulsion, for the size and shape of ice crystals, and melting time. Reduced fat content can increase the brittleness and roughness of ice cream. In addition to shaping the rheological properties of ice cream, dairy fat is also a flavor carrier contributing to their full and creamy taste [Bahramparvar & Tehrani, 2011].

The rise in consumer awareness about the nutritional and biological value of foods, as well as changing nutritional trends, encourage food manufacturers to develop new production technologies. Low-calorie foods containing only natural ingredients are in most significant demand. According to many authors, the energy value of foods can be reduced through the partial replacement of fat with polysaccharides, which effectively imitate fat on account of their unique functional properties [Javidi *et al*., 2016]. The ideal fat replacer should not only decrease a product's energy value, but it should also exert minimal influence on the functional properties and the sensory attributes of ice cream. Polysaccharides, including guar gum [Javidi & Razavi, 2018], maltodextrin and polydextrose [Güzeler *et al*., 2011], fructooligosaccharides [Akalin *et al*., 2008; Soukoulis *et al*., 2010], dietary fiber from cereals and citrus fruit [Soukoulis *et al*., 2010], starch [Sharma *et al*., 2017], and barley β-glucan [Abdel-Haleem & Awad, 2015], are most widely used as fat mimetics in the production of ice cream. β-Glucans are highly interesting fat mimetics in the production of ice cream and other dairy products. Unlike other polysaccharide replacers, they deliver health benefits by decreasing the risk of diet-dependent diseases such as hyperinsulinemia, hyperlipidemia, impaired immunity [Kanagasabapathy *et al*., 2013], and osteoporosis [Aljewicz *et al*., 2018]. β-Glucans isolated from various sources differ in structure, namely the presence and distribution of glycosidic bonds and molecular weight [Stone, 2009]. β-Glucans isolated from mushrooms are characterized by the highest molecular weight ($2–3\times10^6$ g/mol), whereas these isolated from bacteria and oats have a lower molecular weight at 0.66×10^5 g/mol and 0.56×10^5 g/mol, respectively [Survase *et al*., 2007]. Due to their high molecular weight, β-glucans are better thickening agents than other polysaccharides, and they reduce the amount of other ingredients in food production.

Despite the above, there is a general scarcity of published research on the suitability of β-glucans for ice cream production. The effect of the addition level and structure of polysaccharides, including highly purified β-glucan preparations, on

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the functional properties of low-fat ice cream has never been investigated. Therefore, the aim of this study was to determine the influence of β-glucans structure and content level on the functional properties and sensory attributes of low-fat ice cream.

MATERIALS AND METHODS

Materials and sample preparation

The study was performed on control ice cream with 10% fat content (typical fat content of full-fat ice cream on the Polish market). In experimental (low-fat) ice cream, the fat content was reduced to 2.5% in order to determine the possibility of replacing fat with 0.5% and 1% addition of β-glucans. The experimental ice cream was produced with the addition of highly purified (75%) (1–3)(1–4) β-glucan isolated from oats *Avena sativa* L. (Beta Bio Technology, Poland) and highly purified (90%) (1–3) β-glucan (curdlan) isolated from *Agrobacterium* sp. bacteria (Xi'an Lyphar Biotech Co., Ltd, China). The β-glucans content of the preparation was based on a certificate provided by the manufacturer. The addition of (1–3)(1–4) β-glucan was normalized to ensure that β-glucans content was identical to that of the experimental ice cream containing (1–3) β-glucan. Samples of ice cream stored at -18°C for 1 day and 14 days were collected for analysis.

Ice cream production

The following ingredients were used in ice cream production: deionized water, dehydrated milk fat (10% *w/w* in the control ice cream; 2.5% *w/w* in the experimental ice cream; Supply Group BV, Maasland, Netherlands), skimmed milk powder (8.5% *w/w* in the control ice cream; ~17% *w/w* in the experimental ice cream; Gostynin, Poland), whey protein isolate (3% *w/w*; Fonterra, Auckland, New Zealand), sucrose (8% *w/w*; Poch, Gliwice, Poland), glucose in powder (3% *w/w*; Poch, Gliwice, Poland), and lecithin (0.5% w/w; Sigma-Aldrich, Schnelldorf, Germany). The control ice cream contained a stabilizer (0.3% *w/w*, blend of locust bean gum, guar gum, and carrageenan), whereas the experimental ice cream contained 0.5% or 1% of (1–3)(1–4) β-glucan or (1–3) β-glucan. The experimental ice cream was produced based on Patent Application P.418827 [2020]. The prepared sample was high-shear dispersed at 10,000 rpm for 3 min and pasteurized at 95°C for 15 min. Ice cream emulsions were prepared with the use of a multifunctional device (Rpol, Mielec, Poland). After preparation, the mixtures were cooled to 4±1°C in an ice-water bath and aged overnight at 4°C in a cooler. Continuous freezing was performed in a professional ice cream maker (Easy Freeze 1000; Promag, Anzola dell'Emilia, Italy). All samples were frozen at -18°C in 100-mL hinged-lid polypropylene containers and analyzed after 1 and 14 days. The ice cream was produced on the same day.

Analyses of physical properties

Chemical composition analysis

Ice cream samples were analyzed for the content of total solids [AOAC, 2000; method 941.08] and protein [EN ISO 8968–1:2014].

Overrun and melting properties

Overrun (%) was determined according to the method described by Rinaldi *et al*. [2014]. A volume of liquid mix and ice cream was weighed, and percentage of overrun was calculated according to the equation:

$$Overrun\ (\%) = \frac{m_{\text{ice cream mix}} - m_{\text{ice cream}}}{m_{\text{ice cream mix}}} \qquad \text{(Eq. 1)}$$

where: $m_{\text{ice cream mix}}$ was the weight of mix and $m_{\text{ice cream}}$ the weight of ice cream after freezing.

Melting time was determined according to the method described by Granger *et al*. [2005]. The overrun and melting time properties of each ice cream sample were measured in three replications.

Viscosity and flow behavior of emulsions

The viscosity of the ice cream emulsions was determined after 3 h of aging at 4±1°C with the RheolabQC Viscometer (Anton Paar GmbH, Ostfildern, Germany) equipped with a cooling/heating circulator (Model F12-MC, Julabo Labor Technik, Seelbach, Germany) and the CC27 measuring system. The samples were allowed to stand for 3 min before analysis. Twenty-nine viscosity values were measured and recorded (PhysicaRheo Plus software; Anton Paar GmbH, Ostfildern, Germany) at intervals of 5 1/s to create a range of shear rates from 0 to 150 1/s. The viscosity at a shear rate of 6.32 1/s was used for comparison (the value closest to the conditions in the oral environment during chewing). Rheological properties were fitted to two rheological models, including Herschel-Bulkley and Ostwald models. The Herschel-Bulkley model produced the best fit to upward flow curves (results not shown), and it was selected for modeling flow behavior:

Herschel-Bulkley model:

$$\tau = \tau_0 + k\,(\gamma)^n \qquad \text{(Eq. 2)}$$

where: τ is shear stress (Pa), k is the consistency index ($Pa{\cdot}s^n$), γ is shear rate (1/s), τ_0 is yield stress, and n is the flow behavior index (dimensionless). The viscosity of each ice cream sample was measured in three replications.

Texture analysis of ice cream

An instrumental back extrusion test was performed using the TA-XT-plus Texture Analyzer (Stable Micro Systems, Surrey, UK) equipped with a 5-mm diameter stainless steel cylindrical probe. The following test settings were applied: penetrating distance = 15 mm, force = 5 g, probe speed during penetration = 3.3 mm/s, and probe speed before and after penetration = 3 mm/s. Tests were performed immediately after ice cream samples were removed from the freezer, and the time interval for the analysis was standardized. The samples were stored at -12°C for 24 h before analysis. The analyses were performed at 20±1°C. The force-time curves were analyzed using Texture Exponent. The texture of each ice cream sample was measured in six replications.

Color analysis

The instrumental color analysis was performed with the CR-400 Chroma Meter (Konica Minolta, Ramsey, USA). The values of L*, a* and b* color parameters were measured. Individual ice cream samples were placed in a Petri dish. The device was calibrated before the test using the white and black standard included in the measurement set. Sample temperature was equal to the ambient temperature (20±1°C). The color of each ice cream sample was measured in six replications.

Sensory analysis

The sensory attributes of ice cream samples were analyzed in a dedicated laboratory by sensory profiling using a five-point descriptive scale, according to ISO Standard method [EN ISO 13299:2016]. The evaluation was carried out by a panel of eight suitably trained persons with the appropriate sensory sensitivity, according to ISO Standard method [EN ISO 8586:2014]. Before the main sensory test, three 2-h sessions were conducted for the panelists with the use of commercial ice cream, milk, cream, milk powder, and flavor substances. Each sample was coded using a three-digit random number and served in 100 mL transparent hinged-led propylene containers. During the sensory analysis, each of the panelists was provided with a definition card and a product evaluation card. The cards contained a total of 28 attributes. Each attribute was defined. Individual sensory attributes were assessed using a five-point descriptive scale from 1 to 5. The intensity of a given sensory attribute was tested, where 1 denoted the absence of a given attribute, and 5 denote attribute of extreme intensity. The sensory attributes of ice cream samples were analyzed once after 14 days of storage.

Statistical analysis

The results were verified for normal distribution and homogeneity of variance. In physio-chemical analyses, the significance of differences between means was analyzed by Tukey's test and the interactions between factors (storage time, addition level and structure of β-glucans, and the interactions between factors) were determined by two-way ANOVA. At this stage, data were presented as means ± standard deviation. The experiment was performed in duplicate. The results of the sensory evaluation were analyzed using one-way ANOVA. Fisher's LSD tests were carried out to determine statistical differences between mean values within individual attributes. All results were processed in Statistica 13.5 PL software (Statsoft 2017, Krakow, Poland) at 0.05 significance level.

RESULTS AND DISCUSSION

In frozen dairy desserts, the sensory attributes and, consequently, the consumer acceptability of the final product are determined by fat content, the distribution of fat in the product, but also by the functional additives used during production. Other research studies have demonstrated that the application of inulin or oligofructose [Akalin & Erisir, 2008; Karaca *et al.*, 2009] significantly increased ice cream aeration.

The average overrun was determined at 63.53% in the control ice cream and increased significantly by ~17% in the samples containing 0.5% of β-glucans. Higher overrun resulted from an increase in the viscosity of the ice cream mix as well as the fact that the minimal boundary that guarantees the system's stability and the production of a thin film between air bubbles was exceeded. The system was additionally stabilized by the presence of whey proteins and β-glucans that decreased its surface tension [Burkus & Temelli, 2000]. In fresh ice cream, the overrun decreased (approximately 4%) when the content of β-glucans increased from 0.5% to 1%, but the changes were insignificant. Storage time had no significant influence on overrun ($p>0.05$) (Table 1). Despite the above, an increase in overrun is not identical for all polysaccharides. The addition of guar gum and basil seed gum [Javidi *et al.*, 2016] in ice cream production decreased aeration. The above differences could be attributed to variations in the structure and the functional attributes of the analyzed additives, including their hygroscopic properties.

The use of the β-glucans additive resulted in a significant ($p<0.001$) change in the melting time of ice cream (Table 1). In fresh ice cream with 0.5% addition of β-glucans, a significant (about 13 min) shortening of ice cream melting time was observed. A reduction in the melting time was also found in ice-cream with 1% of β-glucans. However, these changes were not significant. A significant increase in the melting time of ice cream was found in ice cream with 0.5% of (1–3)(1–4) β-glucan and 1% of (1–3) β-glucan after storage. The change of melting time during storage was most probably caused by slow absorption of free water by polysaccharide and formation of a more stable polysaccharide network. Overrun, the type of applied ingredients, the size of ice crystals, fat content, and the degree of fat destabilization influence the thermal stability of ice cream and, consequently, its melting time [Rezaei *et al.*, 2015]. Similar results were obtained in a study by Dymitrów *et al.* [2012] where the ice cream melting time increased with a decrease in fat content and a decrease in fat destabilization [Bolliger *et al.*, 2000]. Abdel-Haleem & Awad [2015] also observed that the application of barley (1–3)(1–4) β-glucan in the production of low-fat ice cream decreased the melting time, which was attributed to a higher ice cream freezing temperature. The application of maltodextrin and polydextrose in the ice cream production process also increased the melting time [Roland *et al.*, 1999]. Contrary results were reported by Florowska *et al.* [2013], where the addition of inulin prolonged ice cream melting. The above authors suggested that prolonged melting was associated with the water-binding capacity of polysaccharide molecules. It should also be noted that Florowska *et al.* [2013] analyzed ice cream samples with 20% fat content and 16.5% sugar content. However, the melting time of ice cream is more likely to be influenced by the product's chemical composition and the above factors than the hygroscopic properties of hydrocolloids.

Firmness is yet another structural attribute of ice cream. The texture analysis demonstrated that ice cream firmness was significantly ($p<0.001$) determined by the structure and content level of β-glucans (Table 1). The 0.5% addition of (1–3)(1–4) β-glucan led to a significant decrease in ice cream firmness by 45%. Firmness increased by an additional 102% in the samples containing 1% of (1–3) β-glucan in comparison to ice cream containing 0.5% of (1–3) β-glucan.

TABLE 1. Overrun, melting time, rheological, and texture parameters of ice cream with different β-glucan contents.

Structure	Storage time	Ice cream	β-Glucan content	Rheological properties			Texture parameters				Overrun	Melting time for 30 g of product
				Consistency index (K)	Flow behavior index (n)	Yield stress (tau 0)	Consistency	Firmness	Viscosity index	Cohesiveness		
	(day)		%	(Pa·s^{n})			(g·s)	(g)	(g·s)	(g)	(%)	(min)
(1–3) (1–4) β-Glucan	1	Control (FF)	0	1.37±0.10^{b}	0.73±0.03^{a}	6.61±1.04^{a}	9257±307^{a}	1418±106^{a}	-1080±90^{a}	-168±12^{b}	63.53±3.61^{b}	35.45±3.24^{a}
		Experimental (LF)	0.5	1.02±0.07^{Ac}	0.72±0.04^{Aa}	0.31±0.07^{Ab}	5518±41^{Ab}	782±58^{bA}	-501±3^{Ab}	-132±11^{Ac}	73.45±2.07^{a}	22.05±0.99^{b}
			1	2.18±0.01^{Aa}	0.65±0.00^{Ab}	0.31±0.00^{Ab}	9020±18^{Aa}	1403±154^{Aa}	-1140±106^{Aa}	-267±20^{Aa}	69.94±1.71^{ab}	29.91±3.79^{ab}
	14	Control (FF)	0	1.46±0.16^{b}	0.64±0.01	9.77±1.18^{a}	8377±433	1919±210^{a}	-896±19^{ab}	-195±0	63.54±0.70^{c}	35.47±3.23^{b}
		Experimental (LF)	0.5	1.32±0.02^{Ac}	0.67±0.00^{A}	0.48±0.00^{Ab}	6881±1192^{A}	808±57^{Ab}	-875±72^{Ab}	-177±18^{A}	73.61±0.84^{a}	48.74±3.06^{Aa}
			1	2.43±0.38^{Aa}	0.63±0.03^{A}	0.00±0.00^{b}	8981±1973^{A}	1141±296^{Ab}	-1039±81^{Aa}	-213±24^{A}	69.12±1.50^{b}	26.43±3.36^{Ac}
(1–3) β-Glucan	1	Control (FF)	0	1.37±0.10^{c}	0.73±0.03^{a}	6.61±1.04^{a}	9258±307^{b}	1418±106^{b}	-1080±90^{a}	-168±12^{a}	63.53±3.61^{b}	35.45±3.24^{a}
		Experimental (LF)	0.5	15.09±0.96^{Bb}	0.31±0.01^{Bb}	0.00±0.00^{Bb}	15498±400^{Bc}	1585±89^{Bb}	-1385±16^{Bb}	-270±30^{Bb}	75.61±1.95^{a}	23.52±3.61^{b}
			1	45.41±2.17^{Ba}	0.23±0.01^{Bc}	0.00±0.00^{b}	25356±1596^{Ba}	3205±274^{Ba}	-2395±8^{Bc}	-434±1^{Bc}	71.67±0.80^{a}	30.58±1.65^{a}
	14	Control (FF)	0	1.46±0.16^{c}	0.64±0.01^{a}	9.77±1.18^{a}	8377±433^{b}	1919±210^{b}	-896±19^{b}	-195±0^{c}	63.54±0.70^{c}	35.47±3.23^{a}
		Experimental (LF)	0.5	17.54±1.22^{Bb}	0.24±0.05^{Bb}	0.00±0.00^{Bb}	15640±75^{Ba}	2024±10^{Bb}	-1711±5^{Ba}	-235±10^{Bb}	74.05±1.21^{b}	23.52±3.61^{Bb}
			1	1936.65±5.44^{Ba}	0.01±0.00^{Bc}	0.00±0.00^{b}	17355±2075^{aB}	2695±167^{Ba}	-1694±135^{Ba}	-344±10^{Ba}	69.46±0.92^{a}	35.02±1.62^{Ba}
ANOVA	Content (C)			≤0.001	≤0.001	<0.001	≤0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Structure (S)			≤0.001	≤0.001	>0.05	≤0.001	<0.001	<0.001	<0.001	>0.05	0.008
	Time (T)			≤0.001	≤0.001	0.002	≤0.001	>0.05	0.002	0.013	>0.05	<0.001
	C·S·T			≤0.001	≤0.001	>0.05	≤0.001	>0.05	0.003	0.012	>0.05	<0.001

FF – Full fat; LF – Low fat. The values represent mean and standard deviation for n=3 (only texture parameters n=6). Each time is calculated separately. ^{abc}Mean values in a column with different superscript letters are significantly different (p≤0.05) – factor content. ^{AB}Mean values in a column with different superscript letters are significantly different (p≤0.05) – factor structure.

TABLE 2. Color (L*, a*, b*, ΔE) characteristics of ice cream.

Ice cream	β-Glucan structure	β-Glucan content (%)	Color analysis			
			*L	*a	*b	ΔE
Control (FF)		0	88.44 ± 0.51^{a}	-2.95 ± 0.12^{a}	12.44 ± 0.39^{a}	0
Experimental (LF)	(1–3)(1–4)	0.5	85.97 ± 0.97^{b}	-2.87 ± 0.17^{Aa}	7.82 ± 1.21^{Bc}	5.24
		1	83.27 ± 0.31^{Bc}	-3.59 ± 0.07^{b}	10.12 ± 0.49^{b}	5.71
	(1–3)	0.5	85.70 ± 0.14^{b}	-3.46 ± 0.09^{Bb}	12.05 ± 0.31^{A}	2.82
		1	85.40 ± 0.25^{Ac}	-3.76 ± 0.05^{c}	11.83 ± 0.47	3.21

The values represent mean and standard deviation for n=3. ^{abc} Mean values in column with different superscript letters are significantly different ($p\leq0.05$) – factor content. ^{AB} Mean values in column with different superscript letters are significantly different ($p\leq0.05$) – factor structure. Legend: FF – full fat; LF – low fat; L* – brightness, values between 0 (black) and 100 (white); a* – value between -120 (red) and +120 (green) on the red and green; b* – value between -120 (blue) and +120 (yellow) on the blue and yellow; ΔE – total colour difference.

Like in the current study, the addition of inulin (2–9%) to ice cream in low-fat ice cream decreased firmness, which was attributed to the higher overrun of ice cream and inulin's higher water absorption capacity and lower freezing temperature [Bahramparvar & Tehrani, 2011]. Contrary results were reported by Javidi *et al.* [2016] and Soukoulis *et al.* [2010], where the addition of other hygroscopic fat replacers, including basil seed gum, guar gum, fructooligosaccharides, and maltodextrin, to ice cream increased their firmness. The application of β-glucans with a linear structure increased the melting time and aeration, decreased elasticity, and increased instrumentally measured firmness relative to the samples containing β-glucans with a branched structure. ANOVA analysis revealed that consistency and the viscosity index were significantly determined by the structure and content level of the added β-glucans. The samples containing (1–3) β-glucan were characterized by a higher viscosity index, which was proportional to the content of the applied additive. Our results corroborate the findings of other authors [Hu *et al.*, 2015]. In contrast, the 5% addition of modified starch caused a significant decrease (by approx. 80%) in the viscosity index of ice cream. The consistency changed significantly ($p\leq0.001$) by -40% and +67% after the addition of 0.5% of (1–3)(1–4) and (1–3) β-glucans relative to the control ice cream. In ice cream containing 1% of (1–3) β-glucan, the consistency increased significantly by 173% in comparison with the control ice cream (Table 1).

The analysis of rheological models demonstrated that the Herschel-Bulkley model was characterized by the highest coefficient of determination ($0.99\leq r^2\leq0.999$). The rheological analysis and the back-extrusion tests revealed that the content level and structure of β-glucans, as well as storage time significantly ($p<0.001$) influenced the consistency index of ice cream. The 1% addition of (1–3)(1–4) β-glucan contributed to a significant ($p\leq0.05$) increase (by 60% and 66% in fresh and 14-day stored, respectively) in the consistency index. In fresh samples containing 0.5% and 1% of (1–3) β-glucans, the consistency index increased significantly ($p<0.001$) by 1100% and 3315%, respectively, relative to the control ice cream. The flow behavior index was significantly affected by the structure, content of β-glucan as well as storage time. The 1% addition of (1–3) β-glucan decreased the flow behavior index by -69% ($p<0.001$) relative to the fresh control ice cream (0.734) and by -12% relative to the samples containing (1–3)(1–4) β-glucan. The consistency index increased with an increase in β-glucans content, and similar results were reported by Simsekli & Dogan [2015]. β-Glucans and other polysaccharides bind water molecules, which form small crystals. During the production process, crystals form larger aggregates that absorb water and increase the viscosity index of the ice cream. As a result, the viscosity of low-fat ice cream is equal to higher than that noted in full-fat ice cream [Akalin & Erisir, 2008; Karaca *et al.*, 2009, Rezaei *et al.*, 2015]. The higher value of the consistency index in ice cream containing (1–3) β-glucan also resulted from differences in the β-glucan structure. Unlike (1–3) β-glucan, (1–3)(1–4) β-glucan (similarly to inulin) induces the formation of water gels with considerable viscosity, but lower firmness [Stone, 2009].

Regardless of their level, β-glucans caused significant differences in the color of all experimental samples (Table 2). In comparison with the control ice cream, samples with the addition of (1–3)(1–4) and (1–3) β-glucans were characterized by higher values of ΔE, determined at 5.24% and 2.82% in ice cream containing 0.5% of the additive, respectively, and at 5.71% and 3.21% in ice cream containing 1% of the additive. The addition of β-glucans resulted in significant changes in ice creams color, including decreased values of a* (the exception was ice cream with 0.5% of (1–3)(1–4) β-glucan). Only the (1–3)(1–4) β-glucan caused a significant decrease in the *b parameter. In other studies, the application of soy protein isolate [Akesowan, 2009] and citrus fiber [Dervisoglu & Yazici, 2006] also induced changes in the value of a* (greenness). The value of L* was higher in the control sample than in the experimental samples (containing β-glucans). The decrease in the lightness L* of the experimental ice cream resulted from the application of β-glucans. However, it could also be caused by the interactions between ice cream ingredients (mainly sugars and amino acids), which formed dark pigments (melanoidins) during thermal processing (preparation and pasteurization of the mixture) [Akesowan, 2009] and were undetectable to the naked eye.

Five groups of attributes were evaluated in the sensory analysis of ice cream: appearance, aroma, texture, mouthfeel, and taste. The structure and content level of β-glucans had no significant ($p>0.05$) influence on color intensity or color uniformity of ice cream. Undesirable sensory attributes of appearance, such as discoloration or atypical color, were not observed in any of the examined samples (Table 3). The digital image analysis revealed minor ($p\leq0.05$) changes in color, but these changes were too small to be identified by the panelists during the sensory analysis. Similar observations were made by Surapat & Rugthavon [2003] in whose study, the application of modified starch did not change the color of ice cream. In an experiment conducted by Abdel-Haleem & Awad [2015], the color of the experimental ice cream did not change after the addition of 0.4% of barley β-glucan. The above authors also demonstrated that the 0.4% addition of barley β-glucan and 1% and 2% addition of hulless barley flour to low-fat ice cream did not significantly alter its aroma. In the present study, a reduction in the fat content of ice cream decreased the intensity of the milky or sweet aroma. The milky aroma was most distinctive in the control sample and the sample containing 0.5% of (1–3)(1–4) β-glucan. A moderately perceptible milky aroma characterized the remaining samples. The sweet aroma was less intense in ice cream with the addition of (1–3) β-glucan than in the remaining samples ($p\leq0.05$). Atypical aromas were not detected. The applied β-glucans and other polysaccharides can mask the characteristic milky aroma, but this effect is determined by the content level and purity of the applied additive.

TABLE 3. Mean values of sensory attributes of ice cream with an addition of β-glucans.

Ice cream		Control (FF)	Experimental (LF)				p-Values
β-Glucan structure			(1–3)(1–4) β-Glucans		(1–3) β-Glucans		
β-Glucan content		0%	0.5%	1%	0.5%	1%	
Sensory attributes by group							
Appearance	Color intensity	2.1	2.3	2.4	2.4	2.5	>0.05
	Atypical color	1.0	1.0	1.0	1.0	1.0	>0.05
	Color uniformity	4.1	4.1	4.1	4.1	4.1	>0.05
	Discoloration	1.0	1.0	1.0	1.0	1.0	>0.05
Aroma	Milky	3.1^{a}	3.1^{a}	2.4^{b}	2.4^{b}	2.4^{b}	0.029
	Sweet	2.5^{a}	2.4^{a}	2.3^{a}	2.1^{ab}	1.8^{b}	0.032
	Atypical	1.0	1.0	1.0	1.0	1.0	>0.05
Texture	Firmness	3.3	3.1	3.4	3.4	3.3	>0.05
	Sandiness	1.8	2.0	1.8	1.8	1.8	>0.05
	Ropiness	1.8^{b}	1.8^{b}	2.3^{ab}	2.4^{a}	2.8^{a}	0.002
	Fluffiness	3.0	2.3	2.6	2.4	2.6	>0.05
	Texture uniformity	3.6	3.6	3.6	3.6	3.5	>0.05
	Sogginess	1.8^{b}	2.3^{ab}	2.5^{a}	2.5^{a}	2.8^{a}	0.011
	Creaminess	2.1^{b}	2.6^{ab}	3.0^{a}	3.1^{a}	3.1^{a}	0.027
Mouthfeel	Melting	3.1	3.5	3.0	2.9	2.9	>0.05
	Greasiness	2.5	2.6	2.6	3.0	3.1	>0.05
	Firmness	2.5	2.3	2.3	2.3	2.6	>0.05
	Gumminess	1.0^{c}	1.0^{c}	1.0^{c}	2.3^{b}	2.8^{a}	0.000
	Ice crystallization	1.0	1.0	1.0	1.0	1.0	>0.05
	Wateriness	2.4	2.6	2.3	2.3	2.3	>0.05
	Mouth coating	1.0^{c}	1.9^{b}	2.4^{b}	2.5^{b}	3.5^{a}	0.000
	Viscosity	1.8^{c}	2.0^{c}	2.3^{bc}	2.8^{b}	3.5^{a}	0.000
Taste	Milky	3.8	3.1	2.9	3.0	3.0	>0.05
	Creamy	3.1	3.1	3.0	2.9	2.6	>0.05
	Powdered milk	3.8	3.6	3.5	3.5	3.8	>0.05
	Sweet	3.8	3.9	3.9	3.9	4.1	>0.05
	Cooked	2.3	2.3	2.1	1.9	1.8	>0.05
	Atypical taste	1.0	1.0	1.0	1.0	1.0	>0.05

FF – Full fat; LF – Low fat. ^{a,b,c}– Mean values in a row with different letters are different at $p\leq0.05$. Scale from 1 to 5, where 1 denoted the absence of a given attribute, and 5 denoted attribute of extreme intensity.

The most significant changes in the sensory attributes of the analyzed ice cream were noted in the analysis of texture and mouthfeel. A statistical analysis of mouthfeel revealed differences in three out of the eight evaluated descriptors. The addition of (1–3) β-glucan led to a significant increase in the gumminess and viscosity of ice cream. The relevant increase was higher in the samples containing 1% of β-glucan than in the samples containing 0.5% of β-glucan. Mouth coating, described as a sensation of having a slick coating on the tongue and other mouth surfaces, was most intense in the sample containing 1% of bacterial β-glucan. The addition of β-glucans had no significant effect on the taste of ice cream. The texture analysis revealed that the evaluated ice cream samples did not differ significantly in firmness, sandiness, fluffiness, or uniformity ($p>0.05$). The control sample was characterized by the lowest ropiness, sogginess and creaminess. The addition of β-glucans positively affected the creaminess of consistency. The application of (1–3) β-glucan resulted in higher ropiness in comparison with other samples. Regardless of its addition and structure, β-glucans increased sogginess in all experimental samples. Creaminess generally increases in response to the use of stabilizers [Bahramparvar & Tehrani, 2011], and it is associated with a decrease in the size of polysaccharide crystals that interact and produce a fatty mouthfeel [Franck, 2002]. In turn, ropiness and sogginess increase with a rise in viscosity that accompanies the application of β-glucans. Similar results were reported in the rheological analyses carried out by Florowska *et al.* [2013]. Ice cream viscosity increased with a rise in the content level of β-glucans, which is consistent with the findings of Soukolis *et al.* [2009]. The increase in viscosity also contributed to the presence of mouth-coating. The observed increase in gumminess resulted from the application of a higher content level of the stabilizer [Karaca *et al.*, 2009], in particular in ice cream with 1% addition of (1–3) β-glucan. The applied β-glucans did not led to significant changes in ice cream taste, and they conformed to the requirements imposed on food stabilizers [Kilara & Chandan, 2008]. However, higher sensory viscosity can eliminate the application of bacterial β-glucans from commercial ice cream production. On the other hand, an increase in the creaminess of ice cream containing β-glucans and other polysaccharides increase the application of these additives as fat replacers [Karaca *et al*., 2009]. Nevertheless, consumer acceptance of increased viscosity, gumminess, and creaminess of ice cream produced with the addition of β-glucans should be tested before these additives are applied (Table 3).

CONCLUSIONS

The results of this study indicate that the functional properties of ice cream are influenced by the structure and content level of the β-glucans. The use of glucans resulted in shortening the melting time of ice cream and increasing its aeration. Compared to (1–3)(1–4) β-glucan, (1–3) β-glucan significantly affects hardness, viscosity index, and cohesion. Similarly, significant differences in rheological properties (consistency index, flow behavior index) and sensory attributes were observed between the ice cream produced with the addition of (1–3) β-glucan with a linear structure and (1–3)(1–4) β-glucan with a branched structure. More significant variations in the above parameters were noted in the samples containing 1% of β-glucans than in those containing 0.5% of β-glucans. The application of (1–3)(1–4) β-glucan isolated from oats supported the production of low-calorie ice cream with functional properties that most closely resembled the control ice cream (full-fat, with stabilizing substances). The sensory attributes of ice cream containing (1–3)(1–4) β-glucan were also more similar to those of control ice cream.

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Original research article
Food Chemistry Section

Impact of the Encapsulation Process by Spray- and Freeze-Drying on the Properties and Composition of Powders Obtained from Cold-Pressed Seed Oils with Various Unsaturated Fatty Acids

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Key words: cold-pressed oils, encapsulation, tocopherols, sterols, fatty acids

The aim of this study was to determine the influence of encapsulation methods on fatty acid composition and content of bioactive compounds in cold-pressed oils. Rape, flax, and safflower seed oils (10.2%) were mixed with water (70.0%) and wall components (19.8%) to obtain emulsions, which were then subjected to spray- and freeze-drying. Surface and total oil contents, and changes in contents of fatty acids, sterols, and tocopherols were compared in powders and natural oils.

The spray-drying was a more effective encapsulation method compared to the freeze-drying. Fatty acid composition of the oils was quite stable during the encapsulation process. Sterol degradation was high and only 35–40% of these compounds were determined in powders. In turn, tocopherol losses were dependent both on the encapsulation method and oil type. The encapsulation by freeze-drying allowed the retention of almost all tocopherols of cold-pressed flax and safflower seed oils.

INTRODUCTION

Cold-pressed plant oils are used mainly by the pharmaceutical industry as a raw material for the production of dietary supplements, while their application is limited in the food industry. These oils constitute, first of all, an element of "slow food" [Miele & Murdoch, 2002], which is due to their poor storage stability, related to a high content of polyunsaturated fatty acids, like particularly in flax and safflower seed oils [Dorni *et al.*, 2018; Khattab & Zeitoun, 2013; Tańska *et al.*, 2016].

Cold-pressed oils obtained from such raw materials as rape, flax or safflower seeds are considered to be valuable edible fats from a nutritional point of view. Their value is a result of, among others, a high content of unsaturated fatty acids, oleic acid in rape seed oil (up to 60%), linoleic acid in safflower seed oil (up to 80%), and α-linolenic acid in flax seed oil (up to 60%) [Ghazani *et al.*, 2014; Khalid *et al.*, 2017; Tańska *et al.*, 2016]. A beneficial effect of fatty acids of the rape seed oil on the blood lipid profile consists in reducing the concentration of total cholesterol and the low-density lipoprotein (LDL) fraction, without any effect on the concentration of the high-density lipoprotein (HDL) fraction [Nguemeni *et al.*, 2010]. Additionally, results obtained by Nguemeni *et al.* [2010] support the hypothesis that diet supplementation with rape seed oil can enhance the treatment of ischemic stroke. α-Linolenic acid, which belongs to the *n*-3 family (flax seed oil is a good source of this acid), may help protect against certain infections and in treating conditions including ulcers, migraine headaches, attention deficit/hyperactivity disorder, eating disorders, preterm labour, emphysema, psoriasis, glaucoma, Lyme disease, lupus, and panic attacks [Harper *et al.*, 2006]. The health-promoting properties of the above-mentioned cold-pressed oils are also affected by the native bioactive components they contain, such as: sterols (up to 835 mg/100 g in rape seed oil), tocopherols (up to 60 mg/100 g in flax seed oil), carotenoids (up to 28 mg/100 g in rape seed oil), squalene (up to 4 mg/100 g in flax seed oil), and polyphenols (up to 14 mg/100 g in safflower seed oil) [Ben Moumen *et al.*, 2015; Ghazani *et al.*, 2014].

The use of plant oils in the food industry can be increased by the application of the encapsulation process, which allows extending the oxidative stability of the core of the capsule and guarantees the controlled release of substances from the capsule during the technological process [Aksoylu & Günç Ergönül, 2017]. The most popular method of oil encapsulation is the spray-drying. It is considered to be an optimal solution for oils because it allows for the transformation of emulsion into powder, simultaneously preserving its valuable properties. Because of the large surface area of the droplets, water evaporates almost immediately and the droplets are transformed into particles. Another popular method used

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to prepare powdered oils is encapsulation by freeze-drying. It requires a much longer time but can be done at a very low temperature. During this process, water is frozen, followed by its removal from the sample, initially by sublimation (primary drying) and then by desorption (secondary drying) [Ogrodowska *et al.*, 2017].

Oil in a powdered form can be applied as a functional supplement to dairy products [Wang *et al.,* 2011], bakery products [Gallardo *et al.,* 2013], and fruit and vegetable products [Donsì *et al.,* 2011]. Although encapsulated oils are easier to apply in food products and more resistant to oxidative changes than the cold-pressed oils, there are still few studies that explain the effect of the encapsulation on the native oil components such as unsaturated fatty acids and bioactive compounds.

Our previous work [Ogrodowska *et al.*, 2019] showed that the encapsulation by spray- and freeze-drying had a significant impact on the content of bioactive compounds and oxidative stability of encapsulated bio-oils from evening primrose, borage, and blue weed seeds. We showed that despite the lower process efficiency, the encapsulation by freeze-drying would be more recommended for evening primrose seed oil. In turn, the encapsulated borage and blue weed oils should be produced by spray-drying. We hypothesized that the differences observed were connected to the initial chemical composition of the bio-oils used. Little is known, however, on this kind of a relationship. Generally, previous studies have focused on the physical properties of powders obtained with different encapsulation methods or under different process conditions [Bakry *et al.*, 2016].

In this work, we compared the influence of the most popular encapsulation methods (spray- and freeze drying) on the effectiveness of the encapsulation process and losses of sterols and tocopherols in powders obtained from cold-pressed oils with various unsaturated fatty acid composition.

MATERIALS AND METHODS

Materials

Three types of cold-pressed plant oils were investigated in this study. Seeds of rape, flax, and safflower were from "Szarłat" company (Łomża, Poland). The oils were produced by pressing the raw material on a IBG Monforts & Reiners, Komet CA59G (Nürnberg, Germany) laboratory expeller equipped with a nozzle gaving 4 mm in diameter. The oils were purified by centrifugation on a centrifuge (type 5810R, Eppendorf AG, Hamburg, Germany) operated at 15,000×*g*. Whey protein concentrate was purchased from"Ostrowia" company (Ostrów Mazowiecka, Poland). Maltodextrin (DE 14–22) and guar gum were purchased from Edpol Food & Innovation Company (Łomża, Poland).

Emulsification process

The composition of emulsions was the same for all samples: 19.8% (*w/w*) of wall materials (maltodextrin 15.4%, whey protein concentrate 3.9%, and guar gum 0.5%), 10.2% (*w/w*) of oil, and 70% (*w/w*) of water. The proportion of wall materials and oil loading were established based on the work of Ogrodowska *et al.* [2017]. The oils were blended with aqueous solutions of wall materials at 9,000 rpm for 120 s at 40°C using Thermomix (Vorwerk, Wuppertal, Germany). Then, emulsions were further homogenized at 240 bar (I step) and 40 bar (II step) using a high-pressure laboratory valve homogenizer (Panda 2K, GEA Niro Soavi, Parma, Italy).

Emulsion drying process

The spray-dried powders were prepared using a pilot-plant spray dryer (A/S Niro Atomizer, Copenhagen, Denmark) with disc having 110 mm in diameter as a spraying mechanism at 6400 rpm. The emulsion was pumped into a spray dryer chamber at a constant feeding speed. The inlet temperature was set at 130°C, whereas the outlet temperature at 90°C. The feed flow rate was 77 mL/min and the air flow rate was approx. 400 kg/h.

Freeze-dried powders were prepared using a lyophilizer (Cryolizer Freeze Dryer type B-64, New Brunswick Scientific Co., Inc., New Brunswick, NJ, USA). The emulsions were put into aluminium pans and frozen at -20°C for 24 h. Then, the emulsions were lyophilized, with an ice condenser set at -50°C at a pressure of 0.12 mbar and freeze-drying time reaching 72 h.

Physical characterization of emulsions

Immediately after preparation, 50 mL of an emulsion sample was transferred to a graduated cylinder of 50 mL, sealed, and stored at 25°C for 24 h. The emulsion stability was evaluated by the phase separation observed throughout 24 h and expressed as % of upper phase separation.

The viscosity of the emulsions was measured using a Brookfield RV DV–II+ Pro Extra rheometer (AMETEK Brookfield, Middleboro, MA, USA) equipped with an RV-3 stainless steel spindle (disc with a diameter of 34.69 mm and a height of 1.65 mm). Measurements (100 mL of emulsion) were done at 25°C by a linear increase in shear stress from 0.01 to 1.64 Pa. Viscosities are given as the average viscosity of the linear part of the plot of shear stress *versus* viscosity and are expressed in mPa·s.

Droplet size of emulsions was measured using a Mastersizer 3000 particle size analyzer (Malvern Instruments, Ltd., Worcestershire, UK). Emulsion droplets were suspended in recirculating water (3,000 rpm), reaching an obscuration of 15–18%. Measurements were carried out at 25°C and emulsion droplet size was expressed as Sauter mean diameter D(4,3).

Characterization of encapsulation process effectiveness

The content of surface oil was determined in the oils with the washing method [Ogrodowska *et al.,* 2017]. The surface oil was extracted three times with hexane for 5 min, filtered, and evaporated to dryness in the R-210 type rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) under an N_2 stream.

The total oil content in the powders was determined according to the method described by Ogrodowska *et al.* [2017]. The total oil was extracted with a 2:1 (v/v) chloroform:methanol mixture, then the chloroform phase was isolated and evaporated to dryness in the R-210 type rotary evaporator (Büchi Labortechnik AG) under an N_2 stream.

The process efficiency (PE) of encapsulated oil production was determined as the ratio between the oil covered by wall material and the total oil content in the powder [Kaushik *et al.,* 2015]. The content of oil covered by the wall material was computed from the difference between total oil content and surface oil content in the powder whose values were determined as described above.

Morphological characterization of powders

The morphology of the powders was analyzed using scanning electron microscopy (SEM). The Quanta 200 SEM (FEI Company, Hillsboro, OR, USA) was applied to acquire images of the powder samples. Each powder sample was placed on double-sided adhesive carbon tabs, mounted on SEM tubs, and coated with palladium in a sputter coater. Then, the coated sample was analyzed using the SEM operating an accelerating voltage of 30 kV with 800× (spray-dried sample) or 100× (freeze-dried sample) magnifications.

Fatty acid composition of oils and powders

Fatty acid composition was analyzed in the total oil extracted with a 2:1 (*v/v*) chloroform:methanol mixture.

Fatty acid esters were prepared according to the procedure described in a work of Zadernowski & Sosulski [1978]. The fatty acids were methylated by heating their solution in a chloroform:methanol:sulfuric acid mixture (100:100:1, *v/v/v*) at 70°C for 2 h. Fatty acids methyl esters were analyzed using a GC-MS QP2010 PLUS system (Shimadzu, Kyoto, Japan) equipped with a BPX70 capillary column (25 m × 0.22 mm × 0.25 μm; SGE Analytical Science, Victoria, Australia). Helium was used as the carrier gas with a flow rate of 0.9 mL/min. The temperatures were as follows: the injector – 230°C, the column was programmed in the range from 150°C to 250°C, interface of GC-MS – 240°C, the ion source – 240°C, and the electron energy was 70 eV. Individual fatty acids methyl esters were identified on the basis of their retention times and mass spectra, and results were expressed as the percentage (*w/w*) of each fatty acid in total fatty acids based on their peak areas.

Content of bioactive compounds in oils and powders

Bioactive compounds were analyzed in the total oil extracted with a 2:1 (*v/v*) chloroform:methanol mixture.

