UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Chemii

PRACA DOKTORSKA

WYKONANA W KATEDRZE CHEMII ANALITYCZNEJ
I SPEKTROSKOPII STOSOWANEJ WYDZIAŁU CHEMII
UNIWERSYTETU MIKOŁAJA KOPERNIKA W TORUNIU
ORAZ FABRYCE CUKIERNICZEJ KOPERNIK S.A.

INNOWACYJNE WYROBY CUKIERNICZE JAKO ELEMENT ZRÓŻNICOWANEJ I ZBILANSOWANEJ DIETY PRZYCZYNIAJĄCY SIĘ DO WALKI Z CHOROBAMI CYWILIZACYJNYMI

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Toruń 2023

Podziękowania

Dla mojej Pani Promotor prof. dr hab. Aleksandry Szydłowskiej-Czerniak,
za nieocenioną pomoc naukową podczas pisania niniejszej pracy, cenne rady,
wsparcie i ogromną życzliwość.

Dla Promotora Pomocniczego ś. p. mgr Przemysława Myśliwy Prezesa Zarządu
Fabryki Cukierniczej Kopernik S.A. za umożliwienie rozpoczęcia
studiów doktoranckich oraz ogromne wsparcie.

Dla Promotora Pomocniczego mgr Karoliny Ziółkowskiej – Wachowiak
Prezesa Zarządu Fabryki Cukierniczej Kopernik S.A. za umożliwienie
kontynuacji studiów doktoranckich.

Praca powstała w wyniku realizacji programu „Doktorat wdrożeniowy”
w ramach zatrudnienia w Fabryce Cukierniczej Kopernik S.A. w Toruniu

Spis treści:

Wykaz skrótów i oznaczeń

ABTS	kwas 2,2'-azynobis(3-etylobenzotiazolino-6-sulfonowy)
AC	aktywność przeciwutleniająca (ang. antioxidant capacity)
AGE	końcowe produkty zaawansowanej glikacji (ang. advanced glycation end-products)
BBD	plan Boxa–Behnkena (ang. Box-Behnken design)
C_40%RPC_RO	ciastka z zawartością 40% wytłoków rzepakowych, przygotowane z użyciem oleju rzepakowego (ang. cookies with 40% rapeseed press cake, prepared using rapeseed oil)
CCD	centralny plan kompozycyjny (ang. central composite design)
Co	konszowanie (ang. conching)
ChFrE	ekstrakt z owoców aronii (ang. chokeberry fruit extract)
CUPRAC	metoda oznaczania zdolności redukowania jonów Cu(II) (ang. CUPric reducing antioxidant capacity)
DCh	czekolada deserowa (ang. dark chocolate)
DCh + ChFrE	czekolada deserowa wzbogacona w cynk i ekstrakt z owoców aronii (ang. dark chocolate with zinc and chokeberry fruit extract)
DCh + EFlE	czekolada deserowa wzbogacona w cynk i ekstrakt z kwiatów czarnego bzu (ang. dark chocolate with zinc and elderflower extract)
DCh + EFrE	czekolada deserowa wzbogacona w cynk i ekstrakt z owoców czarnego bzu (ang. dark chocolate with zinc and elderfruit extract)
DPPH	2,2-difenylo-1-pikrylohydrazyl
EB	koncentrat soku z owoców czarnego bzu (ang. elderberry)
EDS	spektroskopia dyspersji energii (ang. energy dispersive spectroscopy)
EFlE / EF	ekstrakt z kwiatów czarnego bzu (ang. elderflower exctract)
EFrE	ekstrakt z owoców czarnego bzu (ang. elderberry exctract)
FC	metoda Folina-Ciocâlteu
FRAP	metoda oznaczania zdolności redukowania jonów Fe(III) (ang. ferric-reducing antioxidant power)

GC	pierniki w czekoladzie deserowej (ang. gingerbread cookies)
GCEF	pierniki w czekoladzie deserowej wzbogaconej ekstraktem z kwiatów czarnego bzu (ang. gingerbread cookies with elderflower extract)
GCEFEB	pierniki w czekoladzie deserowej wzbogaconej ekstraktem z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu (ang. gingerbread cookies with elderflower extract and elderberry extract)
HAT	metody oparte na mechanizmie przeniesienia atomu wodoru (ang. hydrogen atom transfer)
HPLC-FLD	wysokosprawna chromatografia cieczowa z detektorem fluorescencyjnym (ang. high-performance liquid chromatography with fluorescence detection)
MGTP	sproszkowane liście zielonej herbaty matcha (ang. matcha green tea powder)
MOLP	sproszkowane liście moringa (ang. moringa leaves powder)
ROS	reaktywne formy tlenu (ang. reactive oxygen species)
RPC	wytłoki rzepakowe (ang. rapeseed press cake)
RSM	metodologia powierzchni odpowiedzi (ang. response surface methodology)
SAFA	nasycone kwasy tłuszczowe (ang. saturated fatty acids)
SEM	skaningowy mikroskop elektronowy (ang. scanning electron microscope)
SET	metody oparte na mechanizmie przeniesienia pojedynczego elektronu (ang. single electron transfer)
Te	temperowanie (ang. tempering)
TPC	całkowita zawartość związków fenolowych (ang. total phenolic content)
WCh	czekolada biała (ang. white chocolate)
WCh_4%MGTP_Te	czekolada biała z 4% dodatkiem sproszkowanej zielonej herbaty matcha na etapie temperowania (ang. white chocolate with 4% matcha green tea powder added during tempering process)
WCh_4%MOLP_Te	czekolada biała z 4% dodatkiem sproszkowanych liści moringa (ang. white chocolate with 4% moringa leaves powder added during tempering process)

1. Życiorys naukowy

Urodziłem się 20 października 1986 roku w Inowrocławiu. W 2010 roku ukończyłem II Liceum Ogólnokształcące im. Marii Konopnickiej w Inowrocławiu. We wrześniu tego samego roku rozpocząłem jednolite studia magisterskie na Uniwersytecie Ekonomicznym w Poznaniu, na kierunku: Towaroznawstwo. W czerwcu 2015 roku uzyskałem tytuł magistra inżyniera na podstawie obronionej pracy magisterskiej pt.: „*Analiza porównawcza opakowań olejów jadalnych na przykładzie Zakładów Tłuszczowych „Kruszwica" S.A.*". Bezpośrednio po ukończeniu studiów i zdobyciu tytułu magistra podjąłem pracę w sektorze spożywczym, najpierw w Zakładach Tłuszczowych „Kruszwica" S.A., a w 2018 roku w Fabryce Cukierniczej Kopernik S.A. W tym samym roku rozpocząłem studia doktoranckie w ramach programu „doktorat wdrożeniowy" pod opieką prof. dr hab. Aleksandry Szydłowskiej-Czerniak, w Katedrze Chemii Analitycznej i Spektroskopii Stosowanej, Wydziału Chemii, Uniwersytetu Mikołaja Kopernika w Toruniu. Opiekunem pomocniczym z przedsiębiorstwa byli kolejno Prezesi Zarządu Fabryki Cukierniczej Kopernik S.A., mgr Przemysław Myśliwy oraz mgr Karolina Ziółkowska-Wachowiak.

Pod kierunkiem Pani Profesor, prowadziłem badania mające na celu zaprojektowanie i analizę nowych wyrobów cukierniczych o podwyższonym potencjale antyoksydacyjnym. Przedmiotem moich badań były ciastka z dodatkiem wytłoków z nasion rzepaku, czekolady białe z dodatkiem sproszkowanych liści zielonej herbaty matcha (*Camellia sinensis* L.) i sproszkowanych liści moringa (*Moringa oleifera*), czekolady deserowe z dodatkiem ekstraktów z kwiatów i owoców czarnego bzu (*Sambucus nigra* L.), owoców aronii (*Aronia melanocarpa*) oraz pierniki w czekoladzie z dodatkiem ekstraktu z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu. Ponadto wykorzystując metody chemometryczne optymalizowałem parametry procesu mielenia przypraw korzennych (gałki muszkatołowej i imbiru) dodawanych do ciasta piernikowego.

W ramach studiów doktoranckich współpracowałem również z Uniwersytetem Warmińsko – Mazurskim w Olsztynie oraz Uniwersytetem Nauk Przyrodniczych w Poznaniu. Otrzymałem dwa Granty Dziekana Wydziału Chemii UMK, przeznaczone na badania czekolady gorzkiej z dodatkiem steroli roślinnych oraz składników o właściwościach antyoksydacyjnych, a także na optymalizację procesu ekstrakcji związków bioaktywnych z wykorzystaniem rozpuszczalników głęboko eutektycznych.

Na mój dorobek naukowy składa się **5 publikacji**, których jestem współautorem (w **3 pierwszym autorem**), **2 artykuły w monografii pokonferencyjnej** oraz **5 prezentacji** na konferencjach krajowych i międzynarodowych.

W ramach pracy w Fabryce Cukierniczej Kopernik S.A. dokonałem **3 zgłoszeń** w **Urzędzie Patentowym RP**, z czego na **1 wynalazek** został przyznany patent.

Wykształcenie:

10.2018 – obecnie Uniwersytet Mikołaja Kopernika w Toruniu, Wydział Chemii, studia doktoranckie.

09.2005 – 06.2010 Uniwersytet Ekonomiczny w Poznaniu, Wydział Towaroznawstwa, kierunek: Towaroznawstwo, specjalizacja: Menedżer Produktu, tryb dzienny, studia jednolite magisterskie. Temat pracy dyplomowej: *Analiza porównawcza opakowań olejów jadalnych na przykładzie Zakładów Tłuszczowych „Kruszwica" S.A.*

Publikacje wchodzące w skład rozprawy doktorskiej:

(D1) Szydłowska-Czerniak, A., **Poliński, S.**, & Momot, M. (2021). Optimization of Ingredients for Biscuits Enriched with Rapeseed Press Cake—Changes in Their Antioxidant and Sensory Properties. *Applied Sciences, 11*(4), 1558. **IF = 2.838; pkt. MEiN = 100, Q2/Q3**.

(D2) Poliński, S., Kowalska, S., Topka, P., & Szydłowska-Czerniak, A. (2021). Physicochemical, Antioxidant, Microstructural Properties and Bioaccessibility of Dark Chocolate with Plant Extracts. *Molecules*, *26*(18), 5523. **IF = 4.927; pkt. MEiN = 140, Q2**.

(D3) Poliński, S., Topka, P., Tańska, M., Kowalska, S., Czaplicki, S., & Szydłowska-Czerniak, A. (2022). Impact of Bioactive Compounds of Plant Leaf Powders in White Chocolate Production: Changes in Antioxidant Properties During the Technological Processes. *Antioxidants, 11*(4), 752. **IF = 7.675; pkt. MEiN = 100, Q1**.

(D4) Poliński, S., Topka, P., Tańska, M., Kowalska, S., Czaplicki, S., & Szydłowska-Czerniak, A. (2022). Effect of Grinding Process Parameters and Storage Time on Extraction of Antioxidants from Ginger and Nutmeg. *Molecules, 27*(21), 7395. **IF = 4.927; pkt. MEiN = 140, Q2**.

(D5) Topka, P., **Poliński, S.**, Sawicki, T., Szydłowska-Czerniak, A., & Tańska, M. (2023). Effect of Enriching Gingerbread Cookies with Elder (*Sambucus nigra* L.) Products on Their Phenolic Composition, Antioxidant and Anti-Glycation Properties, and Sensory Acceptance. *International Journal of Molecular Sciences*, *24*(2), 1493. **IF = 6.208; pkt. MEiN = 140, Q1/Q2**.

Monografie:

1. **Poliński, S.**, Topka, P., Kowalska, S., Szydłowska-Czerniak, A. Wpływ sposobu przygotowania prób czekolad na ich właściwości antyutleniające. Na pograniczu chemii, biologii i fizyki – rozwój nauk. Tom 2. Wydawnictwo Naukowe Uniwersytetu Mikołaja Kopernika. Toruń 2021. **pkt. MEiN = 20**.

2. **Poliński, S.**, Momot, M., Strzała, M., Topka, P., Szydłowska-Czerniak, A. Analiza konsumencka wybranych czekolad. Na pograniczu chemii, biologii i fizyki – rozwój nauk. Tom 2. Wydawnictwo Naukowe Uniwersytetu Mikołaja Kopernika. Toruń 2021. **pkt. MEiN = 20**.

Publikacje:

Liczba artykułów w recenzowanych czasopismach naukowych indeksowanych w bazie Journal Citation Reports (JCR) – **5**; **ΣIF = 26.575**; **Σpkt. MEiN = 620**
Liczba artykułów monograficznych – **2**; **Σpkt. MEiN = 40**

Udział w konferencjach naukowych (postery):

1. **Poliński S.**; Momot M., Szydłowska-Czerniak A. Krem do wafli na bazie masła shea. XXVII Międzynarodowa Konferencja Naukowa. Postępy w Technologii Tłuszczów Roślinnych, 27th International Scientific Conference, Progress in Technology of Vegetable Fats, Kazimierz Dolny, 22-24 maja 2019, s. 52.

2. **Poliński S.**, Topka P., Kowalska S., Szydłowska-Czerniak A. Wpływ sposobu przygotowania prób czekolad na ich właściwości antyutleniające. Kopernikańskie E-Seminarium Doktoranckie, Sekcja Nauk Chemicznych- Chemia 4, Toruń, 7 września 2020. s. 28.

3. **Poliński S.**, Kowalska S., Topka P., Szydłowska-Czerniak A. Physicochemical, antioxidant, microstructural properties of dark chocolate with plant extracts. Virtual International Conference „Plant productivity and food safety: Soil science, Microbiology, Agricultural Genetics and Food quality", 15-17 września 2021, s. 100.

4. Kowalska S., **Poliński S.**, Topka P., Szydłowska-Czerniak A. Właściwości przeciwutleniające białej czekolady z ekstraktami roślinnymi. XV Kopernikańskie Seminarium Doktoranckie, Toruń, 20-22 czerwca 2022, s. 89.

5. **Poliński S.**, Topka P., Tańska M, Kowalska S., Czaplicki S., Szydłowska-Czerniak A. Impact of bioactive compounds of plant leaf powders in white chocolate production: Changes in antioxidant properties during the technological processes. Second Edition of Virtual International Conference „Plant productivity and food safety: Soil science, Microbiology, Agricultural Genetics and Food quality", 15-16 września 2022, s. 88.

Sumaryczna ilość prezentacji: **5** (**3** na konferencjach międzynarodowych i **2** na konferencjach krajowych)**.**

Granty Dziekana Wydziału Chemii Uniwersytetu Mikołaja Kopernika:

1. Grant Dziekana Wydziału Chemii, Uniwersytet Mikołaja Kopernika w Toruniu, nr 2020/2301. Czekolada z dodatkami funkcyjnymi (z dodatkiem steroli roślinnych, z dodatkiem składników o właściwościach antyoksydacyjnych) – kierownik.

2. Grant Dziekana Wydziału Chemii, Uniwersytet Mikołaja Kopernika w Toruniu. Zastosowanie metod chemometrycznych i zielonych rozpuszczalników do ekstrakcji związków bioaktywnych z dodatków do wyrobów cukierniczych – kierownik.

Projekty badawcze realizowane w ramach pracy w Fabryce Cukierniczej Kopernik S.A.:

1. Manufaktura czekolady. Opracowanie produkcji czekolad ręcznie przygotowywanych z wykorzystaniem różnego rodzaju surowców. Fundusz Badań i Wdrożeń. Oś priorytetowa 1. Wzmocnienie innowacyjności i konkurencyjności gospodarki regionu. Działanie 1.2 Promowanie inwestycji przedsiębiorstw w badania i innowacje. Poddziałanie 1.2.1 wsparcie procesów badawczo-rozwojowych. Regionalny program operacyjny województwa kujawsko-pomorskiego na lata 2014-2020.

2. Opracowanie receptury oraz technologii produkcji pierników z dodatkiem alkoholu na etapie leżakowania. Fundusz Badań i Wdrożeń. Oś priorytetowa 1. Wzmocnienie innowacyjności i konkurencyjności gospodarki regionu. Działanie 1.2 Promowanie inwestycji przedsiębiorstw w badania i innowacje. Poddziałanie 1.2.1 wsparcie procesów badawczo-rozwojowych. Regionalny program operacyjny województwa kujawsko-pomorskiego na lata 2014-2020.

3. Opracowanie receptury oraz technologii produkcji kremu do wafli oraz pierników wzbogaconych o witaminy D i K. Fundusz Badań i Wdrożeń. Oś priorytetowa 1. Wzmocnienie innowacyjności i konkurencyjności gospodarki regionu. Działanie 1.2 Promowanie inwestycji przedsiębiorstw w badania i innowacje. Poddziałanie 1.2.1 wsparcie procesów badawczo-rozwojowych. Regionalny program operacyjny województwa kujawsko-pomorskiego na lata 2014-2020.

4. Wdrożenie innowacyjnej metody produkcji wyrobów nadziewanych. Bank Gospodarstwa Krajowego. Poddziałanie 3.2.2 Kredyt na innowacje technologiczne Programu Operacyjnego Inteligentny Rozwój współfinansowanego ze środków Europejskiego Funduszu Rozwoju Regionalnego.

Przyznany przez Urząd Patentowy Rzeczypospolitej Polskiej Patent:

1. Sposób wytwarzania pierników (P.432951).

Zgłoszenia w Urzędzie Patentowym Rzeczypospolitej Polskiej:

1. Sposób wytwarzania wyrobów czekoladowych.
2. Sposób wytwarzania pierników z nadzieniem.

2. Rozprawa doktorska: Innowacyjne wyroby cukiernicze jako element zróżnicowanej i zbilansowanej diety przyczyniający się do walki z chorobami cywilizacyjnymi.

2.1. Wstęp

Podobnie jak inne kraje regionu, Polska zmaga się z rosnącą śmiertelnością z powodu tzw. chorób cywilizacyjnych, należą do nich choroby układu krążenia oraz nowotwory złośliwe, które stanowią najczęstszą przyczynę zgonów w kraju. Łącznie powodują 65% wszystkich zgonów. Śmiertelność z powodu chorób układu krążenia od lat utrzymuje się na stałym, wysokim poziomie. Rocznie z ich powodu umiera ponad 440 osób na 100 tys. ludności. Zwiększa się natomiast umieralność z powodu nowotworów złośliwych. W ostatnich latach są one przyczyną śmierci ponad 260 osób na 100 tys. ludności rocznie, podczas gdy na początku dekady było to odpowiednio 240 przypadków (GUS 2020).