The content of sterols was determined according to the procedure described in the work of Roszkowska *et al.* [2015]. The oil samples were dissolved in hexane, and a 5α-cholestane solution was added as an internal standard. The oil sample with the 5α-cholestane (internal standard) hexane solution was saponified in 2 M KOH in a methanolic solution at 70°C for 30 min. Sterols were extracted three times with diethyl ether. The collected extracts were evaporated to dryness in the R-210 type rotary evaporator (Büchi Labortechnik AG) under an N_2 stream. Then, the residue was re-dissolved in pyridine and *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% (*w/w*) trimethylchlorosilane (TMCS), and derivatized at 60°C for 60 min. The mixture was re-dissolved in heptane and analyzed using a GC-MS QP2010 PLUS system (Shimadzu, Kyoto, Japan) equipped with a ZB-5ms capillary column (30 m × 0.25 mm × 0.25 μm; Phenomenex, Torrance, CA, USA). Helium was used as the carrier gas with a flow rate of 0.9 mL/min. The temperatures were as follows: the injector – 230°C, the column was programmed in the range from 70°C to 310°C, the interface of GC-MS – 240°C, the ion source – 220°C, and the electron energy was 70 eV. The sterols were identified on the basis of their retention times and mass spectra. Quantitative analysis of individual compounds was made based on the internal standard method with the use of 5α-cholestane.

The content of tocopherols was determined according to the procedure described in the work of Czaplicki *et al.* [2011]. The oil samples diluted in hexane were analyzed using an Agilent Technologies 1200 series apparatus with a fluorescence detector (Santa Clara, CA, USA). Individual compounds were separated on a LiChrospher Si60 column (250 mm × 4 mm, 5 μm; Merck, Darmstadt, Germany) with the use of 0.7% (*v/v*) iso-propanol solution in *n*-hexane as a mobile phase and the flow rate set at 1 mL/min. The fluorescence detector was used with excitation and emission wavelengths of 296 nm and 330 nm, respectively. Quantitative analysis of individual tocopherols was done based on external calibration curves prepared for α-, γ-, and δ-tocopherol. The sum of β- and γ-tocopherols was calculated as γ-tocopherol.

Statistical analysis

The results obtained were analyzed statistically using Statistica 12.0 PL software (StatSoft, Kraków, Poland). The differences between mean values were determined using the analysis of variance (ANOVA) with Tukey's test at $p \le 0.05$ significance level.

RESULTS AND DISCUSSION

Characteristics of encapsulation effectiveness of cold-pressed oils

Table 1 presents the contents of surface oil and total oil, and process efficiency for cold-pressed oils encapsulated using the spray- and freeze-drying methods. The samples obtained as a result of spray-drying, regardless of oil type, had a much lower content of surface oil (2.91–5.30%, *w/w*) compared to those obtained by freeze-drying (10.25–10.76%, *w/w*). The total oil content in the powders obtained as a result of spray-drying ranged from 28.97% (*w/w*) for rapeseed oil to 29.85% (*w/w*) for safflower seed oil. In turn, the freeze-dried samples had by 1–3% lower content of total oil. The process efficiency for the powders obtained as a result of spray-drying ranged from 82.23% to 89.97% (*w/w*). A high content of surface oil and a similar content of total oil in the samples encapsulated by freeze-drying in comparison to those obtained by spray-drying resulted in the low efficiency of their encapsulation process (62.66–64.86%, *w/w*).

The results obtained correspond to the data presented by other authors. Gallardo *et al.* [2013], who spray-dried emulsions composed of flax seed oil and Arabic gum or its mix with maltodextrin and whey protein isolate, determined the surface oil and the total oil contents, as well as the process efficiency in the following ranges: 1.9–2.7% (*w/w*), 22.1–32.9% (*w/w*) and 87.8–91.4% (*w/w*), respectively. In turn, the use of maltodextrin and methyl cellulose for flax seed oil encapsulation brought about an increase in the surface oil content (24.5%, *w/w*), which resulted in a low PE (25.5%, *w/w*). The surface

TABLE 1. Surface and total oil contents, and efficiency of the encapsulation process of cold-pressed oils.

Type of cold-pressed oil	Emulsion drying method	Surface oil content (%, *w/w*)	Total oil content (%, *w/w*)	Process efficiency (%, *w/w*)
Rape seed oil	SD	2.91 ± 0.09^{bC}	28.97 ± 0.07^{aB}	89.97 ± 0.30^{aA}
	FD	10.57 ± 0.24^{aA}	28.64 ± 0.10^{bA}	63.08 ± 0.95^{bA}
Flax seed oil	SD	3.68 ± 0.03^{bB}	29.74 ± 0.03^{aA}	87.63 ± 0.25^{aB}
	FD	10.76 ± 0.40^{aA}	28.82 ± 0.54^{bA}	62.66 ± 0.68^{bA}
Safflower seed oil	SD	5.30 ± 0.17^{bA}	29.85 ± 0.58^{aA}	82.23 ± 0.92^{aC}
	FD	10.25 ± 0.47^{aA}	29.18 ± 0.18^{bA}	64.86 ± 1.40^{bA}

Mean values in columns, for each type of oil separately (lowercase letters) and for each emulsion drying method separately (uppercase letters), denoted by the same letter do not differ statistically significantly at p>0.05 (Tukey test). SD – spray-drying, FD – freeze-drying.

oil content (4.73–5.57%, *w/w*), similar to the values presented in our study, was also determined by Goyal *et al.* [2015], who used lactose, milk proteins, and sodium caseinate to encapsulate refined flax seed oil by spray-drying. In turn, Chang *et al.* [2016] obtained a broad range of surface oil contents for spray-dried canola oil: from 2.4% (*w/w)* (the coat containing lentil protein isolate, maltodextrin, and sodium alginate) to 11.5% (*w/w*) (the coat containing lentil protein isolate, maltodextrin, sodium alginate, and lecithin). Carneiro *et al.* [2013] reported that the encapsulation efficiency was significantly influenced by the type of wall material used. In their experiment, PE determined for flax seed oil encapsulated by spray-drying varied from 62.3% (sample coated with whey protein concentrate and maltodextrin) to 95.7% (*w/w*) (sample coated with Hi-Cap waxy maize and maltodextrin). On the other hand, Umesha *et al.* [2013] studied the influence of oil and wall material proportion in emulsion on characteristics of garden cress seed oil encapsulation by spray-drying. They found that an increase in the oil/wall material ratio in the range from 0.1 to 0.75 decreased the PE by increasing the surface oil content. Those authors obtained a significantly higher surface oil content (7.8–13.6%, *w/w*) and a lower PE (47.5–73.3%, *w/w*) for an oil/wall material ratio of 0.5, that is comparable to the present study. Also Tonon *et al.* [2012] showed a relationship between oil loading and encapsulation efficiency of flax seed oil. They suggested lower oil loading (10–40%, *w/w*) in the encapsulation by spray-drying than in the present study. In turn, Hasani *et al.* [2015] confirmed the influence of different wall materials on the freeze-drying efficiency. In their study, fish oil was encapsulated with PE ranging from 70.74% for a maltodextrin, sodium caseinate, whey protein concentrate, and modified starch (Hi-Cap 100) mixture to 88.55% (*w/w*) for a mixture without sodium caseinate. The review of different methods of encapsulation presented by Ruiz Ruiz *et al.* [2017] showed that the key parameter in any of these processes was the selection of wall material. They reported that protein or polysaccharides were primarily used as shell materials in the spray-drying method.

Although most studies indicate the strong impact of wall material composition and oil loading on the process efficiency and the surface oil content, the emulsification method and drying parameters will also have an effect on the encapsulation process [Aksoylu & Günç Ergönül, 2017; Heinzelmann *et al.,* 2000]. Nonetheless, literature data shows that PE decreased with an increasing surface oil content for both methods of powder production [Calvo *et al.*, 2011; Karaca *et al.*, 2013], as was noted in the present study.

As was pointed out in the present study, the oil samples encapsulated by freeze-drying had a higher surface oil content than the encapsulated by spray-drying. This phenomenon was also noticed by others researchers [Anwar & Kunz, 2011; Karaca *et al.*, 2013; Quispe-Condori *et al.,* 2011]. This is mainly due to the difference in the dehydration mechanism in these methods, which greatly affects the microstructure and integrity of the capsule wall. As a result of encapsulation by freeze-drying, the porous and irregular structure of flakes is observed (Figure 1). In particular, those particles of a larger size are easily destroyed, which can lead to powder structure opening and to oil leakage. Silva *et al.* [2016] explained that ice crystals are formed during the freezing stage preceding encapsulation, which contributes to the rupture of the emulsion droplets, thus releasing oil from the particle to the surface.

The results in Table 1 also show the effect of oil type on the encapsulation efficiency. In the case of encapsulation by spray-drying, the highest process efficiency was obtained for the rape seed oil, whereas the lowest was for the safflower seed oil. In turn, for freeze-drying, the safflower seed oil was slightly more effectively encapsulated than rape and flax seed oils. This property was strongly negatively correlated with the surface oil content. However, the total oil content differed only slightly among the samples (up to 4%) and the differences noted for each drying method separately were not statistically significant (p>0.05). Only sparse literature data address the encapsulation process of different oils under the same conditions. Shivakumar *et al.* [2012] found that safflower seed oil was effectively encapsulated by spray-drying at a higher percentage (up to 70% (*w/w*) total oil and 15% (*w/w*) free oil) compared to flax seed oil (up to 65% (*w/w*) of total oil and 19% (*w/w*) of free oil). In turn, Calvo *et al.* [2012] studied the influence of olive oil quality on the spray-drying effectiveness and observed no significant differences among monovarietal and blend-type extra-virgin olive oils. Mikulcová *et al.* [2017] studied the effect of oil type (refined and unrefined hemp seed oils) on the distribution of particles in an emulsion and showed that both oils performed similarly under high-intensity emulsification. The differences in surface oil content and process efficiency of the oil pow-

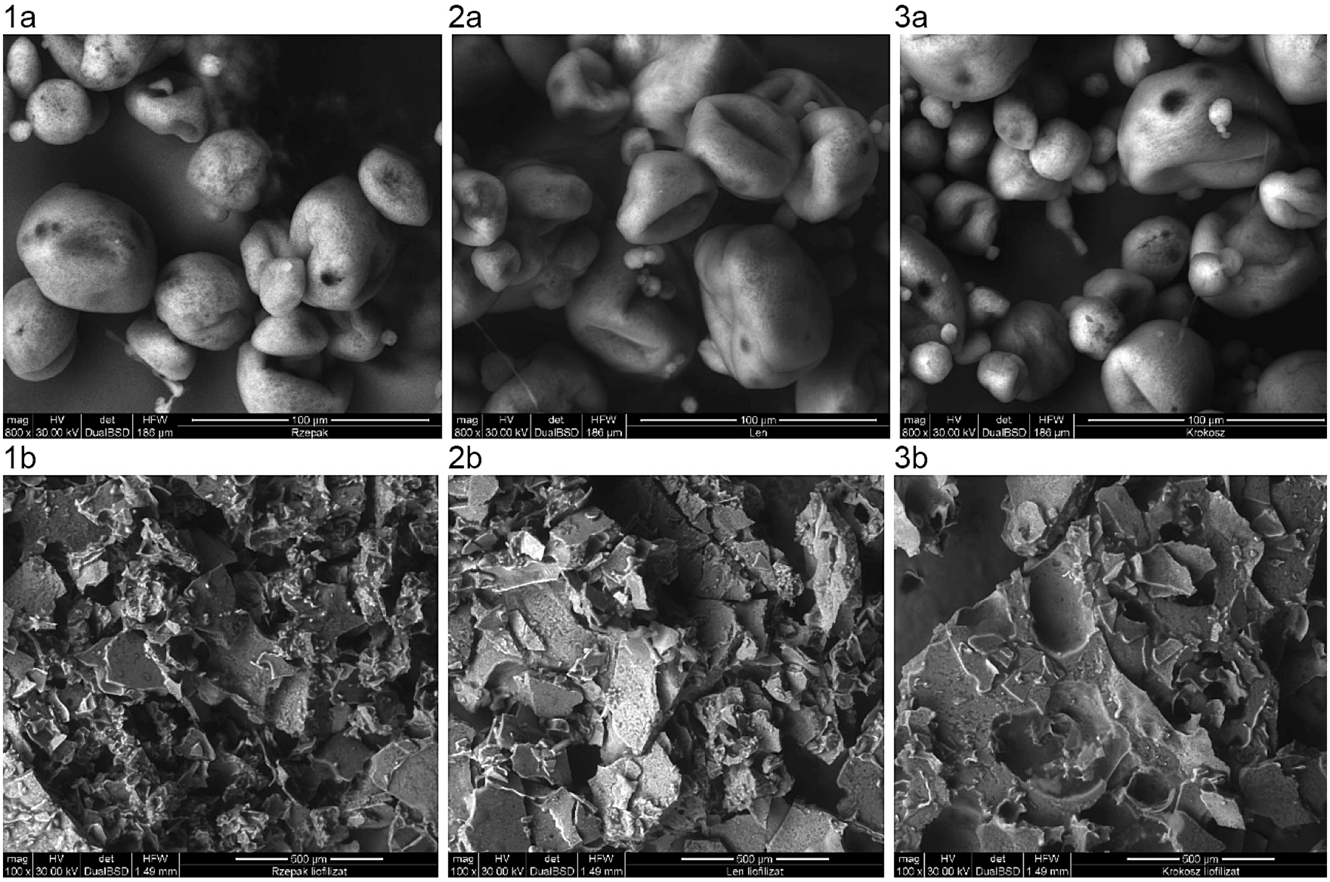

FIGURE 1. Scanning electron microscopy images of powders from cold-pressed rape (1), flax (2), and safflower (3) seed oils obtained by spry-drying (a, mag 800×) and freeze-drying (b, mag 100×) showing differences in powder morphology.

ders obtained by spray-drying were probably due to oil composition. The oils differ significantly in their fatty acid composition and unsaponifiable compounds content (including tocopherols), which could affect the emulsion droplet size and viscosity. Lindenstruth & Müller [2004] reported that the production process parameters (pressure and temperature) had a lesser influence on the emulsion properties than the composition of oil and surfactant phase. In their opinion, if a dispersed phase with small droplet sizes is needed, unsaturated components in the oil phase should be avoided. As shown by Tonon *et al.* [2012], the process efficiency decreased exponentially with droplet size and increased linearly with emulsion viscosity. This phenomenon was also observed in our study. It was noticed that the drying of the emulsion prepared with rape seed oil, which was characterized by a larger droplet size and greater viscosity (1.28 mPa·s), was more effective than the drying of emulsion with flax and safflower seed oils, whose emulsion viscosities were at 1.01 and 1.03 mPa·s, respectively (Table 2). This difference may be related to the fatty acid composition of the oils. Yalcin *et al.* [2012] reported that viscosity of the various vegetable oils depended on their fatty acid composition and increased as chain length and degree of saturation of fatty acids increased. Our results are in agreement with the finding from this study. Rape seed oil had the lowest amount of polyunsaturated fatty acids (PUFA) and the highest amount of monounsaturated fatty acids (MUFA) from all analyzed oils (Table 3). Badke *et al.* [2019] also found a correlation between encapsulation efficiency and the chemical composition of encapsulated oil. According to their suggestion, when the core material is highly hydrophobic, it is necessary to use surfactants which will increase the affinity of the core with the shell, enhancing the encapsulation efficiency. Barbosa *et al.* [2005] reported that the encapsulation efficiency depended also on the emulsion stability. In their study, the lower amount of non-encapsulated material on particles surface was obtained from a more stable emulsion. However, in the present study, the results obtained for the encapsulation efficiency were not affected by the emulsion stability. All emulsions were stable over the 24-h storage at room temperature, showing no phase separation (Table 2).

TABLE 2. Characteristics of basic parameters of emulsions obtained from cold-pressed oils.

Type of cold-pressed oil	Stability after 24 h (%, *v/v*)	Viscosity (mPa×s, *w/w*)	Droplet size D[4,3] (µm)
Rape seed oil	0.00 ± 0.01^a	1.28 ± 0.02^a	8.19 ± 0.34^a
Flax seed oil	0.00 ± 0.00^a	1.03 ± 0.01^b	4.58 ± 0.49^b
Safflower seed oil	0.00 ± 0.02^a	1.01 ± 0.01^b	4.37 ± 0.06^b

Mean values in columns, for each type of oil separately, denoted by the same letter do not differ statistically significantly at p>0.05 (Tukey test).

TABLE 3. Fatty acid composition of natural and powdered cold-pressed oils expressed as area percentage of each fatty acid in total area of all fatty acids and their changes after drying process.

Type of cold-pressed oil	Emulsion drying method	Fatty acids (area %)								
		Myristic C14:0	Palmitic C16:0	Stearic C18:0	Oleic C18:1 *n-9*	Linoleic C18:2 *n-6*	Linolenic C18:3 *n-3*	ΣSFA	ΣMUFA	ΣPUFA
Rape seed oil	-	n.d.	4.23±0.13^{b}	1.26±0.26^{b}	67.23±1.56^{a}	18.61±0.08^{a}	8.67±1.24^{a}	5.49±0.40^{b}	67.23±1.56^{a}	27.28±1.16^{a}
	SD	n.d.	5.09±0.06^{a}	1.92±0.04^{a}	68.28±0.58^{a}	17.31±0.43^{b}	7.41±0.25^{b}	7.01±0.10^{a}	68.28±0.58^{a}	24.71±0.68^{b}
		*n.d.**	*+20.3*	*+52.4*	*+1.6*	*-7.0*	*-14.5*	*+27.7*	*+1.6*	*-9.4*
	FD	n.d.	4.80±0.18^{a}	1.87±0.01^{a}	67.73±0.25^{a}	17.74±0.44^{b}	7.86±0.01^{ab}	6.67±0.19^{a}	67.73±0.25^{a}	25.61±0.44^{b}
		n.d.	*+13.5*	*+48.4*	*+0.7*	*-4.7*	*-9.3*	*+21.5*	*+0.7*	*-6.1*
Flax seed oil	-	n.d.	5.55±0.35^{c}	3.26±0.04^{c}	16.53±0.06^{a}	11.26±0.13^{b}	63.41±0.33^{a}	8.81±0.39^{b}	16.53±0.06^{a}	74.67±0.45^{a}
	SD	n.d.	7.49±0.05^{a}	4.19±0.03^{a}	17.63±0.22^{a}	13.35±0.48^{a}	57.35±0.18^{c}	11.68±0.08^{a}	17.63±0.22^{a}	70.70±0.30^{c}
		n.d.	*+34.9*	*+28.5*	*+6.6*	*+18.7*	*-9.6*	*+32.3*	*+6.6*	*-5.3*
	FD	n.d.	6.46±0.28^{b}	3.72±0.37^{b}	17.17±0.16^{a}	11.34±0.01^{b}	61.33±0.82^{b}	10.17±0.65^{a}	17.17±0.16^{a}	72.67±0.81^{b}
		n.d.	*+16.4*	*+14.1*	*+3.9*	*+0.7*	*-3.3*	*+15.4*	*+3.9*	*-2.7*
Safflower seed oil	-	0.34±0.05^{ab}	6.80±0.07^{c}	2.50±0.27^{a}	11.99±0.17^{b}	77.69±0.47^{a}	0.69±0.01^{a}	9.64±0.29^{a}	11.99±0.17^{b}	78.38±0.46^{a}
	SD	0.46±0.12^{a}	7.80±0.01^{a}	2.56±0.08^{a}	18.73±0.90^{a}	69.13±1.14^{c}	1.34±0.28^{a}	10.82±0.02^{a}	18.73±0.45^{a}	70.46±0.43^{c}
		+35.3	*+14.7*	*+2.4*	*+56.2*	*-11.0*	*+94.2*	*+12.2*	*+56.2*	*-10.1*
	FD	0.29±0.06^{b}	7.11±0.06^{b}	2.44±0.08^{a}	18.34±0.23^{a}	71.03±0.13^{b}	0.81±0.05^{a}	9.83±0.05^{a}	18.34±0.23^{a}	71.84±0.18^{b}
		-14.7	*+4.6*	*-2.4*	*+53.0*	*-8.6*	*+17.4*	*+2.0*	*+53.0*	*-8.3*

Mean values in columns, for each type of oil separately, denoted by the same letter do not differ statistically significantly at $p>0.05$ (Tukey test).
SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids, SD – spray-drying, FD – freeze-drying, n.d. – not detected.
* Lines in italics present percentage changes in concentrations of individual compounds and their groups in the powdered oils relative to the natural cold-pressed oils.

Fatty acid composition in cold-pressed oils and oil powders

The fatty acid composition of the examined cold-pressed oils is shown in Table 3. The major fatty acid in the natural rape seed oil was monounsaturated oleic acid (C18:1 *n-9*) with the percentage share of 67.23 area %. The contents of C18:2 *n*-6 and C18:3 *n*-3 acids in this oil were 18.61 and 8.67 area %, respectively. The content of saturated fatty acids (SFA) was low, and accounted for 5.49 area %. The results obtained for natural cold-pressed oil correspond to the results obtained by other researchers [Lewinska *et al.*, 2015; Roszkowska *et al.*, 2015]. Flax and safflower seed oils had a high level of polyunsaturated fatty acids, which accounted for 78 area % of all fatty acids. In turn, C18:3 *n*-3 acid prevailed in the flax seed oil, and its share was 63.41 area %. The second polyunsaturated fatty acid, C18:2 *n*-6, in this oil accounted for 11.26 area %. In turn, saturated fatty acids (SFA) and MUFA reached, on average, 10 area % and 17 area % in this oil. A similar composition of fatty acids in cold-pressed flax seed oil was reported in other studies [Khattab & Zeitoun, 2013; Tańska *et al.*, 2016]. C18:2 *n*-6 was the major acid in safflower seed oils (77.69 area %), while the share of C18:3 *n*-3 did not exceed 1.5 area %. A similar content of linoleic acid (76.58%) in safflower seed oil was reported by Dorni *et al.* [2018].

In rape seed oil powders obtained both as a result of encapsulation by spray- and freeze-drying, a reduction was observed (by 4.7–14.5%) in the percentage share of polyunsaturated fatty acids: linoleic (C18:2 *n-6*) and α-linolenic (C18:3 *n-3*). However, considerable changes were observed in saturated fatty acids: palmitic (C16:0) and stearic (C18:0), whose shares increased to 13.5–20.3% and 48.4–52.4%, respectively. Changes in percentage shares of PUFA only slightly affected the ratio of *n*-6 to *n*-3 fatty acids. In the natural rape seed oil, this ratio was at 2.01:1, while in the encapsulated rape seed oils it reached 2.03:1. In the encapsulated flax seed oil, C18:3 *n*-3 acid share was reduced by 9.6% (for spray-drying method) and by 3.3% (for freeze-drying method). The content of C18:2 *n*-6 was the highest in the oil encapsulated by spray-drying. Saturated fatty acids accounted for, on average, 10% and 17% in flax seed oils. The contents of SFA and MUFA increased after the encapsulation process, but statistically significant changes ($p \leq 0.05$) were found only for SFA. Quispe-Condori *et al.* [2011] also encapsulated flax seed oil, using the spray- and freeze-drying methods. Although they observed no change in the α-linolenic acid content after spray-drying process, the content of this acid decreased during freeze-drying. During encapsulation of flax seed oil by spray-drying, Barroso *et al.* [2014] also observed an increase in the content of SFAs and a decrease in linolenic acid content. The drying of an emulsion with safflower seed oil resulted in greater losses of PUFAs in comparison to rape and flax seed oils; these losses reached 10.1% with the spray-

-drying and 8.3% with the freeze-drying method. Additionally, noticeably greater changes were observed in the content of MUFAs in comparison to other oils. The share of those acids increased as a result of drying process by more than 50%, while in rape and flax seed oils their increase did not exceed 7%. The changes in the percentage share of fatty acids probably resulted from the drying temperature applied. However, it is not confirmed by the research conducted by Kwon *et al.* [2004] who did not observe any changes in the fatty acid composition of oils obtained as a result of safflower seed roasting at 140–180°C.

When comparing the results for encapsulated oils with the results for crude oils, a decrease in the percentage of the PUFAs was observed. The highest degradation of PUFAs was observed in spray-dried safflower seed oil powder (10.11%), while the lowest degradation was in freeze-dried flax seed oil powder (2.68%). Hue & Nyam [2018], who studied the encapsulation of kenaf seed oil by freeze-drying, noted a lower decrease of the PUFA share, which was 2.29%. Calvo *et al.* [2010] also observed only small changes in the fatty acid composition of extra-virgin olive oil during freeze-drying; including a decrease in the PUFA share (up to 4.3%) and an increase in the MUFA share (up to 1.3%). In contrast, the spray-drying of an emulsion with extra-virgin olive oils performed by Calvo *et al.* [2012] significantly modified the fatty acid composition. In that study, the PUFA share was reduced by 5.3–69.6% depending on the type of olive oil. Additionally, generally greater PUFA losses were found for oil samples along with higher percentage shares. This contradicts the results of our study, in which twofold greater changes were recorded for the encapsulated rape seed oils than for the encapsulated flax seed oils that were clearly richer in PUFAs. Wang *et al.* [2018] also reported no significant effect of the spray-drying method on the composition of fatty acids in peony seed oil characterized by a high PUFA share (39.24% (*w/w*) of C18:3 *n*-3 and 26.96% (*w/w*) of C18:3 *n*-6). Rubilar *et al.* [2012] also showed a reduction in MUFAs in flax seed oil as a result of the spray-drying process. In their study, the content of linolenic acid decreased slightly during the process, but the contents of oleic and linoleic acids decreased by almost 50%. In the work of Ogrodowska *et al.* [2017], regardless of the draying method and processes temperature used, the PUFA losses in pumpkin seed oil ranged from 6.00 to 7.15%. The differences in the PUFA losses observed between the studied oils are likely to result from not only the content of prooxidants and antioxidants but also the droplet size of emulsions prepared with their use. This is consistent with our results where the emulsion containing safflower seed oil had particles with the smallest diameter D[4,3] (Table 2), and after drying the greatest degradation of PUFAs was observed.

Hogan *et al.* [2003] showed an antioxidative effect of 100 mg/kg α-tocopherol addition on fish oil subjected to the spray-drying, while 500 mg/kg of α-tocopherol contributed to a significant increase in the peroxide and anisidine values in encapsulated oil compared to the control sample. In turn, Coupland *et al.* [1996] demonstrated that the rate of lipid oxidation increased as the mean diameter of the droplet decreased because the droplet surface was increasing, which increased the contact between the interface, oxygen and radicals in the solution and thus enabled the unsaturated lipid located on the interface to oxidize easily. Furthermore, they also suggested that the ratio of oxidizable-to-nonoxidizable compounds in emulsion droplets affected the rate at which the lipid oxidation proceeds.

A greater influence of the encapsulation by spray-drying on fatty acid composition compared to freeze-drying observed for the studied oils could be explained by the oil oxidation rate during the processes. The spray-drying requires using high temperatures, which can affect the labile components of oils. Considering even such factors as the cooling effect of water evaporation from the dried emulsion or the short time of its residence in the drying chamber, hydroperoxide formation is high as a result of accelerated oxidation [Aghbashlo *et al.,* 2013]. Anwar & Kunz [2011] also confirmed that even though spray-drying only needed a few seconds to produce a desirable size of granules, it had a high chance of lipid degradation by oxidation due to the high drying temperature in the process. Timilsena *et al.* [2017] reported that complex coacervation-based microencapsulation better protected PUFAs-rich oils than a simple spray drying. They explained that the higher encapsulation efficiency in the complex coacervation-based microencapsulation resulted from the better surface-active nature of the complex coacervates.

Content of sterols in cold-pressed oils and oil powders

The sterol content in the examined cold-pressed oils is presented in Table 4. All examined oils contained Δ5-avenasterol, campesterol, β-sitosterol, and Δ7-avenasterol. Additionally, brassicasterol (37.32 mg/100 g of oil) was present in rape seed oil and Δ7-sitosterol in flax and safflower seed oils (115.34 and 0.79 mg/100 g of oil, respectively).

The total sterols in rape seed oil amounted to 505.46 mg/100 g of oil. A higher content of sterols, ranging from 596.4 to 772.5 mg/100 g of oil, was presented in work by Krajlić *et al.* [2013], who examined rape seed oil obtained from seeds cultivated in Croatia. In turn, Roszkowska *et al.* [2015] found 338.6–411.8 mg/100 g sterols in commercial cold-pressed rape seed oils available in Poland. Both the spray- and the freeze-drying processes resulted in the similar degradation of sterols, by 61.34% and 61.93%, respectively (Table 4).

Flax seed oil had the highest content of total sterols (477.82 mg/100 g of oil) compared to the other oils tested. A similar content of total sterols in flax seed oil was determined by Zhang *et al.* [2018] and Tańska *et al.* [2016]. The results obtained (Table 4) demonstrated a higher degradation of total sterols in freeze-dried flax seed oil (by 65.82%) than in the spray-dried oil (by 59.91%).

The lowest total sterol level (303.97 mg/100 g of oil) in comparison to the other oils under analysis was determined in safflower seed oil. This result agrees with the findings reported by Nogala-Kalucka *et al.* [2010]. The process of safflower seed oil powder production contributed to significant sterol losses, amounting to 59.01% (spray-drying method) and 60.85 % (freeze-drying method).

β-Sitosterol was the major sterol in the examined cold-pressed oils, which has been confirmed in other studies

TABLE 4. Content of sterols in natural and powdered cold-pressed oils and their changes after drying process.

Type of cold-pressed oil	Emulsion drying method	Sterols (mg/100 g of oil)							Total (mg/100 g of oil)
		Brassicasterol	Δ5-Avenasterol	Campesterol	β-Sitosterol	Δ7-Avenasterol	Δ7-Sitosterol	Others	
Rape seed oil	-	37.32 ± 4.60^{a}	18.98 ± 2.30^{a}	177.88 ± 4.92^{a}	237.02 ± 4.29^{a}	26.65 ± 2.33^{a}	n.d.	7.60 ± 1.57^{a}	505.46 ± 8.26^{a}
	SD	18.20 ± 0.54^{b}	6.06 ± 1.10^{b}	71.47 ± 3.40^{b}	89.87 ± 3.04^{b}	5.91 ± 0.41^{c}	n.d.	2.68 ± 0.47^{c}	195.43 ± 7.49^{b}
		-51.23*	-68.04	-59.82	-62.08	-77.80	-	-64.66	-61.34
	FD	15.49 ± 0.80^{c}	7.02 ± 0.31^{b}	67.40 ± 3.06^{c}	88.92 ± 3.70^{b}	10.50 ± 0.54^{b}	n.d.	3.08 ± 0.27^{b}	192.41 ± 2.75^{b}
		-58.50	-63.02	-62.11	-62.48	-60.58	-	-59.49	-61.93
Flax seed oil	-	n.d.	27.48 ± 0.56^{a}	98.91 ± 2.09^{a}	159.41 ± 1.44^{a}	64.23 ± 1.78^{a}	115.34 ± 0.35^{a}	12.44 ± 0.61^{a}	477.82 ± 4.14^{a}
	SD	n.d.	2.46 ± 0.09^{b}	37.59 ± 2.56^{b}	80.54 ± 0.10^{b}	14.48 ± 0.51^{b}	44.22 ± 2.15^{c}	12.24 ± 0.85^{a}	191.53 ± 3.40^{b}
		-	-91.05	-62.00	-49.48	-77.45	-61.66	-1.68	-59.91
	FD	n.d.	2.81 ± 0.41^{b}	34.71 ± 0.43^{c}	71.97 ± 4.56^{c}	1.18 ± 0.08^{c}	49.05 ± 4.17^{b}	3.60 ± 0.40^{b}	163.33 ± 2.68^{c}
		-	-89.78	-64.91	-54.85	-98.16	-57.47	-71.11	-65.82
Safflower seed oil	-	n.d.	10.35 ± 0.59^{a}	98.47 ± 2.79^{a}	130.63 ± 4.10^{a}	15.54 ± 0.64^{a}	0.79 ± 0.01^{a}	3.73 ± 0.05^{a}	303.97 ± 5.60^{a}
	SD	-	1.07 ± 0.13^{b}	25.01 ± 0.16^{b}	74.60 ± 1.20^{b}	9.01 ± 0.04^{b}	0.22 ± 0.02^{b}	3.12 ± 0.02^{b}	122.86 ± 5.99^{b}
		-	-89.59	-74.60	-42.89	-41.98	-72.98	-16.55	-59.01
	FD	n.d.	1.23 ± 0.16^{b}	24.71 ± 1.509^{b}	71.35 ± 0.83^{c}	6.96 ± 1.05^{c}	0.20 ± 0.01^{b}	3.50 ± 0.28^{a}	112.62 ± 2.87^{b}
		-	-88.13	-74.90	-45.38	-55.16	-74.42	-6.23	-60.85

Mean values in columns, for each type of oil separately, denoted by the same letter do not differ statistically significantly at $p>0.05$ (Tukey test). SD – spray-drying, FD – freeze-drying, n.d. – not detected.

* Lines in italics present percentage changes in contents of individual compounds and their total content in the powdered oils relative to the natural cold-pressed oils.

[Czaplicki *et al.*, 2016; Nogala-Kalucka *et al.*, 2010; Rudzińska *et al.*, 2009]. The highest content of β-sitosterol was determined in rape seed oil (237.02 mg/100 g of oil), and the lowest one in oil obtained from safflower seed oil (130.63 mg/100 g of oil). Encapsulated oil production resulted in the significant degradation of β-sitosterol, although the range of these changes did not depend on the method applied, but on oil type. The greatest losses of this component (about 63%) were found for rape seed oil, while the smallest ones in the safflower seed oil (on average 44.5%). The flax and safflower seed oils were also characterized by the highest degradation of Δ5-avenasterol (by 88.13–91.05%). Additionally, the greatest degradation of brassicasterol was observed in the safflower seed oil, which was 88.94% (spray-dried sample) and 89.51% (freeze-dried sample). Hue & Nyam [2018] reported that the unsaturated bond in chemical structures was the major difference among various sterols and the main reason for the degradation of the sterols. β-Sitosterol has one double bond [Hue & Nyam, 2018], while brassicasterol and avenasterol have two double bonds in their molecules; and this may affect the rate of their degradation [Gawrysiak-Witulska *et al.*, 2015]. Our results generally confirmed this phenomenon, with the exception of brassicasterol in the rape seed oils and Δ7-avenasterol in the spray-dried safflower seed oil.

The available literature does not include many studies describing the effect of the powder production process on degradation of biologically active substances, including sterols. The studies on those compounds have been conducted mainly in the context of their changes under the effect of applied technological operations or, generally, of heating. Sterols are among the compounds which are subject to degradation as a result of heat factors, which results in a variety of oxidative derivatives (oxysterols) formed [Rudzińska *et al.*, 2009]. Hernández Sánchez e*t al.* [2016] found that during spray-drying, emulsion drops were in contact with hot air. During the process, at the beginning water is removed, drying liquid drops remain at the air wet bulb temperature (*e.g.* about 40°C) and their temperature may then start increasing at the end of drying when the air current has reached a temperature close to its outlet temperature (*e.g.* 94–110°C). Dry particles may therefore be exposed, for a very short time, to high temperatures, which can create favorable conditions for the formation of oxidation products. On the other hand, Soupas *et al.* [2006] reported that more sterol oxides were generated during storage of milk powder than during spray-drying.

Considering only the total content of sterols in the analyzed samples, it should be noted that the process of encapsulated oil production by freeze-drying contributed to their greater degradation. An opposite relationship was found in the experiment of Kim *et al.* [2014] who determined the content of bioactive compounds in rice bran subjected to different heat treatments. They observed that the sterol content in a freeze-dried and then dry-heated sample was slightly lower than in the control sample. Furthermore, the sterol content linearly decreased as the heating temperature or time increased. Kwon *et al.* [2004], who studied the effect of heat

TABLE 5. Content of tocopherols in natural and powdered cold-pressed oils and their changes after drying process.

Type of cold-pressed oil	Emulsion drying method	Tocopherols (mg/100 g of oil)			Total (mg/100 g of oil)
		α	β + γ	δ	
Rape seed oil	-	31.60±0.18^{a}	33.56±0.95^{a}	0.07±0.01^{a}	65.16±1.13^{a}
	SD	26.29±1.73^{b}	28.67±2.23^{b}	n.d.	54.95±3.97^{b}
		*-16.8**	*-14.6*	*-100.0*	*-15.7*
	FD	21.80±2.22^{c}	27.71±2.64^{b}	0.05±0.01^{a}	49.51±0.42^{c}
		-31.0	*-17.4*	*-26.5*	*-24.0*
Flax seed oil	-	5.43±0.50^{a}	41.83±1.06^{a}	0.09±0.01^{a}	47.26±0.57^{a}
	SD	5.40±0.51^{a}	33.91±1.29^{b}	n.d.	39.31±0.78^{b}
		-0.6	*-18.9*	*-100.0*	*-16.8*
	FD	3.34±0.34^{b}	41.73±0.02^{a}	0.04±0.01^{a}	45.07±0.36^{a}
		-38.5	*-0.2*	*-49.4*	*-4.6*
Safflower seed oil	-	54.58±2.99^{a}	5.52±0.08^{a}	n.d.	60.11±3.06^{a}
	SD	24.71±4.02^{b}	5.01±0.10^{a}	n.d.	29.72±4.12^{b}
		-54.7	*-9.3*	*n.d.*	*-50.5*
	FD	54.28±0.72^{a}	5.03±0.02^{a}	n.d.	59.31±0.74^{a}
		-0.9	*-8.9*	*n.d.*	*-1.3*

Mean values in columns, for each type of oil separately, denoted by the same letter do not differ statistically significantly at $p>0.05$ (Tukey test). SD – spray-drying, FD – freeze-drying, n.d. – not detected. * Lines in italics present percentage changes in contents of individual compounds and their total content in the powdered oils relative to the natural cold-pressed oils.

pre-treatment on the functional constituents of rice germ, reported that free sterols were degraded more easily when a high temperature or vacuum was applied because of their low boiling points. However, Hue & Nyam [2018] observed that loss of total sterols in kenaf seed oil was 59.76% during storage, while 32.86% during freeze-drying.

Content of tocopherols in cold-pressed oils and powders obtained from them

The tocopherol content in the examined cold-pressed oils is presented in Table 5. Tocopherols are the main antioxidants of rape seed oil. The analyzed rape seed oil samples contained tocopherols at the levels of 49.51–65.16 mg/100 g of oil. The oils had high amounts of α- and β+γ-tocopherols, whereas δ-tocopherol content was low. In natural rape seed oil, α-tocopherol was present at the level of 31.60 mg/100 g of oil, β+γ-tocopherol at 33.56 mg/100 g of oil, and δ-tocopherol at 0.07 mg/100 g of oil. These results concur with earlier reported findings, but most of the authors have reported that rape seed oil had high contents of α- and γ-tocopherols, not α- and β+γ-tocopherols [Azadmard-Damirchi *et al.*, 2010; Ghazani *et al.,* 2014]. Rape seed oil powder production resulted in the greatest changes in the δ-tocopherol content, which was subject to complete degradation as a result of spray-drying. On the other hand, after encapsulation by freeze-drying, its content was reduced by 26.5%. However, freeze-drying proved to be a less favorable method for rape seed oil powder production in the case of α-tocopherol. Its content in the freeze-dried oil was lower by 31.0%, while in the spray-dried oil its decreased by 16.8%. However, in both encapsulated samples, the content of β+γ-tocopherol was at a similar level (approx. 28 mg/100 g of oil).

The content of total tocopherols in the natural flax seed oil was determined at 47.26 mg/100 g of oil, with prevailing β+γ-tocopherol, accounting for 88.5%. This oil contained also α-tocopherol (5.43 mg/100 g of oil) and δ-tocopherol (0.09 mg/100 g of oil). Similar contents of tocopherols were presented by Tańska *et al.* [2016], who characterized six samples of cold-pressed flax seed oil. A higher content of total tocopherols in flax seed oil, reaching 79.4 mg/100 of oil, was obtained by Bozan & Temelli [2008].

As a result of flax seed oil spray-drying, β+γ-tocopherol decreased by 18.93%. However, the freeze-drying process did not affect the degradation of those flax seed oil homologues. An opposite dependency was found for α-tocopherol, the degradation of which (by 38.5%) occurred only after freeze-drying.

The content of tocopherols in natural safflower seed oil was comparable to that found in the rape seed oil and amounted to 60.11 mg/100 g of oil. However, the tocopherol profile of this oil was different, with α-tocopherol found a major homologue, with the content reaching 54.58 mg/100 g of oil, *i.e. ca*. 90% of total tocopherols occurring in this oil. Safflower seed oil also contained β- and γ-tocopherols, the sum of which was 5.52 mg/100 g of oil. A similar tocopherol content was reported by Ben Moumen *et al.* [2015] who characterized the lipid fractions of safflower seeds cultivated in Morocco, Spain and India. As a result of spray-drying, the content of α- and β+γ-tocopherols in safflower seed oil was reduced by 54.7% and 9.3%, respectively (Table 5). Freeze-drying affected the content of β+γ-tocopherol to a degree comparable to spray-drying, but it did not affect the content of α-tocopherol.

The encapsulation of flax and safflower seed oils by spray-drying had a greater impact on the degradation of total tocopherol content compared to the freeze-drying. Furthermore, the highest decrease in the sum of tocopherols was observed in encapsulated safflower seed oil (50.5%). However, an opposite relationship was found in the case of rape seed oil. Gawrysiak-Witulska *et al.* [2015] studied the effect of drying temperature on changes in individual homologues of tocopherols in the oil from yellow-seeded rape and found that during drying at 40°C and 60°C, losses of α-, β- and γ-tocopherols did not exceed 2%, while δ-tocopherol losses reached 6%. Increasing air temperature to 100°C and 120°C caused similar losses (23%) in the main tocopherol homologues in rape seed (α-and γ-tocopherols). Also Hernández Sánchez *et al.* [2016] found only 10% degradation of α-tocopherol added to the oil before spray-drying process. A slight effect of the freeze-drying process on the tocopherol content in encapsulated oils was confirmed for walnut and kenaf seed oils [Calvo *et al.*, 2011; Hue & Nyam, 2018]. The powders they obtained had similar contents of tocopherols compared to the raw oils. In contrast, Calvo *et al.* [2010] observed that the content of tocopherols slightly increased in encapsulated olive oil produced by freeze-drying. A similar trend was observed for a freeze-dried rice sample in an experiment performed by Kim *et al.* [2014]. The authors suggested that a significant amount of tocopherols was lost, being bound to proteins or phospholipids, and that applying thermal energy could break the linkages.

CONCLUSIONS

Based on the research conducted, it was shown that the encapsulation efficiency and surface oil content depend not only on the encapsulation method but also on oil composition. The composition of oil, especially fatty acid composition, has a significant influence on the emulsion properties. In case of the spray-drying method, the use of rape seed oil allowed obtaining a higher encapsulation efficiency and lower surface oil content than the use of flax and safflower seed oils. For PUFAs, only a slight effect of the applied encapsulation method on their degradation was observed and it depended on oil type. In turn, sterol degradation did not depend on the drying method applied. In total, the losses of those bioactive substances in all types of oils were high and reached 60–66%. In most cases, the native tocopherols of the studied oils were degraded to a significantly lesser extent than sterols (up to 24%). The only exception was safflower seed oil encapsulated by spray-drying, which contained 50% less total tocopherols.

The results of our experiment show that the encapsulated rape, flax, and safflower seed oils had lower contents of bioactive compounds compared to the crude oils, regardless of the encapsulation method (spray- or freeze-drying). However, the degradation rate of the studied components is comparable to losses occurring in crude plant oils during culinary processes. Our results suggest that detailed analysis of oil composition is necessary before the encapsulation process to enable the appropriate choice of the components of wall materials and drying parameters.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Evaluation of the Antioxidant and Cytotoxic Activities on Cancer Cell Line of Extracts of Parasitic Plants Harvested in Tunisia

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Weeds thrive in agricultural environments. However, in certain areas of the world, they are consumed by humans as food, and they can represent a source of valuable active ingredients of ethnomedical interest. In this study, three North African parasitic plants of the Orobanchaceae family, *Cistanche violacea*, *Orobanche crenata* and *Orobanche lavandulacea*, normally considered as weeds, were studied to compare their biological activities. The antioxidant activity of hexane, ethyl acetate, acetone, methanol, and water extracts obtained by sequential maceration was estimated by DPPH, ABTS and FRAP assays. The metabolic profiles of aqueous extracts were studied by ^{1}H NMR spectroscopy. The oils extracted using hexane as solvent were analyzed by GC-FID and HPLC-DAD to characterize the fatty acid profile. The cytotoxic effect of the oil from the parasitic plants was also studied on B16F10 melanoma cancer cells. All extracts showed antioxidant activity and differences between extracts obtained using different solvents as well as for different plants have been highlighted. The highest total phenolic content was determined in *O. crenata* acetone extract (3.53±0.20 mmol GAE/g) and in *O. lavandulacea* methanol extract (2.29±0.02 mmol GAE/g). ^{1}H NMR spectroscopy revealed the presence of amino acids, organic acids and carbohydrates. The major fatty acids in the oils were 18:2 *n*-6, 16:0, 18:1 *n*-9, and 18:3 *n*-3. *O. lavandulacea* revealed to be a highly cytotoxic species for B16F10 melanoma cells. These data suggest that, despite the extreme climate conditions and the damage that can result, the studied desert parasitic plants can represent a novel and precious source of metabolites, with promising biological activities.

INTRODUCTION

The term ‘weed’ defines a plant that does not have any function considered useful for agricultural production and causes damage to existing plants. The harmfulness of weeds appears mainly in the following aspects: competition for solar radiation, water and nutrients, depreciation of the crop in the presence of fragments of pests, development of pests and diseases. Sometimes the term ‘plant parasite’ is incorrectly used as a weed equivalent. Thus, parasitic plants are plants that are often deemed undesirable. In the most disadvantaged areas of the world there is a need to look for less expensive food sources [Khattak *et al*., 2006]. Conventional vegetables grown in fields are expensive for most people. Parasitic plants could prove to be a good alternative for expensive vegetables [Ceccanti *et al*., 2018]. There is evidence dating back many centuries about the use of some parasitic plants as food in times of famine or as a source of precious drugs widely used in folk medicine. Nevertheless, very little is known about the richness of secondary metabolites of parasitic plants and their potential use in human nutrition.

In North Africa, especially in Tunisia, holoparasitic plants are restricted to a few botanical families: Cuscutaceae [El Mokni *et al*., 2016], Cynomoriaceae [Ben Attia *et al*., 2018; Zucca *et al*., 2019], Orchidaceae [El Mokni *et al*., 2010], Rafflesiaceae and Orobanchaceae [El Mokni *et al*., 2015]. These plants lack of chlorophyll and depend on their hosts for carbon and/or mineral nutrition. However, parasitic plants can cause a serious threat to several food crops and spontaneous vegetation, requiring careful monitoring of their agronomical, ecological and economic impacts [Nickrent & Musselman, 2004]. Orobanchaceae is an unconventional edible parasitic plant family, similar to the asparagus [Nada & El-Chaghaby, 2015], which is part of the flora of Tunisia. Despite they can negatively impact food crops, these plants also have some medicinal uses [Zhang *et al*., 2018]. Contemporary pharmacological studies, in fact, have progressively validated the traditional uses of Orobanchaceae [Wang *et al*., 2017].

In southern Tunisia, three common holoparasitic plant species, belonging to the Orobanchaceae, are: *Cistanche violacea* (Desf.) Hoffmanns & Link, *Orobanche crenata* Forssk, and *Orobanche lavandulacea* Rchb. *Cistanche violacea*, synonym of *Phelypaea violacea* Desf. is a species of angiosperms Eudicotyledonous, endemic to North Africa (*i.e.* Tunisia, Libya, Algeria, Morocco, Western Sahara, Egypt, Saudi Ara-

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bia) [Baba Aissa, 2011]. Ecologically, this species is localized on heavy and salty soils. It is an obligate parasite species of Chenopodiaceae and Zygophyllaceae. In Saharan areas, *C. violacea* parasites *Zygophyllum cornutum* and *Limoniastrum guyonianum*. In Libya, the fat and fleshy underground part is consumed during famine, and the Tuaregs eat this species as asparagus. The genus *Cistanche* is considered the most valuable species of the Orobanchaceae with a strong resistance to harsh environmental conditions.

Orobanche crenata is a parasitic herbaceous species, without chlorophyll and hermaphrodite. At the end of the flowering (Spring season) the plant becomes russet and the flowers become more consistent. It is more common in countries adjacent to the Mediterranean. It extends east to Pakistan and India, and north to Europe. The host range of *O. crenata* is restricted to Fabaceae and Apiaceae and to a relatively sporadic occurrence in a few other families, such as Asteraceae and Cucurbitaceae. The wild hosts are mainly in these same families.

Orobanche lavandulacea synonym: *Phelipanche lavendulacea* (Rchb) Pomel, is a parasitic species with flower stalks averaging 15 to 60 cm high and 0.4 to 0.7 cm wide. *Orobanche lavandulacea* is a very rare taxon and has a steno-Mediterranean distribution, from Morocco and Spain on the west to Syria and Jordan, and along the Black Sea in the east (including also Canary Islands, France, Italy, Croatia, Greece, Cyprus, Algeria, Tunisia, Egypt, Turkey) [Sánchez Pedraja *et al*., 2005]. It appears to be a strict parasite of the Fabaceae family in the Mediterranean region [Mèdail *et al*., 2011]. *Orobanche lavandulacea* has been described in certain ethnicities as a medicinal plant with great qualities.

The biological activity of these species has scarcely been studied and little is known in terms of their phytochemistry.

FIGURE 1. Real images of *Cistanche violacea* collected in Dahar el Maztouria, *Orobanche crenata* and *Orobanche lavandulacea* collected in Dahar Cheneni, Tataouine, Tunisia. The distance between the two collection points is about 25 km. The flag on the map indicates the collection area.

The main reason may be related to the fact that these plants have mostly been recognized as weeds that cause agricultural issues [Prider *et al*., 2009] without prospecting for any economically profitable applications. Only a few investigations report the chemical and biological activities of these species [Bougandoura *et al*., 2016; Debouba *et al*., 2012]. The lipid profiles of the seeds of twenty-one *Orobanche* spp. (including *O. crenata* and *O. lavandulacea*) have been characterized [Velasco *et al*., 2000]. Phytochemical studies on different *Cistanche* spp. have isolated several chemical groups, including phenylethanoid glycosides, iridoids, and lignans (present in free and glycosidic forms), polysaccharides, free amino acids, ashes and minerals [Bougandoura, 2016; Deyama *et al*., 2006; Ebringerová *et al*., 1997; Wang *et al*., 2012]. Bougandoura *et al.* [2016] isolated a new iridoid and a new phenylethanoid glycoside with five known compounds from an aqueous-methanol extract of the aerial parts of *C. violacea*. The phenolic content, antioxidant power, and antimicrobial activities of *O. crenata* have been recently studied [Genovese *et al*., 2019], and its potential as a food prospect for human nutrition has also been shown [Renna *et al*., 2015].

In the present study, our goal was to extend the knowledge of three holoparasitic plants growing in the Dahar Natural region, in the southern of Tunisia (Figure 1), and to evaluate some aspects of their phytochemistry and antioxidant properties. Our findings can contribute to increase the perspectives for application of these plants in different areas such as in nutraceutical and functional food production.

MATERIALS AND METHODS

Chemicals and instrumentation

Deuterium oxide (D_2O, 99.9%), sodium 3-trimethylsilylpropionate-2,2,3,3,-d4 (TSP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and standards of fatty acids and fatty acid methyl esters were purchased from Sigma–Aldrich (Milan, Italy). Methanolic HCl (3 N) was purchased from Supelco (Bellefonte, PA, USA). The cyanidin-3-*O*-glucoside (C3G) standard was acquired from Extrasynthese (Genay, France). Ultrapure water (18 mΩ) was obtained with a Milli-Q Advantage A10 System apparatus (Millipore, Milan, Italy). All other reagents, standards and solvents were of HPLC grade and used without further purification. All the spectrophotometric analyses were performed using UltroSpec 2100 pro (Amersham Bioscience, Milan, Italy).

Plant material

The plant species were harvested during the spring of 2017, from the region of Tataouine, south-eastern Tunisia. The species, all belonging to the family of Orobanchaceae and identified in the South Tunisian Flora, are: *C. violacea*, from the site named Dahar El Maztouria (DMM 32.853200, 10.446700), *O. crenata*, and *O. lavandulacea,* from Dahar Cheneni (DMM 32.918700, 10.268200, Figure 1). Plants were authenticated by Prof. Mohamed Chaieb based on the following flora: Pottier-Alapetite [1979, p. 1990], Flora Medcheklist (1984, 1986, 1989) [Greuter *et al*.], Dobignard & Chatelain [2010] and the Flora of Chaieb & Boukhris [1998]. A specimen of each collected species was deposited in the Laboratory of Botany at the University of Sfax, Tunisia, under number Tun. Flo. 39/2017. For each species, we collected the whole plant. During collection, samples were kept at approximately 4–8°C in a portable refrigerator (using dry ice to better control the temperature). Then, they were taken back to the laboratory where they were gently cleaned, and the residual soil was removed. The specimens (between 15 and 20 for each species) were cut into slices, approximately 0.5 cm thick, and freeze-dried (Telstar LyoQuest, Milan Italy). Samples were milled and prepared for the extraction step.

Preparation of extracts

Freeze-dried samples were extracted by cascade maceration using different solvents with increasing polarity. Briefly, 25 g of powder was mixed with 150 mL of *n*-hexane. After 24 h of incubation at 25°C in the dark, the supernatant was recovered by filtration. The organic solvent was removed using a rotary evaporator under vacuum (Rotavapor Buchi R200, Cornaredo, Italy). Then, the residues were recovered, and the process was repeated in cascade using ethyl acetate, acetone, methanol and water in turn as the solvent. After the last extraction process, water was removed using a freeze-dryer (Telstar LyoQuest). The dry extracts obtained were stored at -20°C. Three independent samples were extracted for each species to ensure statistical analysis of the results. Before each analysis, the extracts have been re-dissolved in the same solvents in stock solutions ranging 2–10 mg/mL, and then drastically diluted in water (or methanol) to ensure similar experimental conditions during the assays.

Oil extraction

Oil extractions were performed using a known amount of each plant. The powdered plant was transferred into a cellulose extraction thimble and inserted into a Soxhlet assembly fitted with a 100 mL flask. Fifty milliliters of *n*-hexane were added, and the whole assembly was heated for 6 h using a heating mantle. The extracts were concentrated using a rotary evaporator. The dry extracts obtained were stored at -20°C.

Determination of total phenolic content by the Folin-Ciocalteu method

Total phenolic content was determined using the Folin-Ciocalteu reagent [Slinkard & Singleton, 1977] by reference to the calibration curve obtained using gallic acid as a standard (linear range from 0.05 to 0.6 mM). The results were calculated in equivalents of gallic acid per gram of extract (mmol GAE/g).

Determination of flavonoids

The total flavonoid content contained in the extracts was estimated using a previously described method [Zucca *et al*., 2010]. Catechin was used for calibration (linearity range of 0.1–0.6 mM), and the results were calculated in catechin equivalents per gram of extract (mmol CE/g).

Determination of anthocyanins

The total content of anthocyanins was determined as previously described [Delazar *et al*., 2010; Zucca *et al*., 2016] according to the differential pH method. Five hundred μL of extract was mixed with 0.5 mL of each buffer solution. The absorbances were measured at 510 nm and 700 nm with respect to the distilled water as a reference. Final absorbance was calculated using equation (1).

$$A = (A\,\lambda_{510} - A\,\lambda_{700})\ \text{pH } 1.0 - (A\,\lambda_{510} - A\,\lambda_{700})\ \text{pH } 4.5 \quad (1)$$

The molar extinction coefficient of C3G was used in the Lambert-Beer equation to calculate the anthocyanin content. It was expressed in mg equivalents of cyanidin-3-*O*--glucoside per gram of extract (mg C3G/g).

Measurement of antioxidant activity

DPPH radical scavenging activity

The antiradical activity was determined using the spectrophotometric method involving the deep purple 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [Brand-Williams *et al*., 1995]. A total of 700 μL of DPPH radical (25 mg/L) was mixed with 300 μL of each extract. The sample was mixed with a vortex for 30 s and then stood at room temperature for 30 min. The absorbance was measured at 515 nm. Trolox was used as a standard (linear range 5–50 μM), and the results were calculated in Trolox milliequivalents per gram of extract (mmol TE/g).

Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) assay was performed according to the Benzie & Strain [1996] method with some modifications. The test consisted of reacting 770 μL of water and 30 μL of each extract with 200 μL of the FRAP solution in glass hemolysis tubes. After 6 min of standing at room temperature and centrifugation for 10 min at 10,000 rpm, the absorbance of the supernatant was measured at 593 nm. FRAP was calculated in Fe(II) mmol per gram of extract (mmol Fe(II) / g).

Trolox Equivalent Antioxidant Capacity

The Trolox equivalent antioxidant capacity (TEAC) was determined using the spectrophotometric assay with a stable radical cation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet +}$) [Re *et al*., 1999]. Two solutions were prepared: a solution of ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (7 mM) and a solution of potassium peroxydisulfate (2.45 mM). ABTS radical cation was generated by mixing the two solutions in equivalent amounts. The mixture reacted for at least 12 h in the dark at room temperature. The obtained solution was diluted with 75 mM potassium phosphate buffer, pH 7.4, to a final absorbance of 0.70±0.05 measured at 734 nm. Then, 1 mL of $ABTS^{\bullet +}$ solution was mixed with 10 μL of extract solution and stood for 6 min. A decrease in absorbance at 734 nm was finally detected. Trolox was used as a standard, and the results were expressed in Trolox milliequivalents per gram of extract (mmol TE/g).

NMR SPECTROSCOPY

The aqueous extracts were dried under vacuum. A total of 10 mg of each dried extract was resuspended in 600 μL of D_2O containing the internal standard (TSP) added at a known concentration (1 mM) and vortexed. Then, 650 μL of the resulting solution was transferred to a 5 mm OD NMR tube.

^{1}H NMR analysis was performed on a spectrometer (Varian UNITY INOVA 500, Palo Alto, CA, USA) operating at 499.84 MHz. The spectra were acquired at 300 K from 256 scans collected over a spectral width of 6000 Hz. A 45° pulse, an acquisition time of 2.3 s and a relaxation time of 4 s were used. The removal of the water peak was obtained by applying a pre-saturation technique with low power radiofrequency irradiation for 1.5 s. A line broadening of 0.3 Hz was applied to all free induction decays (FIDs) prior to Fourier transformation. The phase and baseline of the spectra were manually corrected using MestReNova software (version 8.1.2, Mestrelab Research SL, Santiago de Compostela, Spain), and ^{1}H NMR chemical shifts were referred to the TSP resonance at δ 0.00 ppm.

The molecular composition of the extracts, expressed in mol%, was estimated by measuring the area under the NMR peaks assigned to the specific functional groups of molecules and normalizing the integrals to the sum of the total spectra to compensate for possible differences in the extraction yields. The MHz library in Chenomx NMR suite version 7.5 (evaluation edition, Chenomx Inc., Edmonton, AB, Canada) was used for data interpretation.

Fatty acid analysis

The fixed oils of the plant species (3 mg, in EtOH solution) obtained by oil extraction were subjected to mild saponification as previously reported [Rosa *et al*., 2012]. Dried saponifiable fractions were dissolved in acetonitrile with 0.14% (v/v) acetic acid and then analyzed by high-performance liquid chromatography (HPLC-DAD). A portion of dried fatty acids after saponification was methylated with 3N methanolic HCl (at room temperature) as reported, and fatty acid methyl esters (FAME) were analyzed by gas chromatography (GC-FID). All solvents were evaporated under vacuum.

FAME were measured on a gas chromatograph HP--6890 (Hewlett-Packard, Palo Alto, CA, USA) with a flame ionization detector (GC-FID) and equipped with a cyanopropyl methyl-polysiloxane HP-23 FAME column. FAME were identified with standard compounds and quantified as a percentage of the total fatty acids (FA). The unsaturated fatty acids (UFA) composition of plant oils was obtained with an 1100 HPLC-DAD system (Agilent Technologies, Palo Alto, CA, USA). UFA (detected at 200 nm) were eluted with $CH_3CN/H_2O/CH_3COOH$ (75/25/0.12, *v/v/v*) as the mobile phase at a flow rate of 2.3 mL/min using an Agilent Technologies XDB-C_{18} Eclipse column. UFA were identified using standard compounds (linear calibration curves, correlation coefficients > 0.995) and conventional UV spectra; chromatogram data were recorded and integrated with an Agilent OpenLAB Chromatography data system.

Tumor cell cultures

The B16F10 cell line from mouse melanoma was obtained from the Interlab Cell Line Collection (ICLC) (IRCCS Az. Osp. Univ. San Martino, Genova, Italy). Sub-

TABLE 1. Phenolic composition and antioxidant activity of the *C. violacea*, *O. crenata*, and *O. lavandulacea* extracts (data are expressed per gram of dry extract).

	Plant name	Hexane	Ethyl acetate	Acetone	Methanol	Water
Extraction yield (g/100 g DM)	*C. violacea*	1.4±0.2	1.0±0.3	1.7±0.6	26.4±0.7	27.3±1.1
	O. crenata	1.3±0.4	0.5±0.1	2.4±0.5	11.8±0.2	18.5±0.6
	O. lavandulacea	1.1±0.1	0.5±0.1	3.8±0.4	5.5±0.3	15.8±0.3
$DPPH^{\bullet}$ scavenging activity (mmol TE/g)	*C. violacea*	2.44 ± 0.02^{d}	1.14 ± 0.01^{g}	7.16 ± 0.17^{a}	0.89 ± 0.01^{gh}	1.10 ± 0.008^{g}
	O. crenata	0.64 ± 0.01^{hi}	2.40 ± 0.17^{d}	5.30 ± 0.02^{c}	0.50 ± 0.06^{i}	0.33 ± 0.006^{i}
	O. lavandulacea	2.00 ± 0.03^{e}	6.80 ± 0.35^{b}	1.61 ± 0.05^{f}	1.43 ± 0.01^{fg}	0.30 ± 0.003^{i}
TEAC (mmol TE/g)	*C. violacea*	15.71 ± 0.2^{d}	6.35 ± 0.1^{efg}	42.01 ± 0.6^{a}	13.01 ± 0.01^{d}	12.70 ± 0.03^{d}
	O. crenata	4.70 ± 0.1^{gh}	20.06 ± 0.4^{c}	14.14 ± 0.2^{d}	3.34 ± 0.09^{gh}	1.52 ± 0.02^{h}
	O. lavandulacea	8.65 ± 1.0^{ef}	23.71 ± 1.5^{b}	8.57 ± 1.0^{ef}	9.08 ± 0.80^{ef}	5.67 ± 0.30^{fg}
FRAP (mmol Fe(II)/g)	*C. violacea*	1.26 ± 0.02^{fg}	1.26 ± 0.04^{fg}	4.84 ± 0.10^{b}	0.20 ± 0.09^{j}	0.19 ± 0.006^{j}
	O. crenata	0.40 ± 0.02^{i}	28.67 ± 0.07^{a}	3.24 ± 0.06^{c}	0.73 ± 0.01^{h}	0.44 ± 0.008^{i}
	O. lavandulacea	1.09 ± 0.03^{g}	1.31 ± 0.04^{ef}	1.53 ± 0.02^{d}	1.49 ± 0.02^{de}	0.32 ± 0.006^{ij}
Total phenolic content (mmol GAE/g)	*C. violacea*	0.60 ± 0.09^{f}	1.0 ± 0.02^{d}	1.50 ± 0.04^{c}	0.68 ± 0.04^{ef}	0.69 ± 0.07^{ef}
	O. crenata	0.50 ± 0.01^{f}	1.71 ± 0.03^{c}	3.53 ± 0.20^{a}	2.12 ± 0.01^{b}	1.43 ± 0.09^{c}
	O. lavandulacea	0.52 ± 0.01^{f}	2.07 ± 0.06^{b}	0.93 ± 0.02^{de}	2.29 ± 0.02^{b}	1.09 ± 0.01^{d}
Total flavonoid content (mmol CE/g)	*C. violacea*	0.07 ± 0.010^{i}	0.79 ± 0.03^{de}	0.80 ± 0.08^{de}	0.23 ± 0.01^{g}	0.47 ± 0.04^{f}
	O. crenata	0.02 ± 0.001^{j}	6.78 ± 0.20^{a}	0.75 ± 0.10^{de}	0.45 ± 0.03^{f}	0.64 ± 0.05^{ef}
	O. lavandulacea	0.15 ± 0.009^{h}	4.53 ± 0.20^{b}	0.37 ± 0.04^{f}	2.39 ± 0.08^{c}	0.92 ± 0.02^{d}
Total anthocyanin content (mg C3G/g)	*C. violacea*	6.6 ± 0.4^{bc}	5.3 ± 0.5^{c}	21.6 ± 1.2^{a}	0.27 ± 0.01^{g}	0.70 ± 0.03^{f}
	O. crenata	4.7 ± 0.2^{cd}	22.4 ± 0.3^{a}	4.3 ± 0.3^{d}	8.36 ± 0.40^{b}	3.22 ± 0.07^{e}
	O. lavandulacea	3.7 ± 0.2^{de}	21.2 ± 0.4^{a}	3.6 ± 0.3^{de}	4.7 ± 0.10^{cd}	5.26 ± 0.10^{c}

Data are expressed as mean ± SD (n =3). Mean values for the same analysis having different letters are significantly different ($p<0.05$; One–way ANOVA followed by the Bonferroni Multiple Comparisons Test). DM: dry material. TEAC: Trolox equivalent antioxidant capacity. FRAP: Ferric reducing antioxidant capacity. GAE: Gallic acid equivalents. CE: Catechin equivalents. C3G: cyanidin-3-*O*-glucoside.

cultures of B16F10 cells were cultured in T-75 culture flasks and passed with a trypsin-EDTA solution. The cells were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 units/mL) and streptomycin (100 μg/mL) (Invitrogen) in a 5% CO_2 incubator at 37°C.

MTT test of cell viability

The cytotoxic effect of oils was evaluated in cancer B16F10 cells by the MTT assay. Cancer B16F10 cells, cultured according to Rosa *et al.* [2015], were exposed to different aliquots of oils (50–500 μg/mL, from a 20 mg/mL solution in EtOH) in complete culture medium for 24 h. Treated cells were compared to untreated cells (control cells, receiving no treatment) and vehicle-treated cells (incubated for 24 h with an equivalent volume of EtOH; maximal final concentration, 1.2%) for viability. After incubation, B16F10 cells were subjected to the MTT assay as previously reported. The color change was measured at 570 nm with a microplate reader (Infinite 200, Tecan, Mannedorf, Switzerland); the absorbance is proportional to the number of viable cells.

Statistical analysis

GraFit 7 (Erithacus Software, London, United Kingdom), R 2.5.1 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad INSTAT (GraphPad Software, San Diego, CA, USA) were used for data analysis. The Pearson correlation coefficient (r) was calculated using GraphPad INSTAT. One-way analysis of variance (ANOVA) and the Bonferroni multiple comparison test were used to assess the statistical significance of the differences. All the analyses were performed at least in triplicate (unless otherwise stated), and the data are reported as mean ± standard error of the mean (SEM) or standard deviation (SD).

RESULTS AND DISCUSSION

As it could be seen in Table 1, the yield of the different extractions varied considerably depending on the plant and the solvent used. *C. violacea* gave the best yield for the aqueous and methanolic extracts, with 27.3% and 26.4%, respectively. The lowest yield was obtained for both *Orobanche* ethyl acetate extracts (0.5%). These data suggest that the majority of the compounds extracted present high polarity.

Quantification of total phenolics, flavonoids and anthocyanins, and antioxidant activity

Total phenolics content of the extracts varied greatly (from 0.50 mmol GAE/g for the *O. crenata* hexane extract to 3.53 mmol GAE/g for the *O. crenata* acetone extract). The two *Orobanche* species were richer in phenolics compared to the *Cistanche* species. However, the total phenolic contents of *O. crenata* and *O. lavandulacea* extracts were quite different. The acetone extract of *O. crenata* was the richest in phenolics, whereas the *O. lavandulacea* showed higher (or similar) total phenolic content for the ethyl acetate and methanol extracts.

As flavonoids constitute a significant portion of phenolics in *Cistanche* spp. [Debouba, 2012], they have also been quantified in our samples. The highest total flavonoid content was observed in the ethyl acetate extracts of *O. crenata* and *O. lavandulacea* with 6.78 and 4.53 mmol CE/g, respectively (both statistically higher than the values determined for the other extracts at $p<0.05$).

Among flavonoids and their derivatives, anthocyanins are colored compounds (orange, purple to blue). Although widely involved in the coloring of petals, they are also found in many tissues. Their synthesis in foliar organs is often activated by stress (cold, deficiencies, senescence, *etc*.). All studied parasite species were well colored and exhibited significant amounts of anthocyanins. Thus, the highest total anthocyanin contents were present in the ethyl acetate extract of *O. crenata* (22.43 mg C3G /g), acetone extracts of *C. violacea,* and ethyl acetate extract of *O. lavandulacea* (21.59 and 21.17 mg C3G/g, respectively, $p<0.05$ in comparison with all the other data). In this case, a different distribution of compounds among the solvents could be seen, possibly suggesting a different anthocyanin composition among the three studied species. Such data need however confirmation by more precise chromatographic quantifications.

Several *in vitro* methods have been recommended to evaluate the antioxidant activity of plant extracts [Schlesier *et al.*, 2002; Zucca *et al.*, 2013]. There are, in fact, many methods differing in their analytical principles and experimental conditions, and antioxidant molecules give different contributions to the total antioxidant potential [Craft *et al*., 2012]. In the present study, we used DPPH, ABTS (TEAC), and FRAP assays to evaluate the *in vitro* antioxidant activity of *C. violacea*, *O. crenata*, and *O. lavandulacea* extracts (Table 1). The extracts with the highest free radical scavenging activity ($DPPH^{•}$ and $ABTS^{•+}$) also showed the highest reducing ability of Fe(III) (FRAP). This probably reflects the presence of antioxidant molecules in our extracts that can intervene by two types of reaction mechanisms [Craft et al., 2012]. For the FRAP, the reduction of Fe(III) is based exclusively on an electron transfer. Regarding the DPPH and ABTS assays, these radicals can, in fact, be neutralized either by direct reduction *via* electron transfers or by radical scavenging *via* a transfer of a hydrogen atom. The present study shows that all the plants investigated have antioxidant activities that vary considerably from one species to another. The highest antioxidant activity, based on all the tests combined, was obtained with the *C. violacea* species, followed by the two *Orobanche* species.

TABLE 2. Correlation coefficient (r) calculated between the content of phenolic groups and the results antioxidant assays.

	DPPH	TEAC	FRAP
C. violacea			
Total phenolics	0.8445	0.7823	0.9187*
Total flavonoids	0.4356	0.3470	0.5714
Total anthocyanin	0.9791*	0.9104*	0.9975*
O. crenata			
Total phenolics	0.8155	0.4101	0.0116
Total flavonoids	0.2025	0.8118	0.9964*
Total anthocyanin	0.0910	0.7448	0.9623*
O. lavandulacea			
Total phenolics	0.4492	0.5345	0.3584
Total flavonoids	0.8210	0.8765	0.2560
Total anthocyanin	0.9439*	0.9660*	0.1246

* statistical significant ($P<0.05$).

The highest TEAC was recorded for the following extracts: the acetone extract of *C. violacea* with 42.01 mmol TE/g, the ethyl acetate extract of *O. lavandulacea* with 23.71 mmol TE/g, and the ethyl acetate extract of *O. crenata* with 20.06 mmol TE/g. The last mentioned extract also had the highest FRAP (28.67 mmol TE/g). The DPPH assay showed lower values than TEAC; this could be probably due to some different affinity to the two radicals among the chemicals of the extracts.

The results (Table 2) showed that the antioxidant capacity evaluated through all these methods correlated with the contents of phenolic compounds, confirming their predominant role in this context. The highest correlation coefficient values were more frequently obtained for correlation between results of antioxidant assays and total anthocyanin contents, thus suggesting their predominant contribution in the antioxidant capacity of the samples.

Most of the published studies on the plant species selected for this study is limited to the assessment of the antioxidant capacity of essential oils. Very little has been reported in the literature on the antioxidant activity of non-volatile extracts in the plant. Particularly, we found little reports on the total phenolic content of *C. violacea* and its antioxidant activity [Bouchouka, 2016]. This species, harvested in Tunisia, was the subject of a single study on the antioxidant capacity of the aqueous and methanolic extract with the DPPH assay whose IC_{50} values were 6.38 and 17.15 μg/mL for the flowers and the bulb, respectively [Debouba *et al*., 2012]. The same study showed that the flowers had the strongest iron reducing and the bulb had the strongest chelating effect. Concerning the *C. violacea* that grows in Algeria, only one recent investigation has focused, for the first time, on the anti-inflammatory activity of a few isolated chemical constituents of the ethyl acetate fraction of the aqueous/methanolic extract of aerial parts of the plant [Bougandoura *et al*., 2016]. Regarding the two *Orobanche*, to the best of our knowledge, there is no published investigation on the phytochemical content and antioxidant activity of *O. lavandulacea*, whereas the study of Nada & El-Chaghaby [2015] showed that *O. crenata* harvested from Egypt could be a potential source of nutrients, antioxidants, and antibacterial compounds.

On the other hand, in the present work, we also found a good positive correlation between the results of three assays used to evaluate the antioxidant capacity. This presumably indicates that in an extract, the phenolics provide the radical scavenging activity (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$) and possibly are themselves responsible for the ability to reduce ferric ions. Some authors report that the antioxidant capacity of natural substances could have a reciprocal correlation with their reducing capacity [Guettaf *et al*., 2016] and that the latter can, in turn, serve as an important indicator of their potential for antioxidant activity [Wang *et al.*, 2012].

^{1}H NMR spectroscopy

The chemical profiles of the aqueous extracts of the three selected species were characterized by ^{1}H NMR spectroscopy. Representative spectra are shown in Figure 2. Spectral resonances of metabolites were attributed by using literature data [HMDB, 2019]. The high field region of the spectra (0–3.0 ppm) revealed the presence of amino acids such as alanine, asparagine, and valine and organic acids including acetate, citrate, malate, malonate, and succinate. The middle spectral region of all extracts (3.0–5.5 ppm) was dominated by the signals from carbohydrates (mannitol, glucose, fructose, sucrose, and fucose). In addition, betaine and choline were also identified in this portion of the spectrum. The low field region of the spectra (5.5–9 ppm) showed the weakest signals, mainly from aromatic compounds.

The relative abundances of 16 metabolites (five carbohydrates, six organic acids, three amino acids, and two osmoprotectants) were determined by considering easily integrated peaks. The results are shown as mol% in Table 3. Inspection of the table shows that mannitol was the most abundant carbohydrate in all the samples. Concerning the class of organic acids, all extracts were particularly rich in malic acid, especially *O. lavandulacea*. Higher levels of acetic acid were found in *O. crenata* and *O. lavandulacea* than in *C. violacea*. The accumulation of asparagine was significantly higher in *C. violacea* than in *O. lavandulacea*, while the presence of this amino acid was not revealed in the extract of *O. crenata*. Finally, the betaine content was much higher in the *C. violacea* extract than in the two *Orobanche* species.

It is known that the content of soluble sugars and other carbohydrates, free amino acids, organic acids and osmoprotectants in plants may act as metabolic signals in response to several environmental factors, such as drought, salinity and nutrient imbalances. Tolerance to abiotic stresses is very complex due to both the complexity of interactions between stress factors and various molecular, biochemical and physiological phenomena affecting plant growth and development. Although the presented NMR results are not enough to compare the metabolism of the three plants under investigation, the presence of the abovementioned differences in composition is a clear indication of different biological adaptabilities to the harsh environment of the Dahar region.

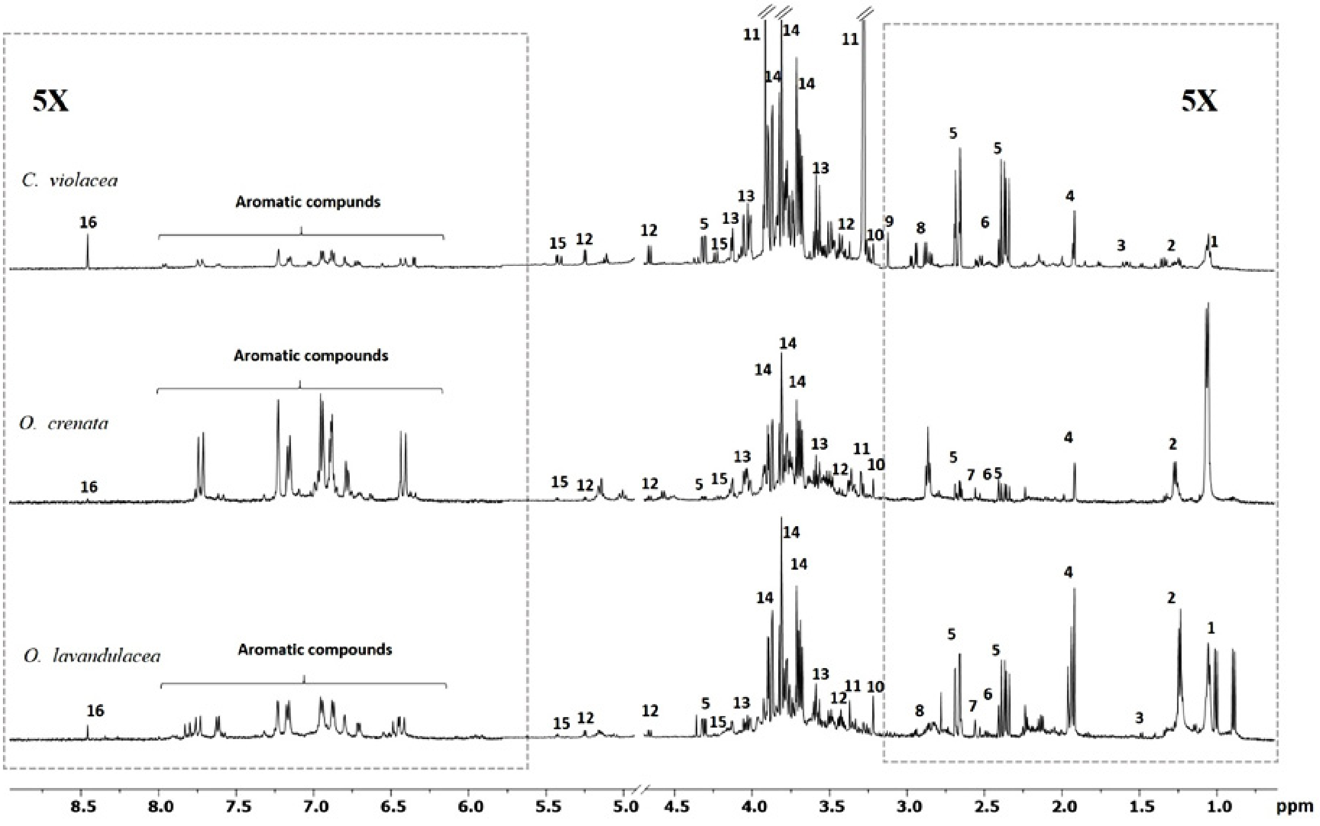

FIGURE 2. Stack-plot of ^{1}H NMR spectra of the aqueous extracts of *C. violacea*, *O. crenata,* and *O. lavandulacea*. Key: (1) valine, (2) fucose, (3) alanine, (4) acetate, (5) malate, (6) succinate, (7) citrate, (8) asparagine, (9) malonate, (10) choline, (11) betaine, (12) glucose, (13) fructose, (14) mannitol, (15) sucrose, (16) formate.

TABLE 3. Composition (mol %) of the aqueous extracts of *C. violacea*, *O. crenata* and *O. lavandulacea* estimated by the analysis of the corresponding ^{1}H NMR spectra.

Metabolite	*Cistanche violacea*	*Orobanche crenata*	*Orobanche lavandulacea*
Carbohydrates			
Glucose	8.22±0.43^{a}	7.58±1.04^{a}	7.25±2.30^{a}
Fructose	14.72±1.67^{b}	30.43±1.96^{a}	15.35±1.12^{b}
Sucrose	2.06±0.35^{a}	2.16±0.32^{a}	1.40±0.09^{b}
Mannitol	16.81±2.86^{c}	39.99±2.62^{a}	34.87±1.00^{b}
Fucose	0.27±0.02^{b}	1.10±0.11^{a}	1.32±0.46^{a}
Amino acids			
Alanine	0.14±0.02^{b}	nd	0.60±0.12^{a}
Asparagine	2.64±0.84^{a}	nd	1.31±0.72^{b}
Valine	0.08±0.01^{b}	nd	0.46±0.21^{a}
Organic acids			
Acetate	0.62±0.18^{b}	2.40±0.55^{a}	3.50±1.14^{a}
Citrate	nd	1.49±0.09^{a}	1.52±0.31^{a}
Formate	0.69±0.10^{a}	0.67±0.07^{a}	1.12±0.68^{a}
Malate	15.68±0.71^{b}	9.70±0.75^{c}	25.46±5.09^{a}
Malonate	0.96±0.01	nd	nd
Succinate	0.23±0.01^{b}	0.69±0.04^{a}	0.51±0.17^{a}
Other compounds			
Betaine	36.59±0.17^{a}	0.67±0.04^{b}	0.38±0.01^{c}
Choline	0.28±0.01^{b}	0.87±0.13^{a}	0.84±0.08^{a}

Data are expressed as mean values ± standard deviations (SD), n=3; nd.: not detectable; Mean values in the same row having different letters are significantly different (P<0.05; One–way ANOVA followed by the Bonferroni Multiple Comparisons Test).

TABLE 4. Fatty acid composition (% of total fatty acids) obtained by GC-FID analysis of *C. violacea* (CvO), *O. crenata* (OcO) and *O. lavandulacea* (OlO) SE oils.

Fatty acid	CvO	OlO	OcO
12:0	9.49±0.35^{a}	0.57±0.03^{c}	1.92±0.21^{b}
14:0	7.98±0.72^{a}	4.36±0.04^{b}	8.41±1.09^{a}
16:0	28.66±1.60^{b}	33.36±1.13^{a}	32.07±1.26^{a}
16:1 *n-7*	1.89±0.40^{b}	2.48±0.09^{a}	2.79±0.12^{a}
18:0	4.37±0.58^{a}	4.09±0.86^{a}	3.36±0.93^{a}
18:1 *n-7*	1.01±0.3^{b}	1.67±0.04^{a}	1.20±0.32^{ab}
18:1 *n-9*	9.71±0.88^{a}	9.63±0.13^{a}	6.88±0.71^{b}
18:2 *n-6*	30.78±1.08^{b}	23.95±0.32^{c}	34.36±1.47^{a}
18:3 *n-3*	0.33±0.36^{c}	18.92±0.25^{a}	8.29±0.77^{b}
SFA	50.49±3.13^{a}	42.69±0.62^{b}	46.04±2.66^{ab}
MUFA	12.61±1.29^{ab}	13.79±0.07^{a}	10.86±0.93^{b}
PUFA	34.11±1.44^{b}	42.87±0.29^{a}	42.66±2.23^{a}

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. All data are expressed as mean values ± standard deviations (SD); (n = 4). Mean values in the same row having different letters are significantly different (P<0.05; One–way ANOVA followed by the Bonferroni Multiple Comparisons Test).

Fatty acid profiles of parasitic plant oils

The GC-FID and HPLC-DAD analyses allowed us to obtain qualitative information on the individual FA composing the three oil extracts: *C. violacea* (CvO), *O. crenata* (OcO), and *O. lavandulacea* (OlO). Table 4 shows the composition of FA (expressed as % of total FA, g/100 g) of the oils obtained by GC-FID analysis. The content of saturated FA (SFA) in CvO was approximately 50%, which essentially contained lauric acid 12:0, myristic acid 14:0, palmitic acid 16:0, and stearic acid 18:0, with contents of 9.49%, 7.98%, 28.66%, and 4.37%, respectively. The CvO content of monounsaturated FA (MUFA) was approximately 12.61%, which was mainly composed of oleic acid 18:1 *n*-9 (9.71%) and palmitoleic acid 16:1 *n*-7 (1.89%), while the amount of polyunsaturated FA (PUFA) was 34.11%, which was mainly represented by the essential FA linoleic acid (18:2 *n*-6) and α-linolenic acid (18:3 *n*-3) (3.78% and 0.33%, respectively).