Jednym z istotnych czynników zwiększających ryzyko zachorowania na choroby układu krążenia oraz nowotwory jest stres oksydacyjny (Allen i Bayrakturan, 2009; Di Filippo i wsp., 2006; Griendling i wsp., 2021; Hayes i wsp., 2020). Stres oksydacyjny w uproszczeniu można zdefiniować jako brak równowagi między produkcją reaktywnych form tlenu (ROS) a niską aktywnością mechanizmów antyoksydacyjnych w organizmie.

Kluczowym czynnikiem mającym wpływ na stres oksydacyjny jest dieta. Od naszych nawyków żywieniowych w dużej mierze zależy ryzyko powstania stresu oksydacyjnego. Badania wykazały, że dieta śródziemnomorska bogata w dużą ilość składników o właściwościach antyoksydacyjnych ma znaczący wpływ na redukcję stresu oksydacyjnego (Dai i wsp., 2008). Niezwykle istotne ze zdrowotnego punktu widzenia jest to, żeby produkty o właściwościach antyoksydacyjnych były spożywane regularnie. Ze względu na dostępność określonych produktów oraz zwyczaje żywieniowe nie każda dieta jest wystarczająco bogata w składniki o właściwościach antyoksydacyjnych. Niedobór tych składników w diecie może być potencjalnie zrekompensowany poprzez wprowadzenie do niej produktów o potwierdzonym wysokim potencjale przeciwutleniającym.

Jednym z nieodłącznych elementów codziennej diety większości mieszkańców Polski są różnego rodzaju wyroby cukiernicze. Z uwagi na fakt, że spożycie wyrobów cukierniczych ma w większości przypadków podłoże hedonistyczne, niezwykle istotne jest, aby wprowadzane

do diety wyroby cukiernicze cechowały się walorami sensorycznymi na co najmniej takim samym poziomie jak dotychczas spożywane produkty. Obecne na polskim rynku produkty cukiernicze o właściwościach prozdrowotnych najczęściej odbiegają od standardowych produktów pod kątem sensorycznym, a ich konsumpcja jest ściśle związana z właściwościami prozdrowotnymi które posiadają.

Warunkiem realnego wpływu wyrobów cukierniczych na redukcję stresu oksydacyjnego, a tym samym na ograniczenie ryzyka zachorowania na większość chorób cywilizacyjnych, w tym choroby układu krążenia i choroby nowotworowe jest systematyczne ich spożywanie. W związku z tym nie mogą być to wyroby kupowane okazjonalnie. Sytuacja, w której konsument kupowałby dany wyrób cukierniczy głównie ze względu na jego walory sensoryczne, natomiast jego właściwości prozdrowotne stanowiłyby drugorzędny motyw zakupowy, byłaby optymalna z punktu widzenia przedstawionej w niniejszej pracy idei. Analogiczne rozwiązanie zastosowano wprowadzając w Polsce nakaz wzbogacania tłuszczów do smarowania w witaminy A i D, w związku z ich niedoborem w diecie. Konsument kupując tłuszcz do smarowania kieruje się chęcią zaspokojenia określonych potrzeb, które nie są najczęściej w żaden sposób związane z obecnością witamin w produkcie. Jednakże przy okazji, najczęściej zupełnie nieświadomie, uzupełnia niedobory witamin A i D.

Opracowanie oraz wdrożenie do sprzedaży pierników wzbogaconych o naturalne składniki charakteryzujące się właściwościami antyoksydacyjnymi jest samo w sobie nowością, gdyż do tej pory nie odnotowano wdrożenia tego typu.

Badania właściwości antyoksydacyjnych opracowanych wyrobów cukierniczych zostały przedstawione w czterech pracach opublikowanych w czasopismach indeksowanych w bazie JCR (ang. Journal Citation Report). Natomiast w kolejnym artykule również opublikowanym w czasopiśmie indeksowanym w JCR, opisana została optymalizacja procesu mielenia dwóch z pięciu przypraw dodawanych do ciasta piernikowego będącego przedmiotem rozprawy doktorskiej.

2.2. Cele i założenia rozprawy doktorskiej

Nadrzędnym celem moich badań było zaprojektowanie, analiza i wdrożenie wyrobów cukierniczych:

- z dodatkiem składników o właściwościach antyoksydacyjnych,

- o właściwościach organoleptycznych takich samych lub lepszych w stosunku do wyrobów dostępnych na rynku, co jest kluczowe dla konsumentów,
- stanowiących element zróżnicowanej i zbilansowanej diety przyczyniający się potencjalnie do walki z chorobami cywilizacyjnymi,
- możliwych do wdrożenia w regularnej produkcji w skali przemysłowej.

Zadania badawcze zawarte w cyklu publikacji stanowiących osiągnięcie naukowe dotyczyły:

- Zastosowania narzędzi chemometrycznych, planu centralnego-kompozycyjnego (CCD) z dwoma zmiennymi niezależnymi i planu Boxa–Behnkena (BBD) z trzema zmiennymi niezależnymi oraz metodologii powierzchni odpowiedzi (RSM) do:
    - optymalizacji składu ciastek wzbogacanych w wytłoki rzepakowe (RPC) i tłuszcze różniące się zawartością nasyconych kwasów tłuszczowych (SAFA),
    - optymalizacji procesu mielenia i przechowywania imbiru i gałki muszkatołowej – głównych przypraw dodawanych do ciasta piernikowego;
- Oznaczania całkowitego potencjału antyoksydacyjnego za pomocą zmodyfikowanych metod analitycznych opartych na reakcjach pomiędzy przeciwutleniaczami a modelowymi rodnikami: DPPH (2,2-difenylo-1-pikrylohydrazyl), ABTS (kwas 2,2'-azynobis(3-etylobenzotiazolino-6-sulfonowy)) lub jonami metali: CUPRAC (ang. CUPric reducing antioxidant capacity) i FRAP (ang. ferric-reducing antioxidant power) oraz zawartości związków fenolowych metodą Folina-Ciocâlteu (FC) w przyprawach i zaprojektowanych nowych wyrobach cukierniczych:
    - ciastkach wzbogacanych w wytłoki rzepakowe i różnego rodzaju tłuszcze (olej rzepakowy, margaryna, tłuszcz kokosowy),
    - czekoladach deserowych z dodatkiem ekstraktów: z owoców czarnego bzu, kwiatów czarnego bzu i owoców aronii,
    - czekoladach białych wzbogaconych sproszkowanymi liśćmi zielonej herbaty matcha i sproszkowanymi liśćmi moringa,
    - pierników w czekoladzie deserowej z ekstraktem z kwiatów czarnego bzu i nadzieniem zawierającym koncentrat soku z owoców czarnego bzu;
- Analizy poszczególnych związków o właściwościach antyoksydacyjnych (kwasów fenolowych, flawonoidów, tokoferoli, karotenoidów) w:
    - ekstraktach przypraw zmielonych w optymalnych warunkach,

- czekoladach białych wzbogaconych sproszkowanymi liśćmi zielonej herbaty matcha i sproszkowanymi liśćmi moringa,
- piernikach w czekoladzie deserowej wzbogaconej ekstraktem z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu;

- Oznaczania biodostępności czekolad deserowych fortyfikowanych ekstraktami roślinnymi z wykorzystaniem trójfazowego procesu trawienia *in vitro*;
- Oznaczania zwartości końcowych produktów zaawansowanej glikacji (AGE) suplementowanych pierników;
- Zastosowania skaningowej mikroskopii elektronowej (SEM) z spektrometrem EDS do badania struktury i składu pierwiastkowego czekolad deserowych z dodatkiem ekstraktów roślinnych;
- Analizy sensorycznej proponowanych funkcjonalnych wyrobów cukierniczych:
  - ciastek wzbogacanych wytłokami rzepakowymi,
  - białych czekolad fortyfikowanych sproszkowanymi liśćmi zielonej herbaty matcha i sproszkowanymi liśćmi moringa,
  - pierników w czekoladzie, pierników w czekoladzie z dodatkiem ekstraktu z kwiatów czarnego bzu oraz pierników w czekoladzie z dodatkiem ekstraktu z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z czarnego bzu.

2.3. Problemy badawcze

2.3.1. Wyroby cukiernicze o właściwościach antyoksydacyjnych

Współczesne czasy charakteryzują się intensywnym rozwojem nauki i medycyny, jednocześnie w istotny sposób rośnie świadomość społeczna dotycząca wpływu zachowań żywieniowych na zdrowie człowieka. Na popularności zyskują produkty o właściwościach prozdrowotnych, w tym także wyroby cukiernicze. Wyroby funkcjonalne stały się w ostatnim czasie również przedmiotem dużego zainteresowania naukowców. Na szczególną uwagę zasługują prace dotyczące właściwości antyoksydacyjnych wyrobów cukierniczych.

Jako że czekolada jest popularnym produktem cukierniczym i jednocześnie stanowi doskonałą matrycę dla różnego rodzaju dodatków, znaczną grupę publikacji stanowią te dotyczące właściwości antyoksydacyjnych czekolad suplementowanych. Wśród dodatków

o właściwościach antyoksydacyjnych zastosowanych do wzbogacania czekolady można wymienić ekstrakt z cynamonu (Muhammad i wsp., 2021), suszone śliwki, rodzynki, morele, żurawinę, papaję (Komes i wsp., 2013), sproszkowane owoce czarnego bzu, aronii, jagód, malin, jeżyn, granatu i sproszkowane kwiaty czarnego bzu (**D2**; Żyżelewicz i wsp., 2021), sproszkowane liście herbaty matcha (*Camellia sinensis* L.), moringa (*Moringa oleifera*) i sakura (**D3**; Martini i wsp., 2018). Jednocześnie na uwagę zasługuje fakt, że czekolady zawierające w swoim składzie proszek kakaowy, a więc zasadniczo wszystkie czekolady z wyjątkiem czekolady białej, same w sobie charakteryzują się znaczącym potencjałem antyoksydacyjnym (Todorovic i wsp., 2015, Vinson i wsp., 2006). Jest to w dużej mierze związane z obecnymi w proszku kakaowym licznymi związkami fenolowymi takimi jak: epikatechiny, katechiny oraz kwercetyna i jej pochodne (Gil i wsp., 2021; Mikołajczak i Tańska, 2021; Todorovic i wsp., 2017). Z kolei zawartość związków fenolowych w proszku kakaowym uzależniona jest zarówno od obszaru geograficznego, z którego pochodzą ziarna kakaowca (Febrianto i Zhu, 2022) jak i od procesów jakimi są one poddawane (Gültekin-Özgüven i wsp., 2016; Ioannone i wsp., 2015).

Istnieją liczne publikacje naukowe opisujące prozdrowotne właściwości produktów powstałych w wyniku przetwarzania ziaren kakaowca. Do najistotniejszych z punktu widzenia medycyny należą właściwości przeciwnowotworowe (Giannandrea, 2009; Martin i wsp., 2013) oraz właściwości związane z przeciwdziałaniem chorobom układu krążenia (Cinar i wsp., 2021; Kerimi i Williamson, 2015; Kwok i wsp., 2016). Obie te grupy chorób zaliczane są do chorób cywilizacyjnych, w związku z czym można uznać, iż pochodne ziaren kakaowca mogą być istotnym elementem niniejszej rozprawy doktorskiej.

Z punktu widzenia potencjału prozdrowotnego równie ważną kategorią słodyczy są ciastka. Podobnie jak czekolada, stanowią one bardzo dobry nośnik prozdrowotnych dodatków o właściwościach antyoksydacyjnych. Wśród wielu składników dodawanych do ciastek, charakteryzujących się znacznym potencjałem antyoksydacyjnym warto wymienić: sproszkowane pestki oliwek (Bolek, 2020), skórki z winogron (Abreu i wsp., 2019), wytłoki z borówek (Tagliani i wsp., 2019), produkty uboczne powstałe w wyniku przetwarzania jabłek, ananasów i melonów (Toledo i wsp., 2019), sproszkowaną skórkę mango (Ajila i wsp., 2008), okarę (Lee i wsp., 2020), wytłoki rzepakowe (**D1**), kawę rozpuszczalną (Passos i wsp., 2017) i pyłek pszczeli (Krystyjan i wsp., 2015).

Poza zastosowaniem składników dodatkowych o właściwościach antyoksydacyjnych innym skutecznym sposobem na zwiększenie całkowitej aktywności przeciwutleniającej ciastek jest zastąpienie podstawowego składnika jakim jest mąka pszenna, mąką o wyższym potencjale antyoksydacyjnym. Do takich mąk można zaliczyć: odtłuszczoną mąkę sezamową (Mas i wsp., 2022), odtłuszczoną mąkę z chia (Mas i wsp., 2020), mąkę z żołędzi (Parsaei i wsp., 2018), mąkę z siemienia lnianego (Kaur i wsp., 2017) oraz mąkę gryczaną (Jan i wsp., 2015). Jednakże istotną kwestią jest to, iż w przeciwieństwie do wyrobów czekoladowych ciastka poddawane są procesowi wypieku, który wpływa negatywnie na ich potencjał antyoksydacyjny (Nanditha i Prabhasankar, 2008). Aby uniknąć utraty właściwości antyoksydacyjnych przez produkt na skutek działania wysokiej temperatury podczas wypieku, skutecznym rozwiązaniem jest dodanie składników o właściwościach antyoksydacyjnych po wypieku. Jednak z przyczyn technologicznych nie zawsze jest to możliwe. Innym sposobem na ograniczenie negatywnego wpływu procesu wypieku na moc przeciwutleniającą gotowego produktu jest zastosowanie mikroenkapsulacji (Papillo i wsp., 2019; Pasrija i wsp., 2015). Najczęściej stosowaną w przemyśle technologią mikroenkapsulacji ze względu na koszt, użyteczność i skalowalność jest suszenie rozpyłowe (Gharsallaoui i wsp., 2007). Suszenie rozpyłowe pozwala na uzyskanie mikrocząstek, w których składnik aktywny jest chroniony ciągłą i zwartą warstwą środka stabilizującego. (Porras-Saavedra i wsp., 2018). Stworzona w ten sposób bariera chroni składniki aktywne przed wysoką temperaturą panującą podczas wypieku.

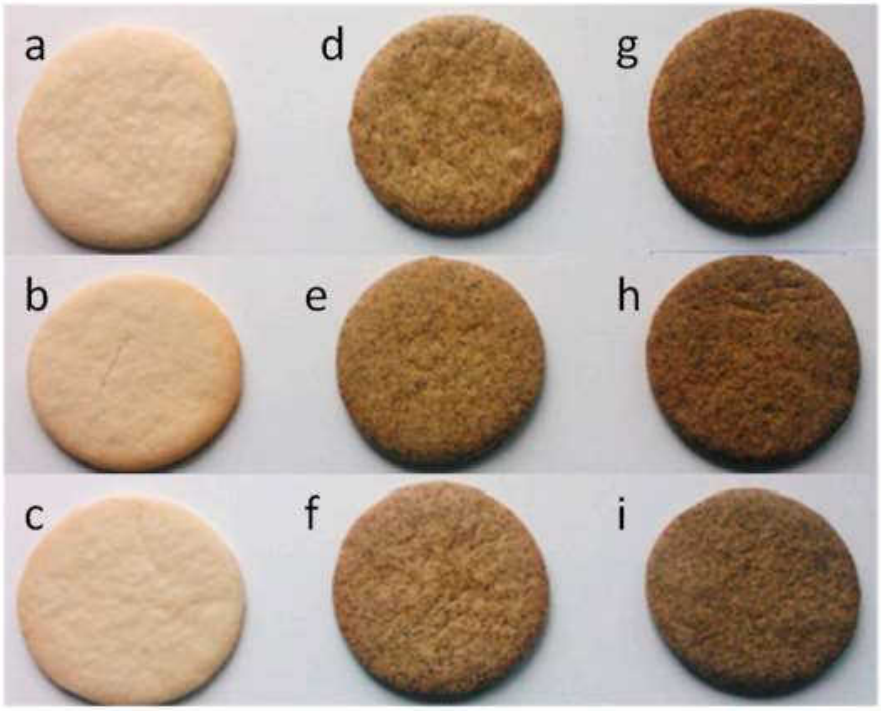
Oprócz wyrobów czekoladowych i ciastek również inne kategorie słodyczy znajdują się w obszarze zainteresowania naukowców zajmujących się badaniami nad właściwościami antyoksydacyjnych. Wśród nich warto wymienić: żelki do produkcji których zastosowano emulsję sacharozoodporną (Pan i wsp., 2022), żelki z dodatkiem zielonego propolisu (Cedeño-Pinos, 2021), żelki z dodatkiem ekstraktu z herbaty (*Camellia sinensis*) (Gramza-Michałowska i Reguła, 2007), cukierki z barwnikami antocyjanowymi z czerwonej kapusty (Rizk i wsp., 2009), cukierki z dodatkiem suszonych owoców figowca pagodowego (*Ficus religiosa*) (Verma i Gupta, 2015), lody z ekstraktami ziołowymi i fruktooligosacharydami (Gremski i wsp., 2019), lody z dodatkiem osadu z wina winogronowego (Hwang i wsp., 2009), lody z dodatkiem winogron, granatów, proszku z nasion sezamu i ich olejów (Akca i Akpinar, 2021), lody z dodatkiem ziół medycznych (Ali i wsp., 2014) oraz lody wzbogacone sokiem z trzciny cukrowej (*Saccharum officinarum* L.) (Ullah i wsp., 2015).

2.3.2. Ciastka wzbogacone w wytłoki rzepakowe

Zbiory rzepaku (*Brassica napus* L.) stanowią ponad 97% zbiorów roślin oleistych w Polsce i wciąż ulegają zwiększeniu (Kapusta, 2022). Natomiast na świecie rzepak jest trzecią rośliną oleistą pod względem wielkości uprawy (po olejowcu gwinejskim i soi). Głównym produktem ubocznym w procesie pozyskiwania oleju z nasion rzepaku są wytłoki rzepakowe mające szerokie zastosowanie w żywieniu zwierząt (Troise i wsp., 2018). Ze względu na całoroczną dostępność, stosunkowo niską cenę oraz walory wytłoków rzepakowych wynikające z dużej zawartość białka, błonnika i związków fenolowych (Fetzer i wsp., 2018) są one obiecującym dodatkiem do żywności, w tym do wyrobów cukierniczych.