The absolute content of the main FA of CvO was detected by HPLC and the results were as follows: 118.5 mg/g, 41.4 mg/g, and 15.2 mg/g of 18:2 *n*-6, 18:1 *n*-9, and 18:3 *n*-3, respectively (Table 5). The two *Orobanche* oils showed different FA profiles compared to CvO, while some differences were observed between them. OlO showed a concentration of approximately 42.69% SFA, mainly 16:0 (33.36%), 14:0 and 18:0 (about 4% each); 13.79% MUFA, mainly 18:1 *n*-9 (9.63%); and 43% PUFA, mainly 18:2 *n*-6 (23.95%) and 18:3 *n*-3 (18.92%). OcO, compared to OlO, showed higher values of SFA (46.04%, with 32.07% of 16:0 and 8.41% of 14:0), a lower amount of MUFA (10.86%, with 6.88% of 18:1 *n*-9), and equal values of PUFA (42.66%), mainly represented by 18:2 *n*-6 (34.36%) and 18:3 *n*-3 (8.29%). The amounts of the main UFA in OlO and OcO determined by HPLC analysis were 172.0 and 201.0 mg/g for 18:2 *n*-6, 143.9 and 54.4 mg/g for 18:3 *n*-3, 81.2 and 47.4 mg/g for 18:1 *n*-9, respectively. All species exhibited a high level of SFA and PUFA. The main fatty acids were linoleic, palmitic and oleic acids in CvO; palmitic, linoleic and α-linolenic acids in OlO; and linoleic, palmitic and lauric acids in OcO. Significant differences were observed among oils in the levels of *n*-3 (ω-3) FA; in particular, OlO emerged as the richest in 18:3 *n*-3 (p<0.05). Moreover, all oils showed high levels of essential FA, which represented 31.1%, 42.9%, and 42.6% of the total FA content, in CvO, OlO, and OcO, respectively. The differences observed in the FA profiles of the oil could be attributable to several factors, such as variances in plant lipid metabolism, the impact of the harvest location (availability of climate, soil and water), and the diversity of metabolic precursors derived from different host plants.

Lipid content and composition are essential factors if a plant is to be considered from a food point of view. High levels of oleic (55–61%), linoleic (27–38%), and palmitic (6–9%)

TABLE 5. Unsaturated fatty acid composition (mg/g of oil) obtained by HPLC-DAD analysis of *C. violacea* (CvO), *O. crenata* (OcO), and *O. lavandulacea* (OlO) oils.

Fatty acid	CvO	OlO	OcO
16:1 *n*-7	2.43±0.54^c	8.62±0.62^b	10.07±0.24^a
18:1 *n*-9	41.44±0.28^c	81.19±4.28^a	47.40±0.32^b
18:2 *n*-6	118.47±2.69^c	172.02±3.59^b	201.00±1.83^a
18:3 *n*-3	15.23±0.46^c	143.86±3.29^a	45.44±0.56^b

All data are expressed as mean values ± standard deviations (SD); (n = 4). Mean values in the same row having different letters are significantly different (P<0.05; One–way ANOVA followed by the Bonferroni Multiple Comparisons Test).

acids have been previously reported in the seeds of *O. crenata* and *O. lavandulacea* [Velasco *et al*., 2000]. In contrast, our result showed that the lipids extracted from the whole plants contained predominantly linoleic and palmitic acids, since the pathways of lipid synthesis/accumulation may vary depending on the tissue [Chapman *et al*., 2012]. Moreover, OlO emerged as the richest in 18:3 *n*-3, an essential fatty acid that has been reported to modulate inflammatory responses in animal models and clinical trials and to reduce the cardiovascular disease [Rosa *et al.*, 2012].

The FA composition of oils makes these parasitic plants suitable for nutritional application because they provide a rich source of functional compounds (essential FA) with potential health benefits.

Cytotoxicity on B16F10 mouse melanoma cells

There is a great interest in the potential anticancer properties of unconventional vegetable oils and several reports have described interesting antimutagenic activities of fixed oils obtained from plants and herbs used in traditional medicine [Rosa *et al*., 2012]. Moreover, the oil extracted from the edible parasitic plant *Cynomorium coccineum* showed a significant cytotoxic effect on melanoma and colon cancer cells. Therefore, in the present study, we tested the cytotoxic effect of oils from the three parasitic species by MTT assay in B16F10 mouse melanoma cells, a metastatic cancer tyrosinase-expressing cell line [Casañola-Martin *et al*., 2014] used to screen natural antitumor extracts and lipid compounds. Figure 3 shows the viability, expressed as % of the control, measured in B16F10 cells after 24 h of incubation in the presence of different concentrations (50–500 μg/mL) of oil extracts. OlO showed the highest cytotoxic effect with respect to CvO and OcO. The treatment with OlO induced a significant reduction in cell viability at a concentration of 50 μg/mL in comparison with the control, with a viability reduction of 95% at 500 μg/mL. The treatment with OcO induced a significant reduction (P<0.001) in cell viability (45%) at only 500 μg/mL. EtOH, used to dissolve oils, was not toxic in cancer cells, and cell viability, measured at the maximal tested dose (1.2%), was 97%. CvO exhibited a significant cytotoxic effect from the 250 μg/mL dose, causing a viability reduction of 14% at the highest tested doses.

On the whole, our results showed that all the plant oils used in this study have a certain cytotoxic activity, which is manifested by the decrease in the viability of the B16F10 cells. None of the parasitic plants belonging to the Orobanchaceae family was previously used for viability tests on B16F10. Only one group reported a certain cytotoxicity of 3'-*O*-methyl isocrenatoside and methyl caffeate extracted from *Orobanche cernua* [Qu *et al*., 2016].

Among three plant species studied, *O. lavandulacea* was revealed to be a highly cytotoxic species for B16F10. In this

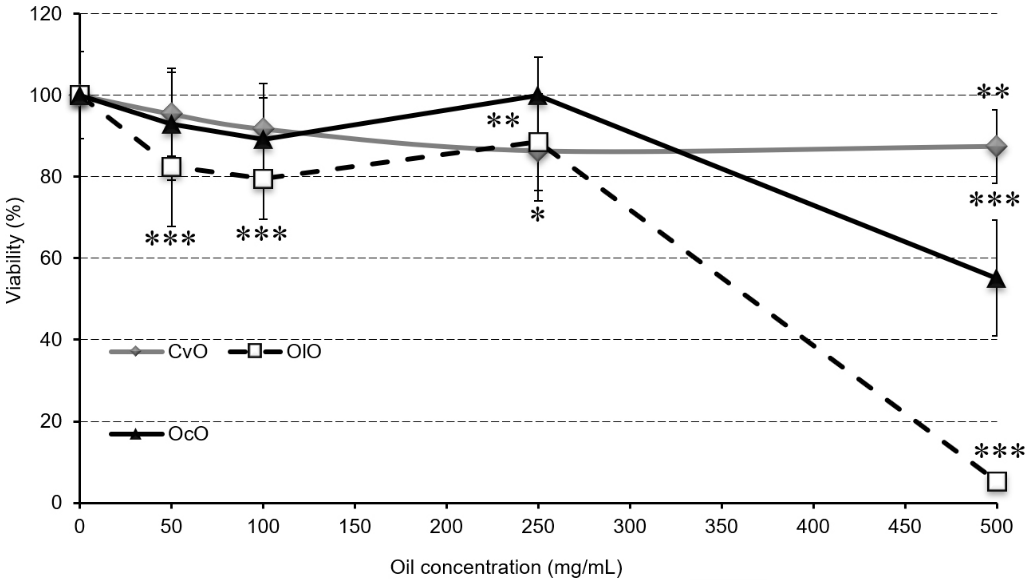

FIGURE 3. Viability, expressed as % of the control, induced by 24 h of incubation with different concentrations (50–500 μg/mL) of oils obtained from *C. violacea* (CvO), *O. crenata* (OcO), and *O. lavandulacea* (OlO) in B16F10 cancer melanoma cells (MTT assay). Three independent experiments were performed, and the data are presented as the mean ± SD; *** P<0.001; ** P<0.01; * P<0.05 *versus* control; (n=16) (one–way ANOVA followed by the Bonferroni Multiple Comparisons Test).

study, OlO emerged as the richest in 18:3 *n*-3 and several plant-based oils rich in α-linolenic acid, such as canola and flax seed oils, have shown the ability to modulate cancer cell growth and death. In particular, the fixed oil obtained from the aerial part of the parasitic plant *C. coccineum* showed a significant growth inhibitory effect on melanoma and colon cancer cells and its antitumor activity has been related to the oil level of 18:3 *n*-3 and the ability of this essential fatty acid to be the precursor in cancer cells for the formation of long chain *n*-3 PUFA (EPA and DHA) with anticancer activity [Rosa *et al*., 2012, 2015]. Nevertheless, further studies are needed to identify the OlO compounds responsible for the observed activity.

CONCLUSIONS

On the whole, these results highlighted the antioxidant activity measured as $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity and the ability to reduce ferric ions, as well as cytotoxic activity on B16F10 cells of three plants of Orobanchaceae family (*C. violacea*, *O. crenata*, and *O. lavandulacea*). Our findings support the role and value of wild foods in agricultural systems, despite the harsh climate conditions of growth. Despite the lack of a complete phytochemical characterization (one of the future required steps), such results could help in the valorization of these plants, contributing in preserving biodiversity. The reported observations, in fact, open the path to other lines of research, with the aim of improving the value of these species, and applications of these plants in several perspectives such as in nutraceutical and food production, in accordance with a possible use of parasitic plants as good alternative for expensive vegetables.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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Original research article
Food Quality and Functionality Section

Approaches for Improvement in Digestive Survival of Probiotics, a Comparative Study

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The aim of this study was to compare approaches commonly recommended in the literature for the improvement of the survival of probiotics in the human digestive tract. The survival of two probiotics, *Lactobacillus casei* and *Bifidobacterium lactis*, in the presence or absence of prebiotics, maize starch, fermented milk and upon encapsulation in calcium alginate-chitosan was evaluated. While *B. lactis* was resistant to stomach juice, but sensitive to duodenal juice, *L. casei* showed an exactly opposite behaviour. In contrast to other published studies, here the overall digestive survivability of probiotics was not improved significantly by prebiotics, maize starch or encapsulation. However, a significant improvement of the overall survivability of *B. lactis* (but not *L. casei*) during *in vitro* digestion was noted in milk and fermented milk, possibly due to reduction of the activity of bile against this probiotic. Summarising, no one method could be universally recommended for the improvement of probiotic survivability. Nevertheless, this research indicated that certain probiotic characteristics, such as susceptibility to bile or acid or ability to utilise matrix components as an energy source, could be used in further research to select the most effective approaches to deliver viable cells into lower parts of the digestive tract.

INTRODUCTION

Delivering live probiotics to the lower part of the human digestive tract, when administered orally, presents a challenge. Low stomach pH and action of bile salt hamper their survival [Bezkorovainy, 2001].

According to multiple studies, the survivability of probiotics in the digestive tract can be improved by application of a food matrix or addition of a prebiotic, or encapsulation [Burgain *et al.*, 2011; Govender *et al.*, 2014; Kingwatee *et al.*, 2014; Sanchez *et al.*, 2014]. As yet, these three methods were not confirmed to ensure effective survival of multiple strains of probiotics. Previous studies have examined the behaviour of only one or very few probiotics without consideration for differences in their survival characteristics [*e.g.* Burgain *et al.*, 2011; Haghshenas *et al.*, 2015; Kingwatee *et al.*, 2014; Lo Curto *et al.*, 2011; Sanchez *et al.*, 2014; Sumeri *et al.*, 2010]. Additionally, these studies varied in the application of *in vitro* digestion models which could be dynamic or static and applied different pH, digestion time, and chemical concentrations at each stage of digestion, making comparison difficult. Therefore, based on such data it is not possible to distinguish the method for oral route delivery of probiotics, with the best protective effect. This is important since many of the commercial probiotic formulations combine multiple strains.

The few existing studies comparing different approaches for the improvement of probiotic survivability focus on combined effects, *e.g.*, encapsulation with addition of elements of food matrix or prebiotic [Fredua-Agyeman & Gaisford, 2015; Shori, 2017]. Such studies also do not allow a conclusion as to whether there is a general method to improve the digestive survivability of probiotics.

Therefore, in this study we compared three methods previously shown to improve probiotic survival and two probiotic strains featuring different sensitivity to digestive juices.

The three compared methods were:

- Food matrix inclusion (fresh and fermented milk, maize starch),
- Different prebiotic concentrations (inulin and FOS),
- Electrospray encapsulation in calcium alginate and chitosan.

The chosen probiotic strains were *Lactobacillus casei* W56 and *Bifdobacterium lactis* W52, which according to the manufacturer should feature different sensitivities to gastro-intestinal conditions. *L. casei* W56 was sensitive to stomach juice but unstable in duodenum juice (unlike *B. lactis* W52).

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Thus, it was possible to test the protective effect of the applied methods to probiotics in stomach and duodenal conditions. Ideally, the method which would be generally recommended for the improvement of the probiotic survival should be effective in both types of digestive juices. Testing whether such an approach was available from those recommended by the literature was the subject of this study.

MATERIALS AND METHODS

Bacterial strains, materials, and reagents

Two probiotic strains used in the study, *B. lactis* W52 and *L. casei* W56, in a powdered form, were obtained from Winclove Probiotics (Amsterdam, Netherlands). Based on in-house experiments conducted by Winclove Probiotics, it was expected that *B. lactis* W52 would survive the *in vitro* digestion better compared to *L. casei* W56. Freeze-dried organisms were kept at 4°C prior to use. Winclove Probiotics supplied commercial prebiotics – Fructo-oligosaccharide (FOS P1) (hereafter FOS), and inulin containing a small quantity of FOS (Inulin and FOS P7) (hereafter inulin), and a probiotic supplement carrier material (Maize Starch) that were used as received. Full fat milk (composition per 100 mL: fat 3.6 g, sugar 4.7 g, and protein 3.4 g; pH 6.8) was purchased from a local supermarket. Sodium chloride, sodium bicarbonate, potassium chloride, calcium chloride, porcine pepsin, porcine pancreatin, and porcine bile used for the digestive assay were obtained from Fisher (Loughborough, UK). Sodium alginate, chitosan, and calcium carbonate used in probiotic encapsulation were purchased from Avonchem limited (Macclesfield Cheshire, UK), Acros Organics (Morris Plains, USA) and Fisher (Loughborough, UK), respectively.

Preparation of samples challenged to *in vitro* digestion

Portions of approximately 0.1 g of probiotic powders were combined with prebiotics (at four levels of concentration: 0.1, 0.5, 1, and 5%, *w/w*) or maize starch (5%, *w/w*) by mixing in sterile tap water. Probiotics were added aseptically. The count of cells in the initial sample was above 8 log cfu/mL (count of viable cells in each sample is presented in the Results and Discussion section).

To allow resuscitation of probiotics, samples were kept for 15 min at room temperature prior to analysis and commencement of the simulation of digestion. The highest concentration of prebiotic was chosen to reflect the dose that was previously shown to have a beneficial effect to the human health [Kellow *et al.*, 2014]. Fermented milk was prepared by the incubation of sterile milk (150 mL) with the addition of ~0.1 g of probiotic powder with either *L. casei* W56 or *B. lactis* W52 for 18 h at 40°C. The pH of milk following fermentation was 4.3 for *L. casei* W56 and 4.7 for *B. lactis* W52. Control samples contained only sterile tap water and probiotics.

For probiotic encapsulation, we have chosen calcium alginate additionally coated with chitosan. Calcium alginate is a frequently used encapsulation material, since it is food-grade and enables release of the probiotics in intestines [Segale *et al.*, 2016]. However, it tends to create porous capsules, which may mean that the probiotics would not be protected from the contact with digestive juices [Burgain *et al.*, 2011]. To minimise this effect and enhance the survival, calcium alginate capsules were coated with chitosan following the example of Shori [2017].

Consideration was also given to the method of capsule preparation. One of the novel methods being increasingly applied in research studies is electrospraying, where voltage is used to extrude a polymer solution through a capillary, resulting in a formation of small droplets [Coghetto *et al.*, 2016]. This method is suitable for use with probiotics as compared to some other methods, such as *e.g.* spray-drying, it does not require high temperatures.

For encapsulation, probiotics were mixed into the 22.5 g/kg sodium alginate solution. They were either added to the sodium alginate as a supplied freeze-dried powder (~0.1 g of powder to 5 mL of alginate; powder) or upon previous resuscitation (broth). The resuscitation was carried out by subsequently:

1. Inoculating 150 mL de Man Rogosa Sharpe (MRS) broth with ~0.1 g of probiotic powder and incubating for 24 h at 37°C (*B. lactis* was grown in MRS broth supplemented with 0.5 g/L L-cysteine),
2. Harvesting cells from 40 mL of the broth by centrifugation at 1500×*g* for 15 min at 25°C,
3. Washing and centrifuging the pellet twice with the saline solution using same settings as in 2., and
4. Re-suspending the pellet in 3 mL of the saline solution and adding to 20 mL of the alginate solution.

Suspensions of probiotics in sodium alginate were then electrosprayed. The electrospraying process has been explained in the previous publication [Zaeim *et al.*, 2017]. The equipment used for electrospraying was provided by Electrospinz Ltd (Blenheim, New Zealand). The set up was composed of a polymer header tank, a hollow needle with a 0.06 mm internal diameter, and a dish collector grounded through a crocodile clip. The solution was electrosprayed at 8 kV and the distance between the needle and the collector was 8 cm. The polymer/ probiotic solution was placed in the header tank and flowed under gravity to the needle. Droplets were electrostatically attracted into a dish collector which contained 500 mM calcium carbonate solution at pH 5.2. About 0.5 g of calcium alginate droplets encapsulating probiotics were obtained during a single 2 h run. Capsules were then filtered through a filter paper (Whatman no 4, Fisher, Loughborough, UK), rinsed with sterile water, and further coated with chitosan.

For coating, 20 mg of chitosan was dissolved in 2 mL of 100 mL/L glacial acetic acid and the pH was raised to ~6.0 by adding 0.5 M NaOH. Alginate micro beads were immersed in the chitosan solution and stirred at 60 rpm for 40 min using an orbital shaker (LSE, Corning, New York, US). This procedure was adapted from a method by Sohail *et al.* [2011].

The coated capsules were then filtered, washed twice with sterile water, and placed in a fresh portion of sterile water. The capsule suspension was stored for up to 2 days at 4°C prior to digestion assay.

Capsules were prepared in triplicate and characterised by means of the optical microscope (MOTIC B1 Advanced Series with Motic Images Plus version 3 software for image analysis). An example image of these capsules is shown in Figure 1. The prepared capsules contained probiotics at a level of >6 log cfu/g.

In vitro digestion model

To investigate the survivability of probiotics in the human digestive tract, a model designed based on several studies was applied. The composition and proportions of the digestive juices were adapted from studies of Marteau *et al.* [1997] and Timmerman *et al.* [2007] and were given in Table 1. The chosen transit times were 30 min in the stomach, 1 h in the duodenum and 2 h in the ileum. The pH during digestion was 2 for stomach and 6.5 for duodenum stage. Ileal juice was simulated by the addition of 11.5 mM of $CaCl_2$ to duodenum juice containing the sample in order to deactivate bile salts. Anaerobiosis during the digestion was created by overlying digestive liquids with 5 mL of mineral oil. Anaerobiosis seemed to be applied only in recent artificial digestion studies on the survival of probiotics, nevertheless it is a realistic condition present in the digestive tract. In our preliminary assessment, we saw a better survival of the microorganisms, especially *L. casei* W56 in the stomach juice, when anaerobiosis was applied. Stomach and duodenal juice were prepared and warmed up to 37°C prior to experimentation. Artificial digestion was carried out at 37°C with a constant agitation on an orbital shaker (LSE, Corning, New York, US) at 60 RPM.

Liquid samples were added in volumes of 5 mL, whilst capsules in quantities of ~0.5 g suspended in 5 mL of sterile deionised water to digestive juices. The pH of digestive juices was measured and re-adjusted upon the addition of the samples.

In vitro digestion of each sample was carried out in triplicate.

Enumeration of probiotics

Enumeration of lactic acid bacteria in the samples was carried out using the ISO 15214:1998 method. Briefly, samples were serially diluted in buffered peptone water (BPW, Oxoid, Basingstoke, England, CM0509). The diluted samples were then transferred in volume of 1 mL to empty Petri dishes and mixed with ~15 mL of de Man, Rogosa, Sharpe agar (MRS, Oxoid, Basingstoke, England, CM0361). Growth of *B. lactis* W52 was encouraged by the addition of 0.5 g/L of L-cysteine into MRS agar. Plates were incubated at 37°C for 72 h in 150 mL/L CO_2, <10 mL/L O_2 and N_2 atmosphere created with the MULTIVAC T200 tray sealer (Multivac, Wolfertschwenden, Germany). The limit of detection for the method was 1 cfu/mL of digestive juice. Results below the limit of detection were included in statistical analysis as 0.5 cfu/mL.

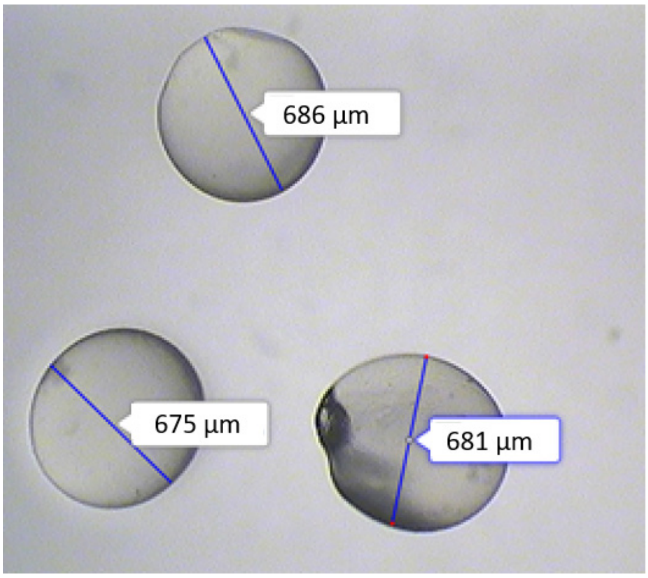

FIGURE 1. Light microscopy image of capsules obtained in the process of electrospraying.

Calcium alginate-chitosan capsules were visually intact through the entire digestion process. Hence, prior to enumeration, cells were released from the capsules. This was carried out by stirring encapsulated probiotics in 10 mL of 0.1 M phosphate buffer at pH 7 at room temperature for 30 min on a magnetic stirrer. The release of the probiotics from capsules has been confirmed by preliminary trials, where the count of viable cells was determined at different time points during mixing.

Statistical analysis

Statistical tests were performed using IBM SPSS Statistics 22. All assumed a significance level of 0.05. Particular tests are mentioned in the Results and Discussion section next to relevant data.

All log reduction values quoted in the text and shown in the figures were corrected for the dilution factors caused by the addition of stomach and duodenum juice to the samples within the digestive assay.

RESULTS AND DISCUSSION

The survival of probiotics during *in vitro* digestion

Survival curves of *L. casei* W56 and *B. lactis* W52 during passage through digestive liquids in control sample, as well as in the presence of prebiotics, food matrices, and in encapsulates was presented in Figure 2. Statistical tests (repeated measures

TABLE 1. Composition of simulated stomach and duodenal juice.

	NaCl	$NaHCO_3$	KCl	$CaCl_2$	Porcine pepsin	Porcine pancreatin	Porcine bile
	(g/L)						
Stomach	6.2	1.2	2.2	0.22	3.2	–	–
Duodenum	5	–	0.6	0.25	–	9	14

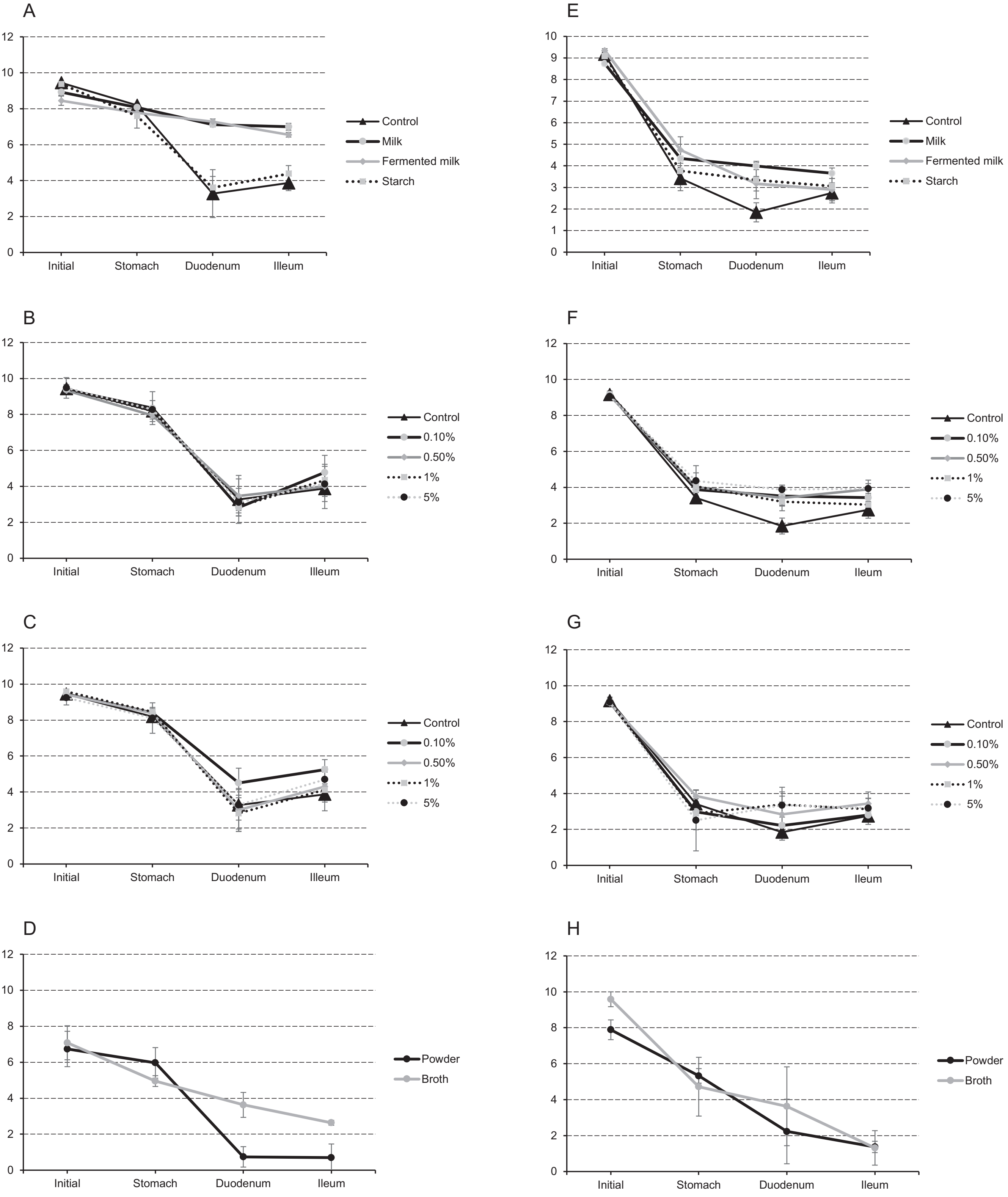

FIGURE 2. Survival during *in vitro* digestion of *B. lactis* W52 (graphs A, B, C and D) and *L. casei* W56 (graphs E, F, G and H) in presence of (A and E) food matrices, (B and F) inulin, (C and G) FOS, and (D and H) in capsules. Errors bars correspond to standard deviation. Counts are expressed per mL of initial solution. Dilutions factors from the addition of juices during the experiment were not compensated for.

ANOVA with Bonferroni post-hoc, results were not presented), indicated that stage in the digestion process had a significant effect on the log reduction of probiotic population. The digestion stage where the largest log reduction occurred was stomach for *L. casei* W56 (mean log reduction ± standard deviation, 4.4±1.1 log cfu) and duodenum for *B. lactis* W52 (3.9±1.9 log cfu). At the same time, *L. casei* W56 seemed relatively resistant to duodenum juice (0.6±1.4 log cfu) and *B. lactis* W52 to stomach juice (0.5±0.5 log cfu). The ileum juice offered the gentlest conditions for both probiotics (0.2±1.1 and -0.7±1.2 log cfu for *L. casei* W56 and *B. lactis* W52 respectively, negative log reduction indicated growth).

For *L. casei* W56, the survival curves representing control, food matrices, and all levels of prebiotics followed a similar pattern. Encapsulated, freeze-dried *L. casei* W56 seemed to reduce at similar rates through all digestive stages, indicating that the approach was able to minimise the effect of stomach juice on the probiotic. However, this trend was not observed in broth-grown, encapsulated *L. casei* W56, where a greater decline of probiotic population in the stomach was observed. The result indicates that the form in which *L. casei* W56 was encapsulated, rather than the encapsulation, had an influence on its survivability in the stomach. In turn, *B. lactis* W52 behaved similarly to the control when probiotic was challenged to digestive assay in the presence of maize starch, prebiotics at all concentration levels as well as within capsules containing probiotic powder. The survival curves of *B. lactis* W52 in the presence of milk and fermented milk as well as upon encapsulation of the broth-grown probiotics, resembled a straight line, meaning that the decline of the probiotics was similar through all the digestion stages. Out of these three treatments, encapsulation of the broth-grown probiotics seemed to feature a steeper decline for the population of *B. lactis* W52 compared to when the probiotic was challenged in milk or fermented milk.

Presented survival curves are real log cfu/mL counts disregarding the dilution of the probiotics by the addition of stomach and duodenum juices. To compare the effectiveness of different approaches, total log reductions were calculated based on the concentration of probiotics in the first (initial) and after passage through the last (ileum) stage correcting for dilution factors resulting from the addition of digestive juices (see Figure 3).

Total log reductions for *L. casei* W56 ranged from 4.2 to 7.4 log cfu (milk and capsules containing broth-grown probiotics, respectively) and for *B. lactis* W52 from 1.0 to 5.1 log cfu (fermented milk and capsules containing probiotic powder, respectively). For *L. casei* W56, there was no treatment which decreased the total log reduction significantly compared to the control. However, encapsulation of broth-grown probiotics caused a significant ($p<0.05$) increase of the total log reduction compared to the control and all other treatments. On the other hand, a significantly ($p<0.05$) greater survival of *B. lactis* W52 was obtained in the presence of milk and fermented milk compared to the control and all the other treatments.

Overall, mean total log reductions were significantly higher for *L. casei* W56 compared to *B. lactis* W52 (paired for treatments t-test, $p<0.05$), indicating that *B. lactis* W52 was more resistant to conditions of the digestive tract than *L. casei* W56. This was in line with the suggestion given by the probiotic provider, as specified in the method section.

The effect of probiotic type on the survival during *in vitro* digestion

This study evaluated survivability of two different probiotic bacteria, *L. casei* W56 and *B. lactis* W52, in human digestive juices. Other works suggest that these two probiotic species might exhibit contrasting survival in human digestive tract, although it should be noted that each of these works used a different *in vitro* digestion design [Fávaro-Trindade & Grosso, 2002; Kingwatee *et al.*, 2014; Lo Curto *et al.*, 2011].

Here, by application of a single *in vitro* digestion design for these two probiotic species, we had an opportunity to verify the difference in their survival. We have found that overall *B. lactis* W52 survived better compared to *L. casei* W56. Nevertheless, the magnitude of the differences in survival was affected by the type of the matrix surrounding probiotics.

The effect of the presence of food matrices on the survival of probiotics during *in vitro* digestion

The presence of a food matrix, such as milk, may substantially improve the survival of probiotics. Several authors noted that fat could potentially enhance probiotic survival. For example, Tompkins *et al.* [2011] showed that probiotics (ProtecFlor®, commercial supplement containing 4 probiotic strains) survived better in 1% fat milk and oats compared to fruit juice and spring water. Furthermore, Lo Curto *et al.* [2011] showed that the digestive survivability of two different probiotics: *L. casei immunitas* and *L. acidophilus johnsonii*, was improved in the presence of the whole milk matrix compared to water by 6.5 and 1 log cfu, respectively. This finding indicated that different probiotics may not be equally protected by fat. In the present study, food matrices containing fat, milk, and fermented milk, improved significantly the survival of *B. lactis* W52 (on average by 3.6 and 3.7 log cfu, respectively), but not that of *L. casei* W56 (on average by 1.3 and 0.0 log cfu, respectively). Since *B. lactis* W52 was sensitive to duodenum juice, while *L. casei* W56 was comparatively resistant to it, results might point at the role of fat in the protection of probiotics from bile. Given that the task of bile is to emulsify the fat to aid its digestion, inclusion of fat into probiotic matrix could mean that the bile salts would not be free to interact with probiotic cells [Begley *et al.*, 2005].

Based on the published literature, it was expected that fermentation of milk might add to the protective effect of the food matrix through:

1. Possible acid adaptation, especially of stomach juice sensitive *L. casei* W56, and
2. Propagation of the probiotic population from logarithmic growth phase into stationary phase.

Improvement of acid resistance might be expected, since the pH of fermented milk was lower than that of fresh milk (4.3 and 6.8, respectively). Nevertheless, in our study, the digestive survival of *L. casei* W56 was on average worse (although not significantly) in fermented milk compared to milk (total log reduction 5.5 and 4.2 log cfu, respectively). In contrast, the development of acid adaptation was demonstrated for *L. acidophilus* LA-5 and *L. rhamnosus* GG which after ex-

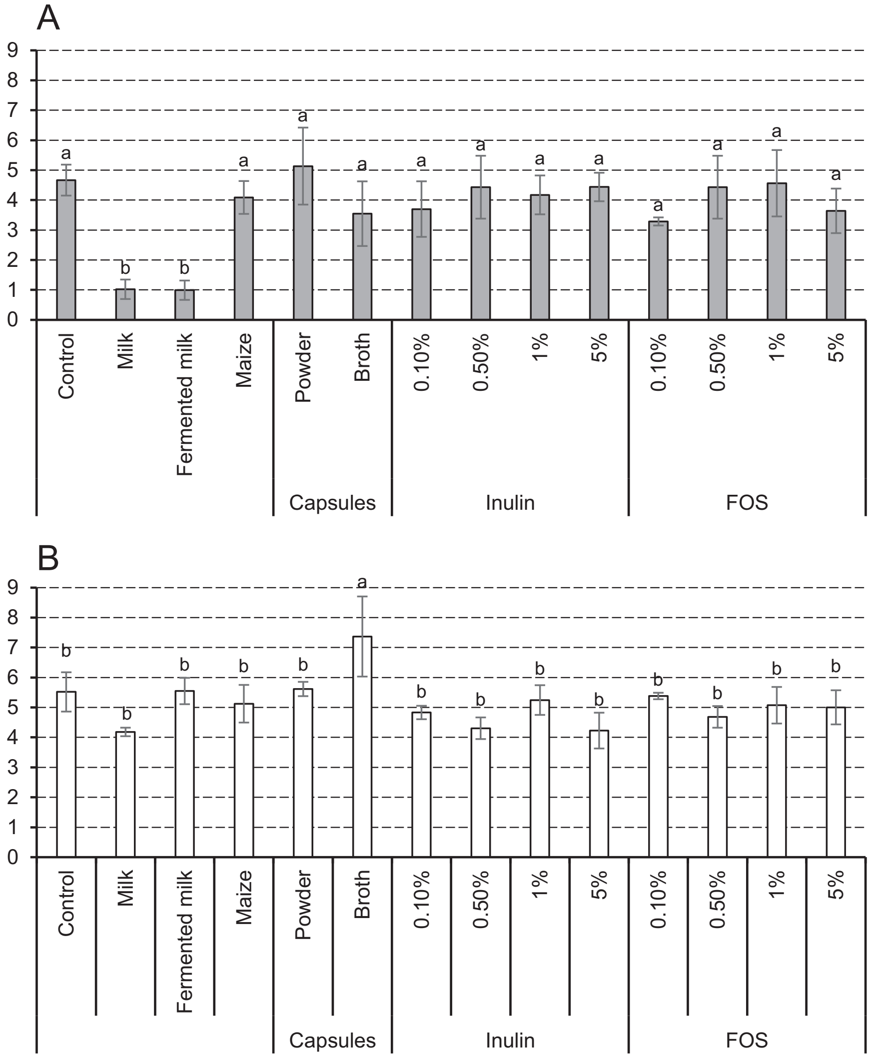

FIGURE 3. Total log reduction for (A) B. *lactis* W52 and (B) *L. casei* W56 in the *in vitro* assay. Columns note mean values and error bars standard deviations. Different letters above different columns in the same graph mean significant differences between different matrices and/ or control (ANOVA and Tukey post-hoc, significance level of 0.05).

posure to low pH (3.5) prior to digestion, showed a slightly better survival in the stomach acid compared to the control (by 0.31 and 0.30 log cfu, respectively [Sumeri *et al.*, 2010]). The same study did not report acid adaptation in these strains, when probiotics were adapted at pH 4.5, which was closer to pH of fermented milk here, and hence supports our finding [Sumeri *et al.*, 2010].

In fermented milk, cells should have reached the stationary growth phase. According to Lo Curto *et al.* [2011], probiotics challenged with digestive juices survive better when in the stationary phase compared to the logarithmic growth phase. Here, this effect was not observed for either of the two tested probiotics. Some possible reasons for different results between current and the Lo Curto *et al.* [2011] study are differences in used probiotic strains, digestive assay design as well as the way in which cells have been grown into the stationary phase. In the cited study, after the addition of probiotics to milk or water, samples were maintained at 4–6°C for 6 days, while here, the milk was fermented for 18h at 40°C and samples were subjected to the digestive assay, without chilling. It is not clear whether cold storage could improve the resistance of probiotics to digestive juices, nevertheless some information in support of this hypothesis can be found in the literature. It is known that the temperature of cell growth will affect the expression of genes and the physiological characteristics of microorganisms [Spano & Massa, 2006]. Additionally, cases of the resistance to multiple stressors upon adaptation to a single stressor have been documented in the literature. For example, acid adaptation of *Bifidobacterium breve* resulted in a better survival during cold storage of probiotic as well as during exposure to bile and hydrogen peroxide [Park *et al.*, 1995]. Overall, these data suggested that adaptation to cold stress could be beneficial in aiding probiotic survival during passage through the upper digestive tract.

Maize starch is used by probiotic manufacturers as a carrier material at a concentration of approx. 90% in the powdered product formulation (Winclove probiotics, private communication). In this study, digestive survival of *L. casei* W56 and *B. lactis* W52 in the presence of maize starch was not improved compared to the control. However, high amylose maize starch has been shown to enhance the survival of *Bifidobacterium* Lafti™ 8B and 13B during exposure to *in vitro* as well as *in vivo* (mice) digestion [Wang *et al.*, 1999]. Significantly, both of these probiotics had an ability to utilise amylose from maize starch. The mechanism of the improvement of the digestive survival of probiotics in the presence of food source was well explained by Corcoran *et al.* [2005]. These authors found that metabolizable sugars have been shown to improve the tolerance of *L. rhamnosus* GG to acid by provision of the energy for the exclusion of protons from cells. Summarising, studies suggest that the ability of probiotics to use a present matrix as an energy source might be a factor allowing to improve their digestive survival. This was partly supported by findings in our study. The total log reduction of stomach juice-sensitive *L. casei* W56 was on average lowest (although not significantly) in the presence of milk compared to all other treatments (including fermented milk where the lactose concentration would be lower), suggesting that the lactose present in milk could have aided survival of this probiotic in the stomach juice.

The effect of the encapsulation on the survival of probiotics during *in vitro* digestion

In the present study, we have compared two encapsulation approaches – powder and broth. Most of the reviewed studies have used freshly grown and harvested cells for encapsulation (broth method). Encapsulation of the powder might however make a commercial sense, since the population of encapsulated probiotics declines during storage [Yeung *et al.*, 2016].

Capsules obtained in this study measured between ~500 to 800 μm and on average 660 μm. This was much greater than 100 μm, the limiting size below which survival could not be facilitated [Khosravi Zanjani *et al.*, 2014]. Nevertheless, we found that the applied encapsulation did not improve the overall survival of the studied probiotics using neither of the probiotic strains nor the encapsulation approaches. These data do not generally agree well with the literature reviewed by Shori [2017]. Also, in recently published research papers [Yeung *et al.*, 2016; Zaeim *et al.*, 2017], alginate-chitosan encapsulation is claimed an effective means of protecting probiotics. Nevertheless, overall results show that this protection is limited. For example, Yeung *et al.* [2016] encapsulated *B. infantis* UMA299 into alginate-chitosan using injection-gelation method and observed that the encapsulation provided improved protection to stomach juice (by 1.3 log cfu compared to control), but not duodenum juice. In the present study, we also noted a significant improvement of protection of *L. casei* W56 in stomach juice compared to control when probiotic powder was encapsulated (by 3.2 log cfu; calculated based on data from Figure 2 when corrected for the dilution factor resulting from the addition of the digestive juice to the sample). Nevertheless overall survival of *L. casei* W56 did not improve due to increased sensitivity of the encapsulated probiotic to duodenal and ileal juice (increase of log reduction compared to control by 1.5 and 3.2 log cfu, respectively; calculated based on data from Figure 2 when corrected for the dilution factor resulting from the addition of the digestive juice to the sample). Furthermore, for encapsulates containing broth-grown *L. casei* W56, survival in the stomach or any other digestive stage was not improved, and total log reduction was significantly greater compared to the control (Figure 3).

In another study, authors investigated the survival of *L. plantarum* ATCC 8014 encapsulated into alginate-chitosan using electrospraying [Zaeim *et al.*, 2017]. They have found that overall the survival of probiotic was improved compared to free cells, but only by ~0.9 log cfu. This improvement was of a similar magnitude to the one observed for broth-grown, encapsulated *B. lactis* W52 (total log reduction decreased compared to control by 1.1 log cfu).

The effect of the presence and concentration of prebiotics on the survival of probiotics during *in vitro* digestion

It has been shown that probiotic survival in the digestive juices may be improved using prebiotics proportionally to the applied prebiotic concentration [Haghshenas *et al.*, 2015; Kingwatee *et al.*, 2014; Sanchez *et al.*, 2014]. In the present study, we did not observe the improvement of survival in digestive liquids with increased concentration of either inulin or FOS. Furthermore, the total log reduction seen upon application of prebiotics in our *in vitro* digestion, decreased by the maximum of 1.4 log cfu compared to the control (for *B. lactis* W52 with 0.1% FOS). Clearly, this decrease was lower compared to what could be expected based on the literature (upon application of prebiotic concentration ≥0.1% the log reduction decreased from 2 to ~4 log cfu in studies by Haghshenas *et al.* [2015], Kingwatee *et al.* [2014], and Sanchez *et al.* [2014]).

It is not clear why in this study we have not seen substantial improvement of probiotic survival in the presence of prebiotics. One of the possible reasons could be the ability of probiotics to metabolise substances as an energy source. In this study, we did not focus on probiotic metabolism but on the evaluation of different approaches for the improvement of probiotic survival. Nevertheless, obtained results and published literature data highlight that probiotic metabolism could be one of the factors contributing to probiotic stress resistance and should be a subject of further research [Wang *et al.*, 1999; Corcoran *et al.*, 2005].

CONCLUSIONS

In the present study we have reported survivability of *L. casei* W56 and *B. lactis* W52 in the presence and absence of food matrices, prebiotics and upon encapsulation during simulated passage through selected parts of the human digestive tract. Although improvements in the digestive survival of *B. lactis* W52 were achieved by application of milk and fermented milk, no solution seemed to improve viability of *L. casei* W56. Hence, neither of the examined methods could be recommended as a universal solution for the improvement of probiotic survival during passage through upper parts of the digestive tract.

Findings presented in this work suggested that in a choice of suitable method for the digestive survival improvement, probiotic characteristics play an important role. In the course of this study we have found that the studied probiotics featured a different survival behaviour. While *L. casei* W56 was sensitive to stomach juice, *B. lactis* W52 was relatively resistant to stomach juice but declined in the presence of duodenum juice. Interestingly, *B. lactis* W52 survived the digestion well in the presence of matrices that contained fat (milk and fermented milk, 3.6%). This finding indicated that the interaction of bile with fat might have minimised the losses of *B. lactis* W52 in the duodenum juice.

Another important characteristic of probiotics that may improve their ability to survive through upper digestive tract is the utilisation of the matrix components as an energy source. Prebiotics, starch as well as metabolizable sugars may be used by probiotics as food and consequently provide energy for the removal of protons from cells (as shown for glucose by Corcoran *et al.* [2005]), improving resistance to gastric acid. Although the study presented here did not focus on the characterisation of probiotic metabolism, obtained results highlighted that the ability to utilise surrounding matrix as a food source might be of key interest if the improvement of the probiotic survivability in the human digestive tract is sought.

Our results suggested that probiotic manufacturers could consider focusing on the development of suspension protocols for probiotic powders. Currently, the general guidance for a suspension of probiotic powder is to mix it with water prior to ingestion. Based on the results presented here, we could recommend using whole milk instead. Further research into optimisation of such protocols looking at different, acid-sensitive probiotics may be of benefit.

This research highlighted knowledge gaps in understanding mechanisms governing probiotic survival in the upper gastrointestinal tract. Optimisation of probiotic survival in studies investigating health benefits of probiotics could address to date observed discrepancies between the reports (as noted by *e.g.* Kasińska & Drzewoski [2015]).

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Original research article
Food Quality and Functionality Section

Antioxidant and Renin-Angiotensin System Inhibitory Properties of Cashew Nut and Fluted-Pumpkin Protein Hydrolysates

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Key words: fluted pumpkin, cashew nut, protein hydrolysates, renin, angiotensin converting enzyme, antioxidant

Antioxidant and renin-angiotensin system (RAS)-inhibitory protein hydrolysates derived from the enzymatic hydrolysis of cashew nut (CNP) and fluted pumpkin (FPP) proteins were investigated. The CNP and FPP hydrolysates (CNPH and FPPH) from pepsin or Alcalase treatments were subjected to membrane ultrafiltration using different MWCOs to obtain <1, 1–3, 3–5, 5–10, and >10 kDa peptide fractions. Hydrolysis of protein isolates at similar enzyme levels allowed obtaining peptic hydrolysates with a lower degree of hydrolysis (46.7–48.0%) when compared to the Alcalase-produced hydrolysates (47.7–50.3%). Amino acid composition revealed that CNPH had 28% hydrophobic residues when compared to higher contents (32–35%) in the 3–10 kDa peptide fractions. In contrast, aromatic residues increased from 8% in the FPPH to 9–13% in the peptide fractions. The *in vitro* $^{\bullet}$OH and DPPH$^{\bullet}$ scavenging activities were significantly ($p<0.05$) enhanced by ultrafiltration but potency was inversely related to peptide size. The ferric-reducing power was the highest for the <1 kDa CNPH (2.47) when compared to 1.33 for CNPH and other peptide fractions. Metal chelation ability was significantly ($p<0.05$) enhanced by ultrafiltration only for the CNPH with 21% compared to ~96% for the peptide fractions. ACE inhibition was significantly ($p<0.05$) lower for the Alcalase CNPH and peptide fractions (~87%) compared to ~92% of pepsin-CNPH. However, renin inhibition was significantly ($p<0.05$) increased by ultrafiltration from 45.7 and 62.1% to ~82.4 and 96.5% for FPPH and CNPH, respectively. We conclude that the strong antioxidant properties coupled with RAS inhibition make CNPH and FPPH as well as their low molecular weight peptides potential ingredients to formulate health-promoting foods.

INTRODUCTION

Scientific research has focused heavily on evaluating the nutritional value of the underutilized and underexploited food crops. Crops like cashew nut [Liu *et al*., 2018], *Buccholzia coriacea* [Ijarotimi *et al*., 2018a], African yam bean [Ajibola *et al*., 2016], bambara groundnut [Thammarat *et al*., 2015] and fluted pumpkin seeds [Fagbemi, 2007] have been investigated as functional (foaming and emulsifying) ingredients in industrial applications based on their protein content (15–30%). However, another option is to convert the proteins into value-added bioactive peptides by enzymatic hydrolysis. Bioactive peptides usually contain 2–20 amino acid residues per molecule, and are released upon enzymatic hydrolysis, during food processing or gastrointestinal digestion [Malomo *et al*., 2015]. These peptides may exert diverse physiological effects, such as antihypertensive, antimicrobial, antithrombotic, hypocholesterolaemic, antioxidative, and can also inhibit calmodulin-dependent enzymes [Aderinola *et al*., 2019; Nwachukwu *et al*., 2019; Siddeeg *et al*., 2015;Thammarat *et al*., 2015]. These physiological effects are due to their amino acid composition, peptide sequences and molecular weights.

Fluted pumpkin (*Telfairia occidentalis*), commonly known as *fluted gourd*, *Ugu* and *edikangikong* (in Ibo and Efik, Nigeria languages, respectively) is a tropical plant grown in West Africa with its seeds widely consumed in Nigeria as soup condiments [Fagbemi, 2007]. The fermented seeds have high seed protein contents (66–71%), which made them suitable functional ingredients to formulate marmalade and cookies [Fagbemi, 2007]. Cashew nut (*Anarcadium occidentale*) is a heart-like shaped fruit widely grown in Africa, with annual production of about 5–7 metric tonnes, but cultivated mainly as an export crop in Nigeria [Fagbemi, 2009]. Its proteins have a well-balanced amino acid composition, which is abundant enough to meet the requirements recommended by the FAO/WHO [Liu *et al*., 2018]. Besides, the SDS-PAGE pattern of cashew nut proteins revealed polypeptides of 21–32 and 53 kDa molecular weights (MW) under reducing and non-reducing conditions [Liu *et al*., 2018]. The SDS-PAGE pattern under reducing conditions indicate presence of inter-molecular disulfide bonds in the polypeptide molecules [Liu *et al*., 2018; Malomo

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& Aluko, 2015], which could lead to the production of sulfur-containing peptides with strong antioxidant properties. In addition, the amino acid compositions of fluted pumpkin and cashew nut proteins indicate an abundance of negatively charged amino acids (glutamic and aspartic) that can also contribute to antioxidant properties due to the presence of excess electrons [Udenigwe & Aluko, 2011]. Fluted pumpkin and cashew nut proteins also contain high levels of positively-charged (lysine and arginine) and branched-chain (valine, leucine, isoleucine) amino acids, which are reported to enhance the inhibitory activities of peptides against enzymes involved in hypertension development [Udenigwe *et al*., 2012; Wu *et al*., 2006a, b].

In disease pathogenesis and management systems, free radicals have been implicated to cause oxidative stress, giving rise to unacceptable state of health that reduce the quality of life through development of several chronic diseases. Therefore, it is important to inhibit or scavenge these radicals by using antioxidants [Arise *et al*., 2017]. Although, the body possesses natural self-defense mechanisms (such as antioxidants like glutathione and superoxide dismutase) to fight the free radicals, there exists a concern about the ineffective amounts present during illness and in old age [Thammarat *et al*., 2015]. Therefore, research efforts focused on food protein-derived hydrolysates, and peptides as new sources of natural antioxidants have recently emerged.

Various plant materials including *Linum usitatissimum* [Nwachukwu *et al*., 2019], *Buccholzia coriacea* [Ijarotimi *et al*., 2018b], *Cajanus cajan* [Olagunju *et al*., 2018], *Moringa oleifera* [Aderinola *et al*., 2019], *Vigna subterranean* [Arise *et al*., 2017], *Cucumis melo* [Siddeeg *et al*., 2015], and *Voandzeia subterranea* [Thammarat *et al*., 2015] have been identified as potential antioxidants sources. This was demonstrated by the strong antioxidant activity of their protein hydrolysates, including radical scavenging activity, reducing power and metal ion chelating activities.

Bioactive peptides from food proteins have also exhibited renin-angiotensin system (RAS)-inhibitory activity. Regulation of arterial blood pressure in the human body is mainly achieved through diverse physiological systems [Majumder & Wu, 2015] including the RAS, kinin-nitric oxide system (KNOS), renin-chymase system (RCS) and neutral endopeptidase system (NEPS). The key elements constituting the RAS are both the renin and angiotensin converting enzyme (ACE). The renal renin acts on the zymogen, angiotensinogen from the liver to produce inactive decapeptide, angiotensin-I (AT-I). ACE then, hydrolyzes AT-I by the removal of the dipeptide His-Leu from the C-terminus, to produce angiotensin-II (AT-II), a potent vasoconstrictor octapeptide [Aluko, 2019a; Malomo *et al*., 2015]. High levels of AT-II can lead to excessive vasoconstriction, which causes hypertension, a chronic medical symptom responsible for worldwide health problems because of its ability to trigger cardiovascular complications including peripheral vascular disease and renal dysfunctions [Arise *et al*., 2017]. Thus, RAS inhibitors block the formation of AT-II and potentiate action of the vasodilatory nitric oxide [Malomo *et al*., 2015].

Several potent synthetic ACE inhibitors (captopril, lisinopril, fosinopril, enalapril) are used extensively in the clinical treatment of hypertension but with significant adverse effects (dry cough, skin rashes, headache) on health [Malomo *et al*., 2015]. Recent studies on diverse peptides released from pigeon pea [Olagunju *et al*., 2018], Bambara groundnut [Arise *et al*., 2017], and pistachio [Li *et al*., 2014] proteins have shown ability to modulate RAS functions. Interestingly, studies on food bioactive peptides do not indicate side effects in the hypertension rat model [Udenigwe *et al*., 2017; Girgih *et al*., 2016]. However, previous works have reported on some structural features of potent antioxidant as well as ACE and renin-inhibitory peptides. For example, a quantitative structure-activity relationship (QSAR) study was used to show that amino acids with bulky and hydrophobic side groups are preferred for ACE-inhibitory dipeptides [Wu *et al*., 2006a]. For tripeptides, an aromatic amino acid at the C-terminus with a positively charged amino acid in the middle and a hydrophobic residue at the N-terminus enhanced ACE inhibition. For longer peptides, the ACE-inhibitory activity was dependent on the four amino acid residues present at the C-terminus [Wu *et al*., 2006b]. Meanwhile, the potency of dipeptides that inhibit renin activity was shown to be dependent on a branched-chain amino acid at the N-terminus with tryptophan at the C-terminus [Udenigwe *et al*., 2012]. With respect to antioxidant protein hydrolysates, the presence of sulfur-containing, acidic amino acids, lysine and leucine enhanced free radical scavenging ability [Udenigwe & Aluko, 2011].

Accordingly, more studies are now directed towards understanding how to produce food protein-derived peptides to be used as nutraceuticals for managing chronic diseases. This study, thus aimed to investigate and assess the feasibility of producing enzymatic fluted pumpkin seed and cashew nut protein hydrolysates with *in vitro* antioxidant and RAS--inhibitory activities.

MATERIALS AND METHODS

Materials

Fluted pumpkin seeds and cashew nuts were obtained from the Federal University of Technology, Teaching and Research Farm, Akure, Nigeria, authenticated in the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure, Nigeria. Renin was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) while other enzymes such as Alcalase, pepsin and ACE (rabbit lung) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other analytical-grade reagents were obtained from Fisher Scientific (Oakville, ON, Canada).

Preparation of fluted pumpkin and cashew nuts protein meal

The fluted pumpkin seeds were dehulled and sliced into small pieces according to the previously described method [Fagbemi, 2007]. The sliced seeds were oven dried at 50°C (Plus11 Sanyo Gallenkamp PLC, UK), for 8 h and then pulverized into flour using a coffee grinder. The flour was then defatted continuously for 8 h with a Soxhlet's apparatus using *n*-hexane as a solvent and air-dried for 24 h in a fume hood at room temperature to remove solvent residues. The defatted flour was then passed through 0.5 mm sieves, packed in plastic containers, labeled as the fluted pumpkin protein flour (FPP), and kept at -20^{o}C.

The cashew nuts were cut into halves using the manual cashew kernel cutter according to the previously described method [Fagbemi, 2009]. After cutting, the nuts were removed and dried in an oven at 100°C for 2 h. The covering testas were removed by squeezing and then winnowed to obtain cream color nuts. The nuts were then broken into smaller pieces and defatted using *n*-hexane solvent. The defatted nuts were air-dried for 24 h in a fume hood at room temperature to remove the residual *n*-hexane, and thereafter milled into flour using a blender (Philips laboratory blender, HR2811 model, The Netherlands). The defatted cashew nut flour (CNP) was passed through a sieve with 0.5 mm mesh size, packed into a polythene bag and then stored at -20^{o}C.

Preparation of fluted pumpkin seed and cashew nuts protein isolates

Fluted pumpkin seed and cashew nuts protein isolates (FPPI and CNPI) were produced from FPP and CNP according to the method described by Malomo *et al.* [2014] with slight modifications. Briefly, FPP and CNP were dispersed in deionized water (1:20, *w/v*) and the dispersion was adjusted to pH 10 using 2 M NaOH to solubilize the proteins while stirring at 37^{o}C for 2 h; this was followed by centrifugation (7,000×*g*, 60 min at 4^{o}C). The precipitate was discarded while the supernatant was filtered and thereafter adjusted to pH 4.0 with 2 M HCl to precipitate the proteins; this was followed by centrifugation (7,000×*g*, 60 min at 4^{o}C). The resultant precipitate was re-dispersed in deionized water, adjusted to pH 7.0 with 2 M NaOH, and freeze-dried to obtain the FPPI and CNPI. Protein contents were determined by the Lowry method [Markwell *et al*., 1978] using 0.1 M NaOH to dissolve the samples.

Preparation of fluted pumpkin and cashew nuts protein hydrolysates and ultrafiltration membrane hydrolysate fractions

Fluted pumpkin seeds and cashew nut protein hydrolysates (FPPH and CNPH) were produced according to the method described by Malomo *et al*. [2015] with slight modifications using Alcalase at 50^{o}C, pH 8 for 4 h and pepsin at 37^{o}C, pH 2 for 2 h. FPPI and CNPI (5%, *w/v*, protein weight basis) were each suspended in deionized water in a glass beaker equipped with a stirrer, and adjusted to the appropriate temperature and pH prior to addition of the proteolytic enzyme. Alcalase (>2.4 U/g) and pepsin (>250 units/mg) were added separately to the FPPI and CNPI slurry at a 1–4:100 ratio (E/S). During hydrolysis, the required pH was maintained constant by addition of NaOH and HCl, respectively after which the enzymes were inactivated by adjusting to pH 4.0 with 2 M HCl followed by immersing the reaction vessel in boiling water bath for 10 min. After cooling to room temperature, the undigested proteins were precipitated by centrifugation (3,500×*g*, 60 min at 4^{o}C) and a portion of each supernatant was freeze-dried as the FPPH and CNPH, which were then stored at -20^{o}C. Protein contents of the freeze–dried FPPH and CNPH were determined using the modified Lowry method [Markwell *et al*., 1978]. The supernatants from Alcalase-produced FPPH and pepsin-produced CNPH at 2:100 and 3:100 ratios (E/S), respectively were also sequentially passed through ultrafiltration membranes with molecular weight cut-off (MWCO) of 1, 3, 5, and 10 kDa in an Amicon stirred ultrafiltration cell (Merck KGaA, Darmstadt, Germany). Thus, the retentate from 1 kDa membrane was passed through 3 kDa membrane whose retentate was passed through 5 kDa and the last retentate was then passed through 10 kDa membrane. The permeate from each MWCO membrane was collected as peptide sizes of <1, 1–3, 3–5, and 5–10 kDa, freeze-dried, and stored at –20°C.

Amino acid composition analysis

The amino acid profiles of the fluted pumpkin and cashew seed protein products were determined using the HPLC Pico-Tag system (Waters, Milford, MA, USA) according to the previously described method after samples were digested with 6 M HCl for 24 h [Bidlingmeyer *et al*., 1984]. The cysteine and methionine contents were determined after performic acid oxidation [Gehrke *et al*., 1985] and the tryptophan content was determined after alkaline hydrolysis [Landry & Delhaye, 1992]. The samples were derivatized for 20 min using a solvent mixture containing 95% ethanol:water:triethylamine:phenylisothiocyanate (7:1:l:1, *v/v/v/v*), dried under vacuum and dissolved in buffer A (940 mL of 0.14 M sodium acetate, pH 6.40, containing 0.05% triethylamine, mixed with 60 mL acetonitrile) prior to HPLC separation on the Pico-Tag column using a flow rate of 0.45 mL/min and detection at 254 nm. The gradient was from 10–50% buffer B (60% acetonitrile and 40% water by volume) in buffer A over 10 min.

Determination of the degree of hydrolysis

The degree of hydrolysis (DH) was defined as percentage of α-amino groups of substrates released during hydrolysis and was determined using the trinitrobenzene sulfonic acid (TNBS) method as previously described [Adler-Nissen, 1979] and modified by Malomo *et al*. [2015]. Briefly, CNPI and FPPI were treated under vacuum with 6 M HCl for 24 h and α-total amino groups as L-leucine equivalent was determined. The DH was calculated as the percentage ratio of the L-leucine equivalent of CNPH and FPPH to that of CNPI and FPPI.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activities of FPPH, CNPH, and peptide fractions were determined according to the method described by Girgih *et al.* [2011]. Experimental samples, GSH and 1,10-phenanthroline (3 mM) were each separately dissolved in 0.1 M phosphate buffer (pH 7.4) while $FeSO_4$ (3.0 mM) and 0.01% hydrogen peroxide were each dissolved in distilled water. An aliquot (50 μL) of samples or GSH (reaction mixture concentration of 1 mg/mL) or buffer (blank) was first added to a clear, flat bottom 96-well plate followed by 50 μL of 1,10-phenanthroline and then 50 μL of $FeSO_4$. To initiate the Fenton reaction in the wells, 50 μL of hydrogen peroxide was added to the mixture, the plate was covered and incubated at 37^{o}C for 1 h with shaking. The change in absorbance values of samples (s) and blank (b) were measured using Biotex 542 Powerwave XS microplate-reader (Biotek Instruments Inc., Winooski, Vermont, USA) at 536 nm at 10 min intervals for 1 h. The hydroxyl radical scavenging activity was calculated using the reaction rate (ΔA/min) equation below:

$$\text{OH radical scavenging activity } (\%) = \\ = \left(\frac{(\Delta A_{536}\ /\text{min })\text{b} - ((\Delta A_{536}\ /\text{min })\text{s}}{(\Delta A_{536}\ /\text{min })\text{b}}\right) \times 100$$

2,2-Diphenyl-1-picryhydrazyl radical scavenging assay

The scavenging effect of CNPH, FPPH, and their peptide fractions on 2,2-diphenyl-1-picryhydrazyl free radical (DPPH$^{\bullet}$) was measured according to the method of Aluko & Monu [2003]. FPPH, CNPH and peptide fractions (10 mg) were each dissolved in 1 mL of buffer (0.1 M sodium phosphate buffer, pH 7.0 containing 1% (*w/v*) Triton X-100). DPPH$^{\bullet}$ was dissolved in methanol to a final concentration of 100 μM. A 100 μL aliquot of each sample solution (containing FPPH, CNPH or peptide fractions) was mixed with 100 μL of the DPPH$^{\bullet}$ solution in the 96-well plate to a reaction mixture concentration of 1 mg/mL (0.2 mg/200 μL) and incubated at room temperature in the dark for 30 min. The absorbance values of the blank, reduced glutathione (GSH) and samples were measured at 517 nm using a Biotex 542 Powerwave XS microplate-reader. The blank consisted of sodium phosphate buffer in place of the peptide sample, while GSH (1 mg/mL) was used as the positive control. The percent DPPH$^{\bullet}$ radical scavenging activity of the samples was determined using the following equation:

$$\text{DPPH}^{\bullet}\text{radical scavenging activity } (\%) = \left(1 - \frac{A_{517}\ of\ sample}{A_{517}\ of\ blank}\right) \times 100$$

Ferric-reducing power

The ferric-reducing power of the FPPH, CNPH, and membrane fractions was determined according to the modified method of Zhang *et al*. [2008]. Experimental sample or GSH (10 mg) was dissolved in 1 mL of 0.2 M phosphate buffer, pH 6.6; an aliquot (250 μL) was mixed with 250 μL of the buffer and 250 μL of 1% potassium ferricyanide solution. The mixture was thoroughly mixed using a vortex machine and heated at 50°C for 20 min. After incubation, 250 μL of 10% trichloroacetic acid was added followed by 50 μL of 0.1% ferric chloride dissolved in double distilled water and then 200 μL of double distilled water was added to give sample concentration of 1 mg/mL reaction mixture. The solution was allowed to stand for 10 min at room temperature, after which it was centrifuged at 1,000×*g* for 10 min. An aliquot (200 μL) of the supernatant was transferred to a clear bottom 96-well plate and the absorbance was measured at 700 nm using a Biotex 542 Powerwave XS microplate reader. The ferric reducing power was presented as absorbance values at 700 nm.

Chelation of ferrous ions

The ferrous ion chelating activity of the FPPH, CNPH, and membrane fractions was determined using a modified method of Xie *et al*. [2008]. Experimental samples and GSH solution were each combined with 0.05 mL of 2 mM $FeCl_2$ and 1.85 mL double distilled water in a reaction tube to give 1 mg/mL reaction mixture concentration. Ferrozine solution (0.1 mL of 5 mM) was added and mixed thoroughly. The mixture was then allowed to stand at room temperature for 10 min from which an aliquot of 200 μL was removed and added to a clear bottom 96-well plate. A blank experiment was also conducted by replacing the sample with 1 mL of double distilled water. The absorbance of blank and sample at 562 nm was measured using a Biotek microplate reader and the ferrous ion chelating activity of the sample compared to that of GSH. The percentage chelating effect (%) was calculated using the following equation:

$$\text{Ferrous ion chelating activity } (\%) = \left(1 - \frac{A_{562}\ of\ sample}{A_{562}\ of\ blank}\right) \times 100$$

Angiotensin-Converting Enzyme (ACE) inhibition assay

The ability of FPPH, CNPH, and membrane fractions to inhibit *in vitro* ACE activity was measured according to a spectrophotometric method using synthetic *N*-[3-(2-furyl) acryloyl]-l-phenylalanyl-glycyl-glycine (FAPGG) as the substrate (Sigma-Aldrich, St. Louis, MO, USA) as reported by Malomo *et al*. [2015]. Briefly, 1 mL of 0.5 mM FAPGG (dissolved in 50 mM Tris-HCl buffer containing 0.3 M NaCl, pH 7.5) was mixed with 20 μL of ACE (20 mU final reaction activity) and 200 μL sample dissolved in same buffer. The rate of decrease in absorbance at 345 nm was recorded for 2 min at room temperature using Varian Cary 50-UV/Visible spectrophotometer (Varian Inc., Belrose, NSW, Australia). The buffer was used instead of sample solutions in the blank experiment, while the sample concentration in the reaction mixture was 1 mg/mL. ACE activity was expressed as the rate of reaction (ΔA/min) and inhibitory activity was calculated as:

$$\text{ACE inhibition } (\%) = 1 - \left({\Delta A/\text{min} - 1(\text{sample})}\Big/{\Delta A/\text{min} - 1(\text{blank})}\right) \times 100$$

where: $\Delta A/\text{min}_{(\text{sample})}$ and $\Delta A/\text{min}_{(\text{blank})}$ represent ACE activity in the presence and absence of the samples, respectively.

Renin inhibition assay

In vitro inhibition of human recombinant renin activity by FPPH, CNPH, and membrane fractions was conducted using the Renin Inhibitor Screening Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the previously described method [Malomo *et al*., 2015]. Prior to the assay, renin buffer was diluted in 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl. The renin protein solution was diluted 20 times with the assay buffer before use and pre-warmed to 37°C prior to initiating the reaction in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA, USA) maintained at 37°C. Before the reaction, (i) 20 μL substrate, 160 μL assay buffer, and 10 μL Milli-Q water were added to the background wells; (ii) 20 μL substrate, 150 μL assay buffer, and 10 μL Milli-Q water were added to the blank wells; and (iii) 20 μL substrate, 150 μL assay buffer, and 10 μL sample (1 mg/mL reaction mixture concentration) were added to the inhibitor wells. The reaction was initiated by adding 10 μL renin to the blank and sample wells. The microplate was shaken for 10 s to mix, incubated at 37°C for 15 min, and the fluorescence intensity (FI) was recorded using excitation and emission wavelengths of 340 and 490 nm, respectively. The percentage renin inhibition was calculated as follows:

$$\text{Renin inhibition (\%)} = 1 - \left({}^{\text{FI sample}}\!/_{\text{FI (control)}} \right) \times 100$$

Statistical analysis

Triplicate replications were used to obtain mean values and standard deviations. Statistical analysis was performed with SAS (Statistical Analysis Software 9.1) using one-way ANOVA. Duncan's multiple-range test was carried out to compare the mean values for samples with significant differences taken at $p<0.05$.

RESULTS AND DISCUSSION

Amino acid composition

The protein contents of the CNPI and FPPI are 84 and 82.6%, respectively, which fall in the ranges (80–90%) previously reported [Malomo *et al*., 2014] for protein isolates from different plant protein sources. The amino acid compositions of peptides present in CNPH and FPPH as well as their membrane fractions are presented in Table 1. The results clearly showed that Glu, Asp, and Arg were present in the highest amounts of 14.66–23.29, 8.71–11.24, and 5.94–20.89 g/100 g, when compared to 3.32–7.15, 3.20–5.00, and 1.40–2.85 g/100 g obtained for other amino acids such as Val, Ile and Trp, respectively. The potentials of Glu and Asp to donate excessive electrons as free radicals scavengers (strong antioxidant) and Arg to work as NO-precursor, a known vasodilator, made them potential tissue protective agents against oxidative stress [Jakubczyk & Baraniak, 2014] and hypertension [Malomo *et al*., 2015]. Similar levels of Val, Ile, Glu, and Arg have been previously reported for cashew nut albumin and globulin fractions [Liu *et al*., 2018]. In this study, the results (3.27–6.25 g/100 g) obtained for Pro, a crucial amino acid that has been reported to contribute to the effectiveness of ACE-inhibitory peptides, were higher than the 1.33–2.25 g/100 g reported for *Phaseolus lunatus* protein hydrolysates [Magana *et al*., 2015]. The results (Table 1) further confirm the protein hydrolysates as rich sources of hydrophobic (HAA) and aromatic (AAA) amino acids that could contribute to the antioxidant and RAS inhibition activities [Magana *et al*., 2015].

TABLE 1. Amino acid composition (g/100 g) of cashew nut (CNPH) and fluted-pumpkin (FPPH) protein hydrolysates and their membrane fractions.

Amino acid	CNPH[1]	CNPH fractions				FPPH[2]	FPPH fractions				Least significant difference ($p<0.05$)
		<1 kDa	1–3 kDa	3–5 kDa	5–10 kDa		<1 kDa	1–3 kDa	3–5 kDa	5–10 kDa	
Asp	11.24	9.10	9.61	10.05	11.12	8.71	10.81	10.62	9.15	11.03	0.97
Thr	3.38	3.14	3.37	4.41	3.91	2.71	3.54	3.59	3.86	3.29	0.03
Ser	4.93	5.37	4.96	5.02	4.99	4.23	5.25	5.74	6.15	4.88	0.87
Glu	18.56	23.29	18.39	18.63	21.42	18.86	21.15	20.15	14.66	18.94	3.42
Pro	5.18	4.12	4.55	3.73	4.38	6.25	4.69	4.89	3.27	4.94	0.77
Gly	4.46	4.21	4.53	3.68	4.36	4.90	4.50	4.42	4.17	4.40	0.84
Ala	4.27	2.98	4.30	4.85	3.94	3.14	3.68	3.41	6.44	4.18	0.06
Cys	0.82	1.95	0.83	0.40	0.40	0.99	0.88	1.05	0.21	0.87	0.11
Val	4.25	5.10	4.57	7.04	7.15	3.32	6.07	5.16	5.97	4.48	0.88
Met	1.37	1.25	1.43	1.38	1.43	1.05	1.49	1.27	1.94	1.41	0.12
Ile	4.24	3.63	4.29	4.50	4.02	3.20	3.75	3.64	5.00	4.33	0.10
Leu	7.90	7.03	7.74	13.56	10.84	6.12	8.76	7.26	10.32	7.96	1.24
Tyr	4.01	3.19	3.88	3.14	3.38	3.45	3.64	3.27	5.00	3.78	0.08
Phe	4.56	4.51	4.65	6.64	5.09	3.10	4.58	4.16	6.02	4.33	0.29
His	2.67	2.39	2.77	1.78	2.01	3.16	2.49	2.78	2.72	2.71	0.12
Lys	3.20	3.96	3.25	2.43	2.64	4.41	3.23	4.41	2.57	3.03	0.14
Arg	13.35	13.44	15.23	5.94	7.10	20.89	9.45	12.28	10.79	13.90	4.21
Trp	1.62	1.40	1.73	2.85	1.84	1.52	2.08	1.91	1.78	1.57	0.08
HAA[3]	28.03	26.06	27.71	35.46	32.16	24.07	29.32	26.68	33.15	28.17	6.20
AAA[4]	10.19	9.10	10.26	12.63	10.31	8.07	10.30	9.34	12.80	9.68	0.94

[1]HAA = Hydrophobic amino acids, [2]AAA = Aromatic amino acids.

Degree of hydrolysis

The bioavailability of peptides released during protein hydrolysis process is related to their structural properties (amino acid composition and sequence), which is dependent on the degree of hydrolysis [Jakubczyk & Baraniak, 2014]. Figure 1 revealed the different degrees of hydrolysis (DH) for the enzymatic treatment of CNPI and FPPI proteins. The CNPI was observed to be more readily hydrolyzed by Alcalase at enzyme to substrate (E/S) ratios of 1–3:100 than the FPPI, which may be due to differences in the primary structure of the two seed proteins. In contrast, similar DH values were obtained for CNPI and FPPI hydrolyzed by pepsin. Generally, for each seed protein, higher DH values were achieved for hydrolysates obtained with Alcalase when compared to pepsin activities. The current outcome is contrary to the findings of Arise *et al*. [2019] where the DH of *Azadirachta indica* seed protein hydrolysates from pepsin activity was higher (27.88%) than the 14.73% obtained for Alcalase hydrolysis. The higher DH observed for the Alcalase-produced hydrolysates (~51%) might be due to the broad and non-specific enzymatic activity when compared to pepsin (47%), which has a narrow proteolytic specificity for peptide bonds formed by amino acids with aromatic or bulky side chains [Aluko, 2019b]. The results are comparable to the 45% DH reported for Alcalase-hydrolyzed horse gram flour [Bhaskar *et al*., 2019]. Decreases in DH with increased E:S may be due to enzyme overcrowding, which could lead to redundancy or antagonistic effects that lowered the rate of proteolysis.

The DH values presented for peptic CNPH (47%) and FPPH (46%) were higher than the values of 14, ~28, and 28% previously reported for hydrolysates obtained by simulated gastrointestinal digestion of pea globulin [Jakubczyk & Baraniak, 2014], neem seed protein [Arise *et al*., 2019], and hemp protein [Malomo *et al*., 2015], all with ACE-inhibitory or RAS-inhibitory activities. The samples preparation, different protein sources, and specificities of the proteases [Jakubczyk & Baraniak, 2014] might have resulted in the differences of DH values obtained for these hydrolysates. The high DH for CNPH and FPPH indicates short peptide lengths, which could enhance inhibition of ACE and renin activities. Overall, the data showed that the CNPI and FPPI were highly susceptible to Alcalase and pepsin proteolysis.

Hydroxyl radical scavenging activity

The hydroxyl radical (•OH) is one of the most damaging reactive oxygen species (ROS) because it reacts with all cellular macromolecules such as proteins, polyunsaturated fatty acids (PUFAs) and nucleic acids to induce severe damages to cells [Jin *et al*., 2016]. Therefore, •OH scavenging is an effective defense strategy of the human body against various diseases elicited by ROS [Zhuang *et al*., 2013]. The ability of CNPH or FPPH and their membrane fractions to scavenge •OH is presented in Figure 2. The •OH scavenging activity (HRSA) of CNPH and FPPH were 58 and 38%, respectively when compared to GSH (57%), a recognized potent physiological antioxidant. However, the HRSA was significantly ($p<0.05$) increased upon membrane filtration and separation into peptides of varying sizes. The results further showed the direct and indirect relationship between the DH (Figure 1) and mo-

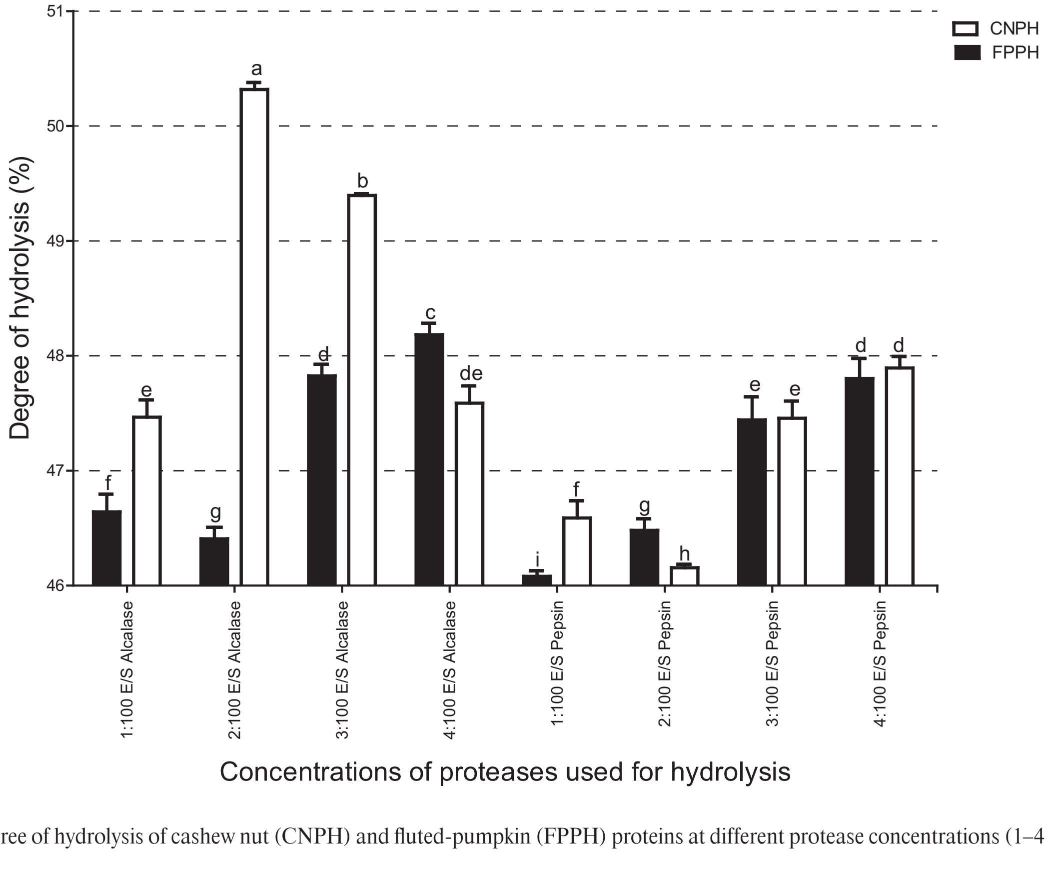

FIGURE 1. Degree of hydrolysis of cashew nut (CNPH) and fluted-pumpkin (FPPH) proteins at different protease concentrations (1–4:100 ratio E/S).

lecular weight sizes, respectively on the HRSA of the protein hydrolysates. For instance, the higher DH of CNPH over FPPH was directly related to their HRSA while the scavenging effect reduced with increase in peptide size. For example, the HRSA values of <1 kDa fractions of CNPH (~99%) and FPPH (95%) were higher than those of the >10 kDa fractions (42 and 38%, respectively). In addition, the CNPH had a higher content of HAA, which may have contributed to the higher HRSA when compared to FPPH.

Previous report [Ketnawa *et al*., 2017] had suggested that the antioxidant activity of peptides is dependent on the molecular mass, hydrophobicity and type of amino acid as well as enzymatic hydrolysis reaction conditions. The current findings (Figure 2) are in agreement with the stronger HRSA for LMW than the HMW peptides of barley glutelin [Xia *et al*., 2012] but are different from the reported trend for *B. coriacea*-derived peptides [Ijarotimi *et al*., 2018b]. Our results are higher than the 23.36, 22.50, 32.3, and 81% HRSA reported for pancreatin-produced pigeon pea [Olagunju *et al*., 2018], black soybean [Ralison *et al*., 2013], 5–10 kDa fractions from *B. coriacea* seed [Ijarotimi *et al*., 2018b], and alfalfa leaf [Xie *et al*., 2008] protein hydrolysates, respectively.

2,2-Diphenyl-1-picryhydrazyl radical scavenging activity

2,2-Diphenyl-1-picryhydrazyl radical scavenging activity (DRSA) is one of the many assays employed to understand the mechanism of antioxidant action of food protein-derived peptides. Figure 3 showed the DRSA of the pepsin-produced (3:100 ratio E/S) CNPH and Alcalase-produced (2:100 ratio E/S) FPPH, which ranged from 40 to 57% when compared to 60% for GSH, a recognized potent physiological antioxidant. Meanwhile, a slightly higher DRSA (41–98%) was observed after the protein hydrolysates were fractionated by ultrafiltration. The improved ability of <1 kDa peptides to scavenge free radicals with maximum efficiency when compared to HMW peptides have been suggested from previous findings on antioxidative potentials of *Parkia speciosa* [Siow & Gan, 2013] and hemp seed [Malomo *et al*., 2015] protein hydrolysates. In addition, the small-size LMW (<1 kDa) peptides may be resistant to endopeptidase degradation in the digestive tract and enhance faster absorption from the gastrointestinal tract into blood circulation, which could promote ability to interact with and scavenge endogenous free radicals [Erdmann *et al*., 2008].

The DRSA values of our peptide fractions (98%) are better than the 32, 56.22, and 53.15% reported for the Alcalase-produced hydrolysates of asparagus extract [Montone *et al*., 2019], *seinat* seed protein albumin, and globulin [Siddeeg *et al*., 2015], respectively. The reason for this might be that CNPH and FPPH peptide fractions probably had higher DPPH affinity and H-atom donating capacity when compared to those of asparagus extract and *seinat* seed protein fractions since DPPH assay involved a reduction mechanism by active compounds [Siddeeg *et al*., 2015]. The high levels of acidic and hydrophobic amino acids may have contributed to the strong DRSA of the protein hydrolysates [Udenigwe & Aluko, 2011].

Ferric reducing power

A reducing power is an effective method for assessing the ability of an antioxidant in electron donation mechanism. Figure 4 shows the reducing power (absorbance) of CNPH, FPPH and their membrane fractions measured at 700 nm. It is noteworthy that an increase in absorbance indicates better reducing power of the test protein sample. The CNPH and its membrane fractions, with the excep-

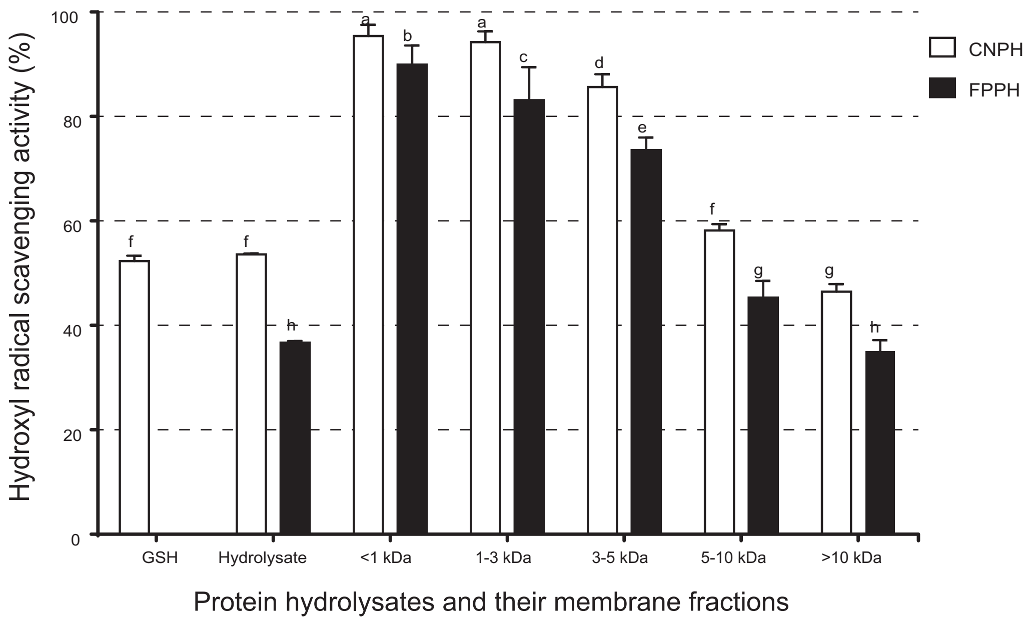

FIGURE 2. Hydroxyl radical scavenging activity of pepsin-produced (3:100 ratio E/S) cashew nut (CNPH) and Alcalase-produced (2:100 ratio E/S) fluted-pumpkin (FPPH) protein hydrolysates and their membrane fractions. Columns with the different letters have mean values that are significantly different at $p<0.05$.