W podrozdziale *2.3.1.* podane zostały przykłady publikacji naukowych dotyczących wzbogacania ciastek w dodatki o właściwościach antyoksydacyjnych. Nie wiele jest jednak danych dotyczących optymalizacji receptur wyrobów cukierniczych suplementowanych produktami ubocznymi pochodzącymi z przemysłu rolno-spożywczego. Dopiero, w publikacji **D1** podjęto próbę określenia wpływu dodatku wytłoków rzepakowych do ciastek na ich właściwości antyoksydacyjne.

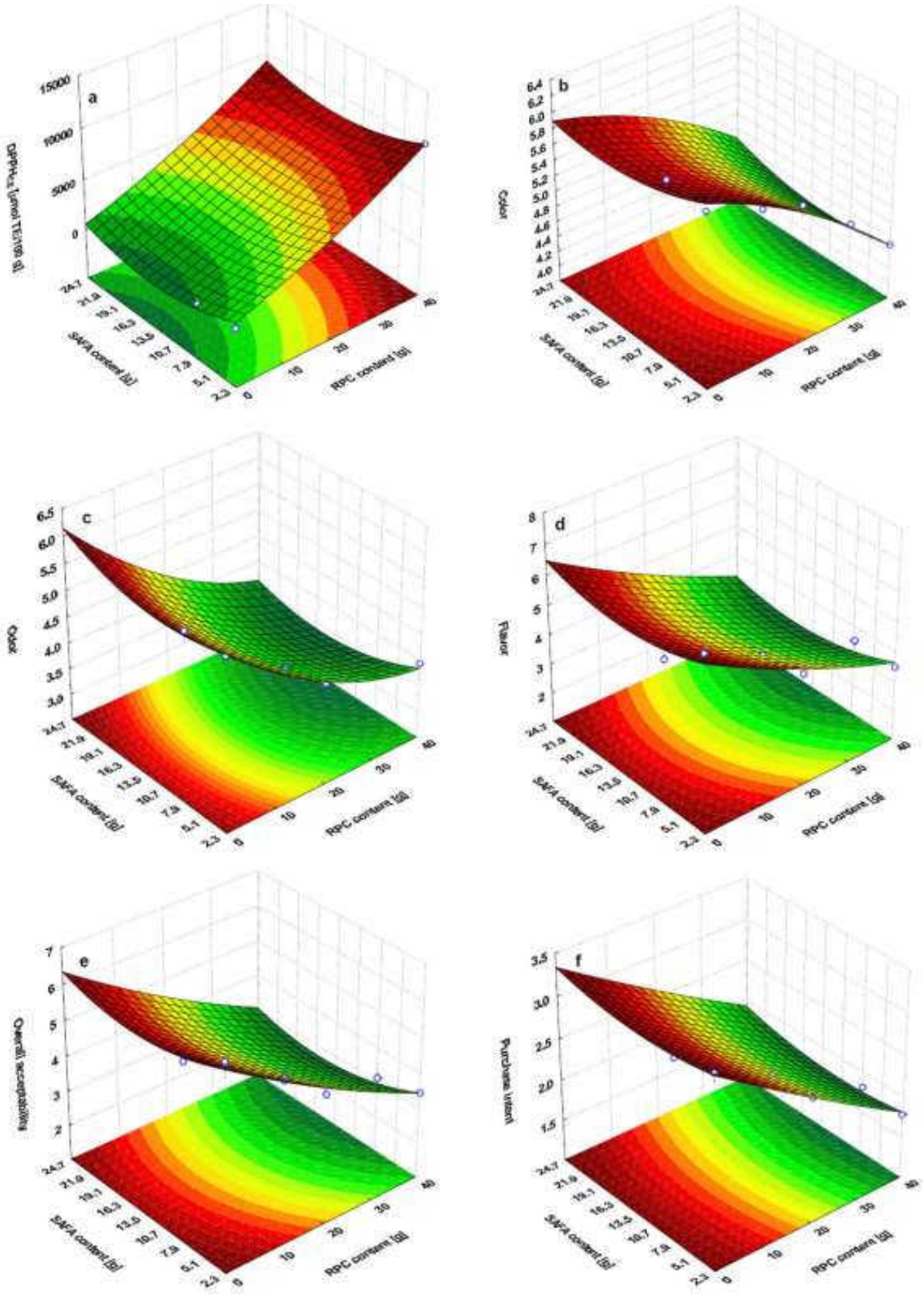
Jednym z zadań badawczych rozprawy doktorskiej była optymalizacja produkcji ciastek przygotowanych na bazie mąki pszennej z dodatkiem RPC, bogatych w związki fenolowe i stanowiących główny produkt uboczny przy produkcji oleju rzepakowego oraz różnych rodzajów tłuszczów roślinnych: oleju rzepakowego, margaryny i oleju kokosowego różniących się zawartością SAFA na 100 g tłuszczu, które wynosiły kolejno: 2,3 g, 7,8 g i 24,9 g (**D1**). Dla każdej receptury zastosowano trzy różne dawki wytłoków rzepakowych (kolejno 0%, 20% i 40%) otrzymując w ten sposób 9 wariantów ciastek, przedstawionych na rysunku 1. Optymalizację składu fortyfikowanych ciastek o zwiększonym potencjale przeciwutleniającym przeprowadzono za pomocą CCD z dwoma zmiennymi (zawartością wytłoków rzepakowych i zawartością SAFA w tłuszczach) powtarzając eksperyment dla punktu centralnego w połączeniu z RSM .



*Rysunek 1. Zdjęcia ciastek przygotowanych z ciasta bez dodatku wytłoków rzepakowych (a-c), z dodatkiem 20% (d-f) i 40% wytłoków rzepakowych (g-i) z zastosowaniem kolejno od góry: oleju rzepakowego, margaryny i tłuszczu kokosowego [**D1**].*

Przygotowane ciastka analizowano pod kątem właściwości antyoksydacyjnych z wykorzystaniem metody DPPH. Największą zdolność zmiatania rodnika DPPH posiadały ciastka zawierające w swoim składzie olej rzepakowy i dodatek 40% RPC, o czym świadczyła najwyższa wartość DPPH równa 10088 µmol TE/100 g. Dla porównania uzyskany wynik DPPH dla ciastek wypieczonych z użyciem oleju rzepakowego niewzbogaconych RPC wyniósł 843 µmol TE/100 g. Otrzymane wyniki potwierdzają znaczący wpływ dodatku RPC na aktywność antyoksydacyjną ciastek.

Natomiast z analizy wyników RSM (rysunek 2a) można wnioskować, iż zmienna niezależna w postaci zawartości SAFA w zastosowanym tłuszczu nie ma istotnego wpływu na zdolność zmiatania wolnych rodników mierzoną metodą DPPH.



*Rysunek 2. Wykresy powierzchni dopasowanych odpowiedzi (RSM) i konturowe dla wyników: (a) DPPH ekstraktów uzyskanych metodą ekstrakcji konwencjonalnej ($DPPH_{CE}$), (b) koloru, (c) zapachu, (d) smaku, (e) ogólnej akceptowalności i (f) zamiaru zakupu ciastek funkcjonalnych wyrażone jako funkcja zawartości wytłoków rzepakowych (RPC) i zawartości nasyconych kwasów tłuszczowych (SAFA) [**D1**].*

Niezależnie od badań właściwości antyoksydacyjnych ciastek bez dodatku i z dodatkiem RPC, przeprowadzona została analiza sensoryczna proponowanych nowych produktów. Wyniki analizy sensorycznej wykazały jednoznacznie, że wraz ze wzrostem zawartości RPC w ciastkach obniżała się w sposób zasadniczy ocena koloru, zapachu, smaku oraz ogólna akceptowalność i chęć zakupu produktu (rysunek 2 b-f). Ponadto dla ciastek bez dodatku RPC uzyskano w pięciopunktowej skali intencji zakupu wyniki w przedziale od 3,19 do 3,33, dla ciastek z dodatkiem 40% RPC wynik intencji zakupu był zdecydowanie niższy i mieścił się w zakresie od 1,78 do 1,90.

Z punktu widzenia właściwości antyoksydacyjnych dodatek do ciastek RPC jest dobrym rozwiązaniem. Wdrożenie tego typu ciastek do przemysłowej produkcji byłoby możliwe zarówno pod kątem technologicznym jak i ze względu na dostępność surowca. Jednak biorąc pod uwagę negatywny wpływ dodatku RPC na cechy sensoryczne produktu, w tym wyczuwalny smak gorzki i trawiasty, wprowadzenie produktu na rynek wymaga dalszych prac nad jego udoskonaleniem.

2.3.3. Biała czekolada wzbogacona w sproszkowane liście zielonej herbaty matcha i sproszkowane liście moringa

Czekolada jest jednym z najpopularniejszych wyrobów cukierniczych na świecie (Voora i wsp., 2019). Czekolada może stanowić produkt gotowy, np. uformowana w postaci tabliczki lub może służyć jako składnik innego wyrobu cukierniczego, np. będąc polewą do ciastek. Najczęściej spotykanymi czekoladami dostępnymi w sprzedaży detalicznej są: czekolada biała, czekolada mleczna, czekolada gorzka i czekolada deserowa. Wśród wszystkich dostępnych rodzajów czekolad, największe właściwości prozdrowotne ma czekolada gorzka ze względu na znaczną ilość polifenoli, głównie epikatechin i katechin (Cooper i wsp., 2007).

Biała czekolada zgodnie Dyrektywą 2000/36/WE Parlamentu Europejskiego i Rady z dnia 23 czerwca 2000 r. odnoszącą się do wyrobów kakaowych i czekoladowych przeznaczonych do spożycia przez ludzi to *wyrób otrzymywany z masła kakaowego, mleka lub z wyrobów mlecznych i cukru, zawierający nie mniej niż 20% masła kakaowego i nie mniej niż 14% suchej masy mlecznej otrzymanej w drodze częściowego lub całkowitego odwodnienia mleka pełnego, półtłustego lub odtłuszczonego, śmietanki, śmietanki całkowicie lub częściowo odwodnionej, masła lub tłuszczu mlecznego, w którym zawartość tłuszczu jest nie mniejsza niż 3,5%*. Ze względu na brak w swoim składzie beztłuszczowej masy kakaowej bogatej

w polifenole, biała czekolada uznawana jest za mniej zdrową niż pozostałe rodzaje czekolad (Lončarević i wsp., 2019). Pomimo, iż czekolada biała nie stanowi sama w sobie produktu funkcjonalnego może nadal być doskonałym nośnikiem dodatków o właściwościach prozdrowotnych.

Proces produkcji czekolady obejmuje takie etapy jak fermentacja, suszenie, prażenie, mielenie ziaren kakaowych, mieszanie wszystkich składników (miazgi kakaowej, cukru, masła kakaowego, emulgatorów, aromatu i ewentualnie składników mlecznych), konszowanie (Co) oraz temperowanie (Te). Główne reakcje chemiczne, takie jak hydroliza protein, hydroliza węglowodanów, fermentacja alkoholowa i reakcje Maillarda zachodzą podczas fermentacji, suszenia, prażenia ziarna kakaowego i konszowania masy czekoladowej. Reakcje te są najważniejsze dla rozwoju smaku i aromatu (Barišić i wsp., 2019). Fermentacja ziarna kakaowego to proces, w którym następuje rozwój drożdży i bakterii w ziarnach kakaowca, proces ten jest prowadzony na plantacjach kakaowych w ramach produkcji ziarna kakaowego. Suszenie ziarna kakaowego przeprowadza się tuż po fermentacji w celu obniżenia wilgotności i zakończenia procesów oksydacyjnych indukowanych podczas fermentacji ziarna kakaowego. Po wysuszeniu, ziarna kakaowe zawierają 6–8% wilgoci. Nieznaczne ilości wody zapobiegają rozwojowi pleśni i sprawiają, że ziarna są bardziej stabilne podczas transportu i przechowywania (Beckett i wsp., 2017; Gutiérrez i wsp., 2017). Następnym etapem obróbki ziarna kakaowego jest prażenie. Jest to proces wysokotemperaturowy, prowadzony zwykle w temperaturze pomiędzy 120 a 140 °C, co ma istotne znaczenie dla zachodzenia reakcji Maillarda. Prażenie zmniejsza zawartość niepożądanych składników, nadaje charakterystyczny dla czekolady aromat i smak oraz odkaża ziarna kakaowe (Afoakwa i wsp., 2008; Beckett i wsp., 2017; Gutiérrez i wsp., 2017). Po uprażeniu, ziarna kakaowca są mielone i mieszane z pozostałymi składnikami czekolady. Kolejnym etapem produkcji czekolady jest konszowanie, czyli proces mieszania i podgrzewania mający na celu wytworzenie płynnej czekolady, odparowanie lotnych kwasów, uzyskanie odpowiedniej lepkości, usunięcie nadmiaru wilgoci i uzyskanie pożądanej barwy (Afoakwa i wsp., 2008; Aprotosoaie i wsp., 2016). Ostatnim etapem obróbki czekolady przed formowaniem lub oblewaniem jest temperowanie polegające na podgrzewaniu i schładzaniu czekolady do odpowiednich, ściśle ustalonych temperatur, dzięki czemu masło kakaowe w czekoladzie krystalizuje w kontrolowany sposób, co skutkuje uzyskaniem stabilnego produktu o pożądanych właściwościach fizycznych i sensorycznych. Poszczególne etapy produkcji czekolady przedstawione zostały na rysunku 3.

Rysunek 3. Etapy produkcji czekolady.

Zarówno matcha (*Camellia sinensis* L.) jak i moringa (*Moringa oleifera*) należą do produktów roślinnych zyskujących w ostatnim czasie na popularności ze względu na swoje właściwości prozdrowotne. Sproszkowana odmiana japońskiej zielonej herbaty, matcha jest szczególnie bogata w bioaktywne składniki, takie jak kwasy fenolowe (głównie kwas chlorogenowy i kwas galusowy), flawonoidy (zwłaszcza flawany, znane również jako katechiny herbaciane oraz garbniki), alkaloidy (metyloksantyny, głównie kofeina) i aminokwasy (teanina, gamma kwas aminomasłowy-GABA), które stanowią nawet do 30% suchej masy surowca (Devkota i wsp., 2021; Koláčková i wsp., 2020).

Moringa to indyjskie zioło lecznicze, którego liście podobnie jak liście zielonej herbaty matcha są bogatym źródłem kwasów fenolowych, takich jak kwas: ferulowy, kwas galusowy i innych kwasów hydroksybenzoesowych, zawiera również aminokwasy egzogenne, minerały (wapń, potas, magnez) i błonnik (Dhibi i wsp., 2022; Meireles i wsp., 2020).

W ramach prowadzonych badań opublikowanych w **D3** przygotowano białe czekolady z dodatkiem sproszkowanych liści zielonej herbaty matcha (MGTP) i sproszkowanych liści moringa (MOLP) w następujących stężeniach: 1%, 2%, 3% i 4%. Ponadto każdy dodatek o różnym stężeniu wprowadzano do czekolady białej w dwóch wariantach: (1) w trakcie procesu konszowania (Co) oraz (2) w procesie temperowania (Te). Dla wszystkich czekolad z dodatkiem MGTP i MOLP oraz dla czekolady białej bez żadnych dodatków przeprowadzono analizę całkowitego potencjału antyoksydacyjnego (AC) metodami DPPH, ABTS, CUPRAC i FRAP a także oznaczono całkowitą zawartość związków fenolowych (TPC). Ponadto dla próbek czekolady z dodatkiem 4% MGTP i MOLP oznaczono zawartość poszczególnych kwasów fenolowych z wykorzystaniem metody wysokosprawnej chromatografii cieczowej

z detektorem fluorescencyjnym (HPLC-FLD). W tabeli 1 przedstawiono zdjęcia i wykaz składników czekolad o zawartości 4% MGTP i MOLP dodanych na etapie temperowania.

Tabela 1. Zdjęcia oraz wykaz składników badanych czekolad białych.

	Czekolada biała	**Czekolada biała wzbogacona w sproszkowane liście herbaty matcha**	**Czekolada biała wzbogacona w sproszkowane liście moringa**
Kod	WCh	WCh_4%MGTP_Te	WCh_4% MOLP_Te
Zdjęcie			
Wykaz składników	cukier, tłuszcz kakaowy, mleko pełne w proszku, serwatka w proszku, emulgator (lecytyna sojowa), aromat	cukier, tłuszcz kakaowy, mleko pełne w proszku, serwatka w proszku, sproszkowane liście herbaty matcha 4%, emulgator (lecytyna sojowa), aromat	cukier, tłuszcz kakaowy, mleko pełne w proszku, serwatka w proszku, sproszkowane liście moringa 4%, emulgator (lecytyna sojowa), aromat

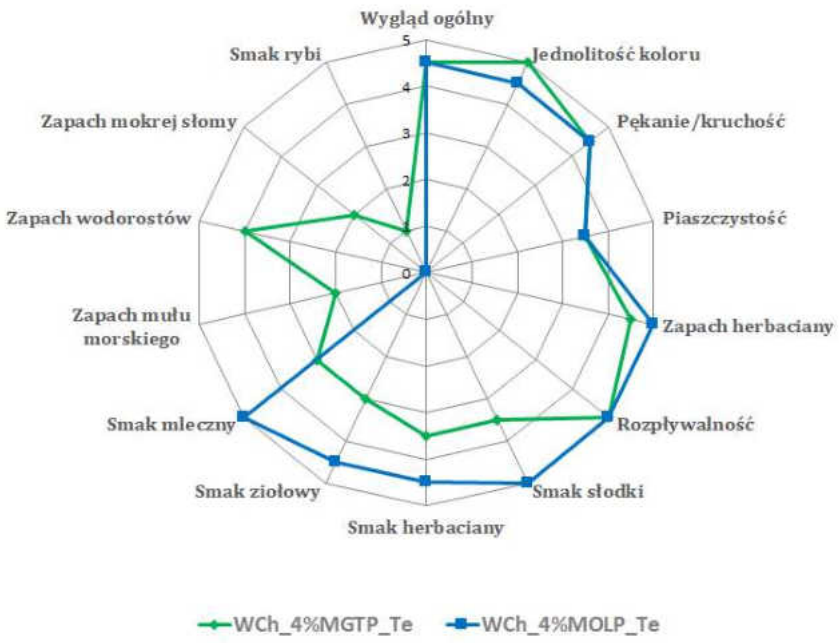
Najwyższe wyniki AC niezależnie od zastosowanej metody analitycznej i TPC uzyskano dla czekolady wzbogaconej w 4% MGTP (DPPH = 11,85 mmol TE/100 g, ABTS = 13,88 mmol TE/100 g, CUPRAC = 10,35 mmol TE/100 g, FRAP = 3,18 mmol TE/100 g, TPC = 229 mg GA/100 g) jak i dla czekolady po dodaniu 4% MOLP (DPPH = 1,50 mmol TE/100 g, ABTS = 2,15 mmol TE/100 g, CUPRAC = 2,40 mmol TE/100 g, FRAP = 0,59 mmol TE/100 g, TPC = 40.88 mg GA/100 g) w trakcie procesu temperowania (Te). Na uwagę zasługuje fakt, iż w obu przypadkach odnotowano wielokrotny wzrost ilości związków o właściwościach antyoksydacyjnych w stosunku do białej czekolady bez dodatków (DPPH = 0,21 mmol TE/100 g, ABTS = 0,43 mmol TE/100 g, CUPRAC = 0,53 mmol TE/100 g, FRAP = 0,08 mmol TE/100 g, TPC = 6,84 mg GA/100 g). W przypadku czekolad z dodatkiem MGTP i MOLP w ilości 4% był to odpowiednio wzrost 33- i 5-krotny. Zastosowanie kilku metod analitycznych oznaczenia AC pozwoliło na uzyskanie bardziej wiarygodnych wyników potwierdzających istotny wpływ stosowanych roślinnych dodatków na potencjał przeciwutleniający czekolady białej.