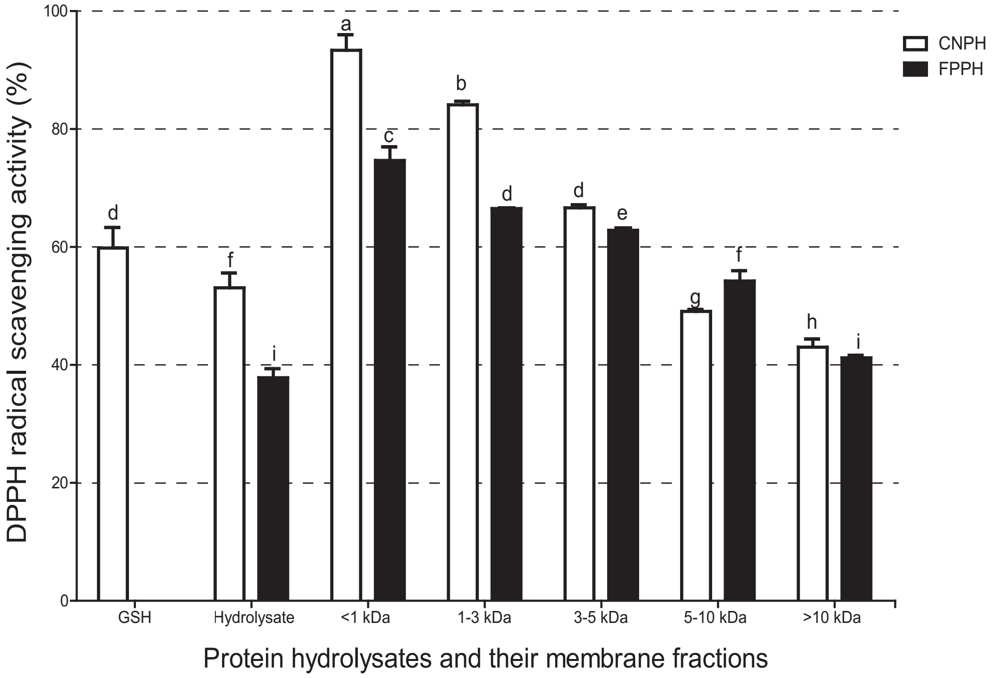

FIGURE 3. DPPH radical scavenging activity pepsin-produced (3:100 ratio E/S) of cashew (CNPH) and Alcalase-produced (2:100 ratio E/S) fluted-pumpkin protein hydrolysates and their membrane fractions. Columns with different letters have mean values that are significantly different at p<0.05.

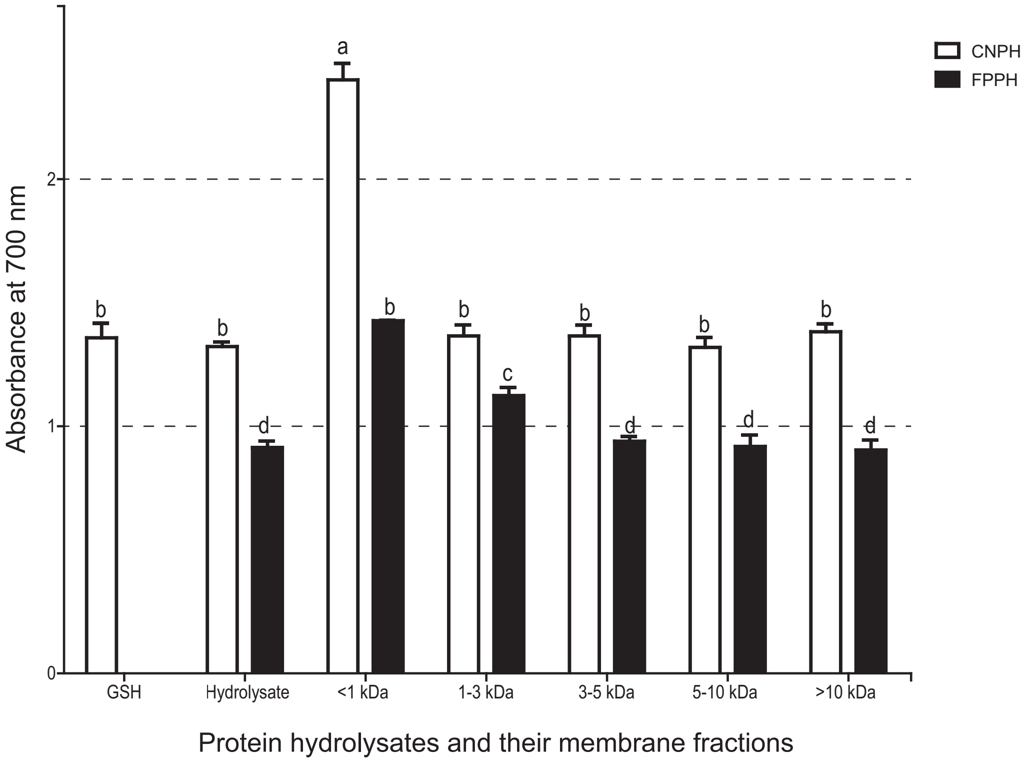

FIGURE 4. Ferric reducing power of pepsin-produced (3:100 ratio E/S) cashew nut (CNPH) and Alcalase-produced (2:100 ratio E/S) fluted-pumpkin (FPPH) protein hydrolysates and their membrane fractions. Columns with the different letters have mean values that are significantly different at p<0.05.

tion of <1 kDa fraction, exhibited similar absorbance value (~1.4) compared to GSH (depicting same reducing power ability) whereas, the reducing powers of the membrane fractions from FPPH decreased at an increased molecular sizes (Figure 4). The current observation contradicts the previous study [Girgih *et al*., 2011] that reported an increase in reducing power of the hemp protein hydrolysate fractions at increased peptide sizes. The ferric reducing power of the pepsin-produced (3:100 ratio E/S) CNPH and its fractions (1.45–2.47) was higher than those of Alcalase-produced (2:100 ratio E/S) FPPH and its membrane fractions (1.00–1.46), with better reducing ability (2.47 and 1.46) from their respective <1 kDa fractions. The differences in the reducing power exhibited by CNPH and FPPH may be related to the increased availability of hydrogen ions [Kong & Xiong, 2006] produced during CNPI hydrolysis. Notably, the presence of reducing compounds caused reduction of Fe^{3+}/ferricyanide complex to ferrous (Fe^{2+}) through electron donation [Jemil *et al*., 2017], thereby increasing the absorbance of the reaction. This is exemplified by the higher cysteine content of the <1 kDa peptides, which also displayed the highest absorbance value. The results are consistent with previous finding that sulfur-containing amino acids enhance ferric reducing power properties of food protein hydrolysates [Udenigwe & Aluko, 2011].

The current study further supported the findings from a previous study on African yam bean seed protein hydrolysate [Ajibola *et al*., 2013] that the highest reducing power values did not correspond to the higher HAA contents. For instance, the highest reducing power (2.47 and 1.46) but lowest HAA (26.06 and 29.32%) values were obtained for the <1 kDa fractions compared to the lowest reducing power (1.45 and 1.00) but highest HAA (35.46–33.15%) values of 3–5 kDa fractions from CNPH and FPPH, respectively as evident in Figure 4 and Table 1. However, the previous study of Pownall *et al*. [2010] on the dependence of reducing power of pea seed protein hydrolysate fractions on its total HAA contents rather than their peptide sizes is different from present findings.

Ferrous ion chelating activity

Active metals have been suggested to be involved in several key pathogenic processes exhibited in cardio- and/or neurodegenerative diseases in the biological system [Mundi & Aluko, 2014]. For instance, the reaction between redox-active ferrous ion (Fe^{2+}) and H_2O_2 can generate $^{\bullet}OH$, which has been implicated in various oxidant-induced metabolic disorders [Aderinola *et al*., 2018]. Therefore, employing chelating agents such as CNPH and FPPH to inactivate ferrous ions and prevent formation of nontoxic metal complexes could play an important role in preventing oxidative injuries in the body. The results show no effect of peptide size on ferrous ion chelation ability of the samples (Figure 5). However, fractionation of the CNPH led to improved fer-

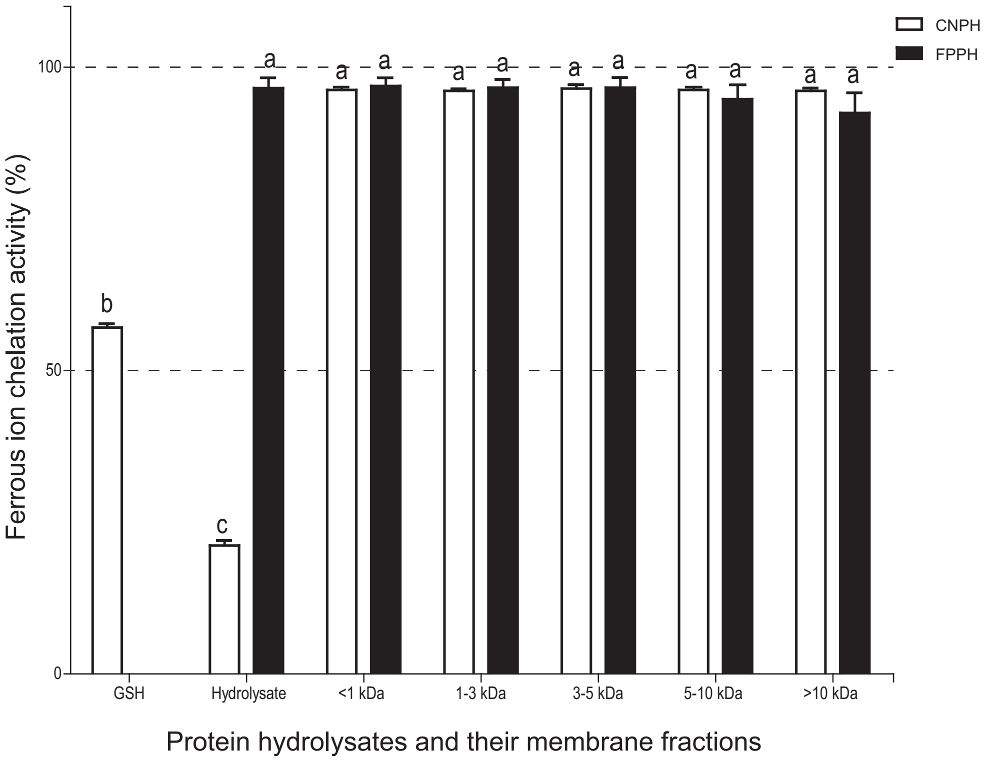

FIGURE 5. Ferrous ion chelation activity of pepsin-produced (3:100 ratio E/S) cashew nut and Alcalase-produced (2:100 ratio E/S) fluted-pumpkin (FPPH) protein hydrolysates and their membrane fractions. Columns with the different letters have mean values that are significantly different at $p<0.05$.

rous ion chelation, which suggests stronger synergistic effects for the peptide fractions. The results obtained in this work show stronger ferrous ion chelation for the peptide fractions when compared to the 15.7–38.5% values (also at 1 mg/mL) reported for similar hemp seed peptides peptides [Girgih *et al.*, 2011]. The differences may be due to variations in the type of protein substrates and proteolytic enzymes used for each work.

The degree of protein hydrolysis and type of amino acid have been shown to alter ferrous ion chelation activity of isolated peptides [Jamdar *et al.*, 2010]. However, the present study is contradictory to the previous report of Jamdar *et al.* [2010] because even though the CNPH had a higher DH, the metal chelating ability was lower than that of FPPH. Moreover, there were no significant ($p>0.05$) differences between the ferrous ion chelation ability of all the peptide fractions.

In vitro activities against the renin-angiotensin converting enzymes system

The CNPI and FPPI were each subjected to enzymatic hydrolysis using Alcalase or pepsin at different concentrations (E/S ratio of 1–4:100) and the hydrolysates evaluated for ACE and renin inhibitions. As shown in Figure 6, the ACE-inhibitory activity of pepsin-CNPH samples was mostly better than the Alcalase-CNPH. In contrast, ACE-inhibitory activity of Alcalase-FPPH was similar to those obtained pepsin-FPPH, except that the 4:100 E/S pepsin-FPPH was significantly lower. The results indicate that intrinsic differences in the primary structure of the proteins may have led to the production of peptides with different amino acid sequences and hence the observed variations in ACE-inhibitory activity of CNPH and FPPH.

The CNPI was hydrolyzed using 3:100 (E/S) pepsin for 2 h, and the hydrolysate passed through membranes of different sizes, and was then tested for ACE-inhibitory activity. The results show that membrane separation did not improve the ACE-inhibitory activity of the CNPH (Figure 7). This is because there were no significant differences ($p > 0.05$) between the CNPH and the <1, 1–3, 3–5 and 5–10 kDa peptide fractions. However, ACE-inhibitory activity was significantly ($p<0.05$) less for >10 kDa peptides when compared to CNPH. The results (Figure 7) are consistent with previous reports [Montone *et al.*, 2019; Ratnayani *et al.*, 2019] that have showed higher ACE-inhibitory potency of low molecular weight peptides when compared to peptides of bigger sizes.

The FPPI was hydrolyzed with 2:100 (E/S) Alcalase for 4 h and the hydrolysate also passed through membranes of different sizes. As shown in Figure 7, membrane fractionation led to decreased potency of the FPPH peptides against ACE, except 1–3 kDa peptides. Moreover, the smaller-size peptides (<5 kDa) exhibited significantly ($p <0.05$) stronger ACE inhibition than the bigger (>5 kDa) peptides. The results suggest that a strong peptide synergy exists within the protein hydrolysates (pepsin-CNPH and Alcalase-FPPH), which favored a high rate of ACE inhibition. Membrane separation reduced this peptide synergy and hence most of the peptide fractions exhibited weaker ACE inhibition than their respective hydrolysates. Similar findings of reduced peptide potency against ACE as a result of membrane ultrafiltration have also been reported [da Cruz *et al.*, 2016]. Results from a previous study support the current observations about the direct dependency of peptide bioactivities on their amino acids content. For instance, highly acidic amino acids (Asp and Glu) led to a net negative charged peptide with improve antioxidant and effective ACE inhibitory action [Nourmohammadi *et al.*, 2017]. This was attributed to chelation of the zinc atoms in the active center of ACE, which reduced catalytic activity. Similarly, the presence of aromatic and hydrophobic amino acids has been shown to contribute to potency of ACE-inhibitory peptides [Wu *et al.*, 2006a].

In contrast to the ACE inhibition results, Figure 8 shows that the maximum renin inhibition was achieved using 2:100 (E/S) Alcalase (63%) and 4:100 (E/S) pepsin (48%) to prepare CNPH and FPPH, respectively. The present findings showing lower renin-inhibitory activity of the hydrolysates when compared to ACE-inhibitory activity are similar to previous reports on canola [Alashi *et al.*, 2014] but contrary to these on hemp [Malomo *et al.*, 2015] protein hydrolysates. For the CNPH, the results suggest that initial increase in Alcalase concentration from 1–2:100 (E/S) promoted the production of peptides with structural features that enhanced renin inhibition. However, further increases in Alcalase concentration may have led to hydrolysis and structural inactivation of the active peptides, hence the reductions in renin-inhibitory activity at enzyme levels >2:100 (E/S). The opposite effect was obtained for the FPPH whereby the highest renin inhibition was obtained at 4:100 (E/S) pepsin. Therefore, it is possible that at 1–3:100 (E/S) pepsin, the level of FPPI proteolysis was not sufficient to produce strong renin-inhibitory peptides when compared to the 4:100 (E/S) pepsin. Differences in enzyme specificity (peptide cleavage points) may have contributed to the varied outcome between Alcalase and pepsin digestions. After subjecting the two hydrolysates to membrane filtration process, the renin inhibition activities of their membrane fractions were significantly ($p<0.05$) improved as evident in results presented in Figure 9. However, there were no significant differences between the renin-inhibitory activities of the CNPH peptide fractions. In contrast, the 5–10 kDa FPPH peptide fraction exhibited significantly ($p<0.05$) higher renin-inhibitory activity when compared to the other fractions. The results suggest that for the renin inhibition, peptide composition may be more important than peptide size for the FPPH.

Differences in catalytic mechanisms between ACE and renin might have also contributed to the different results observed for CNPH and FPPH inhibitory activities. For instance, the pepsin-produced CNPH and Alcalase-produced FPPH had higher ACE (Figures 6 and 7) but lower renin (Figures 8 and 9), thus revealing the two peptides to have higher affinity for ACE than renin. A previous study [Olagunju *et al.*, 2018] had attributed the low inhibition of renin activity to reduced accessibility to inhibitors as a result of its (renin) more folded protein conformation, which contrasts the more open conformation and easier accessibility of ACE to potential inhibitors.

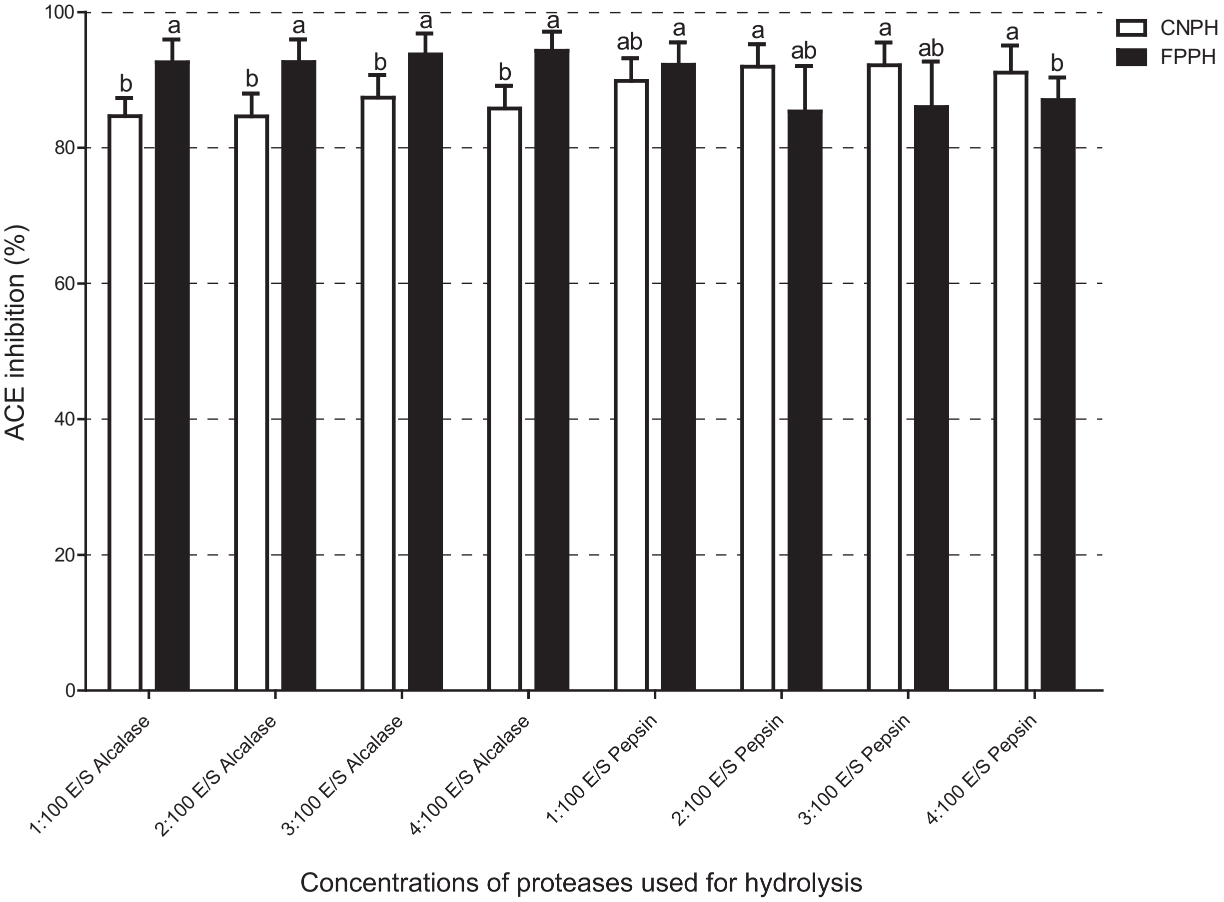

FIGURE 6. ACE inhibition properties of cashew nut (CNPH) and fluted-pumpkin (FPPH) protein hydrolysates produced at different protease concentrations (1–4:100 ratio E/S). Columns with the different letters have mean values that are significantly different at $p<0.05$.

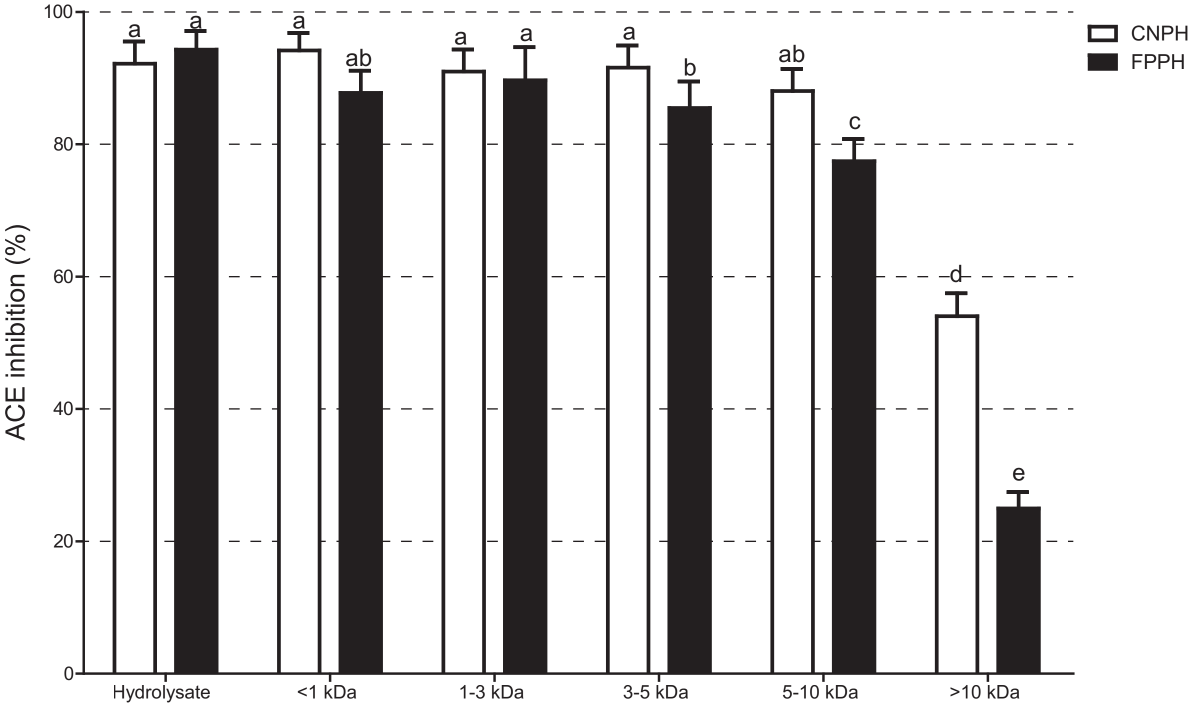

FIGURE 7. ACE inhibition properties of pepsin-produced (3:100 ratio E/S) cashew nut (CNPH) and Alcalase-produced (2:100 ratio E/S) fluted-pumpkin (FPPH) protein hydrolysates and their membrane fractions. Columns with the different letters have mean values that are significantly different at $p<0.05$.

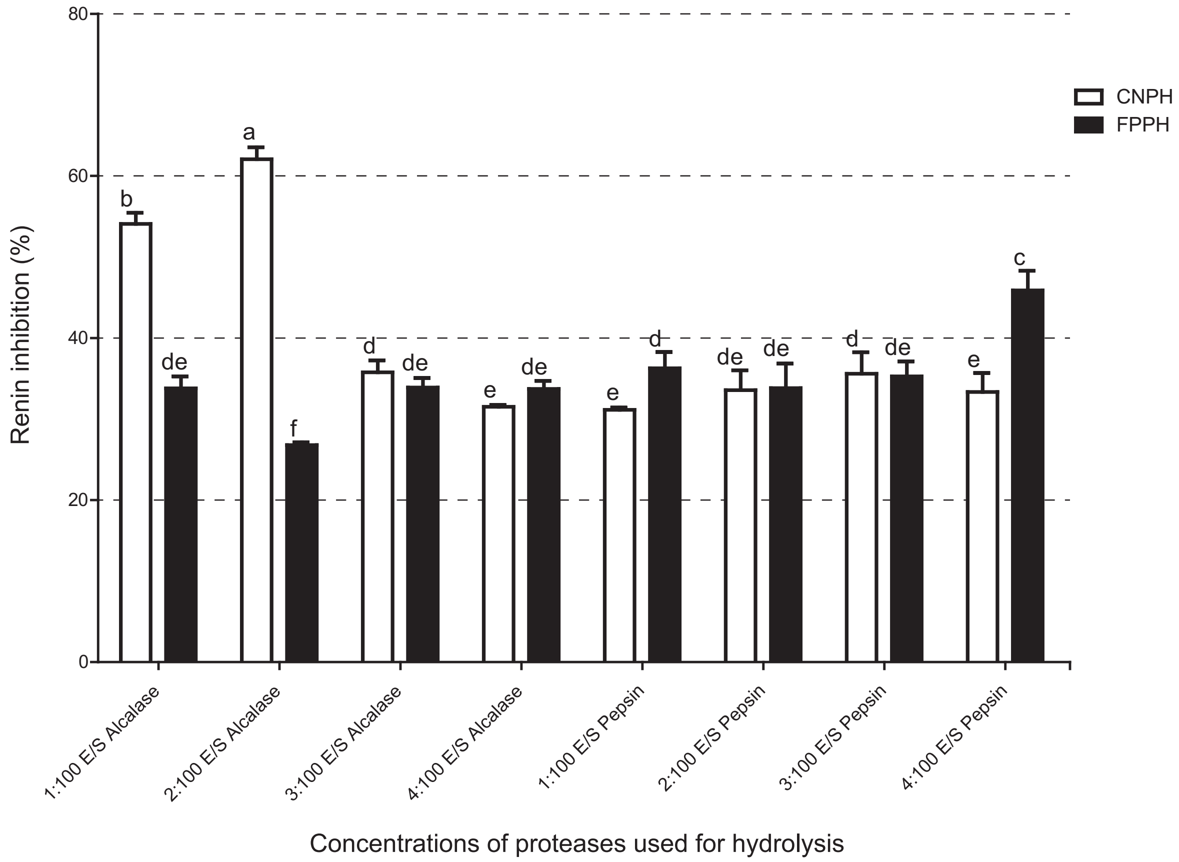

FIGURE 8. Renin inhibition properties of cashew nut (CNPH) and fluted-pumpkin (FPPH) protein hydrolysates produced at different protease concentrations (1–4:100 ratio E/S). Columns with the different letters have mean values that are significantly different at p<0.05.

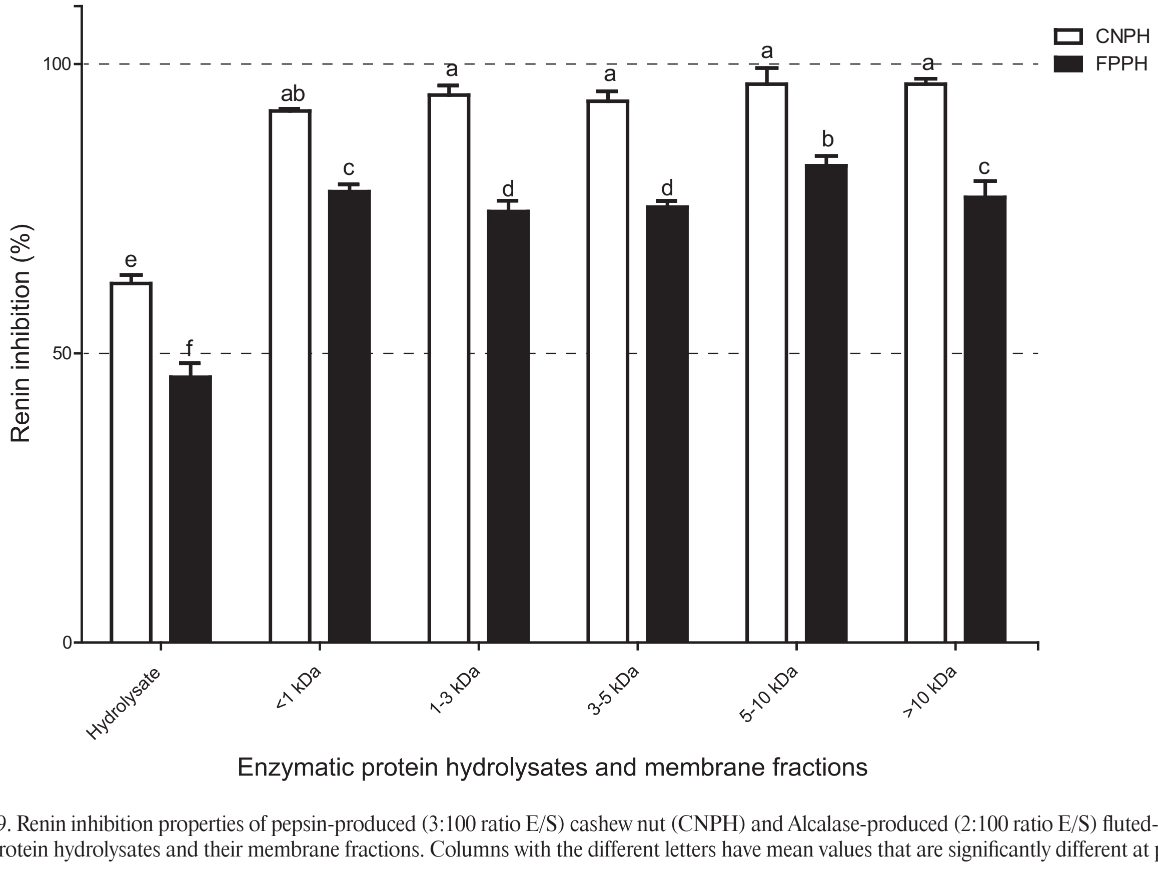

FIGURE 9. Renin inhibition properties of pepsin-produced (3:100 ratio E/S) cashew nut (CNPH) and Alcalase-produced (2:100 ratio E/S) fluted-pumpkin (FPPH) protein hydrolysates and their membrane fractions. Columns with the different letters have mean values that are significantly different at p<0.05.

CONCLUSIONS

The protease-hydrolysis of CNPI and FPPI yielded peptides with *in vitro* antioxidant and RAS-inhibitory activities, which provides new peptide tools for nutritional improvement of human health. Both CNPH and FPPH contain LMW peptides with potentially fast absorption in the gastrointestinal tract. The strongest scavenging of hydroxyl and DPPH$^{\bullet}$ radicals as well as ferric ion reducing capacity were obtained for the <1 kDa peptide fraction. Stronger ACE inhibition by <1 kDa peptides when compared to bigger peptides was observed only when the CNPI was hydrolyzed by pepsin for 2 h. In contrast, CNPI hydrolysis by pepsin for 2 h yielded <1 kDa peptides with weaker renin inhibitory activity than the bigger peptides. Thus, the potency of peptide fractions may be dependent not only on the type of protease but the length of released peptides. Overall, the strong ACE and renin inhibitions suggest that these protein hydrolysates could be used as suitable ingredients for the formulation of health-promoting functional foods and nutraceuticals.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

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Original research article
Food Quality and Functionality Section

Investigations of Volatile Organic Compounds in Berries of Different *Actinidia kolomikta* (Rupr. & Maxim.) Maxim. Accessions

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Key words: berries, clone, cultivar, *A. kolomikta*, volatile compounds

The composition of volatile organic compounds contributes to fruit flavour and is an important element of fruit quality. Berries of sixteen cultivars and female clones of *Actinidia kolomikta* were studied by GC-MS with headspace solid phase microextraction method. In total, 89 compounds were separated and identified. These compounds were classified as terpenes, esters, alcohols, aldehydes, anhydrides, diazoles, hydrocarbons, and ketones. Among volatile organic compounds, esters were the most abundant. Significant differences in the diversity of volatile organic compounds were found among *A. kolomikta* cultivars and clones. Based on these results, we selected potential accessions for the breeding of new cultivars. Summarising the results of this study, the accessions of *A. kolomikta* were grouped according to the main flavour compounds.

INTRODUCTION

The taste of fruit is determined not only by the ratio between sugars and acids but also by a specific blend of different volatile components. There is an increasing interest in natural flavourings that may be used in food and nutraceutical products instead of synthetics [Henare, 2016]. Fruit volatile organic compounds (VOCs) represent a large group of chemical substances with a low molecular weight and a high vapour pressure under ambient conditions. In general, VOCs in fruits account for a very small part of the total weight [Dudareva *et al*., 2006; Jiang & Song, 2010]. VOCs are released from different parts of plants and play an important role in plant interactions with the biological environment [Rodríguez *et al*., 2013]. The fruit flavour properties of different plant species and cultivars depend on the unique blend of VOCs [Jiang & Song, 2010]. According to Negre-Zakharov *et al.* [2009], there are four major classes of VOCs, which are classified by their metabolic origin, namely, terpenoids, phenylpropanoids/benzenoids, fatty acids derivatives, and amino acid derivatives.

Within the genus *Actinidia* Lindl., various chemical substances determine the distinct flavour and the fragrance of berries. The most comprehensive studies of VOCs in this genus were accomplished for cultivars of *A. deliciosa* (A.Chev.) C.F. Liang et A.R. Ferguson and *A. chinensis* Planch. There are numerous studies regarding the quantitative and qualitative characterisation of VOCs in kiwifruit [Cheng *et al*., 2011; Garcia *et al*., 2012b; Mota *et al*., 2012]. For example, over 80 volatile compounds were identified in *A. deliciosa* cultivar 'Hayward'. Interestingly, cultivars 'Hayward' and 'Hort16A' (*A. chinensis*) differ in only a few compounds and the specific aroma of their fruit is mainly attributable to the proportions of the VOCs [Wang *et al*., 2011]. Temporal variations in the VOC composition in kiwifruit depending on the harvest date were observed as well [Mota *et al*., 2012; Wang *et al*., 2011].

The characterization of bound volatile compounds of *A. eriantha* Benth. resulted in detection of major compound classes, *i.e.* alcohols, benzenoids, and phenolics. The precursors of bound compounds, including linoleic, linolenic and benzoic acids and coniferyl alcohol, were found as well [Garcia *et al*., 2012a]. Alcohols, terpenoids, and benzenoids classes were confirmed as the most abundant VOCs in *A. arguta* (Siebold & Zucc.) Plant. ex Miq. [Garcia *et al*., 2011]. In this study, eugenol, raspberry ketone, and 4-vinylguaiacol were identified in berries of *A. arguta* for the first time. Other authors recorded ethyl butanoate and ethyl hexano-

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ate as the most abundant in *A. arguta* berries [Crowhurst *et al*., 2008]. Extracts of berries of the above-mentioned species contained different monoterpenes with such dominant esters as ethyl butanoate, hexanoate, 2-methylbutanoate, and 2-methylpropanoate, as well as the aldehydes: hexanal and hex-E2-enal [Matich *et al*., 2003]. In *A. arguta* berries, the intense fruity aroma was associated with high amounts of ethyl butanoate, whereas the floral aroma could be linked to other VOCs, *i.e.* methyl and ethyl benzoate [Lindhorst & Steinhaus, 2016].

Berries of *A. kolomikta* (Rupr. & Maxim.) Maxim. are an excellent source of biologically active compounds. They accumulate various organic acids, dietary fibres, carotenoids, minerals, flavonoids, and other valuable substances which determine their health-promoting properties [Latocha *et al*., 2010; Paulauskienė *et al*., 2014; Zuo *et al.*, 2012]. The winter-hardy *A. kolomikta* accessions can be distinguished by higher amounts of ascorbic acid and phenolic compounds, and a higher antioxidant capacity from commercial *A. chinensis* and *A. deliciosa* cultivars [Chesoniene *et al*., 2004; Wang *et al*., 2018]. On the other hand, consumers prefer the delicious and aromatic berries of *A. kolomikta* for fresh consumption. This can be explained by the fact that *A. kolomikta* produces more palatable berries with green and edible skin [Chesoniene *et al*., 2004]. The role of VOCs of horticultural plants in their resistance to fungal and bacterial diseases or pests is of fundamental and practical interest. Yet, information on the qualitative and quantitative composition of VOCs in *A. kolomikta* is scarce. Another problem related with cultivation of *A. kolomikta* is the short shelf life of berries, which can be extended using edible polymer coatings or breeding more suitable cultivars [Drevinskas *et al*., 2017]. We hypothesised that *A. kolomikta* berries accumulate a variety of specific VOCs that could provide a pleasant berry aroma and (or) could be responsible for disease and pest resistance. Thus, the main aims of this study were: to determine the main groups of VOCs present in berries of different accessions of *A. kolomikta* and to determine composition of VOCs; as well as to compare the accessions of *A. kolomikta* in order to select these most suitable for consumption and also for the breeding of new cultivars.

MATERIALS AND METHODS

Plant material

A total of sixteen *A. kolomikta* accessions, including Lithuanian and Russian cultivars as well as female clones, were selected for investigations from the experimental collection of the Botanical Garden of the Vytautas Magnus University, Kaunas, Lithuania (Table 1). This collection is located in the central region of Lithuania (latitude 54º87'15"N and longitude 23º91'08"E). The altitude of collection is 76 m above sea level. The average temperature is 15.9–17.8°C in July and 18.1–20.3°C in August. The average rainfall is 60––90 mm in July and 2–16 mm in August. The accessions were previously selected in this collection according to the different ripening time and berry weight [Chesoniene, 2000]. Characteristics of accessions investigated are presented in Table 1. At least three plants represented each accession.

TABLE 1. Characteristics of *A. kolomikta* accessions investigated in this study.

Cultivar or female clone	Origin	Ripening time	*Berry weight, g
Cultivars			
'Landė'	Lithuania	early	3.70±0.17^{b}
'Lankė'	Lithuania	early	1.87±0.07^{ij}
'Anykšta'	Lithuania	late	2.95±0.06^{d}
'VIR- 1'	Russia	early	2.61±0.05^{e}
'VIR-2'	Russia	medium-early	2.41±0.14^{ef}
'Sentiabrskaja'	Russia	late	2.28±0.07^{fg}
'Krupnoplodnaja'	Russia	early	4.22±0.10^{a}
'Matovaja'	Russia	early	2.37±0.11^{ef}
'Aromatnaja'	Russia	early	1.97±0.15^{ij}
'Pavlovskaja'	Russia	early	3.77±0.13^{b}
'Paukštės Šakarva'	Lithuania	early	3.26±0.12^{c}
Female clone			
F1	Lithuania	early	3.71±0.08^{b}
F8	Lithuania	early	2.23±0.14^{fgh}
F ELE	Lithuania	late	2.09±0.10^{ghi}
F2M2	Lithuania	medium-early	1.80±0.09^{j}
F9	Lithuania	medium-early	2.01±0.08^{hij}

*Means whose are followed by the same letters in column showed no significant difference at p≤0.01.

Berries were randomly picked from different plants of each accession at the technical maturity stage, mixed and transported immediately to the laboratory. Technical maturity stage usually starts at the third week of July (early accessions), at the first week of August (medium-early accessions), and at the second week of August (late accessions). The average weight of a berry was measured by using an analytical balance with a precision of 0.01 g (model DJ-150E, ISHIDA company, Kyoto, Japan). For accession, three replicates of 50 berries were estimated.

Berries were stored at -80ºC until analysis. Before analysis, they were lyophilised at -54ºC for 48 h using a Heto LyoLab 3000 lyophiliser (Bad Grund, Germany).

Headspace Solid-Phase Microextraction

The analyses were performed according to the previous studies of Mota *et al.* [2012] and Dong *et al.* [2019] with modifications. One commercial fibre was used to extract volatiles. According to the recommendations of the supplier (Supelco, Bellefonte, Pennsylvania, USA), the fibre coated with PDMS/DVB as a stationary phase and 65-μm film thickness are the most adaptable to determine the compounds in the kiwi matrix. Lyophylised berries of each accession were crushed together and approximately 0.3 g of each sample was loaded in a 10-mL vial and then sealed with a metal cap and PTFE/silicone septa (ROTH, Karlsruhe, Germany). The fibre was exposed to the headspace at 50ºC for 10 min. Afterwards, the fibre was pulled into the hollow needle sheath and the SPME device was removed from the vial and inserted into the injection port of the GC system for thermal desorption at 260°C for 1 min. All samples were analysed in triplicate.

Gas Chromatography/Mass Spectrometry analyses

For HS-SPME analysis, we used a Shimadzu GC-2010 gas chromatograph and mass spectrophotometer GC-MS-QP2010, and workstation software GC-MS solution version 2.71 (Shimadzu Corporation, Okayama, Japan). The column used for analyses was an RTX-5MS (30 m×0.25 mm× 0.25 μm) from Restek (Bellefonte, Pennsylvania, USA). The injector port was heated to 260°C. The carrier gas was helium 5.0 (AGA, Latvia), delivered at a constant flow of 1.5 mL/min. The oven temperature was set at 50°C for 2 min, and then temperature was increased at 8°C/min to 280°C and held for 2 min. Ionisation was maintained off in the fifth min. The electron ionisation detector was maintained at 70 eV. A scan was used from 40 to 400 m/z.

A comparison of MS fragmentation pattern with those of pure compounds and mass spectrum database was performed using the National Institute of Standards and Technology (NIST) MS 08 spectral database. The accessions were grouped according to the flavouring VOCs based on the data from the TGSC Information System (http://www.thegoodscentscompany.com).