Należy podkreślić, że również w niefortyfikowanej WCh obecne były kwasy fenolowe, ich całkowita zawartość wynosiła 363,1 µg/100 g. W tej czekoladzie dominował kwas synapinowy, który stanowił około 66% wszystkich kwasów fenolowych. Ponadto WCh zawierała duże ilości kwasów: wanilinowego, p-OH-benzoesowego i protokatechowego (kolejno 45,35, 32,6 i 21,42 µg/100 g). Kwasy: gentyzynowy, chlorogenowy, kawowy, ferulowy i p-kumarowy również oznaczono w kontrolnej próbce czekolady jednakże w niższych stężeniach, nieprzekraczających 12 µg/100 g. Wzbogacenie czekolad MGTP i MOLP spowodowało znaczący wzrost kwasów fenolowych. Dodatek 4% MGTP zwiększył całkowitą zawartość kwasów fenolowych ponad 8-krotnie, podczas gdy WCh z 4% MOLP wykazała jedynie 3-krotnie większe ilości kwasów fenolowych ogółem. Suplementacja MGTP wzbogaciła czekolady kwas chlorogenowy (odpowiednio 1693 µg/100 g dla WCh_4%MGTP_Co i 1684 µg/100 g dla WCh_4%MGTP_Te) i kwas galusowy (odpowiednio 744 WCh_4%MGTP_Co i 797 µg/100 g dla WCh_4%MGTP_Te) niezależnie od etapu produkcji, podczas którego dodano MGTP. Natomiast w WCh z dodatkiem 4% MOLP oznaczono największe ilości kwasów: p-OH-benzoesowego, synapinowego, protokatechowego i chlorogenowego od 158,29 do 266 µg/100 g. Dla WCh wzbogaconej w MGTP istotny okazał się etap produkcji, na którym dodano MGTP, całkowita zawartość kwasów fenolowych w WCh_4%MGTP_Co wyniosła 3124 µg/100 g a dla WCh_4%MGTP_Te 3202 µg/100 g. W przypadku dodatku MOLP nie stwierdzono istotnych różnic w całkowitej zawartości kwasów fenolowych i zawartości poszczególnych kwasów fenolowych pomiędzy WCh suplementowanymi na etapie Co i Te.

Dominujące związki lipofilowe oznaczone metodą HPLC w analizowanych czekoladach to ɣ-tokoferol (627 µg/100 g), α-tokoferol (105,0 µg/100 g) i *trans*-β-karoten (52,49 µg/100 g). Dodatek 4% MOLP do WCh podczas procesu Te spowodował znaczący wzrost zawartości karotenoidów (do 11339 µg/100 g). Jednocześnie w WCh suplementowanych MGTP w ilości 4% zawartość tych związków była około 4 razy niższa niż po dodatku MOLP w tej samej ilości, niezależnie od etapu procesu, na którym miała miejsce suplementacja. We wszystkich wzbogaconych czekoladach najliczniejszą grupę karotenoidów stanowiła luteina (izomery *cis* i *trans*), stanowiąc 73–86% wszystkich tych związków. Oba dodatki zwiększyły zawartość *trans*-β-karotenu w czekoladach, z MOLP 25-krotnie zaś z MGTP 2-krotnie. Stwierdzono również, że MOLP spowodował wzrost stężenia zeaksantyny (do 1333 µg/100 g) i *cis*-karotenu (do 282 µg/100 g), natomiast MGTP wzbogaciła WCh o luteoksantynę (do 161 µg/100 g) i zeaksantynę (do 99 µg/100 g). Natomiast ogólna zawartość

tokoferoli w suplementowanych próbkach była niższa w porównaniu do kontrolnej w wyniku ich niższych poziomów w proszkach z liści. Nieco wyższe stężenie β-tokoferolu odnotowano w czekoladach z dodatkiem MOLP (wzrost o ok. 8%). Analizując wpływu etapu suplementacji na profil przeciwutleniaczy lipofilowych stwierdzono, że generalnie więcej karotenoidów pozostało w produkcie, gdy oba proszki z liści dodano podczas procesu Te. W przypadku tokoferoli nie odnotowano istotnej zależności między etapem produkcji, podczas którego następuje suplementacja a zawartością poszczególnych tokoferoli.

Równolegle do analiz właściwości antyoksydacyjnych przeprowadzona została analiza sensoryczna czekolad z dodatkiem MGTP i MOLP w ilości 4%. Opracowany podczas badań profil sensoryczny obu czekolad zaprezentowany został na rysunku 4.



*Rysunek 4. Średnie oceny sensoryczne poszczególnych atrybutów białej czekolady z 4% dodatkiem sproszkowanej zielonej herbaty matcha (WCh_4%MGTP_Te) i sproszkowanych liści moringa (WCh_4%MOLP_Te) wprowadzanych w trakcie procesu temperowania [**D3**].*

Z punktu widzenia niniejszej pracy istotne są zwłaszcza negatywne wyróżniki, które mogłyby zniechęcić potencjalnego konsumenta do regularnego spożywania proponowanego produktu. W przypadku czekolady z dodatkiem MGTP wyczuwalne były zapachy wodorostów, mułu morskiego, mokrej słomy i rybi (rysunek 4). Ich obecność nie wyklucza możliwości

wprowadzenia produktu na rynek, jednakże w znaczny sposób ogranicza grono potencjalnych konsumentów.

W czekoladzie z dodatkiem MOLP nie odnotowano negatywnych wyróżników, co jest niewątpliwie istotne w kontekście akceptowalności i chęci zakupu przez większość konsumentów. Nie mniej jednak w obu wariantach wzbogaconej WCh zidentyfikowano specyficzne wyróżniki takie jak smak i zapach herbaciany oraz smak ziołowy (rysunek 4). Nie są to typowo negatywne wyróżniki, jednakże w odniesieniu do czekolady mogą wpływać negatywnie na intencję zakupu. Obecność powyższych wyróżników determinuje ściśle określoną grupę konsumentów, którzy preferują tego typu charakterystyczne zapachy i smaki w połączeniu z czekoladą.

Biorąc pod uwagę właściwości antyoksydacyjne czekolad z dodatkiem MGTP i MOLP można jednoznacznie stwierdzić, że są to potencjalnie wysoko perspektywiczne dodatki do wyrobów cukierniczych. Również pod kątem technologicznym surowce te mogłyby znaleźć szerokie zastosowanie, ze względu na łatwość aplikacji. Zważywszy jednak na specyficzny smak powyższych dodatków, ograniczeniu ulega liczba potencjalnych konsumentów wyrobów cukierniczych nimi suplementowanymi, co z kolei skłania do poszukiwania innych rozwiązań, w większym stopniu spełniających założenia rozprawy doktorskiej.

2.3.4. Czekolada deserowa wzbogacona w ekstrakty z owoców i kwiatów czarnego bzu oraz ekstrakt z owoców aronii

Czekolada deserowa jest wyrobem cukierniczym, który sam w sobie może stanowić element diety, mający znaczenie dla ogólnego poziomu zdrowia na całym świecie, ze względu na obecność w głównym składniku czekolady deserowej, jakim jest miazga kakaowa, takich bioaktywnych komponentów jak: ksantyny (kofeina i teobromina), związki fenolowe (kwasy fenolowe i flawonoidy), sterole, fosfolipidy, witaminy, minerały (K, Mg, Cu, Fe, P) i błonnik pokarmowy (Todorovic i wsp., 2015, Vinson i wsp., 2006). Ponadto podobnie jak czekolada biała, czekolada deserowa może być wzbogacana różnego rodzaju dodatkami, w tym ekstraktami roślinnymi (Belščak-Cvitanović i wsp., 2012, 2015). Natomiast zasadniczą różnicą między czekoladą deserową oraz czekoladą białą z sensorycznego punktu widzenia jest fakt, że wzbogacanie ekstraktami roślinnymi w znacznie mniejszym stopniu wpływa na kolor czekolady deserowej niż na kolor czekolady białej.

Z doniesień literaturowych wynika, że czarny bez i aronia ze względu na swoje liczne właściwości prozdrowotne są istotnym elementem medycyny tradycyjnej w wielu krajach (Anusha Siddiqui i wsp., 2022; Kokotkiewicz i wsp., 2010; Milena i wsp., 2019; Park i wsp., 2016). Zarówno owoce jak i kwiaty czarnego bzu (*Sambucus nigra* L.) charakteryzują się wysoką zawartością związków bioaktywnych takich jak fenole, antocyjany i inne posiadające silne właściwości przeciwutleniające, przeciwbakteryjne, przeciwwirusowe, przeciwdepresyjne, przeciwnowotworowe, przeciwzapalne, hipoglikemiczne, immunomodulujące oraz zdolność do redukcji tkanki tłuszczowej i stężenia lipidów (Domínguez i wsp., 2020). Podobnymi właściwościami charakteryzują się owoce aronii (*Aronia melanocarpa*), to jedne z najbogatszych źródeł związków bioaktywnych, w tym: flawonoli (pochodnych izoramnetyny, mirycetyny, kemferolu i kwercetyny), kwasów fenolowych (głównie kwasu chlorogenowego i kwasu neochlorogenowego), proantocyjanidynów (spolimeryzowanych pochodnych katechin, zwłaszcza epikatechin) i antocyjanów (przede wszystkim pochodnych cyjanidyny). Chociaż owoce te są rzadko używane do bezpośredniego spożycia ze względu na cierpki i gorzki smak, dzięki dużej zawartości związków fenolowych mogą być szeroko stosowane do produkcji naturalnych proszków i suplementów diety o właściwościach prozdrowotnych (Sidor i wsp., 2019).

Zakres przeprowadzonych badań opublikowanych w **D2** obejmował określenie całkowitej aktywności antyoksydacyjnej (AC) oraz zawartości polifenoli (TPC) w czekoladach deserowych (DCh) wzbogaconych w cynk oraz sproszkowane ekstrakty z owoców czarnego bzu (EFrE), kwiatów czarnego bzu (EFlE) i owoców aronii (ChFrE). Zostały również przeprowadzone badania biodostępności w trakcie trawienia z wykorzystaniem modelu przewodu pokarmowego *in vitro*. Ponadto określono wpływ dodatku EFrE, EElE oraz ChFrE na właściwości fizykochemiczne (zawartość suchej masy, tłuszczu, lepkość) czekolady deserowej. Przeprowadzono, także analizę obrazowania za pomocą skaningowego mikroskopu elektronowego (SEM) z przystawką do mikroanalizy rentgenowskiej EDS w celu określenia zmian kształtów cząstek czekolady po wzbogaceniu w poszczególne ekstrakty roślinne z jednoczesną oceną składu chemicznego gotowych próbek czekolady. W tabeli 2 przedstawiono zdjęcia i wykaz składników badanych czekolad.

Tabela 2. Zdjęcia oraz wykaz składników badanych czekolad deserowych.

	Czekolada deserowa	**Czekolada deserowa wzbogacona w cynk i sproszkowany ekstrakt z owoców czarnego bzu**	**Czekolada deserowa wzbogacona w cynk i sproszkowany ekstrakt z kwiatów czarnego bzu**	**Czekolada deserowa wzbogacona w cynk i sproszkowany ekstrakt z owoców aronii**
Kod	DCh	DCh + EFrE	DCh + EFlE	DCh + ChFrE
Zdjęcie				
Wykaz składników	cukier, miazga kakaowa, tłuszcz kakaowy, emulgator (lecytyna sojowa), aromat	cukier, miazga kakaowa, tłuszcz kakaowy, ekstrakt z owoców czarnego bzu 5%, emulgator (lecytyna sojowa), mleczan cynku 0,0065%, aromat	cukier, miazga kakaowa, tłuszcz kakaowy, ekstrakt z kwiatów czarnego bzu 5%, emulgator (lecytyna sojowa), mleczan cynku 0,0065%, aromat	cukier, miazga kakaowa, tłuszcz kakaowy, ekstrakt z owoców aronii 5%, emulgator (lecytyna sojowa), mleczan cynku 0,0065%, aromat

Czekolada deserowa z dodatkiem aronii (DCh + ChFrE) okazała się najbogatszym źródłem antyoksydantów (DPPH = 942,7 µmol TE/g, ABTS = 3592,0 µmol TE/g, CUPRAC = 12945,7 µmol TE/g, FRAP = 350,7 µmol TE/g, TPC = 70,9 mg GA/g). Natomiast, z uwagi na fakt, iż ekstrakt z kwiatów czarnego bzu (EFlE) zawierał więcej związków o właściwościach antyoksydacyjnych w porównaniu z ekstraktem z owoców czarnego bzu (EFrE), czekolada suplementowana ekstraktem z kwiatów czarnego bzu (DCh + EFlE) cechowała się istotnie wyższymi wartościami DPPH = 363,3 µmol TE/g, ABTS = 1211,0 µmol TE/g, CUPRAC = 3752,3 µmol TE/g, FRAP = 102,2 µmol TE/g, TPC = 17,9 mg GA/g niż próba DCh + EFrE (DPPH = 149,3 µmol TE/g, ABTS = 672,6 µmol TE/g, CUPRAC = 3118,5 µmol TE/g, FRAP = 96,0 µmol TE/g, TPC = 12,8 mg GA/g). Warto zaznaczyć, że dodatek roślinnych ekstraktów do czekolady deserowej podwyższył jej właściwości antyoksydacyjne, co potwierdzają znacząco niższe wyniki AC dla próby kontrolnej DCh (DPPH = 144,2 µmol TE/g, ABTS = 433,9 µmol TE/g, CUPRAC = 2985,4 µmol TE/g, FRAP = 66,5 µmol TE/g, TPC = 11,7 mg GA/g).

Przygotowane próby czekolad bez i z ekstraktami roślinnymi poddano działaniu trójfazowego procesu trawienia *in vitro* symulującego warunki w jamie ustnej, żołądku i jelitach. Należy podkreślić, że ilość TPC w badanych próbkach nie zmniejszyła się w sposób istotny po przeprowadzeniu symulacji trawienia. Spadek TPC wyniósł od 3% dla DCh + EFlE do 16% dla DCh. Świadczy to o tym, że badane produkty mogą być doskonałym źródłem biodostępnych związków fenolowych. W znacznie większym stopniu, trawienie *in vitro* wpłynęło na całkowity potencjał antyoksydacyjny badanych próbek. Obniżenie wartości AC wahało się od 2% dla DCh + EFrE przy zastosowaniu metody DPPH do 79% dla DCh + ChFrE używając metod ABTS i CUPRAC.

Zważywszy na uzyskane wyniki AC i TPC dla badanych czekolad można jednoznacznie stwierdzić, że zarówno czekolady deserowe przed i po dodaniu sproszkowanych ekstraktów roślinnych są bardzo cennym źródłem związków fenolowych charakteryzujących się wysoką biodostępnością. Jednocześnie czekolady te odznaczały się wysoką aktywnością antyoksydacyjną co potencjalnie czyni z nich istotny element diety. Na uwagę zasługuje fakt, iż odnotowano znaczne różnice zarówno w AC jak i TPC pomiędzy poszczególnymi czekoladami. Wyniki wskazują jednoznacznie, iż dodatek sproszkowanych ekstraktów z owoców czarnego bzu, kwiatów czarnego bzu i owoców aronii w ilości 5% całkowitej masy wzbogacanej czekolady spowodował znaczący wzrost AC i TPC. Jednocześnie największe wartości AC i TPC niezależnie od zastosowanej metody badawczej, odnotowano dla DCh + ChFrE. Jednakże charakterystyczny cierpki smak aronii przekładający się w istotny sposób na smak DCh + ChFrE wpływa niekorzystnie na potencjalną możliwość wdrożenia produktu na rynek. W odróżnieniu od ChFrE, EFlE charakteryzował się słodkim, lekko kwiatowym aromatem, który jest pozytywnie odbierany przez konsumentów. Biorąc pod uwagę zarówno aspekt sensoryczny jaki właściwości antyoksydacyjne DCh + EFlE, to właśnie ta czekolada wydaje się mieć największy potencjał rynkowy.