Statistical analysis

Data for different classes of VOCs were processed using the Tableu 9.3.0 (Tableau Software, Seattle, Washington, USA). The concept horizontal bar plot was adapted with the intent to be clearer, and differences to be more visible [Jones, 2014]. To present the diversity of VOCs, peak areas of GC-MS separations were used. R statistical computing environment [R Core Team, 2020] was used for clustering analysis of *A. kolomikta* accessions, *i.e.* packages hclust function and Ward D2. The dendrogram revealed relationships among the accessions according to the content of VOCs. Duncan's multiple range test was used to compare mean values of berry weight based on ANOVA at p≤0.01 (R software environment).

RESULTS AND DISCUSSION

VOCs belonging to eight different classes have been identified in berries of *A. kolomikta*. We classified these VOCs from a chemical point of view as terpenes, esters, alcohols, aldehydes, anhydrides, diazoles, hydrocarbons, and ketones. In total, 89 VOCs were detected in berries of different *A. kolomikta* accessions, *i.e.* terpenes (11), esters (30), alcohols (10), aldehydes (3), anhydrides (3), diazoles (1), hydrocarbons (19), and ketones (12). The statistical analysis of VOCs belonging to the different classes was accomplished by peak area and peak height and showed the distribution of these compounds as well as confirmed the separation of six compounds attributed to esters, terpenes, and ketones (Figure 1).

While numerous studies have been done to substantiate the diversity of volatile compounds in other species of *Actinidia,* this study reports that berries of different *A. kolomikta* accessions were also distinguished by a variety of VOCs. The composition of VOCs was specific to different accessions and varied strongly (Figures 2–6). The greatest diversity of VOCs, namely representatives of seven chemical classes, was determined in berries of the female clone F1, the cul-

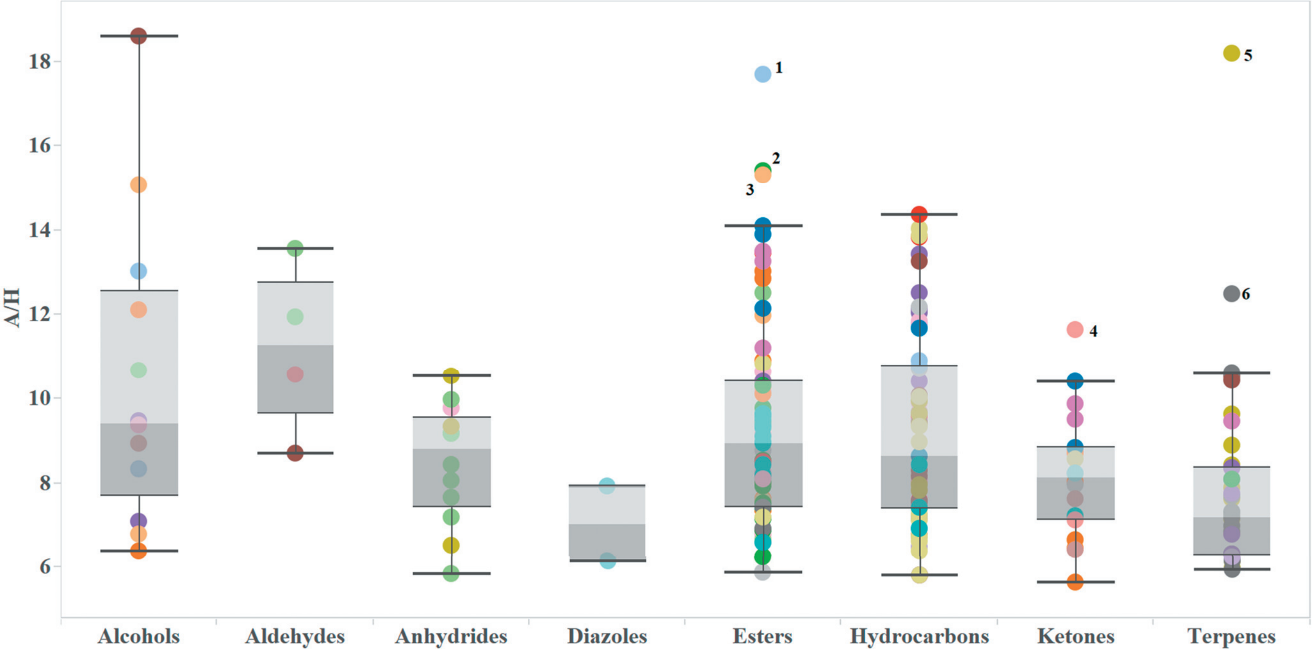
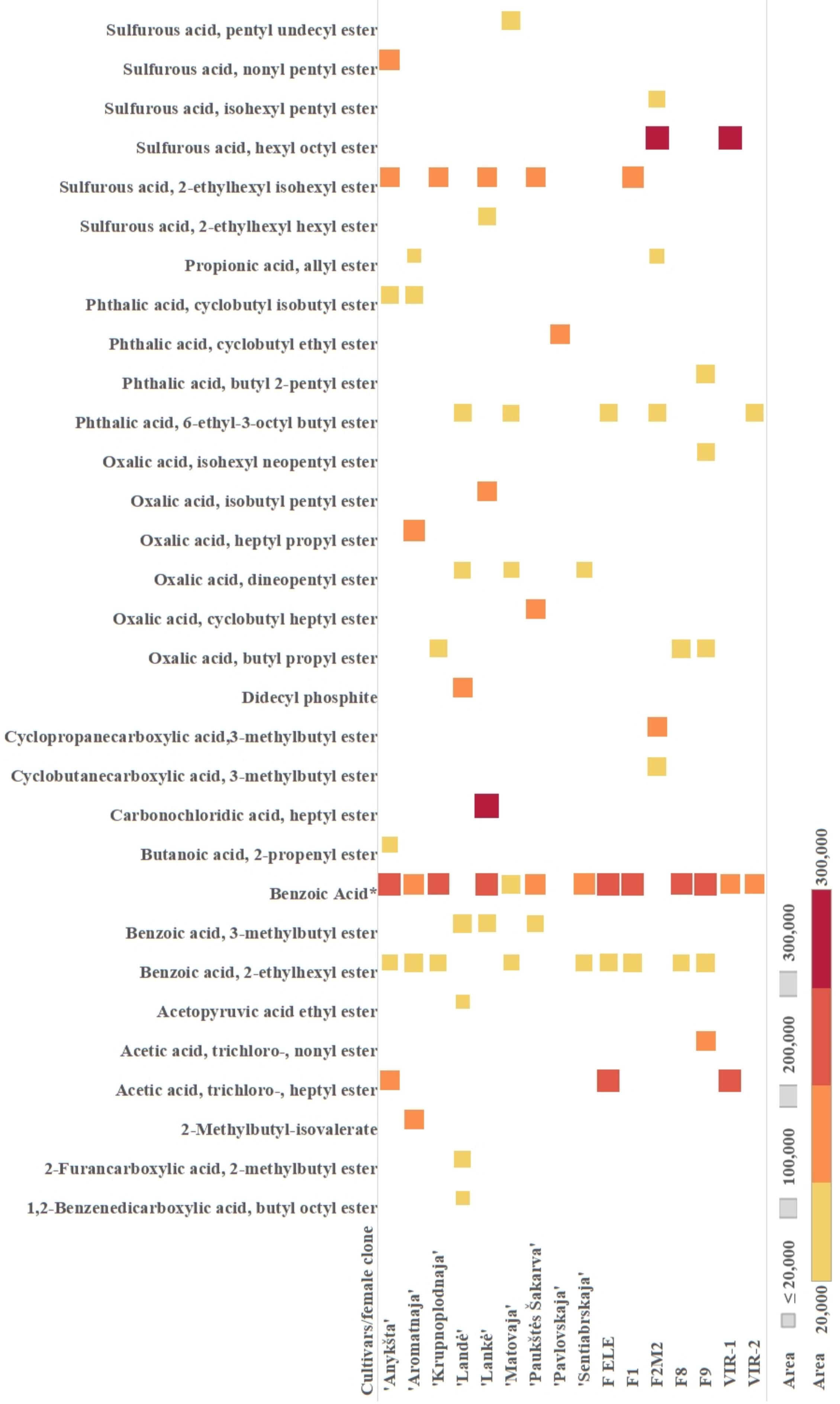


FIGURE 1. Different classes of VOCs according peak area (A) and peak height (H) ratio in berries of *A. kolomikta.* Outlying VOCs: 1 – oxalic acid, cyclobutyl heptyl ester; 2 – oxalic acid, heptyl propyl ester; 3 – oxalic acid, isobutyl pentyl ester; 4 – 6-methyl-5-hepten-2-one; 5 – 3,5,5-trimethyl-1-hexene; 6 – 3,5-dimethyl-1-hexene.



*Benzoic acid <2-[[[4-(4-hydroxy-4-methylpentyl)-, 3-cyclohexen-1-yl]methylene]amino]-, methyl-> ester

FIGURE 2. Diversity of esters in berries of *A. kolomikta* accessions. The size and the colour intensity of squares indicate the content of esters.

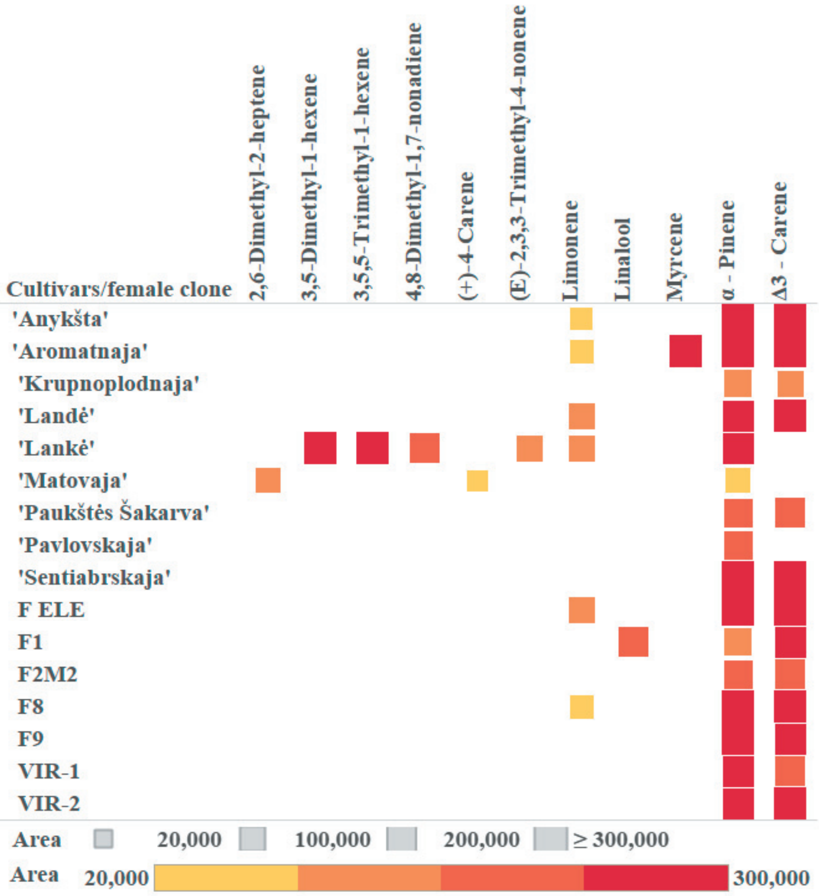


FIGURE 3. Diversity of terpenes in berries of *A. kolomikta* accessions. The size and the colour intensity of squares indicate the content of terpenes.

tivars 'Lankė' and 'Paukštės Šakarva', while the cultivar 'VIR-1' accumulated the smallest number of VOCs classes (four classes). The largest number of different VOCs accumulated was found for the Lithuanian cultivar 'Landė' – 23 compounds and for the Russian cultivar 'Matovaja' – 20 compounds. Berries of both cultivars were also distinguished by a great variety of VOCs classes, including hydrocarbons, terpenes, alcohols, ketones, and esters. *A. kolomikta* accessions contained from one ('Pavlovskaja') to eight (F2M2 and F9) esters, from one ('Pavlovskaja') to six ('Lankė) terpenes, and from one (cultivars 'Anykšta', 'Aromatnaja', 'Krupnoplodnaja', 'Paukštės Šakarva', F8, F ELE) to six ('Landė') ketones. Berries of the cultivar 'VIR-1' did not accumulate hydrocarbons, whereas VOCs belonging to the alcohols were not detected in berries of cultivars 'Aromatnaja', 'Sentiabrskaja', 'VIR-1' and clones F8 and F9. The diversity of aldehydes and anhydrides was not significant, *i.e.* these VOCs were found in berries of three accessions. Other authors determined more aldehydes in *A. arguta* and *A. deliciosa* [Garcia *et al*., 2012b; Matich *et al*., 2003]. In turn, berries of cultivars 'Krupnoplodnaja' and 'Paukštės Šakarva' accumulated 1*H*-imidazole from diazoles class.

Esters were the most abundant class of VOCs in the accessions of *A. kolomikta* (Figure 2). For example, benzoic acid <2-[[[4-(4-hydroxy-4-methylpentyl)-,3-cyclohexene-1-yl] methylene]amino]-,methyl-> ester was the most common because it was determined even in thirteen accessions. Its highest content was determined for the accessions F ELE, 'Anykšta', F1 and F9. Matich *et al*. [2003] reported ethyl butanoate, hexanoate, 2-methylbutanoate, and 2-methylpropanoate among the the most common esters in the berries of *A. arguta.* In turn, Crowhurst *et al*. [2008] confirmed butyl acetate to be the major ester in *A. eriantha* that was responsible for a pineapple-like aroma. Benzoic acid, 2-ethylhexyl ester was common enough, however, its peak areas were considerably smaller. The highest content of carbonochloridic acid heptyl ester was found in the cultivar 'Lankė' whose berries were unique for oxalic acid, izobutyl pentyl ester and sulphurous acid, 2-ethylhexyl hexyl ester. Relatively small contents were characteristic of the unique esters, namely 1,2-benzenedicarboxylic acid, butyl octyl ester and acetoperuvic acid ethyl ester ('Landė'). Six esters of sulphurous acid were separated and four of them, *i.e.* 2-ethylhexyl ester, isohexyl pentyl ester, nonyl pentyl ester, and pentyl undecyl ester, were unique to the accessions 'Landė', 'Anykšta', 'Matovaja' and F2M2, respectively. Sulphurous acid, 2-ethylhexyl isohexyl ester was the most common of sulphurous acid esters. Arulkumar *et al.* [2018] found that this ester had characteristic antimicrobial and antioxidative properties, which were demonstrated in the *in vitro* experiments. Wang *et al*. [2011] have reported that sulphur compounds were found in gold kiwifruit ('Hort16A') and could to be one of the most important contributors to the flavour of this cultivar.

FIGURE 4. Diversity of hydrocarbons in berries of *A. kolomikta* accessions. The size and the colour intensity of squares indicate the content of hydrocarbons.

Rodríguez *et al*. [2013] reported that ester fraction is the main contributor to the aroma of different fruits. Our results confirmed esters as the most abundant class of VOCs because they were found in berries of all accessions investigated. Other authors have determined esters to be one of the main classes of VOCs in berries of *A. deliciosa* and *A. chinensis* [Garcia *et al*., 2012b; Matich *et al*., 2003] and illustrated their impact on the characteristic flavours of different *Actinidia* species [Henare, 2016; Wang *et al*., 2011]. Our results correspond to the investigations of *A. arguta* where the VOCs identified in its berries mainly consisted of esters [Garcia *et al*., 2011]. Differently from the above-mentioned *Actinidia* species, alcohols were the most numerously represented VOCs in berries of *A. eriantha* [Garcia *et al*., 2012a]. Interestingly, we determined that twenty esters out of the thirty found were accession-specific (Figure 2).

Berries of *A. kolomikta* accessions accumulated different levels of α-pinene, a terpenes class compound (Figure 3). Other terpene, Δ^3-carene, was common also and it was found in most of the accessions. In terms of the distinctness of the accessions studied, the cultivar 'Lankė' was exceptional due to the highest variety of terpenes. The smallest amounts were characteristic of (+)-4-carene which was unique to the cultivar 'Matovaja'. The VOCs α-pinene, Δ^3-carene, and limonene were more common compared to other terpenes, which were found only in individual accessions investigated. Matich *et al*. [2003] reported α-pinene, limonene, and linalool in *A. arguta* berries which is in agreement with our results. Terpenoids were referred as the most abundant VOCs for the *A. deliciosa* cultivar 'Hayward' also [Garcia *et al*., 2013]. Nieuwenhuizen *et al*. [2010] noted terpenes as very important secondary metabolites and emphasized their essential role in plants interacting with the biotic environment, including pollinator attraction, direct and indirect defence against insects, bacteria, and fungi. Other studies confirmed that terpenes demonstrate antioxidant and antidiabetic properties as well as antimicrobial or antifungal activities [Kupska *et al*., 2016; Wang *et al*., 2008].

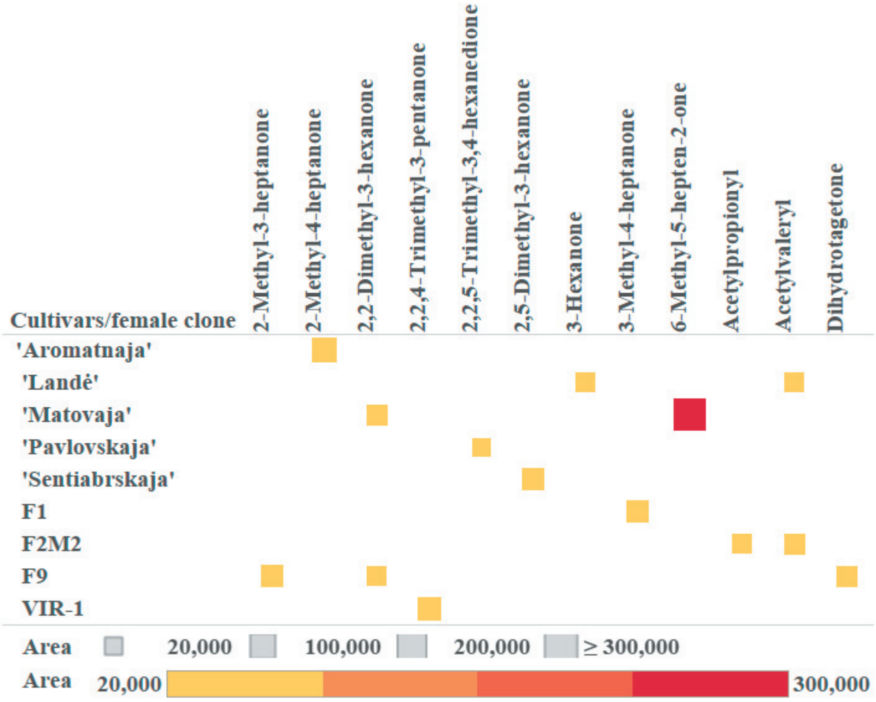


FIGURE 5. Diversity of ketones in berries of *A. kolomikta* accessions. The size and the colour intensity of squares indicate the content of ketones.

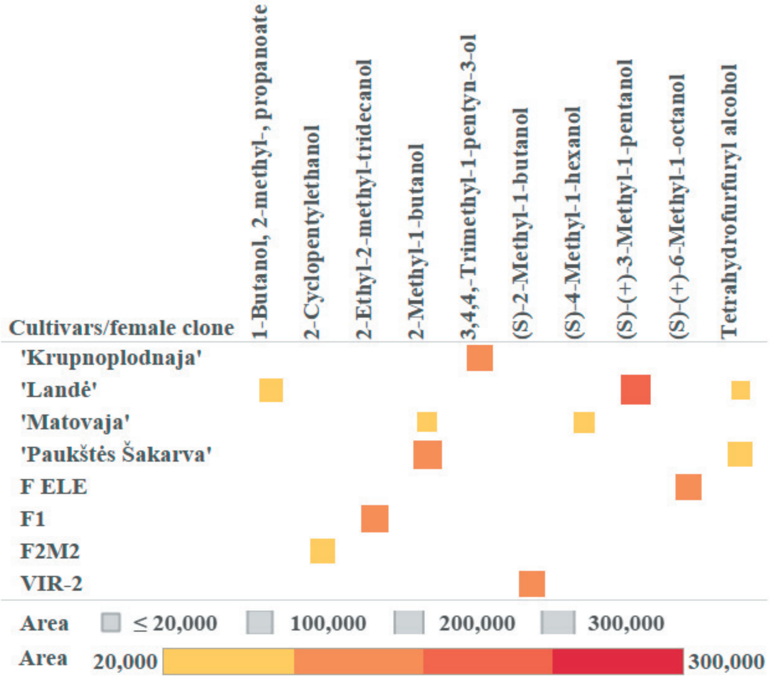


FIGURE 6. Diversity of alcohols in berries of *A. kolomikta* accessions. The size and the colour intensity of squares indicate the content of alcohols.

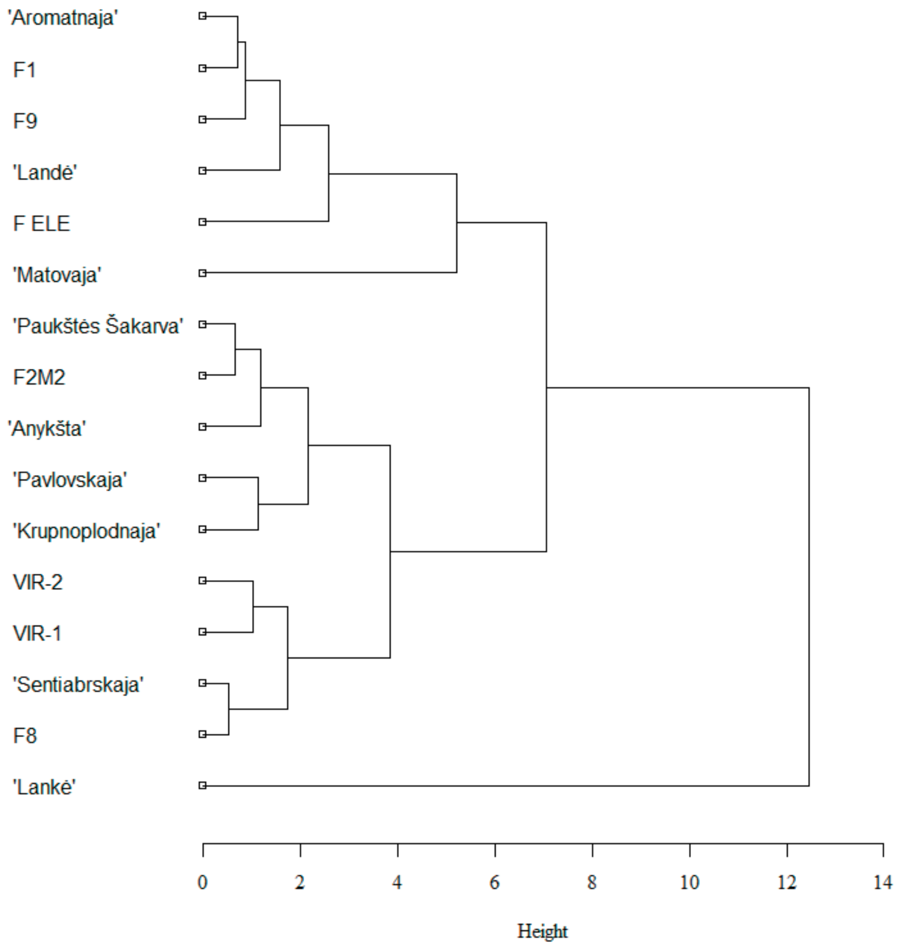


FIGURE 7. Similarity of cultivars and female clones of *A. kolomikta* depending on the content of VOCs

The hydrocarbons 1-iodononane; 2-iodo-3-methylbutane; 2,2-dimethylbutane; 2,6,11-trimethyldodecane and 2,4-dimethyldocosane were common in berries of *A. kolomikta* (Figure 4). Other hydrocarbons were accession-specific. Interestingly, no hydrocarbons were determined in berries of the Russian cultivar 'VIR-1'.

Ketones were detected in all samples at much lower levels whereas the cultivar 'Matovaja' was characterised by the high content of 6-methyl-5-hepten-2-one (Figure 5). Alcohols were found in berries of eight accessions (Figure 6). Our results have shown that the majority of ketones and alcohols were unique and have been found in one particular accession, but not in the others.

The dendrogram of *A. kolomikta* accessions was constructed and revealed the similarity of cultivars and female clones according to the content of VOCs (Figure 7). The fifteen accessions were classified into two main clusters and the cultivar 'Lankė' was distinctly separated from all the other accessions due to its unique VOCs. This cultivar accumulated several unique volatiles (four esters, four terpenes, and one hydrocarbon) and no alcohols and ketones. The high similarity comparing VOCs composition was determined for 'Sentiabrskaja' and F8 for 'Paukštės Šakarva' and F2M2 as well as for 'Aromatnaja' and F1.

The quantitative and qualitative composition of VOCs depends not only on the genotype properties but also on extraction and detection methods. The composition of volatiles in berries of *Actinidia* species have been analysed by different authors using different methods, so this can cause some differences in the results obtained. For comparative reasons, we have created a diagram which is based on the data obtained from TGSC information system and studies of other authors [Garcia *et al*., 2012a; Garcia *et al*., 2013; Lindhorst & Steinhaus, 2016]. We grouped the accessions of *A. kolomikta* according to the tentative aroma active compounds (Figure 8). Benzoic acid <2-[[[4-(4-hydroxy-4-methylpentyl)-,3-cyclohexene-1-yl]methylene]amino]-,methyl->ester; benzoic acid, 2-ethylhexyl ester; α–pinene; Δ^3– carene, and butanoic acid anhydride were ascertained as the main constituents of *A. kolomikta* berry aroma.

Summarizing the results, it can be stated that both a high variety of VOCs and unique compounds were characteristic of *A. kolomikta* berries. Successful use of these berries in human nutrition depends on their flavour properties, which are coherent with quantitative and qualitative composition of VOCs.

Some of these compounds have already been investigated for their effects on human health [An *et al*., 2016; Drummond, 2013; McGhie, 2013] and berry pleasantness [Jiang & Song, 2010; Negre-Zakharov *et al*., 2009; Rodríguez *et al*., 2013]. On the other hand, emission of VOCs by berries is related to a wide range of ecological functions, including their

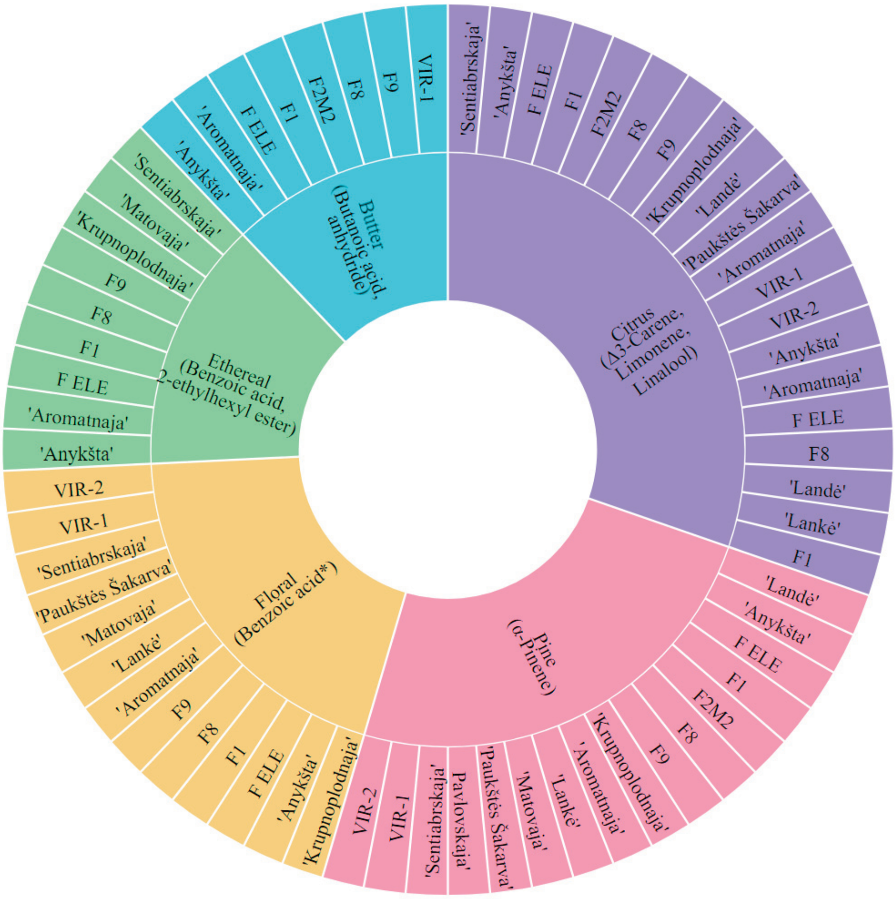


FIGURE 8. The flavour compounds in berries of *A. kolomikta* accessions.
Benzoic acid* – benzoic acid <2-[[[4-(4-hydroxy-4-methylpentyl)-,3-cyclohexene-1-yl]methylene]amino]-,methyl->ester.

role in interacting with pests or microbes [Dudareva *et al*., 2006]. These affirmations provide the basis for more intensive use of *A. kolomikta* berries not only in food but also in pharmaceutical and cosmetic industries. Further studies on the VOCs accumulated in berries of *A. kolomikta* should focus on the biological activities of these important secondary metabolites.

CONCLUSIONS

Investigation of sixteen accessions of *A. kolomikta* revealed the large quantity of VOCs accumulated in berries. All identified compounds were classified as esters, alcohols, hydrocarbons, terpenes, aldehydes, anhydrides, ketones, and diazoles. This study has demonstrated that esters are the most abundant class of VOCs in berries of *A. kolomikta*. While some VOCs are common to almost all accessions, other compounds are specific to only one or a few cultivars or female clones. The accessions F_1, 'Lankė', and 'Paukštės Šakarva' accumulated a large variety of VOCs classes while berries of the cultivars 'Landė' and 'Matovaja' were distinguished by the highest levels of VOCs. The species *A. kolomikta* has a great potential to be considered as a highly valuable source of VOCs which determine the attractiveness of specific cultivars or female clones for consumers. The diversity of VOCs contributing to the flavour of berries is particularly important for the breeding of new cultivars with more attractive and useful properties.

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Original research article
Food Quality and Functionality Section

Effect of Core Temperature on the Oxidation of Lipids and Proteins During Steam Cooking of Large-Mouth Bass (*Micropterus salmoides*)

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Key words: large-mouth bass, steam cooking, lipid oxidation, protein oxidation, volatile compounds, *in vitro* digestibility

Steam cooking is a popular way of preparing fish and the end temperature plays a key role in the quality of the cooked fish. In this study, the lipid and protein oxidation, and the related changes in volatile compounds and *in vitro* digestibility of large-mouth bass (*Micropterus salmoides*) steam cooked to a core temperature of 45°C°C, 55°C, 65°C, 75°C, and 85°C were investigated. Steaming caused a significant increase in the peroxide value (PV) and the thiobarbituric acid-reactive substances (TBARS) value, accompanied by the decreased proportion of unsaturated fatty acids like oleic acid and linoleic acid, which was related to the lipid oxidation and the increase in volatile aldehydes as indicated by the partial least squares analysis. The protein oxidation can be reflected by the significant decrease of total thiol groups, combined with the aggregation as shown in SDS-PAGE and the increase in particle size at pre-digestive phase. And the aggregation of proteins further caused the decreased digestibility of fish meat at the gastric phase, especially when the core temperature was above 75°C. Furthermore, steaming significantly decreased the aerobic count, and no coliform or generic *E. coli* was detected in steamed samples. Thus, the core temperature of 65–75°C was recommended for the consideration of food oxidation and microbial safety.

INTRODUCTION

Cooking is used to eliminate foodborne microorganisms, especially pathogens, and to improve the sensory properties of foods. Steaming as a traditional cooking method helps in maintaining the nutrition and original flavor of food, and also reducing the generation of harmful compounds compared with other cooking methods [Hu *et al*., 2018; Maulvault *et al*., 2012].

Cooking also leads to the formation of reactive oxygen species (ROS), which contribute to the oxidation of lipids and proteins [Sobral *et al*., 2018]. With the development of free radical chain reactions, lipids are oxidized in three simultaneous phases of initiation, propagation and completion, which not only reduces their nutritional value, but also causes the loss of other lipo-soluble bioactive compounds and the impaired eating quality [Chaiyasit *et al*., 2007; Guyon *et al*., 2016]. The oxidation of lipids first gives rise to the primary products such as hydroperoxides and conjugated dienes, and these compounds will be further decomposed, which are related to the increase of some volatile compounds like aldehydes, ketones, and alcohols [Souza & Bragagnolo, 2014].

As for protein oxidation, it can be induced by the ROS directly or by the secondary products of oxidative stress, and is also an important issue in food research [Soladoye *et al*., 2015]. Thermal treatments can further intensify the oxidative modification of protein, for instance, cysteine oxidation can lead to the formation of intermolecular disulfide, and the oxidation of basic amino acids induces the formation of carbonyl groups, the cross linking of carbonyls and free amino groups promotes the content of protein aggregates, which are considered as poor substrates for proteases and may impair the digestibility of protein [Hu *et al*., 2018; Sun *et al*., 2011]. In addition, the protein oxidation products, like kynurenines and α-aminoadipic semialdehyde, present a negative impact on human health [Kjærsgård *et al*., 2006]. Some studies suggested that the accumulation of oxidized proteins in the human body, which had the cytotoxic and mutagenic potential, would contribute to the aging and age-related diseases [Berlett & Stadtman, 1997; Estévez & Luna, 2017]. Therefore, the appropriate cooking condition and the control of food oxidation are crucial during the domestic cooking.

According to recent studies, researches have often focused on the effects of cooking methods on food oxidation, particularly for the livestock and poultry [Lorenzo & Domínguez, 2014; Silva *et al*., 2016]. Hu *et al*. [2017] investigated the lipid and protein oxidation of farmed sturgeon (*Acipenser gueldenstaedtii*) under different cooking methods and con-

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cluded that compared with frying and roasting, steaming significantly decreased the formation of carbonyls and increased the amounts of free thiols. However, protein and lipid oxidation of fish during the steaming process and their correlations with the changes of other physicochemical properties have received much less attention.

Large-mouth bass (*Micropterus salmoides*) is an important commercial fish species in China, because of its rapid growth and high market value [Yuan *et al*., 2014]. The growth rate of large-mouth bass yield has reached the highest among all the freshwater fish species in recent years, with the annual production estimated at 457,000 tons in 2017. It is generally prepared by steaming and is also favored by consumers for its high nutritional value and excellent taste. As mentioned before, protein oxidation is undesired. Generally, a lower degree of cooking leads to a lower level of food oxidation. But insufficient cooking poses a threat to human health due to pathogens. The hypothesis of this study was that large-mouth bass could be steam-cooked to optimal degree, so that the fish is safe to eat, while the food oxidation is kept at a relatively low level. In order to test this hypothesis, lipid and protein oxidation of large-mouth bass were monitored when steamed to different core temperatures, and their potential relationships with the formation of volatile compounds and *in vitro* digestibility were explored.

MATERIALS AND METHODS

Materials

Live large-mouth bass (25±1 cm in length, 378±27 g in weight) was purchased from Vanguard Market (Wuxi, Jiangsu, China) in May and June, 2018. The bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Fisher Scientific (Shanghai, China). Standards for 37 fatty acid methyl ester mixture (C4-C24), 14% boron trifluoride-methanol, α-amylase (from *Bacillus licheniformis*, 720 U/mg protein, CAS: 9000–85–5), pepsin (from porcine gastric mucosa, 3706 U/mg protein, CAS: 9001–75–6), pancreatin (from porcine pancreas, CAS: 8049–47–6), bile salts, and 2,4,6-trimethylpyridine were obtained from Sigma--Aldrich (Shanghai) Trading Co., Ltd. (Shanghai, China). The 5,5'-dinitrobis[2-nitrobenzoic acid] (DTNB), ethylenediaminetetraacetic acid (EDTA), urea, acrylamide, and bisacrylamide were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). All other chemicals used were of analytical grade and were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Preparation of samples

The fish were killed after head stunning, internal organs and scales were removed, then transported on ice to the laboratory within half an hour. After rinsing with tap water, they were randomly divided into 6 groups, the first uncooked group was the control, and the other 5 groups were steam cooked to different core temperatures. Each group contained twelve fish for the measurement of related indicators.

Briefly, a thermometer (K-type, UT 322, Uni-Trend Technology Co., Ltd., Dongguan, Guangdong, China) was inserted into the center of the fish sample (the position where the temperature changed the slowest). Then the fish were each placed on a porcelain dish and cooked to a core temperature of 45°C, 55°C, 65°C, 75°C, and 85°C at 100°C steam using a steam oven (Model S226, Hangzhou Robam Appliances Co., Ltd., Hangzhou, Zhejiang, China), and the corresponding average steaming time was 9.7, 12.2, 14.3, 18.2, and 22.5 min, respectively. When the core temperature reached the target value, samples were taken out from the oven and cooled at room temperature to around 25°C. The cooking loss was measured with the whole fish, then the fish muscle was immediately used for the determination of pH, differential scanning calorimetry (DSC) profile, protein solubility, volatile compounds, *in vitro* digestibility, and microbiological analysis. Some of the samples were lyophilized using a vacuum freeze drier (BenchTop Pro, SP Scientific, Warminster, PA, USA) for the analysis of peroxide value (PV), thiobarbituric acid-reactive substances (TBARS) value, and the composition of fatty acids, and the reminder was stored at -70°C until the determination of the carbonyl contents, total thiols, and SDS-PAGE analysis, a maximum of three weeks.

Cooking loss

Samples were weighed before and after steaming, the surface was dried before weighing, and the cooking loss was calculated as:

$$\text{Cooking loss (\%)} = \frac{\text{raw weight (g)} - \text{cooked weight (g)}}{\text{raw weight (g)}} \times 100$$

pH

The pH was measured using a S220 Seven Compact pH/ion meter (Mettler-Toledo Inc., Columbus, OH, USA) with a suspension resulting from homogenizing 5-g samples at 21,200 rpm with 45 mL distilled water for 1 min (T10, IKA-Werke, Staufen, Germany) and mixing for 30 min on a magnetic stirrer (RO 10, IKA-Werke, Staufen im Breisgau, Germany).

Peroxide value

The PV was measured using the method of Mi *et al*. [2016] with a slight modification. A ~0.5 g lyophilized sample was mixed with 15 mL of a chloroform-methanol solution (2:1, *v/v*) for lipid extraction. Then an aliquot from the chloroform layer (1 mL) was reacted with 50 μL ferrous chloride (3.5 g/L) followed by reaction with 50 μL potassium thiocyanate (300 g/L). The absorbance of the reaction mixture was measured at 500 nm after 5 min, and ferric chloride solution (10.0 μg/mL) was used for the standard curve. The results are expressed as milliequivalents of peroxide per kilogram of dry weight (meq peroxide/kg dry weight).

Thiobarbituric acid-reactive substances

TBARS method was used to estimate the content of thiobarbituric acid reactive substances, with malondialdehyde (MDA) as the standard [Ganhão *et al*., 2011]. Briefly, 1 g of a lyophilized sample was homogenized at 21,200 rpm with 15 mL of a 10% trichloroacetic acid solution and filtered through qualitative filter paper (92410441s, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The filtrate (5 mL) was reacted with 5 mL of 0.02 M TBA at 100°C for 40 min, and then measured at 532 nm after cooling for 15 min. The results are expressed as mg MDA/kg dry weight.

Fatty acid composition

Lipids from the lyophilized sample (0.5 g) were extracted for 3 h using the chloroform-methanol method of Flaskerud *et al.* [2017], and nitrogen was used to limit the exposure to oxygen. Then, the chloroform layer was separated and dried under nitrogen. After saponification using a 0.5 mol/L sodium hydroxide-methanol solution at 65°C for 30 min and methyl esterification using 14% boron trifluoride-methanol at 70°C for 10 min, 4 mL of hexane was used to extract the fatty acid methyl esters.

Fatty acid analysis was done on a GC-2010 Plus gas chromatograph (Shimadzu Co., Tokyo, Japan) with a DB-Wax column (0.25 mm×30 m, 0.25 μm; Restek International, Bellefonte, PA, USA). The initial temperature of the column oven was held at 100°C for 3 min, increased at 5°C/min to 180°C and 3°C/min to 240°C. Fatty acids were identified by comparison of their retention times with a mixture of standards containing all the fatty acids identified in this experiment, and 5 mg/mL glyceryl triundecanoate was used as the internal standard.

Volatile compounds

Volatile compounds of large-mouth bass were determined using Headspace SPME-GC/MS according to the method of Yu *et al.* [2018]. Two grams of minced fish sample were mixed with 50 μg/mL internal standard 2,4,6-trimethylpyridine and 4 mL saturated NaCl solution, and headspace extraction using a SPME fiber (50/30 μm, DVB/CAR/PDMS, Supelco, Bellefonte, PA) was proceed at 45°C for 30 min. Then, it was analyzed by a SCION SQ-456 GC/MS system (Bruker Daltonic Inc., Billerica, MA, USA) equipped with a DB-WAX column (30 m × 0.25 mm × 0.25 μm; Agilent, Palo Alto, CA, USA). Xcalibur software was used to screen out the volatile substances with a matching degree greater than 800 based on the Wiley 7 and NIST 2005 databases.

Carbonyl content

Carbonyl content was determined using the 2,4-dinitrophenylhydrazine (DNPH) method following the procedure of Zhang *et al.* [2018]. About 2 g of samples were dispersed and diluted to 4 mg/mL using a buffer containing 0.6 M NaCl, 20 mM sodium phosphate buffer, and 8 M urea (pH 7.5), then a 2 mL diluent was used for the measurement of the carbonyl content. The absorbance was measured at 370 nm and the carbonyl content was expressed as nmol/mg protein with a molar extinction coefficient of 22,000 M^{-1} cm^{-1}.

Total thiols

Diluent solutions prepared for the measurement of carbonyls were used for the total thiols analysis using the method of Benjakul *et al.* [1997] with some modification by Zhou *et al.* [2018]. Diluent solution of 1 mL was mixed with 9 mL buffer containing 0.6 M NaCl, 20 mM sodium phosphate buffer, 8 M urea (pH 7.5), and 1 mL reagent (0.1% DTNB in 0.2 M Tris-HCl buffer, pH 8.0). Samples were kept in the dark at 40°C for 25 min and then measured at 412 nm, and the total thiol content was calculated using a molar extinction coefficient of 13600 M^{-1} cm^{-1}. The results are expressed as nmol/mg protein.