2.3.5. Optymalizacja parametrów mielenia imbiru i gałki muszkatołowej

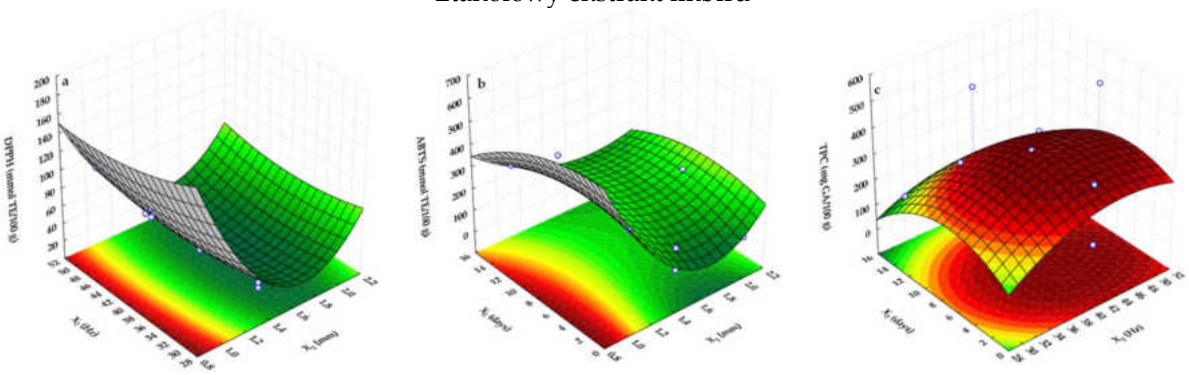
Wspólną cechą charakterystyczną przypraw jest ich pozytywny wpływ na zdrowie człowieka, co między innymi wynika z obecności w nich różnego rodzaju związków o właściwościach antyoksydacyjnych (Hinneburg, 2006; Shahidi and Ambigaipalan, 2015; Srinivasan, 2014). Wiele przypraw, takich jak: cynamon, wanilia, gałka muszkatołowa, imbir, kolendra, goździki, anyż, kardamon znajduje zastosowanie w wyrobach cukierniczych nadając

im specyficzny smak. Podstawowym procesem technologicznym jakim poddawane są przyprawy przed użyciem jest mielenie (Rish, 1997). Od procesu rozdrabniania natomiast w dużym stopniu zależą właściwości antyoksydacyjne zmielonych przypraw (Barnwal i wsp., 2014; Gao i wsp., 2020; McKee i wsp., 1993).

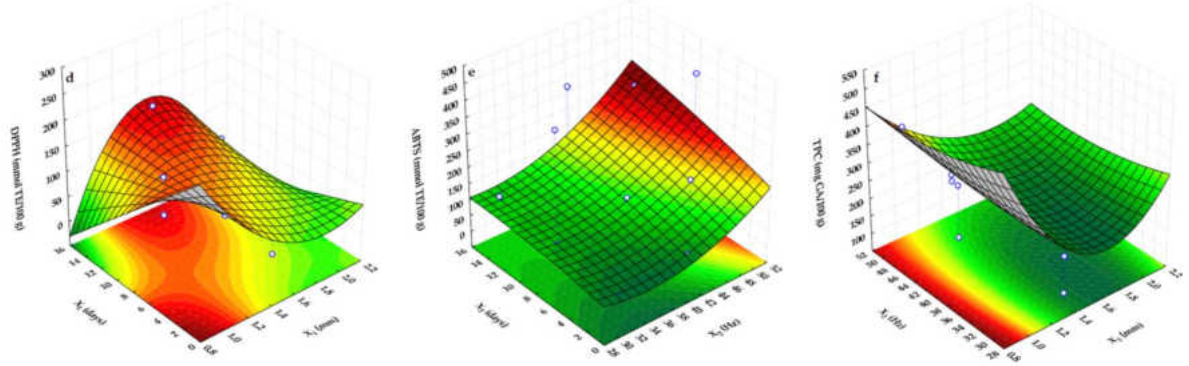
W ramach niniejszej rozprawy doktorskiej przeprowadzona została optymalizacja parametrów mielenia (rozmiaru oczek sita młynka, prędkości mielenia) i czasu przechowywania imbiru oraz gałki muszkatołowej w celu określenia najbardziej odpowiednich warunków przygotowania przypraw o wysokim potencjale antyoksydacyjnym. Wyniki tych badań opublikowane zostały w pracy **D4**. Proces mielenia przeprowadzono zgodnie z planem Boxa-Benhena (BBD) składającym się z 3 czynników niezależnych, ustalonych na 3 poziomach. Obie przyprawy zmielono z wykorzystaniem sit o średnicach oczek 1,0, 1,4 i 2,0 mm. Mielenie przebiegało przy trzech prędkościach mielenia wyrażonych poprzez ustawienia falownika na poziomie 30, 40 i 50 Hz. Ponadto zastosowano 3 różne ramy czasowe pomiędzy rozdrabnianiem a pobieraniem próbek, pobieranie bezpośrednio (0 dni) oraz 7 i 14 dni po mieleniu. Sprawdzono także wpływ dwóch rozpuszczalników, etanolu i mieszaniny etanolu z wodą w stosunku 1:1 stosowanych do ekstrakcji antyoksydantów z przypraw zmielonych zgodnie z planem BBD na potencjał antyoksydacyjny przygotowanych ekstraktów.

Analiza wykresów powierzchni dopasowanych odpowiedzi (RSM) przedstawionych na rysunku 5 jednoznacznie wskazuje na istotny wpływ poszczególnych parametrów niezależnych (średnicy oczek sita, prędkości mielenia, czasu przechowywania) na wyniki DPPH, ABTS oraz TPC w ekstraktach rozdrobnionych przypraw. W ramach przeprowadzonych badań określono optymalne warunki mielenia imbiru i gałki muszkatołowej. Optymalny rozmiar oczek sita w celu przygotowania bogatych w antyoksydanty ekstraktów: etanolowo-wodnego imbiru, oraz etanolowo-wodnego i etanolowego gałki muszkatołowej to 1,0 mm, natomiast dla etanolowego ekstraktu imbiru to 2,0 mm. Optymalna prędkość mielenia dla etanolowo-wodnego i etanolowego ekstraktu imbiru oraz etanolowo-wodnego ekstraktu gałki muszkatołowej, a także etanolowego ekstraktu gałki muszkatołowej to kolejno: 50, 43, 40 i 41 Hz. W przypadku czasu przechowywania pomiędzy rozdrabnianiem a pobieraniem próbek do przygotowania ekstraktów optymalne warunki to 9 dni i 1 dzień kolejno dla etanolowego i etanolowo-wodnego ekstraktu imbiru oraz 7 dni dla obu ekstraktów gałki muszkatołowej.

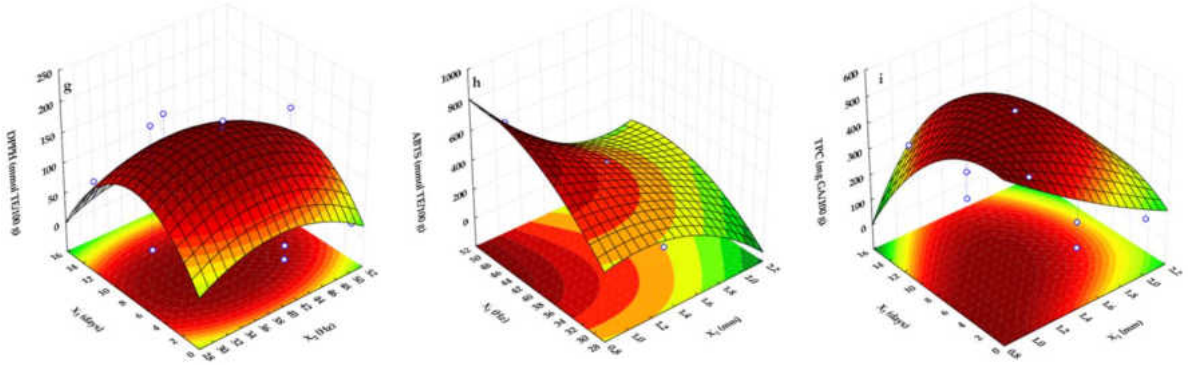
Etanolowy ekstrakt imbiru



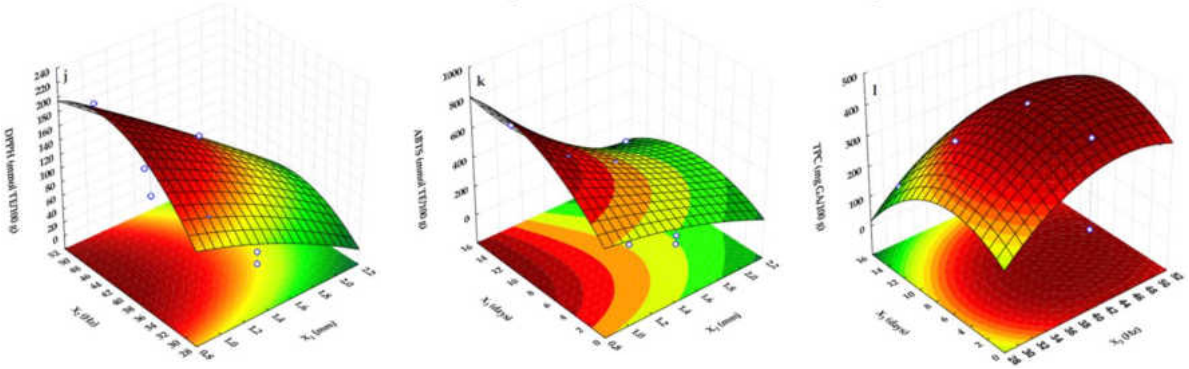
Etanolowo-wodny ekstrakt imbiru



Etanolowy ekstrakt gałki muszkatołowej



Etanolowo-wodny ekstrakt gałki muszkatołowej



*Rysunek 5. Wykresy powierzchni dopasowanych odpowiedzi (RSM) i konturowe pokazujące wpływ rozmiaru oczek w sicie młynka (X_1), prędkości mielenia (X_2) i czasu przechowywania zmielonych przypraw (X_3) na potencjał antyoksydacyjny rozdrobnionych przypraw oznaczony metodą DPPH (a,d,g,j), ABTS (b,e,h,k) i TPC (c,f,i,l) [**D4**].*

W badanych ekstraktach zidentyfikowano jedenaście kwasów fenolowych, niezależnie od zastosowanego ekstrahenta, głównym kwasem fenolowym w ekstraktach z kłącza imbiru był kwas wanilinowy (do 25%) podczas gdy w ekstraktach z gałki muszkatołowej największą ilość stanowił kwas protokatechowy (do 38%). Ekstrakt etanolowy z kłącza imbiru zawierał również znaczne ilości kwasów: p-OH-benzoesowego, elagowego, ferulowego i galusowego oraz mniejsze ilości kwasu salicylowego, p-kumarowego, protokatechowego, syryngowego, synapinowego i kawowego. Natomiast w ekstrakcie etanolowo-wodnym otrzymanym z tej przyprawy nie zidentyfikowano kwasu kawowego. Ekstrakt etanolowo-wodny z kłącza imbiru okazał się również bogatszy w kwasy: p-kumarowy, protokatechowy i synapinowy w porównaniu z ekstraktem etanolowym. W ekstraktach z gałki muszkatołowej stężenie kwasów kawowego i elagowego były poniżej granicy wykrywalności stosowanych metod analitycznych. Oprócz kwasu protokatechowego w ekstraktach tych w większych ilościach występowały kwasy: wanilinowy, ferulowy, i p-OH-benzoesowy (w ekstrakcie etanolowym).

Otrzymane wyniki wieloczynnikowego modelu procesu mielenia pozwoliły na projektowanie potencjału antyoksydacyjnego zmielonych przypraw dodawanych do ciasta piernikowego wykorzystywanego do produkcji pierników będących efektem niniejszej rozprawy doktorskiej, przyczyniając się istotnie do zwiększenia ich właściwości funkcjonalnych korzystnie wpływających na zdrowie człowieka.

2.3.6. Pierniki w czekoladzie deserowej wzbogaconej ekstraktem z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu

Fabryka Cukiernicza Kopernik S.A. jest najstarszym producentem pierników w Polsce i jednocześnie jednym z najstarszych w Europie. Produkcja pierników nieprzerwanie pozostaje głównym przedmiotem działalności Spółki aż do czasów współczesnych. Obecnie Fabryka Cukiernicza Kopernik S.A. jako lider polskiego rynku pierników wyznacza kierunki rozwoju przy jednoznacznym zachowaniu tradycyjnych receptur.

Pierniki są produktem, który jest silnie zakorzeniony w tradycji, kulturze i literaturze europejskiej (Honeyman, 2007). Obecnie ta kategoria wyrobów cukierniczych dostępna jest niemal w każdym sklepie i chociaż największe spożycie przypada na okres zimowy to coraz częściej konsumenci sięgają po te wyroby cukiernicze w ciągu całego roku kalendarzowego.

Ze względu na obecność przypraw korzennych, takich jak: cynamon (*Cinnamomum verum*), imbir (*Zingiber officinale* Roscoe), gałka muszkatołowa (*Myristica fragrans*), goździki (*Syzygium aromaticum*), kolendra (*Coriandrum sativum*) charakteryzujących się szeroką gamą właściwości prozdrowotnych (Agbogidi i Azagbaekwe, 2013; da Silva i wsp., 2019; Kumar i wsp., 2012; Mechchate i wsp., 2021; Srinivasan, 2017) pierniki same w sobie mają pozytywny wpływ na zdrowie konsumentów. Ponadto znaczna część pierników dostępnych na rynku oblana jest czekoladą, w tym czekoladą deserową charakteryzującą się wysokim potencjałem antyoksydacyjnym, co wykazano w artykule **D2**.

Biorąc pod uwagę powyższe aspekty, uzasadnionym wydaje się być wybór pierników jako produktu o właściwościach prozdrowotnych, którego regularne spożywanie przyczyni się do obniżenia ryzyka zachorowania na choroby cywilizacyjne. Z tych względów zasadne jest wprowadzenie funkcjonalnych pierników na rynek zgodnie z założeniami rozprawy doktorskiej.

Ciasto piernikowe przygotowywane jest z mąki pszennej, mąki żytniej, przypraw korzennych oraz syropu piernikowego powstałego na bazie cukru inwertowanego oraz karmelu. Następnie ciasto piernikowe poddawane jest procesowi leżakowania, podczas którego nabiera odpowiednich właściwości plastycznych i wypiekowych. Po procesie leżakowania ciasto piernikowe jest ponownie mieszane, na tym etapie dodawane są również środki spulchniające w postaci wodorowęglanu amonu. Z tak przygotowanego ciasta piernikowego formowane są określone kształty pierników, a następnie są one wypiekane w piecu tunelowym, w temperaturze sięgającej 260 °C. Wypieczone pierniki są nadziewane, studzone, oblewane czekoladą i pakowane. Uproszczony schemat produkcji pierników przedstawiony został na rysunku 6.

Rysunek 6. Uproszczony schemat produkcji pierników.

W produkcie opracowanym w ramach niniejszej rozprawy doktorskiej zastosowano dodatek ekstraktu z kwiatów czarnego bzu (EF) do czekolady deserowej oraz nadzienie z dodatkiem koncentratu z owoców czarnego bzu (EB). Zakres przeprowadzonych badań obejmował określenie właściwości przeciwutleniających i antyglikacyjnych pierników w czekoladzie deserowej wzbogaconej ekstraktem z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z czarnego bzu (GCEFEB) w porównaniu do pierników nienadziewanych w czekoladzie deserowej wzbogaconej w ekstrakt z kwiatu czarnego bzu (GCEF) oraz pierników nienadziewanych w czekoladzie deserowej (GC), a wyniki z tych badań zostały opublikowane w **D5**. W celu wykazania biodostępności produktów z czarnego bzu dla organizmu człowieka analizowano frakcje fenolową wolną (wyekstrahowaną 80% metanolem) i związaną (hydrolizowaną 2 M wodorotlenkiem sodu). Ponadto w ramach badań przeprowadzono analizę sensoryczną pierników. W tabeli 3 zestawiono zdjęcia i wykaz składników badanych pierników.

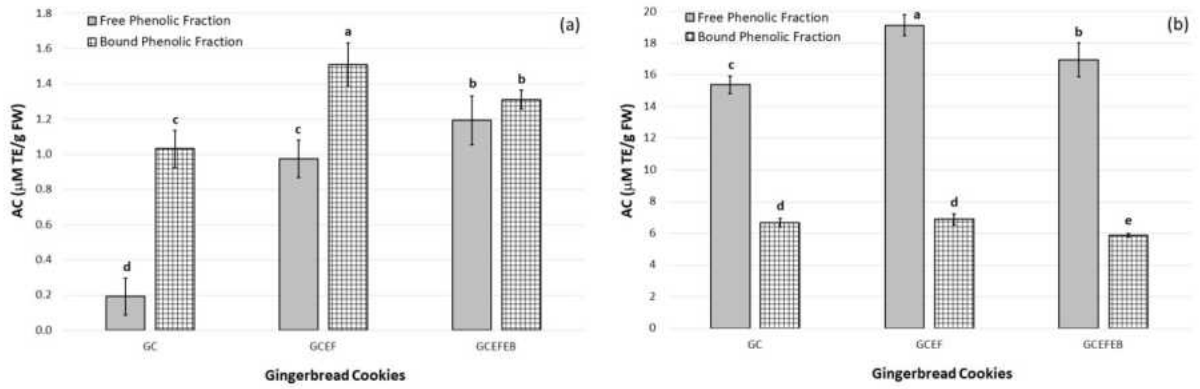
Tabela 3. Zdjęcia oraz wykaz składników badanych pierników.

	Pierniki w czekoladzie deserowej	**Pierniki w czekoladzie deserowej wzbogaconej ekstraktem z kwiatów czarnego bzu**	**Pierniki w czekoladzie deserowej wzbogaconej ekstraktem z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu**
Kod	GC	GCEF	GCEFEB
Zdjęcie			
Wykaz składników	mąka pszenna, czekolada 31% [cukier, miazga kakaowa, masło kakaowe, emulgator (lecytyna sojowa), aromat], cukier, mąka żytnia, barwnik (karmel), przyprawy, substancja spulchniająca (wodorowęglan amonu), sól, regulator kwasowości (kwas cytrynowy)	mąka pszenna, czekolada 31% [cukier, miazga kakaowa, masło kakaowe, emulgator (lecytyna sojowa), aromat], cukier, mąka żytnia, barwnik (karmel), przyprawy, substancja spulchniająca (wodorowęglan amonu), ekstrakt z kwiatów czarnego bzu 0,31%, aromat, sól, regulator kwasowości (kwas cytrynowy)	mąka pszenna, czekolada 25% [cukier, miazga kakaowa, masło kakaowe, emulgator (lecytyna sojowa), aromat], nadzienie 19% [cukier, zagęszczony przecier jabłkowy, koncentrat soku z owoców czarnego bzu 0,4%, regulator kwasowości (kwas cytrynowy), aromat, substancja konserwująca (sorbinian potasu), substancja żelująca (pektyny)], cukier, mąka żytnia, barwnik (karmel), przyprawy, substancja spulchniająca (wodorowęglan amonu), ekstrakt z kwiatów czarnego bzu 0,25%, aromat, sól, regulator kwasowości (kwas cytrynowy)

Wyniki analizy chemicznej pierników wykazały, że dodatek produktów z czarnego bzu miał zasadniczo pozytywny wpływ na zawartość związków fenolowych, najkorzystniejsze okazało się łączne zastosowanie do produkcji pierników czekolady z dodatkiem EF i nadzienia zawierającego EB. Stwierdzono wyraźne różnice w TPC badanych pierników GC, GCEF oraz GCEFEB. Najwyższy wynik TPC w wolnej frakcji fenolowej oznaczono w GCEF (277,44 μg/g), natomiast najniższy w GC (236,34 μg/g). Odnotowano około 4-krotnie mniejszą zawartość TPC w związanej frakcji fenolowej niż w wolnej frakcji fenolowej: odpowiednio 67,68 μg/g w GC, 67,77 μg/g w GCEF i 68,79 μg/g w GCEFEB. W badanych frakcjach fenolowych pierników zidentyfikowano sześć kwasów fenolowych, a kwas ferulowy był głównym kwasem fenolowym we wszystkich wolnych frakcjach fenolowych i stanowił 54% wolnych frakcji fenolowych w GC i GCEF oraz 52% w GCEFEB. Kwas ferulowy dominował także w związanych frakcjach fenolowych w GC (29%) i GCEF (28%), podczas gdy kwas p-kumarowy stanowił największą ilość w GCEFEB (29%). Całkowita zawartość kwasów fenolowych wahała się od 13,24 μg/g w frakcji fenolowej związanej dla GC do 18,40 μg/g w wolnej frakcji fenolowej GCEF. Ponadto w badanych frakcjach fenolowych zidentyfikowano czternaście flawonoidów. Największe stężenie flawonoidów oznaczono we frakcji fenolowej związanej GCEFEB (27,39 μg/g), a najmniejsze we frakcji fenolowej wolnej GC (9,63 μg/g). 3-O-glukozyd izoramnetyny był głównym flawonoidem we wszystkich wolnych frakcjach fenolowych, odpowiednio 55% w GC, 37% w GCEF i 52% w GCEFEB, podczas gdy związana frakcja fenolowa zawierała najwięcej epikatechiny, odpowiednio 85% w GC, 89% w GCEF i 90% w GCEFEB.

Badając wpływ produktów z czarnego bzu na zawartość związków fenolowych w piernikach stwierdzono, że EF i EB zwiększyły ilość związanych kwasów fenolowych oraz flawonoidów. Z kolei zawartość wolnych kwasów fenolowych była zbliżona w GCEF lub niższa w GCEFEB w porównaniu z GC. Wynika to prawdopodobnie z tego, że te bioaktywne związki są wiązane przez węglowodany i białka. Ponadto kwas ferulowy i kwasy hydroksybenzoesowe wydają się być bardziej wrażliwe na obróbkę cieplną, co jest zgodne z wynikami Liazid i wsp. (2007). Po dodaniu EF i EB odnotowano znaczny wzrost zawartości związanego kwasu p-kumarowego (do 97%) i p-hydroksybenzoesowego (do 55%). W przypadku flawonoidów dodatki te zwiększyły w piernikach zawartość wolnego 3-O-glukozydu kwercetyny (o 149% po wzbogaceniu EF i o 212% po dodaniu EF i EB) oraz związanych form epikatechiny (o 26% w GCEF i 11% w GCEFEB) i apigeniny (odpowiednio o 62% i 71%).

Wpływ dodatku ekstraktu z kwiatów czarnego bzu EF i EB na AC pierników przedstawiony został na rysunku 7.



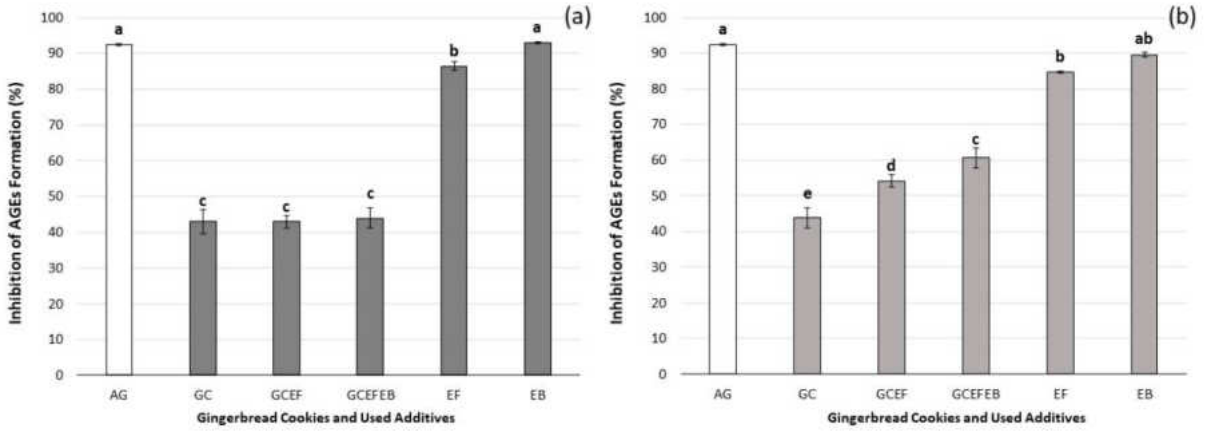
*Rysunek 7. Wyniki aktywności antyoksydacyjnej ($\bar{x} \pm SD$, wartość średnia ± odchylenie standardowe) pierników w czekoladzie bez dodatków (GC), z dodatkiem ekstraktu z kwiatów czarnego bzu (GCEF) jak również nadzieniem zawierającym koncentrat soku z owoców czarnego bzu (GCEFEB), oznaczonej metodami DPPH (a) i ABTS (b). Literami (a–e), osobno dla każdej metody badawczej, oznaczono statystycznie istotne różnice w wynikach aktywności antyoksydacyjnej (jednokierunkowa analiza wariancji ANOVA i test Tukeya, $p \leq 0,05$) [**D5**].*

Na uwagę zasługuje fakt, iż wystąpiły znaczące różnice w wynikach AC tych samych frakcji fenolowych, oznaczonych za pomocą dwóch różnych metod analitycznych. Wyniki DPPH związanej frakcji fenolowej były wyższe niż w wolnej frakcji fenolowej (1,03–1,51 μM TE/g vs. odpowiednio 0,19–1,19 μM TE/g), podczas gdy zastosowanie metody ABTS dało odmienne rezultaty (5,88–6,89 μM TE/g vs. odpowiednio 15,37–19,12 μM TE/g). Warto zauważyć, że wyniki ABTS w wolnej frakcji fenolowej były ponad 14-krotnie wyższe niż uzyskane z wykorzystaniem metody DPPH i 4-krotnie wyższe niż w związanej frakcji fenolowej.

Różnice między AC wolnych i związanych frakcji fenolowych w trzech rodzajach badanych pierników były zbliżone stosując test ABTS, natomiast odmienne wykorzystując metodę DPPH (rysunek 7). Stwierdzono, że w przypadku próby GCEFEB występowały najmniejsze różnice między wynikami DPPH w wolnych i związanych frakcjach fenolowych. Ponadto GCEF charakteryzował się najwyższą wartością AC wolnych i związanych frakcji fenolowych mierzoną metodą ABTS, w przeciwieństwie do wyników uzyskanych w teście DPPH, gdzie wolna frakcja GCEF miała niższą zdolność zmiatania rodników $DPPH^{\bullet}$ niż GCEFEB. Te różnice między wynikami otrzymanymi za pomocą testów DPPH i ABTS mogą być związane z powinowactwem zastosowanych metod analitycznych do antyoksydantów hydrofilowych i hydrofobowych. Metoda ABTS ma zastosowanie zarówno do lipofilowych jak

i hydrofobowych antyoksydantów, podczas gdy hydrofobowy charakter rodnika DPPH• ogranicza oznaczanie hydrofilowych przeciwutleniaczy testem DPPH. Ponadto kationorodniki $ABTS^{\bullet+}$ są bardziej reaktywne niż rodniki $DPPH^{\bullet}$. Reakcja przeciwutleniaczy z $ABTS^{\bullet+}$ obejmuje zarówno przeniesienie atomu wodoru (HAT), jak i transfer pojedynczego elektronu (SET), w przeciwieństwie do reakcji z rodnikami $DPPH^{\bullet}$, która opiera się głównie na mechanizmie HAT [**D2**].

Końcowe produkty zaawansowanej glikacji (AGE) powstają w żywności poddanej obróbce termicznej w wyniku nieenzymatycznej reakcji Maillarda (Foerster i Henle, 2003). Obecność AGE prowadzi do stresu oksydacyjnego oraz w sposób bezpośredni do stanów zapalnych, cukrzycy, przewlekłej choroby nerek i chorób neurodegeneracyjnych (Uribarri i wsp., 2015). Właściwości EF, EB oraz badanych pierników (GC, GCEF i GCEFEB) hamujące powstawanie AGE zostały określone za pomocą modelu BSA-glukozy i przedstawione na rysunku 8.



Rysunek 8. Hamujące działanie pierników w czekoladzie bez dodatków (GC), z dodatkiem ekstraktu z kwiatów czarnego bzu (GCEF), z dodatkiem ekstraktu z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu (GCEFEB); suchego ekstrakt z kwiatu czarnego bzu (EF); koncentratu soku z owoców czarnego bzu (EB); roztwór aminoguanidyny (AG, jako kontrola pozytywna; 1 mM/L) przeciwko tworzeniu się AGE oznaczonych jako wolne (a) i związane (b) frakcje fenolowe. Literami (a–e), osobno dla każdej frakcji fenolowej, oznaczono statystycznie istotne różnice (jednoczynnikowa analiza wariancji ANOVA i test Tukeya, $p \leq 0,05$).

Wyniki przeprowadzonych badań wskazują, iż wzbogacenie czekolady EF, którą oblewane są pierniki GCEF oraz nadzienie z EB zastosowane w GCEFEB nie zwiększało istotnie aktywności hamującej tworzenie się AGE w wolnych frakcjach fenolowych w porównaniu z piernikami kontrolnymi (GC). Natomiast związane frakcje fenolowe pierników

(GCEF i GCEFEB) charakteryzowały się znacząco wyższymi wartościami aktywności hamującej. Najwyższą zdolność hamowania tworzenia się AGE zaobserwowano we frakcji związanej otrzymanej z pierników wzbogaconych EF i EB (rysunek 8). Zjawisko to może być związane z hydrolizą, która spowodowała uwolnienie większej ilości związków charakteryzujących się aktywnością hamującą powstawanie AGE. Zwiększoną zawartość epikatechiny i/lub naringeniny zauważono we frakcji związanej. Wcześniej opublikowane badania wykazały również, że epikatechina i naringenina, jako główne flawonoidy w diecie, mogą opóźniać powstawanie AGE [Kim i wsp., 2015; Teng i wsp., 2018]. Ponadto inne związki fenolowe pochodzące z ekstraktów roślinnych również hamowały powstawanie AGE. Ich mechanizm działania głównie polega na opóźnianiu wytwarzania wolnych rodników w procesie glikacji [Błaszczak i wsp., 2021; Wang i wsp., 2009].

Właściwości sensoryczne opracowanego produktu są niezwykle ważne z punktu widzenia rozprawy doktorskiej. Warte odnotowania jest, że dodatek poszczególnych surowców (EF i EB) do pierników w istotny sposób wpłynął na właściwości sensoryczne gotowych produktów (tabela 4).

Tabela 4. Średnie oceny sensoryczne ($\bar{x}$ ± SD) barwy, zapachu, tekstury, smaku i ogólnego poziomu akceptacji pierników w czekoladzie bez dodatków (GC), z dodatkiem ekstraktu z kwiatów czarnego bzu (GCEF) oraz nadzieniem zawierającym koncentrat soku z owoców czarnego bzu (GCEFEB).

Próbka	Barwa	Zapach	Tekstura	Smak	Ogólny poziom akceptacji
GC	7,38 ± 1,31 [a]	7,64 ± 0,90 [b]	3,38 ± 1,14 [c]	2,88 ± 1,08 [b]	3,17 ± 0,91 [b]
GCEF	7,24 ± 0,94 [a]	8,38 ± 0,62 [a]	5,10 ± 0,80 [b]	6,98 ± 0,69 [a]	7,16 ± 0,67 [a]
GCEFEB	7,22 ± 0,93 [a]	8,39 ± 0,67 [a]	7,10 ± 1,00 [a]	7,71 ± 1,03 [a]	7,97 ± 0,95 [a]

n = 112; ($\bar{x}$ ± SD — wartość średnia ± odchylenie standardowe); wartości oparte na 9-punktowym hedonicznym systemie ocen, z punktami skrajnymi, 1 — „bardzo nie lubię" i 9 — „bardzo lubię". Litery (a–c) w tej samej kolumnie oznaczają statystycznie istotne różnice (jednoczynnikowa analiza wariancji ANOVA i test Tukeya, p ≤ 0,05).

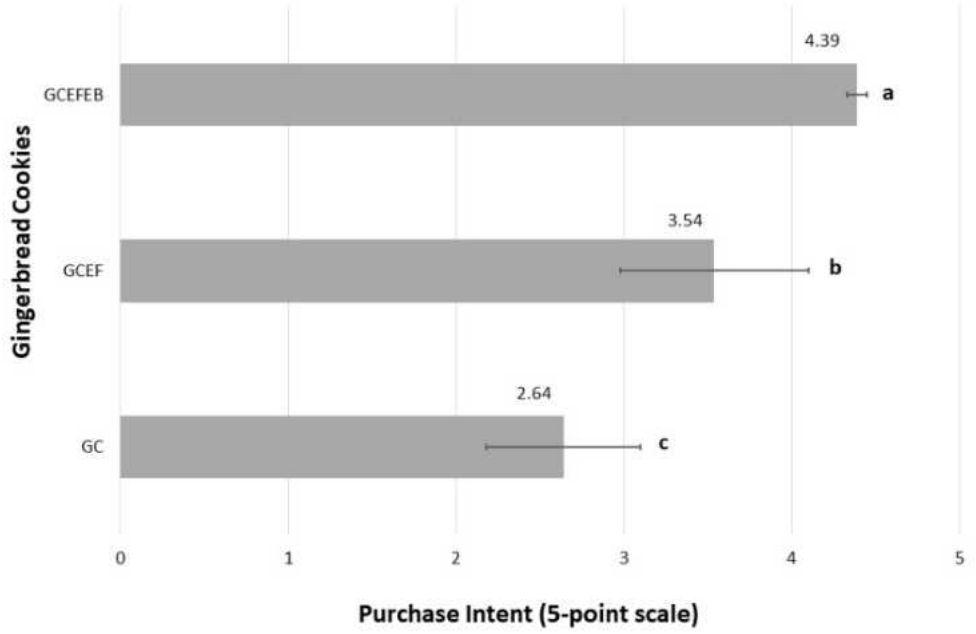
Należy podkreślić, że w przypadku takich atrybutów jak smak i tekstura wykazano znaczne różnice między GC, GCEF i GCEFEB. Najwyższe wyniki dla tych wyróżników uzyskano w przypadku GCEFEB, kolejno 7,71 dla smaku i 7,10 dla tekstury w 9-punktowej skali. Nieco niższą ocenę smaku odnotowano w GCEF (6,98), natomiast bardzo niską punktacją (2,88) cechowały się GC. Tekstura GCEF i GC również była znacznie niżej oceniona niż tekstura GCEFEB, a uzyskane wyniki wynosiły odpowiednio 5,10 dla GCEF, 3,38 dla GC i 7,10 dla GCEFEB. W przeciwieństwie do atrybutów smaku i tekstury, barwa i zapach

analizowanych trzech wyrobów GC, GCEF i GCEFEB były porównywalne, a uzyskane oceny kształtowały się na poziomach: 7,22, 7,24 i 7,38 dla koloru oraz 8,39, 8,38 i 7,64 dla zapachu. Niezależnie od powyższych ocen, dla każdego z badanych pierników oceniono ich ogólny poziom akceptacji przez konsumentów (tabela 4). Najwyższą notę (7,97), która odpowiadała klasyfikacji „bardzo lubię" uzyskał GCEFEB, nieco niższy wynik (7,16) otrzymał GCEF, oraz znacząco niższą ocenę (3,17) odnotowano dla GC, co odpowiadało klasyfikacji „umiarkowanie nie lubię".

W literaturze można znaleźć również inne przykłady, w których dodatek do piernika proszków roślinnych pozytywnie wpłynął na jego cechy sensoryczne. Ghendov-Mosanu i wsp. (2020) wprowadzili do piernika 2% i 4% proszku z miazgi owoców dzikiej róży i zaobserwowali, że ten dodatek poprawiły jego ogólne właściwości, ale ze względu na specyficzny zapach i smak dzikiej róży preferowane było niższe stężenie. Przeciwne wyniki opisane zostały przez Tańską i wsp. (2016), mianowicie stwierdzono, że dodatek wytłoków z czarnego bzu do ciastek kruchych powodował obniżenie ich walorów sensorycznych. Ciastka charakteryzowały się bardziej wyczuwalnym smakiem i aromatem oraz były bardziej kwaśne.

Kluczowa z punktu widzenia niniejszej rozprawy doktorskiej jest intencja zakupu opracowanego produktu. GCEFEB uzyskał najwyższy wynik oceny sensorycznej spośród badanych pierników (4,39 w 5-stopniowej skali) i został zakwalifikowany do kategorii odpowiedzi respondentów „z pewnością kupiłbym" (rysunek 9).

Jak można zauważyć, zarówno EF, jak i EB pozytywnie wpłynęły na intencję zakupu badanych pierników. Warto podkreślić, że wyniki ogólnego poziomu akceptacji (tabela 4) były skorelowane z intencją zakupu, co jest bardzo cenną informacją w kwestii potencjalnej komercjalizacji nowego produktu. Odnotowana intencja zakupu GCEFEB wskazuje na duży potencjał rynkowy tego produktu. W tym samym badaniu, produkt GCEF cechował się umiarkowanym wynikiem (3,54 pkt.) i został zakwalifikowany do kategorii „prawdopodobnie kupiłbym". Najniższą intencję zakupu (2,64 pkt.) zadeklarowano dla GC, co sugeruje, że produkt w takiej postaci nie powinien być wprowadzany na rynek.