SDS-PAGE

The protein extract from the carbonyl determination was used for SDS-PAGE. Samples were diluted to 1 mg/mL using 2× SDS-PAGE sample buffer (0.5 M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, and 0.04% bromophenol blue) with or without 5% β-mercaptoethanol (β-ME), and 10 μL of protein sample were used in electrophoresis with 4% stacking gel and 8% resolving gel. Then, the gels were stained in a 0.1% (*w/v*) Coomassie Brilliant Blue R-250 solution for 3 h and destained with a methanol-acetic acid solution (5% methanol and 7.5% acetic acid, *v/v*) until the bands were clear for around two days.

Differential scanning calorimetry

Raw and steamed samples from the dorsal part (~10–15 mg) were weighed and hermetically sealed into aluminum pans, thermograms were obtained using the DSC (214 Polyma, Netzsch-Gerätebau GmbH, Selb, Germany). Samples were first equilibrated at 25°C for 5 min, and then heated to 100°C at the rate of 10°C/min, a sealed empty pan was used as the reference. The denaturation temperatures and enthalpies were analyzed using Proteus software (Netzsch-Gerätebau GmbH), and results from 35 to 90°C are shown.

Protein solubility

The extraction of water-soluble and salt-soluble protein components was carried out as described by Visessanguan *et al.* [2004]. Then, the concentration of the water-soluble fraction, salt-soluble fraction, and total protein was determined using the BCA protein assay kit, and the results are expressed as a percentage of the water-soluble and salt-soluble fraction in the total protein, respectively.

***In vitro* digestibility**

Both the raw and steam-cooked samples were minced finely using an electric mincer (JYL-C51V, Joyoung Co., Ltd., Jinan, Shandong, China) and then digested under simulated gastrointestinal conditions according to the method of Minekus *et al.* [2014]. Samples were collected at the end of the gastric phase and intestinal phase for further analysis.

Particle size distribution of samples at different digestive phases (pre-digestive, gastric, and intestinal phases) were analyzed using a Microtrac S3500 (Microtrac Inc., Montgomeryville, PA, USA). Briefly, samples were homogenized using an Ultra Turrax homogenizer (T10, IKA-Werke) at 21,200 rpm for 1 min and 1 mL of dispersed solutions (2 mg/mL) were used for measurement. The refractive index was set to 1.46 and particles were considered as non-spherical. The volume-weighed mean diameter $D_{4,3}$ ($D_{4,3} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$) was recorded to describe the mean particle size.

Furthermore, an inverted fluorescence microscope (Eclipse Ti-U, Nikon Corp., Tokyo, Japan) was used to observe the microstructure changes of fish protein at different digestive phases. Samples were stained with 0.32 mg/mL fluorescein isothiocyanate (FITC) and kept in the dark for

TABLE 1. Some physicochemical properties of the raw and steamed muscle samples with different core temperatures of large-mouth bass.

Indexes		Core temperature						*p* value
		Control (~25°C)	45°C	55°C	65°C	75°C	85°C	
Cooking loss (%)		–	5.0 ± 0.6^{d}	6.7 ± 0.6^{c}	8.4 ± 0.4^{b}	9.9 ± 1.3^{ab}	11.1 ± 1.2^{a}	<0.001*
pH		7.20 ± 0.09^{a}	7.18 ± 0.14^{a}	7.26 ± 0.04^{a}	7.13 ± 0.04^{a}	7.06 ± 0.16^{a}	7.13 ± 0.20^{a}	0.513
Peroxide value (meq peroxide/kg dry weight)		0.9 ± 0.2^{c}	1.3 ± 0.3^{c}	1.2 ± 0.2^{c}	1.9 ± 0.1^{b}	3.3 ± 0.6^{a}	2.4 ± 0.2^{b}	<0.001
TBARS (mg MDA/kg dry weight)		1.0 ± 0.1^{c}	3.8 ± 1.4^{b}	4.1 ± 0.3^{b}	5.0 ± 0.4^{b}	5.4 ± 0.5^{b}	8.9 ± 1.4^{a}	<0.001
Carbonyls (nmol/mg protein)		3.0 ± 0.8^{b}	4.2 ± 1.1^{ab}	5.4 ± 1.3^{a}	5.2 ± 1.0^{ab}	6.2 ± 1.8^{a}	5.9 ± 1.0^{a}	0.057
Total thiols (nmol/mg protein)		85 ± 5^{a}	70 ± 3^{b}	71 ± 8^{b}	69 ± 3^{b}	63 ± 2^{bc}	61 ± 4^{c}	<0.001
Water-soluble protein (%)		5.36 ± 0.17^{a}	2.17 ± 0.06^{b}	1.46 ± 0.11^{c}	1.51 ± 0.12^{c}	1.41 ± 0.04^{c}	1.36 ± 0.07^{c}	<0.001
Salt-soluble protein (%)		9.90 ± 0.84^{a}	0.83 ± 0.03^{b}	0.65 ± 0.16^{b}	0.50 ± 0.06^{b}	0.59 ± 0.02^{b}	0.57 ± 0.04^{b}	<0.001
$D_{4,3}$ (μm)	Pre-digestive phase	120 ± 20^{c}	280 ± 20^{b}	370 ± 50^{a}	350 ± 60^{ab}	360 ± 60^{ab}	350 ± 20^{ab}	<0.001
	Gastric phase	80 ± 10^{c}	110 ± 10^{b}	111 ± 8^{b}	120 ± 10^{b}	150 ± 10^{a}	150 ± 20^{a}	<0.001
	Intestinal phase	33 ± 4^{b}	60 ± 10^{a}	80 ± 6^{a}	72 ± 7^{a}	70 ± 20^{a}	69 ± 8^{a}	<0.001

Mean values denoted with different letters in superscripts in the same row are statistically significantly different ($p<0.05$). * The analysis of significance was done in the steamed groups from 45°C to 85°C.

20 min. Each sample was observed using a 20× magnification objective and analyzed using Nikon NIS-Elements BR software (Nikon Corp., Tokyo, Japan).

Microbiological analysis

For the microbiological analysis, the fish sample (5.00 g) was first mixed with sterile 45 mL of 0.85% NaCl solution and then homogenized using a blender (SCIENTZ-09, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, Zhejiang, China) for 2 min at room temperature. The aerobic count was measured using the 3M Aerobic Count Plate (3M Microbiology, St. Paul, MN, USA), and total coliforms and generic *E. coli* were determined using the 3M Coliform/*E. coli* Count Plates (3M Microbiology, St. Paul, MN, USA). One milliliter of each diluted solution (10^{-1} through 10^{-3}) was plated in triplicate, the aerobic count plates were incubated at 30°C for 72 h following the ISO Method 4833:2003, and coliform/*E. coli* count plates were incubated at 35°C for 24 h following the AOAC Official Method 998.08. The results are expressed as log CFU/g.

Statistical analysis

Measurements were all done in triplicates. Results were analyzed using the Statistical Package for the Social Sciences Statistics 20 software (International Business Machines Corp., Chicago, IL, USA) with the one-way analysis of variance (ANOVA). Significant differences were identified using the Duncan's test at an α level of 0.05. The partial least squares (PLS) analysis between fatty acids and the related lipid oxidation and degradation indicators (PV, TBARS and volatile compounds) was performed using XL STAT 2015 software (Addinsoft Inc., NY, USA).

RESULTS AND DISCUSSION

Cooking loss and pH

Endpoint temperature of steam cooking showed a significant impact on the cooking loss of large-mouth bass (Table 1). The value increased significantly from 5.0% at 45°C to 8.4% at 65°C ($p<0.05$), while the difference was not significant between 65°C and 75°C ($p>0.05$), and the cooking loss reached the maximum of 11.1% at the temperature of 85°C. This could be related to the structural changes of fish meat. Li *et al.* [2013] observed the transverse shrinkage of muscle fibers in duck breast meat below 50°C, when the temperature was above 60°C, the fibers and connective tissue shrank longitudinally with the significant decrease in sarcomere length. The shrinkage pressure accelerated the extrusion of water and the increase of cooking loss, which could further influence the juiciness and eating quality of fish meat. But steaming did not influence the pH ($p>0.05$), the control and steamed samples all had pH values slightly above 7 (Table 1).

Lipid oxidation

Lipid oxidation is an important reaction that occurs during fish cooking. Such oxidation is believed to proceed *via* a free radical chain reaction depending on the oxidative environment and muscle characteristics [Lund *et al.*, 2011]. PV can be used to show the level of primary lipid oxidation products during steaming. PV increased significantly with the increase of core temperature ($p<0.001$) and reached the maximum at 75°C (3.3 meq peroxide/kg dry weight), and then decreased significantly at 85°C (2.4 meq peroxide/kg dry weight) as shown in Table 1 ($p<0.05$). The degradation of primary oxidation products promoted the generation of secondary products, which could be estimated using TBARS, and the highest value of 8.9 mg MDA/kg dry weight was reached at 85°C. The study of Dong

TABLE 2. The fatty acid composition (% of total fatty acids) of the raw (control) and steamed large-mouth bass with different core temperatures.

Fatty acid	Control (~25°C)	45°C	55°C	65°C	75°C	85°C
C14:0	1.99±0.05^{c}	2.99±0.30^{b}	2.95±0.26^{b}	3.09±0.27^{b}	2.77±0.54^{b}	3.65±0.12^{a}
C15:0	0.28±0.01^{c}	0.47±0.03^{b}	0.44±0.02^{b}	0.46±0.06^{b}	0.44±0.06^{b}	0.64±0.03^{a}
C16:0	17.7±0.3^{b}	21.6±0.5^{a}	21.5±0.6^{a}	23.5±2.7^{a}	22.2±1.3^{a}	23.9±2.7^{a}
C17:0	0.25±0.01^{c}	0.41±0.01^{b}	0.41±0.01^{b}	0.41±0.03^{b}	0.38±0.08^{b}	0.58±0.00^{a}
C18:0	2.87±0.13^{c}	3.21±0.10^{bc}	3.39±0.26^{abc}	3.87±0.57^{a}	3.67±0.22^{ab}	3.47±0.08^{ab}
C20:0	0.22±0.02^{b}	0.21±0.02^{b}	0.20±0.02^{b}	0.23±0.03^{b}	0.23±0.05^{b}	0.32±0.03^{a}
C22:0	0.19±0.01^{ab}	0.14±0.01^{b}	0.14±0.02^{b}	0.22±0.05^{a}	0.19±0.02^{ab}	0.15±0.07^{ab}
∑SFA	**23.5±0.4**^{c}	**29.0±0.3**^{b}	**29.1±0.6**^{b}	**31.8±3.1**^{ab}	**29.9±1.1**^{ab}	**32.7±2.5**^{a}
C16:1*n*-7	4.8±0.5^{b}	8.1±0.2^{a}	8.3±0.9^{a}	7.2±0.8^{a}	7.2±0.9^{a}	7.0±0.4^{a}
C17:1	0.32±0.02^{d}	0.60±0.01^{b}	0.61±0.04^{b}	0.61±0.04^{b}	0.53±0.07^{c}	0.69±0.02^{a}
C18:1*n*-9	27±1^{a}	26±2^{ab}	28±1^{a}	26±3^{a}	27±2^{a}	22±2^{b}
C20:1*n*-9	1.61±0.05^{b}	2.22±0.27^{a}	1.78±0.02^{ab}	2.26±0.42^{a}	2.17±0.34^{a}	2.21±0.29^{a}
C22:1n-9	0.61±0.02^{d}	1.39±0.09^{ab}	1.16±0.16^{bc}	1.63±0.17^{a}	1.41±0.26^{ab}	0.97±0.19^{c}
∑MUFA	**34±2**^{b}	**38±1**^{a}	**40±1**^{a}	**38±2**^{a}	**38±2**^{a}	**33±2**^{b}
C18:2*n*-6	27.62±1.20^{a}	4.63±0.48^{b}	5.07±0.40^{b}	4.21±0.55^{b}	5.16±0.45^{b}	1.79±0.04^{c}
C18:3*n*-3	2.47±0.11^{a}	0.94±0.06^{b}	0.92±0.04^{b}	0.89±0.04^{b}	0.96±0.16^{b}	0.81±0.00^{b}
C18:3*n*-6	0.14±0.01^{b}	0.28±0.01^{a}	0.29±0.03^{a}	0.28±0.02^{a}	0.19±0.06^{b}	0.30±0.03^{a}
C20:2*n*-6	0.56±0.04^{a}	0.36±0.02^{b}	0.35±0.01^{b}	0.33±0.03^{b}	0.37±0.03^{b}	0.32±0.02^{b}
C20:3*n*-3	0.19±0.00^{a}	0.15±0.01^{b}	0.14±0.01^{b}	0.16±0.03^{b}	0.15±0.03^{b}	0.14±0.00^{b}
C20:3*n*-6	0.15±0.03^{b}	0.24±0.01^{a}	0.24±0.01^{a}	0.23±0.03^{a}	0.22±0.04^{a}	0.16±0.01^{b}
C20:4*n*-6	0.37±0.04^{d}	1.12±0.06^{c}	1.11±0.11^{c}	1.17±0.11^{c}	3.35±0.36^{b}	5.08±0.25^{a}
C20:5*n*-3	1.24±0.09^{d}	3.38±0.07^{b}	3.00±0.28^{c}	3.72±0.14^{a}	0.77±0.06^{e}	0.80±0.02^{e}
C22:5*n*-3	1.52±0.05^{b}	3.21±0.18^{a}	2.97±0.16^{a}	2.82±0.65^{a}	3.00±0.68^{a}	3.48±0.94^{a}
C22:6*n*-3	7.8±0.8^{b}	18.6±0.4^{a}	17.3±1.0^{a}	16.5±4.5^{a}	17.9±0.5^{a}	21.1±3.8^{a}
∑PUFA	**42±2**^{a}	**33±1**^{b}	**31±1**^{b}	**30±5**^{b}	**32±2**^{b}	**34±5**^{b}

Mean values denoted with different letters in superscripts in the same row are statistically significantly different ($p<0.05$). SFA: saturated fatty acids. PUFA: polyunsaturated fatty acids. MUFA: monounsaturated fatty acids.

et al. [2018] also found the similar results, that is, a higher temperature with prolonged heating time led to an increased MDA content and generated more carbon-centered radicals in scallop adductor muscle. Therefore, in order to avoid the severe lipid oxidation, the core temperature should not exceed 85°C during the steam cooking of large-mouth bass.

Fatty acid composition

In this study, 22 fatty acids were identified in fish lipid. Results are shown in Table 2. According to the changes during steaming, the core temperature had a great influence on fatty acid composition of large-mouth bass. The proportion of saturated fatty acids increased with the increase of core temperature, especially myristic acid (C14:0), pentadecanoic acid (C15:0), and heptadecanoic acid (C17:0). Most of the monounsaturated fatty acids showed an increasing trend during steaming, but the proportion of oleic acid (C18:1*n*-9) decreased significantly at the core temperature of 85°C. Four polyunsaturated fatty acids, linoleic acid (C18:2*n*-6), linolenic acid (C18:3*n*-3), eicosadienoic acid (C20:2*n*-6), and 8,11,14-eicosatrienoic acid (C20:3*n*-3), decreased significantly in proportion during steaming. In general, the composition of fatty acids at the core temperature of 85°C is quite different from other steaming temperatures. In the study of Bastías *et al.* [2017], salmon fillets processed with steaming treatment showed a higher proportion of omega-3 fatty acids, especially DHA, when compared with oven cooking and microwaving methods. The advantage of steaming in retaining the unsaturated fatty acids was also seen in the research of Shi *et al.* [2019]. Compared with roasting, steaming significantly increased the contents of free EPA, DHA and ARA in tilapia fillets, which might have resulted from the breakdown of triglyceride. Therefore, in order to maintain the nutritious unsaturated fatty acids, steaming method was recommended in fish cooking.

TABLE 3. The volatile compounds of the raw (control) and steamed large-mouth bass with different core temperatures.

Compound	Content (μg/100 g)					
	Control (~25°C)	45°C	55°C	65°C	75°C	85°C
			Aldehydes			
Hexanal	170±20^{d}	260±4^{c}	300±10^{abc}	270±20^{bc}	350±40^{a}	320±50^{ab}
Heptanal	15.2±2.5^{d}	21.3±0.3^{bc}	22.5±3.3^{bc}	18.7±3.9^{cd}	31.2±4.6^{a}	27.3±3.5^{ab}
(Z)-4-heptenal	3.9±0.9^{a}	3.4±0.6^{a}	3.6±1.5^{a}	3.8±0.2^{a}	4.9±0.4^{a}	5.1±1.3^{a}
Octanal	11±2^{c}	14±2^{bc}	13±1^{bc}	12±3^{bc}	19±3^{a}	17±4^{ab}
Nonanal	9.1±1.4^{c}	13.6±0.3^{b}	16.8±1.6^{ab}	14.2±2.8^{b}	20.0±2.6^{a}	17.6±3.3^{ab}
(E)-2-octenal	1.25±0.02^{a}	1.05±0.11^{ab}	1.16±0.21^{ab}	0.88±0.21^{ab}	0.65±0.57^{b}	1.00±0.28^{ab}
Decanal	0.5±0.7^{a}	1.0±1.1^{a}	1.7±1.4^{a}	0.8±1.0^{a}	1.2±1.1^{a}	nd
			Ketones			
2,3-Pentanedione	13±3^{a}	12±1^{a}	13±2^{a}	12±2^{a}	8±8^{a}	11±2^{a}
2-Nonanone	0.6±0.1^{c}	1.6±0.4^{ab}	2.0±0.7^{a}	1.1±0.4^{bc}	1.4±0.3^{ab}	1.4±0.3^{ab}
			Alcohols			
1-Penten-3-ol	nd	nd	14±13^{a}	nd	23±2^{a}	15±13^{a}
1-Pentanol	6±1^{c}	15±2^{ab}	19±2^{a}	9±8^{bc}	15±1^{ab}	8±6^{bc}
1-Hexanol	3±0^{c}	70±40^{ab}	110±40^{a}	50±30^{bc}	10±8^{c}	2±0^{c}
1-Octen-3-ol	23±3^{b}	42±3^{a}	47±9^{a}	39±6^{a}	44±6^{a}	50±10^{a}
1-Heptanol	1±0^{b}	19±10^{a}	26±8^{a}	17±6^{a}	15±6^{a}	13±6^{a}
1-Octanol	2±1^{c}	7±3^{ab}	9±3^{a}	5±2^{bc}	4±0^{bc}	3±1^{bc}
1-Nonanol	1±1^{a}	3±1^{a}	4±2^{a}	5±6^{a}	nd	nd

Mean values denoted with different letters in superscripts in the same row are statistically significantly different ($p<0.05$). "nd" indicates that the substance has not been detected.

Volatile compounds

It is known that lipid oxidation plays a key role in the formation of volatile compounds which contribute to the flavor of cooked fish, and the volatile compounds associated with the lipid oxidation in this study are listed in Table 3. Aldehyde, which was high in content and low in threshold, was an important contributor in the formation of fish flavor. With the increase of the core temperature, the content of hexanal, heptanal, octanal, and nonanal significantly increased during steaming especially at 75°C and 85°C ($p<0.05$), and at the same time provided the grassy, fatty and fruity flavor to the steamed fish. As for alcohols, the content of 1-octen-3-ol and 1-heptanol increased significantly after steaming ($p<0.05$), which generally yield fatty and vegetable-like flavors, could help to enrich a cooked fish odor with less fishy smell. While for 1-pentanol, 1-hexanol, and 1-octanol, the contents decreased when the core temperature was above 65°C. Combined with the sensory analysis in our previous study [Wang *et al*., 2019], when the core temperature reached 75°C, the increased formation of volatile compounds significantly improved the odor attribute and the consumer acceptance, while the fishy smell was still obvious at the core temperature of 65°C with lower odor and taste score.

Furthermore, correlations between fatty acids and related lipid oxidation and degradation indicators (PV, TBARS and volatile compounds) were analyzed using PLS. The results in Table 1 suggest that contents of the following fatty acids C18:2*n*-6, C18:3n-3, C20:2*n*-6, C20:3*n*-3, and also C18:1*n*-9 were negatively related with PV and TBARS, and around the PV and TBARS were mainly volatile aldehydes like octanal, heptanal, nonanal, and hexanal, which also showed negative and close correlations with the unsaturated fatty acids mentioned above. Combined with the location of samples in Figure 1, the proportion of unsaturated fatty acids like C18:2*n*-6 and C18:3*n*-3 was comparatively high in the raw sample as they were very close to the control in distance and had a major negative loading on t1. While steaming caused the oxidation and degradation of these fatty acids and contributed to the increase of PV and TBARS (Table 1), and the formation of volatile compounds (Table 3), as the steamed samples were located quite far from the control. Samples of 45°C and 65°C had small negative loadings on t1 and t2, the sample of 55°C was located in the fourth quadrant and mainly the alcohols such as 1-hexanol and 1-octanol were around it. And the lipid oxidation was much severer at higher temperatures of 75°C and 85°C as they were closer to the indicators of PV and TBARS, and the corresponding aldehydes also increased significantly at these temperatures (Table 3).

Based on the literature and findings from this study, an oxidative degradation pathway of oleic acid, which had a high proportion in fatty acids of large-mouth bass, was proposed as an example (Figure 2). During steaming, heme irons together with heating and oxygen initiated lipid oxidation and generated oleic peroxides. The oleic peroxides were not stable and the C-8, C-9, C-10, and C-11 might undergo scission to produce a series of aldehydes and alcohols, and they generally increased with increased temperature. Among these aldehydes and alcohols, hexanal and 1-hexanol were the dominant ones. At higher core temperatures, 1-hexanol decreased possibly due to further reactions. Similar changes can also occur in linoleic and linolenic acids, and the formation of volatile compounds improved the flavor of fish during steaming [Schaich *et al*., 2013].

Protein oxidation

Oxidative stress can transfer from lipids to proteins. And protein oxidation in fish can be characterized by the formation of carbonylation and the loss of thiol groups (Table 1). In our study, the temperature does play an important role in the oxidation of proteins. The content of carbonyls increased during the steaming process, which doubled at 75°C and 85°C (6.2 and 5.9 nmol/mg protein, respectively) compared to the raw sample (3.0 nmol/mg protein). While the total thiols decreased significantly and reached a minimum of 61 nmol/mg protein at the core temperature of 85°C. Apart from the changes in carbonyl groups and free thiols, the formation of Schiff bases was also found in sturgeon fillets prepared with different cooking methods [Hu *et al*., 2017]. Combined with the proteomic analysis, heating induced the modification of aromatic residues such as tyrosine and tryptophan, and the formation of advanced glycation end-products, which was much severer in the roasted than in the steamed samples. Thus, the appropriate temperature and time should be chosen in fish cooking to avoid the excessive oxidation and the loss of protein nutritional value.

SDS-PAGE

From the results of SDS-PAGE shown in Figure 3, there was no significant difference between the electrophoretic bands of different steam-treated groups, with the addition of β-ME (Figure 3a). While under the non-reducing condition, the intensity of bands such as the myosin heavy chain (MHC, 220 kDa), actin (43 kDa), and the bands between 75–100 kDa decreased significantly with the increase of the core temperatures (Figure 3b). In addition, the amount of proteins that could not enter the gel also increased compared with the reducing groups. Combined with the significant decrease of total thiols (Table 1) especially at 85°C, it could be concluded that the formation of disulfide bridges through free thiols might be the main cause of protein aggregation. However, there were still some proteins at the top of the stacking gel with the reducing conditions (Figure 3a), which suggested that covalent bonds other than

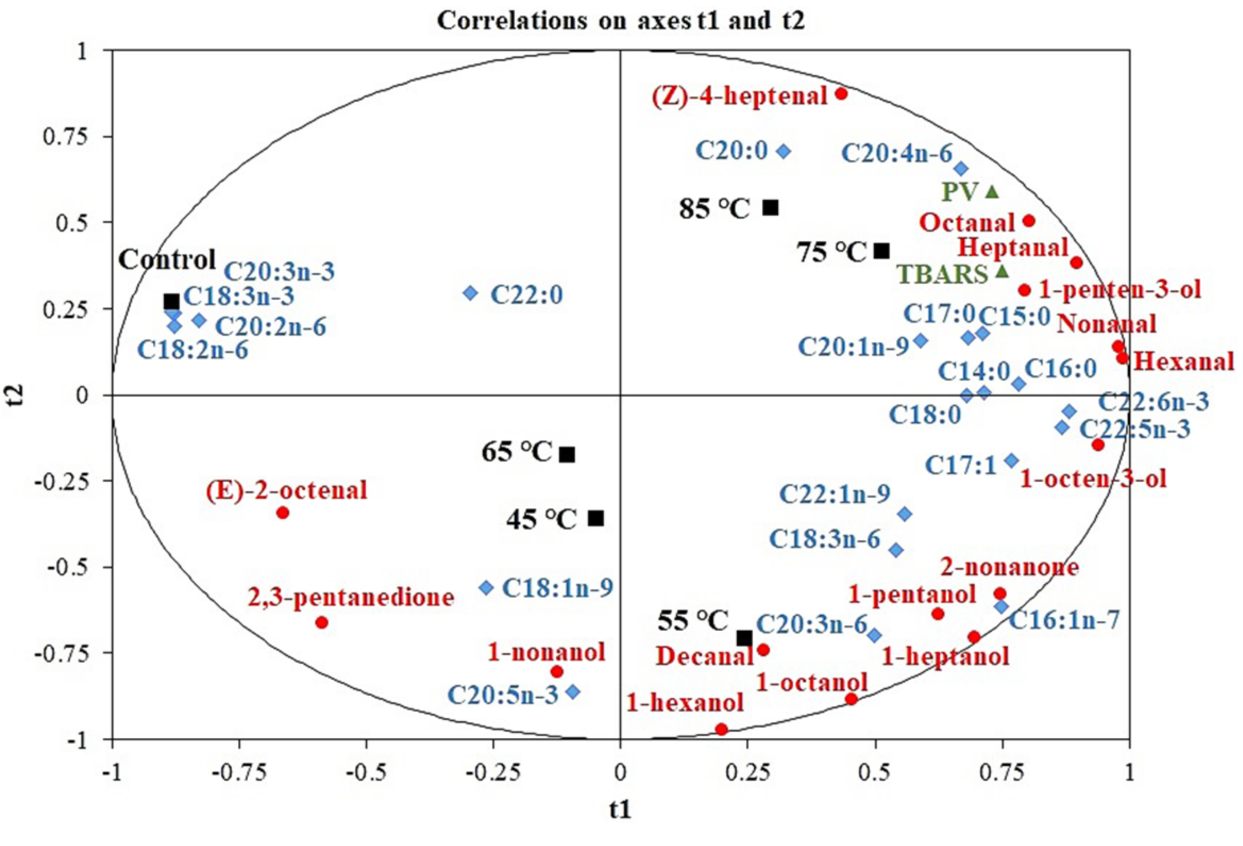

FIGURE 1. Partial least squares (PLS) analysis for the associations of fatty acids (blue) and related lipid oxidation and degradation indicators (PV, TBARS in green and volatile compounds in red) in the raw (control) and steamed large-mouth bass with different core temperatures (black).

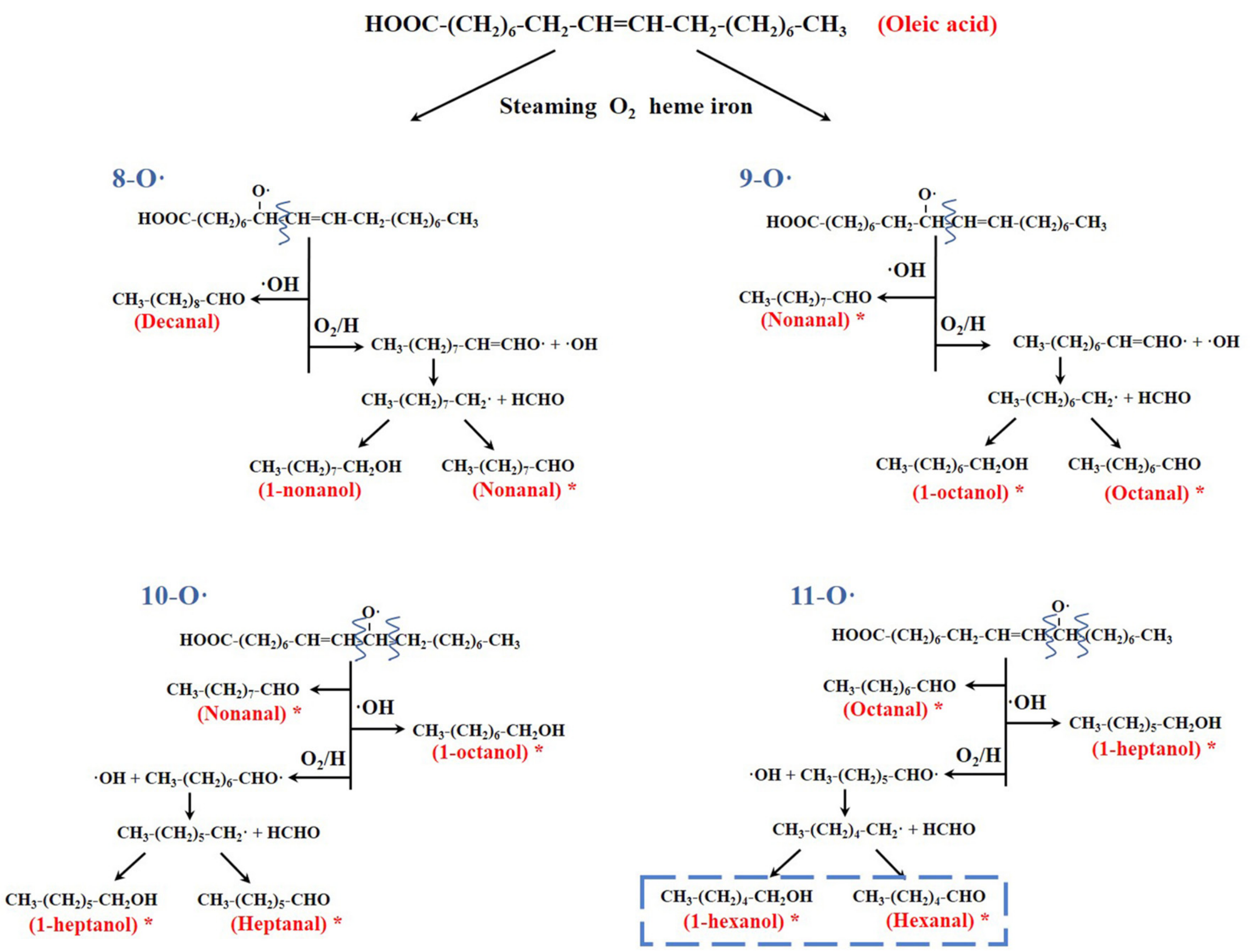

FIGURE 2. Proposed oxidative degradation pathway of oleic acid, modified from Schaich *et al*. [2013]. *Represents the content of this product generally increased after steaming. The dominant products as identified by GC-MS were hexanal and 1-hexanol.

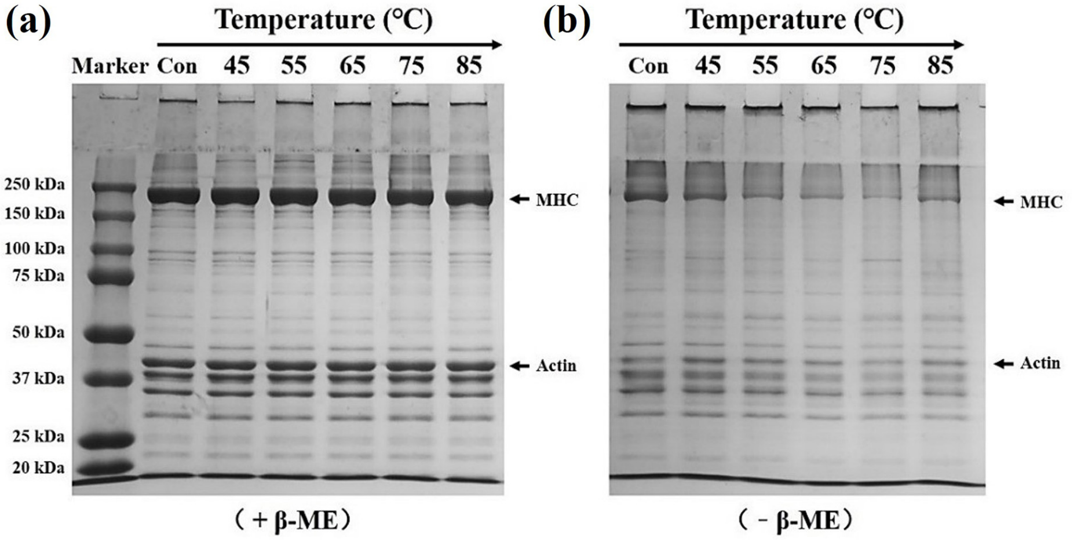

FIGURE 3. SDS-PAGE patterns of the raw (control) and steamed large-mouth bass with different core temperatures. Samples were prepared with (+ β-ME, a) or without (- β-ME, b) 5% β-mercaptoethanol. Positions of the MW marker, myosin heavy chain (MHC), and actin were marked.

disulfides also participated in the physicochemical reactions of protein during steaming. Such cross-linking and aggregation could further impact the solubility of proteins, and the conformational change of proteins might to some extent influence the digestibility of fish meat.

Differential scanning calorimetry and protein solubility

The thermal properties of proteins in fish meat were measured using DSC. Figure 4 shows that before steaming, the raw sample contained two major endothermic peaks. Based on the previous study of Korzeniowska *et al.* [2013], these two thermal transitions of the control could be attributed as follows: Peak 1 might correspond to the myosin and sarcoplasmic protein (47–60°C), and peak 2 might be assigned to actin (67–77°C). The denaturation enthalpy values of peak 1 and peak 2 in the raw sample were 1.21 and 0.43 J/g, respectively. And the disappearance of peak 1 was pronounced when the core temperature reached 55°C, indicating the denaturation of related proteins. Higher temperatures led to the complete denaturation of proteins and no peak was detectable when the core temperature increased to 65°C. Protein denaturation could also be seen in changes of the protein solubility (Table 1), with the significant decreased solubility of both the water-soluble and salt-soluble proteins when applied with steaming ($p<0.001$), and the amount of water-soluble proteins was much less when the core temperature was above 55°C. The protein oxidation and aggregation mainly through disulfide bonds (Figure 3) reduced the binding ability between the protein and water, and thus caused the decreased content of soluble proteins, and the loss of water from the myofibrillar matrix as shown in the result of cooking loss (Table 1).

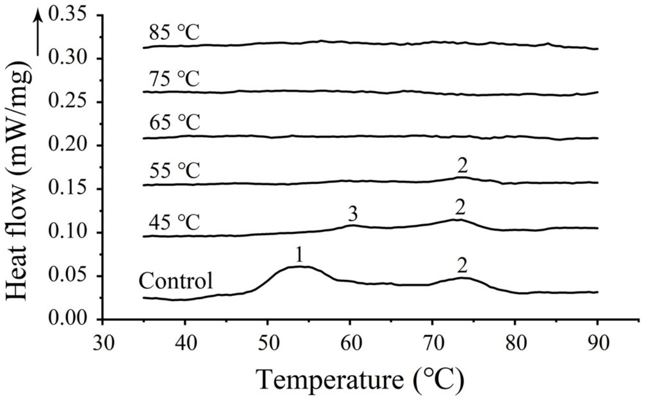

FIGURE 4. DSC thermograms of the raw (control) and steamed large-mouth bass with different core temperatures. Peak 1: myosin and sarcoplasmic protein; Peak 2: actin; Peak 3: sarcoplasmic protein. The arrow pointing up indicated the endothermic reaction.

In vitro digestibility

The oxidation and aggregation of proteins would further affect their gastrointestinal digestion, and in this study the particle size distribution after different digestive phases was used to reflect the aggregation and *in vitro* digestibility of fish protein. Steaming caused a significant increase in particle size ($p<0.001$) compared with the raw sample (Table 1), which was in accordance with the increased aggregation of proteins as indicated by the SDS-PAGE (Figure 3). At the gastric phase, the particle size increased significantly with the increase of core temperature ($p<0.05$), especially above 75°C, indicating that samples were not digested so well when compared with the raw sample. But no difference was found after intestinal digestion ($p>0.05$). Size changes of the fish meat homogenates during *in vitro* digestion were supported by the micrographs (Figure 5). Fish protein aggregated into larger molecules when the core temperature was above 55°C. After digestion, proteins were hydrolyzed by enzymes and the fluorescence intensity of the background increased, while steaming groups still had the large protein not digested fully.

As shown in the study of Wen *et al.* [2015], compared with the core temperatures between 60°C and 70°C, a higher core temperature of 100°C resulted in a significant decrease in the digestibility of pork meat and changed the peptide composition. Some studies suggested that moderate oxidation and conformational changes of protein might improve its digestibility. However, further oxidation and aggregation of sarcoplasmic and myofibrillar proteins reduced the interaction sites between digestive enzymes and proteins, thus resulting in a decline in the overall digestibility of food [Bax *et al*., 2012; Sun *et al*., 2011]. Furthermore, the oxidative modification of proteins and formed potentially toxic products, such as α-aminoadipic acid and HNE-bound protein, could have a harmful implication on human health during the food digestion and adsorption.

Microbiological analysis

From the aspect of food safety, the aerobic count, total coliforms, and generic *E. coli* were determined in this study. The aerobic colony count in ready-to-eat fish food with sat-

TABLE 4. Counts of indicator microorganisms (aerobic count, total coliforms and generic *E. coli*) in raw (control) and steamed large-mouth bass with different core temperatures.

Microorganisms	Counts (log CFU/g)					
	Control (~25°C)	45°C	55°C	65°C	75°C	85°C
Aerobic count	3.2 ± 0.1^{a}	3.0 ± 0.2^{a}	2.6 ± 0.1^{ab}	2.0 ± 0.5^{b}	1.2 ± 0.7^{c}	1.3 ± 0.5^{c}
Total coliforms	1.2 ± 0.3	nd	nd	nd	nd	nd
Generic *E. coli*	nd	nd	nd	nd	nd	nd

Mean values denoted with different letters in superscripts in the same row are statistically significantly different ($p<0.05$). “nd” means not detected.

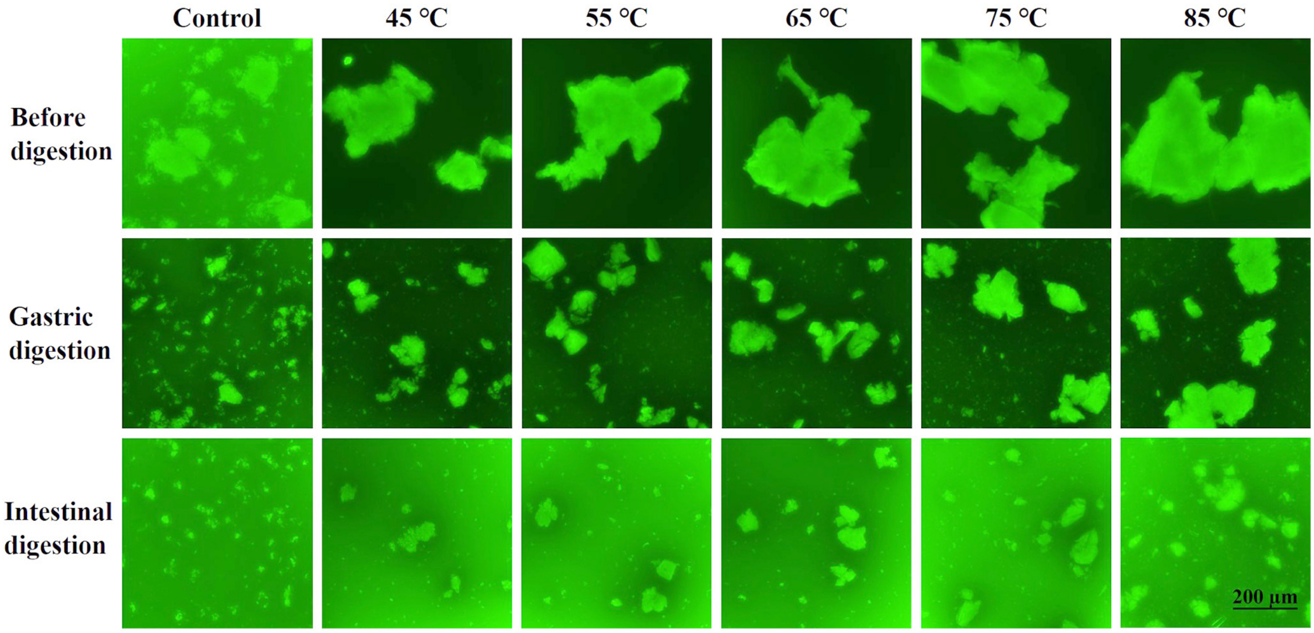

FIGURE 5. Micrographs of the raw (control) and steamed large-mouth bass with different core temperatures at different digestive phases. The magnification was 200 × and the scale bar was 200 μm.

isfactory quality should not exceed 5 log CFU/g [Microbiological Guidelines, 2007]. In our study, the aerobic count in the raw fish meat was only 3.2 log CFU/g (Table 4). It decreased significantly when the steam core temperature reached 65°C ($p<0.05$), and declined to the lowest at 75°C and 85°C, which suggested the effective role of steaming treatment in improving the food safety. The count of total coliforms in the raw fish meat was 1.2 log CFU/g, while they were not detected after steaming, even though the core temperature was only 45°C. And the generic *E. coli* was not detected in both raw and steamed samples. In addition, as recommended by USDA-FDA [2011], the safe minimum internal temperature for fish cooking should be 145°F (around 63°C).

CONCLUSION

Steaming, especially when the core temperature was above 75°C, significantly increased cooking loss of large-mouth bass, which was accompanied with a significant increase of protein oxidation and aggregation, which further caused an increased particle size and decreased *in vitro* digestibility of fish meat. Unpleasant fishy smell often presents in less cooked fish. Analysis of volatile compounds revealed that odor profile was clearly altered when the core temperature increased from 65°C to 75°C, likely due to the oxidative degradation of fatty acids. Core temperature of 65°C or above also enhanced food safety from the microbiological aspect as the aerobic count decreased significantly as compared to the raw samples. In addition, total coliforms and generic *E. coli* were not detected in all steamed fish samples. Therefore, to ensure safety, pleasant flavor and minimal protein oxidation, the core temperature between 65°C and 75°C was recommended for the steamed large-mouth bass.

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CONFLICT OF INTERESTS

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

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Instruction for Authors
Section: Editorial News

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