Rysunek 9. Średnie oceny intencji zakupu pierników w czekoladzie bez dodatków (GC), z dodatkiem ekstraktu z kwiatów czarnego bzu (GCEF), z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu (GCEFEB). Intencję zakupu oparto na 5-stopniowej skali (1 — na pewno nie kupiłbym, 2 — prawdopodobnie nie kupiłbym, 3 — mógłbym kupić lub nie, 4 — prawdopodobnie kupiłbym, 5 — na pewno kupiłbym). Litery (a–c) oznaczają statystycznie istotne różnice (jednoczynnikowa analiza wariancji ANOVA i test Tukeya, $p \leq 0,05$).

2.4. Podsumowanie i wnioski

2.4.1. Podsumowanie i wnioski z rozprawy doktorskiej

Dzięki zastosowaniu wytłoków rzepakowych RPC i zastąpieniu margaryny olejem rzepakowym w ciastkach wypieczonych na bazie mąki pszennej otrzymano produkt o zwiększonym potencjale antyoksydacyjnym (AC). Zawartość RPC była pozytywnie skorelowana z AC jednak im większa była zawartość RPC w ciastkach tym niższa była ich ocena sensoryczna pod względem koloru, zapachu, tekstury, smaku oraz niższa ogólna akceptowalność i intencja zakupu. Zarówno zawartość RPC jak i zawartość SAFA wpłynęły na wyniki DPPH oraz właściwości sensoryczne pieczonych ciastek, jednak ilość RPC miała większy pozytywny wpływ na potencjał przeciwutleniający ciastek niż rodzaj tłuszczu, który został użyty do ich przygotowania. Jednocześnie RPC w większym stopniu wywołał niekorzystne zmiany w właściwościach sensorycznych badanych ciastek niż zawartość SAFA. Ponadto potwierdzono dobrą zgodność przewidywanych właściwości antyoksydacyjnych i sensorycznych zamieszczonych w publikacji **D1** z wynikami eksperymentalnymi co wskazuje na poprawność proponowanych modeli i optymalnych ilości składników do wypieku ciastek

zawierających funkcjonalny dodatek jaki stanowił uboczny produkt (RPC) powstający podczas produkcji oleju rzepakowego.

Przeprowadzone badania przedstawione w publikacji **D3** potwierdziły, że czekolady białe (WCh) wzbogacone w sproszkowane liście zielonej herbaty matcha (MGTP) oraz sproszkowane liście moringa (MOLP) to produkty o charakterystycznych właściwościach sensorycznych i pozytywnych właściwościach prozdrowotnych dzięki obecności bioaktywnych związków fenolowych. Suplementacja WCh na różnych etapach procesu produkcyjnego spowodowała znaczny wzrost AC i TPC oznaczonych przy zastosowaniu różnych metod analitycznych: DPPH, ABTS, CUPRAC, FRAP i FC. Wykazano, że próbki WCh wzbogacone w MGTP miały znacznie większą zawartość związków fenolowych a jednocześnie wykazywały silniejsze właściwości przeciwutleniające niż WCh fortyfikowane MOLP. Suplementacja WCh za pomocą MGTP i MGTP spowodowała również zmiany barwy i właściwości organoleptycznych WCh. Zasadniczo MOLP miał korzystniejszy wpływ na profil sensoryczny WCh niż MGTP. W ramach niniejszej pracy doktorskiej po raz pierwszy zbadano efekt wzbogacenia WCh dodatkami MGTP i MOLP na różnych etapach produkcji czekolady (konszowania - Co i temperowania - Te) na zawartość polifenoli i lipofilowych związków antyoksydacyjnych w WCh. Pomimo, iż dodatek MOLP spowodował mniejszy wzrost zawartości polifenoli niż MGTP to dostarczył znacznie większej ilości przeciwutleniaczy lipofilowych, głównie karotenoidów, o silnych właściwościach przeciwutleniających. Ponadto warto zwrócić uwagę na to, iż etap produkcji czekolady podczas którego dodawane były MGTP i MOLP może być również ważnym czynnikiem decydującym o prozdrowotnym działaniu czekolady. Wyniki uzyskane w badaniach wykazały, że dodanie MGTP i MOLP do WCh w trakcie procesu temperowania (Te) pozwoliło na uzyskanie produktu o wyższej zawartość polifenoli i karotenoidów oraz AC niż w przypadku wprowadzenia tych składników na etapie konszowania (Co).

W ramach przeprowadzonych badań zaprezentowanych w publikacji **D2** wykazano, że wzbogacenie czekolady deserowej (DCh) w roślinne dodatki: ekstrakt z owoców czarnego bzu (EFrE), ekstrakt z kwiatów czarnego bzu (EFlE) i ekstrakt z aronii (ChFrE) miało wpływ na właściwości fizykochemiczne, przeciwutleniające i mikrostrukturalne tych nowych produktów. Ponadto zbadano biodostępność związków antyoksydacyjnych w fortyfikowanych czekoladach za pomocą modelu trawienia *in vitro*. Wzbogacenie DCh ekstraktami roślinnymi zwiększyło jej zdolność do wychwytywania wolnych rodników (wartości $DPPH^{\bullet}$ i $ABTS^{\bullet+}$), zdolność redukującą (CUPRAC i FRAP) oraz ilość związków fenolowych ogółem (TPC) w czekoladach

poddanych i niepoddanych procesowi trawienia. Wzrost ten jest ściśle związany z wysokim potencjałem antyoksydacyjnym zastosowanych roślinnych dodatków. Połączenie obrazowania SEM i analizy EDS ujawniło, że DCh bez i wzbogacona dodatkami roślinnymi wykazywała zauważalne różnice w składzie chemicznym i charakterystyce powierzchni tj.: chropowatość, wielkość ziarna oraz obecność porów lub wypukłości. Potencjalnie EFrE, EFlE i ChFrE mogą być stosowane jako prozdrowotne dodatki do standardowych czekolad uzyskując w ten sposób produkty, które jeszcze w większym stopniu wpływają pozytywnie na zdrowie konsumentów.

Badania nad optymalizacją procesu mielenia imbiru i gałki muszkatołowej zostały przeprowadzone w ramach niniejszej pracy doktorskiej, a ich wyniki opublikowano w artykule **D4**. Określono wpływ parametrów mielenia i czasu przechowywania zmielonych przypraw korzennych na właściwości przeciwutleniające ekstraktów etanolowych i etanolowo-wodnych (1:1) tych przypraw. Zaobserwowano że rozmiar oczek sita miał najbardziej znaczący wpływ na właściwości przeciwutleniające wszystkich badanych ekstraktów z kłącza imbiru oraz etanolowo-wodnych (1:1) ekstraktów z gałki muszkatołowej. Uzyskane eksperymentalne wyniki pokrywały się z przewidywanymi wartościami potencjału antyoksydacyjnego, wskazując że model Boxa-Behnkena można z powodzeniem wykorzystać do optymalizacji warunków mielenia i przechowywania przypraw korzennych. Oprócz parametrów mielenia i przechowywania przypraw korzennych istotna była również polarność rozpuszczalnika użytego jako ekstrahenta. Porównanie właściwości przeciwutleniających ekstraktów przygotowanych za pomocą dwóch powszechnie stosowanych rozpuszczalników wskazało, że mieszanina etanol-woda (1:1) pozwalała na ekstrakcję większej ilości związków fenolowych z kłącza imbiru, natomiast etanol okazał się bardziej odpowiedni do ekstrakcji związków fenolowych z gałki muszkatołowej. Na podstawie uzyskanych badań można również stwierdzić, że ekstrakty z kłącza imbiru charakteryzowały się większą zawartością kwasów fenolowych niż ekstrakty z gałki muszkatołowej. Uzyskane dane mogą zostać wykorzystane w przemyśle spożywczym w ramach produkcji wyrobów zawierających w swoim składzie przyprawy bogate w związki o właściwościach przeciwutleniających.

Badania, których wyniki zawarte zostały w publikacji **D5** dostarczyły istotnych informacji związanych ze wzrostem zawartości związków fenolowych oraz właściwości przeciwutleniających pierników oblanych czekoladą na skutek wzbogacenia jej w ekstrakt z kwiatów czarnego bzu (EF), z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu (EB). Pierniki z tymi dodatkami charakteryzowały się najwyższą zawartością flawonoidów a ich stężenie było istotnie skorelowane z DPPH ($r = 0,82$) i ABTS ($r = 0,68$). Właściwości

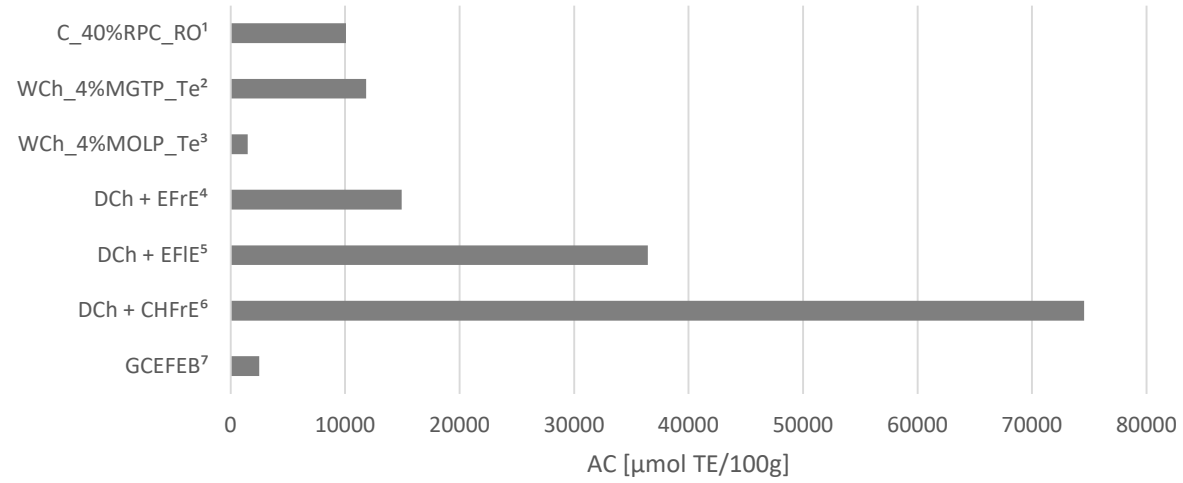
antyglikacyjne fortyfikowanych pierników zostały potwierdzone dla związanej frakcji fenolowej, co prawdopodobnie wynika z wyższej zawartości flawonoidów w tej frakcji, zwłaszcza epikatechiny i naringeniny. Ponadto wykazano pozytywny wpływ dodatków na właściwości sensoryczne i intencję zakupu badanych pierników. Na podstawie wyników z przeprowadzonych badań można stwierdzić, że możliwa jest produkcja pierników, które przy jednoczesnym zachowaniu dobrych właściwości sensorycznych wykazują cechy prozdrowotnej żywności funkcjonalnej ze względu na swoje właściwości antyoksydacyjne i antyglikacyjne.

2.4.2. Podsumowanie i wnioski z wdrożenia

Podczas realizacji badań wchodzących w zakres niniejszej dysertacji, opracowane zostało kilka produktów, zaś do komercjalizacji wybrano pierniki w czekoladzie deserowej wzbogaconej ekstraktem z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z czarnego bzu (GCEFEB). Produkt ten w największym stopniu spełniał założenia rozprawy doktorskiej tzn. regularne jego spożywanie może przyczynić się do ograniczenia zachorowania na choroby cywilizacyjne i jednocześnie uzyskał wysoką ocenę sensoryczną. Ponadto zadeklarowana intencja zakupu wobec tego produktu była wysoka, co oznacza że respondenci są zainteresowani dokonywaniem zakupu i konsumpcją tego produktu.

Na rysunku 10 przedstawiono porównanie potencjału przeciwutleniającego wybranych produktów opracowanych w ramach rozprawy doktorskiej. Jak można zaobserwować, poszczególne produkty różnią się aktywnością przeciwutleniającą. Jednak analizując dane trzeba mieć na uwadze, iż produkty te konsumowane są w różnych ilościach. Przykładowo jednorazowa porcja konsumowanej czekolady jest zdecydowanie mniejsza niż jednorazowa porcja konsumowanych pierników.

Opracowany w ramach rozprawy doktorskiej wybrany do komercjalizacji produkt jakim są pierniki w czekoladzie z dodatkiem ekstraktu z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu (GCEFEB) trafił do sprzedaży w Sklepach Fabrycznych należących do Fabryki Cukierniczej Kopernik S.A. gdzie został bardzo dobrze odebrany przez konsumentów.



Rysunek 10. Aktywność przeciwutleniająca wybranych produktów opracowanych w ramach rozprawy doktorskiej, oznaczona metodą DPPH. [1] Ciastka z zawartością 40% wytłoków rzepakowych i zawartością kwasów tłuszczowych nasyconych w dodanym oleju rzepakowym: 2,3g; [2]Biała czekolada z dodatkiem sproszkowanych liści zielonej herbaty matcha dodanymi na etapie temperowania w ilości 4%; [3]Biała czekolada z dodatkiem sproszkowanych liści moringa dodanych na etapie temperowania w ilości 4%; [4]Deserowa czekolada z dodatkiem ekstraktu z owoców czarnego bzu w ilości 5%; [5]Deserowa czekolada z dodatkiem ekstraktu z kwiatów czarnego bzu w ilości 5% ; [6]Deserowa czekolada z dodatkiem ekstraktu z owoców aronii w ilości 5%; [7]Pierniki w czekoladzie deserowej wzbogaconej ekstraktem z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu.

Rysunek 11. pierniki w czekoladzie deserowej wzbogaconej ekstraktem z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu.

Jednocześnie sprzedaży pierników towarzyszyła kampania informacyjna dotycząca metod zapobiegania i walki ze stresem oksydacyjnym wywołującym poważne konsekwencje dla zdrowia człowieka oraz roli diety, przede wszystkim regularnego spożywania produktów charakteryzujących się wysokim potencjałem antyoksydacyjnym w ochronie przed niekorzystnymi skutkami stresu oksydacyjnego.

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3. Streszczenie rozprawy doktorskiej

Choroby układu krążenia oraz nowotwory złośliwe należące do chorób cywilizacyjnych stanowią najczęstszą przyczynę zgonów w Polsce, dlatego nadrzędnym celem rozprawy doktorskiej było zaprojektowanie i wdrożenie na rynek wyrobów cukierniczych, które mogą stanowić element zróżnicowanej i zbilansowanej diety przyczyniając się do walki z chorobami cywilizacyjnymi. Badania w sposób szczególny koncentrowały się na właściwościach antyoksydacyjnych oraz sensorycznych projektowanych wyrobów cukierniczych.

W ramach rozprawy doktorskiej zaprojektowano: ciastka z wytłokami rzepakowymi, czekolady białe z dodatkiem sproszkowanych liści zielonej herbaty matcha i sproszkowanych liści moringa, czekolady deserowe z dodatkiem ekstraktu z owoców czarnego bzu, ekstraktu z kwiatów czarnego bzu i ekstraktu z owoców aronii oraz pierniki w czekoladzie deserowej wzbogaconej ekstraktem z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu. Ponadto przeprowadzono optymalizację procesu mielenia imbiru i gałki muszkatołowej, znajdujących zastosowanie w produkcji pierników.

W badaniach całkowitego potencjału antyoksydacyjnego innowacyjnych produktów zastosowano szereg metod analitycznych, takich jak: DPPH, ABTS, CUPRAC, FRAP, FC, natomiast poszczególne antyoksydanty (związki fenolowe, tokoferole, karotenoidy) analizowano z wykorzystaniem metod chromatograficznych. Projektowane wyroby cukiernicze poddawane były również analizie sensorycznej w celu określenia możliwości wprowadzenia ich do regularnej sprzedaży. Wykorzystano, także statyczny model układu pokarmowego *in-vitro* w celu określenia biodostępności czekolad deserowych wzbogaconych ekstraktami z owoców i kwiatów czarnego bzu a do zobrazowania wpływu tych ekstraktów na morfologię powierzchni czekolad deserowych wykorzystano skaningową mikroskopię elektronową (SEM). Dodatkowo funkcjonalne zmodyfikowane pierniki przebadano pod kątem właściwości antyglikacyjnych.

Wszystkie zaprojektowane wyroby cukiernicze charakteryzowały się znaczącymi właściwościami antyoksydacyjnymi, jednakże biorąc pod uwagę również właściwości sensoryczne oraz możliwość wprowadzenia do regularnej produkcji, w najlepszym stopniu założenia niniejszej rozprawy doktorskiej spełniają pierniki w czekoladzie deserowej wzbogaconej ekstraktem z kwiatów czarnego bzu, z nadzieniem zawierającym koncentrat soku z owoców czarnego bzu o potwierdzonych właściwościach antyoksydacyjnych i wysokim

ogólnym poziomie akceptacji oraz wysokiej intencji zakupu. I to właśnie ten wyrób cukierniczy został wprowadzony na rynek. Spożywanie tego produktu ze względu na jego właściwości prozdrowotne, w tym właściwości antyoksydacyjne i antyglikacyjne może potencjalnie stanowić element zróżnicowanej i zbilansowanej diety przyczyniający się do walki z chorobami cywilizacyjnymi.

4. Summary of doctoral dissertation

Cardiovascular diseases and malignant neoplasms belonging to civilization diseases are the most common cause of death in Poland. Therefore the primary goal of the doctoral dissertation was to design and market confectionery products that can be part of a varied and balanced diet contributing to the fight against civilization diseases. The research focused in particular on the antioxidant and sensory properties of the designed confectionery products.

As part of the doctoral dissertation, cookies with rapeseed press cakes, white chocolate with powdered matcha green tea leaves and powdered moringa leaves, dark chocolates with elderberry extract, elderberry flower extract and chokeberry extract, gingerbread cookies in dark chocolate enriched with elderberry flower extract, with a filling containing elderberry juice concentrate were designed. In addition, the grinding process of ginger and nutmeg, which are used in gingerbread, production was optimized.

Several analytical methods, such as DPPH, ABTS, CUPRAC, FRAP and FC, were applied to determine the total antioxidant capacity (AC). However individual antioxidants (phenolic compounds, tocopherols, carotenoids) were analyzed using chromatographic techniques. The designed confectionery products were also subjected to sensory analysis to evaluate the possibility of introducing them to regular sale. In addition, a static model of the *in-vitro* digestive system was used to determine the bioavailability of dark chocolates enriched with extracts from elderberries, elderflowers and chokeberries, while scanning electron microscopy (SEM) was used to illustrate the effect of these extracts on the surface morphology of dark chocolates. The modified functional gingerbreads were tested also for anti-glycation properties.

All designed confectionery products were characterized by significant antioxidant properties. However, taking into account the sensory properties and the possibility of introducing them into regular production, the assumptions of this doctoral dissertation are best met by gingerbreads covered in dark chocolate with elderflower extract, with a filling containing elderberry juice concentrate with confirmed antioxidant properties and high overall acceptance level and high level of purchase intention. And it was this confectionery product that was introduced to the market. Consumption of this product due to its health-promoting properties, including antioxidant and anti-glycation properties, can potentially be an element of a varied and balanced diet, contributing to the fight against civilization diseases.

5. Publikacje wchodzące w skład rozprawy doktorskiej

(D1) Szydłowska-Czerniak, A., **Poliński, S.**, & Momot, M. (2021). Optimization of Ingredients for Biscuits Enriched with Rapeseed Press Cake—Changes in Their Antioxidant and Sensory Properties. *Applied Sciences, 11*(4), 1558.

applied sciences

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Optimization of Ingredients for Biscuits Enriched with Rapeseed Press Cake—Changes in Their Antioxidant and Sensory Properties

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Abstract: The optimum formulation for wheat flour (WF)-based biscuits containing the rapeseed press cake (RPC)—the primary by-product of rapeseed oil production rich in phenolic compounds and different types of fats (rapeseed oil, margarine and coconut oil)—was estimated using the central composite design (CCD) with two factors and response surface methodology (RSM). Effects of partial substitution of WF for RPC (0–40 g) in a total flour blend (100 g) and fats with various amounts of saturated fatty acids (SAFA = 2.3–24.9 g) on antioxidant capacity (AC) and sensory characteristics (color, odor, texture, flavor, overall acceptability, and purchase intent scores) of the novel biscuits were investigated. Conventional solid (liquid)–liquid extraction and ultrasound-assisted extraction (UAE) were applied to extract total antioxidants from main ingredients used for the preparation of doughs as well as the baked biscuits. The AC of biscuits and their components were determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The DPPH results were the highest for the RPC flour (DPPH = 15,358–15,630 µmol Trolox (TE)/100 g) and biscuits containing rapeseed oil and 40 g of RPC flour (DPPH = 7395–10,088 µmol TE/100 g). However, these biscuits had lower sensory scores for each attribute and the lowest purchase intent scores. The quadratic response surfaces were drawn from the mathematical models in order to ensure the good quality of the proposed biscuits with RPC. The DPPH results obtained and the mean sensory scores correlate with the predicted values (R^2 = 0.7751–0.9969). The addition of RPC with high antioxidant potential to biscuits and the replacement of margarine or coconut oil by rapeseed oil interfered with their acceptability.

Keywords: rapeseed press cake; fats; biscuits; antioxidant capacity; sensory analysis; consumer acceptance; response surface methodology

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Citation: Szydłowska-Czerniak, A.; Poliński, S.; Momot, M. Optimization of Ingredients for Biscuits Enriched with Rapeseed Press Cake—Changes in Their Antioxidant and Sensory Properties. *Appl. Sci.* **2021**, *11*, 1558. https://doi.org/10.3390/app11041558

Academic Editor: Silvia Mironeasa
Received: 31 December 2020
Accepted: 5 February 2021
Published: 9 February 2021

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1. Introduction

For some time, it has been possible to observe the interest of both producers and consumers in the confectionery market in products with the addition of functional ingredients. One of the categories of functional additions are components with antioxidative properties, which can reduce the level of oxidative stress in cells. Food by-products are mainly considered rich and cheap sources of valuable compounds for supplementation of confectionery and bakery products [1–15]. Recently, the effect of different amounts (5–95%) of agro-industrial by-products such as olive stone powder, okara powder, prickly pear peel, fruit pomaces (rosehip, rowanberry, blackcurrant, elderberry, grape, and blueberry), fruit by-products (pineapple central axis, apple endocarp, melon peels, waste left after goji berry concentrate extraction), cocoa shell, soybean meal and fermented soybean meal, defatted chia flour, and defatted sunflower seed flour on the antioxidant capacity (AC) of biscuits, cookies, and muffins has been analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant

potential (FRAP), β-carotene/linoleic acid, phosphomolybdenum complex, oxygen radical absorbance capacity (ORAC), cupric reducing antioxidant capacity (CUPRAC), and Folin-Ciocalteu (F-C) assays [1,3,5–15]. However, there is little information on the optimization of the dough formulations to maximize functional properties, mainly antioxidant potential of confectionery products enriched with agro-industrial by-products. This knowledge is needed for the improvement of production processes and commercial applications. Response surface methodology (RSM) was only applied to optimize antioxidant capacity determined by ABTS method and total phenolic content (TPC) in the formulation of cookies supplemented with blueberry pomace [7]. Moreover, RSM-based models were proposed to study the effect of replacing WF with organic grape flour (13.06–16.74%), interesterified fat concentration (23.96–34.04%), and sucrose content (11.96–22.04%) on the sensory properties (appearance aroma, flavor, texture, overall impression) of the fortified cookies, and to evaluate the predictive ability of these mathematical models to describe general acceptability of final product [4]. The combined impact of three independent variables, carrot pomace powder (10–20 g), finger millet flour (2.5–7.5 g), and baking time (21–25 min) on general acceptability and physiochemical properties (spread ratio, change in color, amounts of moisture, ash, fat, and fiber, hardness) of the biscuits enriched with waste of carrot juice industry was also optimized by the RSM [2].

Additionally, the AC and TPC of cookies with cocoa shell, soy flour, and green banana flour developed by the simplex centroid design were analyzed by ABTS, FRAP, β-carotene/linoleic acid, phosphomolybdenum complex, and Fast Blue methods. This experimental design was effective for optimization of the acceptance of cookies made with cocoa shell, a by-product of the chocolate industry [11].

However, to the best of our knowledge, there are no reports on the evaluation of the effect of rapeseed press cake (RPC) flour and type of fat on antioxidant properties and sensory quality of baked biscuits. Only the impact of cold pressed RPC, RPC fiber isolate, and RPC alkaline extract on the generation of acrylamide and 5-hydroxymethylfurfural in cookies was investigated. The cookies with RPC had a higher concentration of 5-hydroxymethylfurfural, while the alkaline extract from RPC caused a decrease of acrylamide content in the supplemented cookies [16].

Rapeseed is the third most abundant oil plant worldwide (after palm and soya) and the primary oil seed crop in Poland. During rapeseed processing, several wastes are generated, and RPC is one of the major types of residual biomass from the rapeseed oil industry [16,17]. The RPC can provide a viable and economical source of bioactive compounds, because the varieties grown in Poland have an improved nutritional profile (low amounts of erucic acid and glucosinolates). Nevertheless, glucosinolates and products of their degradation present in RPC can create unique and characteristic flavor, thus, they can be applied as food additives for improvement of the sensory characteristics [18]. Recently, with the increasing interest in circular economy and zero waste, there is intense effort to revalorize food by-products. For this reason, the RPC as natural source of proteins, carbohydrates, crude fiber, lipids, minerals, as well as polyphenols, glucosinolates, and isothiocyanates having antioxidant, antimicrobial and anticarcinogenic properties can be developed for confectionery applications [17,18]. On the other hand, an increase in rapeseed production implies larger domestic supplies of RPC, which would affect the stability of the prices of confectionery enriched with RPC.

Therefore, the aim of this study was to create biscuits with new and attractive for the consumer features that would improve the antioxidant potential and would not cause a deterioration in their sensory properties. The present work is focused for the first time on the optimization of the production of functional and acceptable confectionery products fortified with RPC rich in antioxidants and high quality fats. A central composite experimental design (CCD) and the RSM were used for evaluation of the effects of two independent variables (RPC content—a novel ingredient—and saturated fatty acids (SAFA) content) and their interactions on the response variables: AC determined by the modified DPPH assay and sensory characteristics (color, odor, texture, flavor, overall acceptability, and

purchase intent scores) of wheat flour (WF)-based biscuits. Moreover, in the present study, DPPH results of extracts obtained from ingredients (RPC, WF, rapeseed oil, margarine, and coconut oil) and the baked biscuits by the conventional solid/liquid–liquid extraction and the ultrasound-assisted extraction (UAE) were compared and discussed.

The hedonic method was conducted to evaluate the acceptance, desirability, and preferences of biscuits without and with RPC containing various types of fats.

2. Materials and Methods

2.1. Reagents and Samples

All reagents were of analytical or high performance liquid chromatography (HPLC) grade. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH, 95%), 6-hydroxy-2,5,7,8- tetramethylchromane-2-carboxylic acid (Trolox (TE), 97%) and methanol (99.8%) were purchased from Sigma Aldrich (Poznań, Poland). Redistilled water was used for the preparation of solutions.

The RPC, the primary by-product of the rapeseed oil industry, and the refined rapeseed oil, the final product of a conventional technological process, were provided by a local vegetable oil factory and were stored in an airtight closed poly(ethylene terephthalate) (PET) bag and bottle, respectively, at room temperature. All the other baking ingredients in customary quality, such as WF, refined coconut oil, margarine, sugar, salt, baking powder, and non-carbonated spring water were purchased from the local market. All samples in the original packaging were stored at ambient temperature until treatment and further analysis.

2.2. Biscuits Preparation

Biscuits fortified with RPC were prepared on a laboratory scale according to the following recipe. RPC was milled in the mill (Model FW100, Chemland, Stargard, Poland) and sieved through a mesh sieve of 0.355 mm (Retsch Test Sieve, Haan, Germany) to obtain a uniform size of flour.

The composite flour blends were prepared using various combinations of WF and RPC flour in the ratio of 100:0, 80:20, 60:40, respectively. Three commercial fats such as refined rapeseed oil, margarine, and coconut oil, with a declared SAFA content of 7.5 g/100 g, 26 g/100 g and 83 g/100 g, respectively, were used for preparation of dough formulations. Initially, each flour blend (100 g), salt (1.0 g), and baking powder (2.0 g) were thoroughly mixed. Sugar (45 g) was dissolved in hot spring water (22 mL) and cooled. Finally, 30 g of refined rapeseed oil, margarine and coconut oil containing 2.3 g, 7.8 g and 24.9 g of SAFA, respectively was added separately into mixtures of various powdering ingredients and sugar solution. All ingredients were mixed for 6 min in a hand mixer (5-speed, 500 W, Moulinex, Powermix HM610130, Ecully Cedex, France) at medium speed for 6 min with scraping every 2 min to obtain a homogenous mixtures. Afterwards, solid dough formulations were cooled in a refrigerator at 4 °C for 30 min. Each dough type was kneaded and sheeted to a uniform thickness of 5 mm and cut into circular shapes of 6.5 cm diameter.

The dough pieces were placed on a baking tray with baking paper and baked at 180 °C for 18 min in an electrically heated oven (Electrolux, Warszawa, Poland). Biscuit samples were cooled and stored in airtight plastic containers at ambient temperature before AC and sensory analyses were conducted.

2.3. Determination of Antioxidant Capacity

2.3.1. Samples Preparation by Conventional Solid (Liquid)–Liquid Extraction

Portions of RPC (2.0 g), WF (2.0 g), fats (2.0 g), biscuits (5.0 g of five ground biscuits baked from the same dough formulation), and 70% methanol (20, 15, 5, and 20 mL, respectively), were transferred into Erlenmeyer flasks and shaken using a shaker SK-L 330-Pro (Chemland, Stargard, Poland) at room temperature for 30 min.

Each sample was extracted in triplicate, and the residual samples were separated by centrifugation (centrifuge MPW-54, Chemland, Stargard, Poland, 3120× g, 15 min). The

pooled extracts were filtered using polytetrafluorethylene syringe filters (PTFE, pore size 0.20 µm/diameter 13 mm, Sigma Aldrich, Poznań, Poland) and stored in a refrigerator at 4 °C prior to analysis.

2.3.2. Samples Preparation by Ultrasound-Assisted Extraction

Portions of RPC (2.0 g), WF (2.0 g), fats (2.0 g), biscuits (5.0 g of five ground biscuits baked from the same dough formulation), and 70% methanol (20, 15, 5, and 20 mL, respectively), were transferred into Erlenmeyer flasks and placed in an ultrasonic cleaner bath (5200DTD, Chemland, Poland) with a frequency of 40 kHz, ultrasound input power of 180 W and heating power of 800 W, equipped with a digital timer and temperature controller. The ultrasonic bath's water level was adjusted so that it was slightly higher than the level of samples and solvent into Erlenmeyer flasks. The UAE was performed for 5 min, and the temperature was kept constant at 25 ± 0.3 °C.

The same sample was sonicated in triplicate and centrifuged at 1880× *g* for 15 min (centrifuge MPW-54, Chemland, Stargard, Poland). The supernatants were filtered through a PTFE syringe filter (0.20 µm/13 mm) and collected for subsequent determination of the AC.

2.3.3. DPPH Method

The AC of major ingredients and prepared biscuits were analyzed spectrophotometrically according to a modified DPPH procedure described previously [19]. Briefly, 0.01–0.70 mL of methanolic extracts obtained by conventional solid (liquid)/liquid extraction and UAE were added to 1.99–1.30 mL of methanol and 0.5 mL of DPPH methanolic solution (304.0 µmol/L). The changes in color from deep violet to light yellow were measured at 517 nm against a reagent blank (2 mL of methanol + 0.5 mL of DPPH methanolic solution) after 15 min of reaction using a Hitachi U-2900 spectrophotometer (Tokyo, Japan). The DPPH values were expressed as micromoles of Trolox equivalents (TE) per 100 g of the studied samples.

2.4. Sensory Acceptance Test

Sensory analysis of the baked biscuits was performed using an effective acceptance test with 72 untrained panelists (35 male and 37 female) in the age range of 18–69 recruited among students, staff, and professors of the Faculty of Chemistry, Nicolaus Copernicus University in Toruń, Poland. The sensory test was conducted two days after baking trials using a 9-point hedonic scale (1—"disliked extremely", 2—"disliked very much", 3—"disliked moderately", 4—"disliked slightly", 5—"neither liked nor disliked", 6—"liked slightly", 7—"liked moderately", 8—"liked very much", 9—"liked extremely"), according to Stone and Sidel [20]. The participants were asked to assess the following attributes: liking of color, liking of odor, liking of texture, liking of flavor, and overall acceptability. Additionally, the purchase intent was evaluated using a 5-point scale (1—"certainly would not buy" and 5—"certainly would buy"). For this reason, there was a question: "How likely is it that you will buy this product if it will be available at stores?" at the end of the questionnaire card.

Each untrained panelist evaluated a total of eleven biscuits in an odor-free plastic container with a lid labeled with a 3-digit code in a randomized order to avoid an order effect [21]. Warm dark tea was used by the panelists to rinse the mouth between samples testing.

2.5. Determination of Biscuit Physical Properties

The physical properties, such as diameter, thickness, spread ratio, and weight of biscuits were measured according to the procedures described by Mildner-Szkudlarz et al. [8]. Six biscuits were laid edge to edge and measured for diameter (mm). The biscuits were rotated through 90° and the diameter were remeasured. Than the average value was taken. Thickness was measured by stacking six biscuits on top of each other and taking

average thickness (mm). Spread ratio was calculated by dividing the value of diameter by value of biscuits' thickness. Biscuits' weight was determined using an analytical electronic balance (precision—0.0001 g, model AS 110.R2, Radom, Poland).

2.6. Statistical Analysis and Experimental Design for Optimization

The AC values of the baked biscuits were analyzed by five-fold determination of each methanolic extract obtained by conventional extraction and UAE within the same day using the modified DPPH method. The results obtained were presented as the mean ± standard deviation (SD). All data were statistically evaluated by the analysis of variance (ANOVA) test. A post hoc Duncan's test was applied for the calculation of the significant differences among mean values of characteristic oil parameters at the probability level $p < 0.05$.

The RSM was applied to study the simultaneous effects of the RPC content and SAFA content in fats used for biscuits preparation on their AC determined by DPPH method and sensory characteristics. The levels of RPC and SAFA for a CCD and RSM were determined on the basis of preliminary experiments carried out and were varied from 0 to 40 g and 2.3–24.9 g, respectively. The experimental design used for the analysis was a CCD with two factors and three levels. In this experimental design, the factor levels were coded using "−1" for the lowest level (0 g and 2.3 g for RPC content and SAFA content, respectively), "1" for the highest level (40 g and 24.9 g for RPC content and SAFA content, respectively), and "0" for neutral (middle) level (20 g and 7.8 g for RPC content and SAFA content, respectively). The experiments consisted of 11 runs with two factors and two replicates of the central point for the estimation of pure error. The effect of the two independent variables (RPC content and SAFA content) on the responses (Y_n, Y_1—$DPPH_{CE}$, Y_2—$DPPH_{UAE}$, Y_3—color, Y_4—odor, Y_5—texture, Y_6—flavor, Y_7—overall acceptability, and Y_8—purchase intent) was modeled using a polynomial response surface. The second-order response function for the experiments was predicted by the following equation:

$$Y_n = \beta_0 + \beta_1 RPC + \beta_2 SAFA + \beta_{11} RPC^2 + \beta_{22} SAFA^2 + \beta_{12} RPC \times SAFA \quad (1)$$

where Y_n is one of the eight responses; RPC and SAFA represent the independent variables; β_0 is the constant; β_1, β_2 are the linear-term coefficients; β_{11}, β_{22} are the quadratic-term coefficients; and β_{12} is the cross-term coefficient.

The fitness of the model was evaluated by the determination coefficient R^2, the fraction of the variation explained by the model, and analysis of variance (ANOVA). The F-test was applied to confirm whether the variance explained by the regression model was significantly larger than the variance of the residual and to evaluate the model lack-of-fit (model error).

The effects of two factors (RPC content and SAFA content) and their interactions on DPPH in methanolic extracts of biscuits obtained by conventional extraction and UAE, as well as sensory characteristics, were displayed in surface and contour plots. The chemometric analyses were constructed using the Statistica 8.0 software (StatSoft, Tulsa, OK, USA). However, Fizz software (Biosystemes, Courtenon, France) was applied for the collection of all sensory data.

3. Results and Discussion

3.1. Antioxidant Properties of Biscuits' Ingredients

A low-cost and simple DPPH assay based on spectrophotometric mixed-mode (having both electron transfer (ET) and hydrogen atom transfer (HAT) mechanisms) was proposed as the most suitable method to determine the AC of ingredients used in the preparation of functional biscuits. This assay was chosen due to the fact that DPPH radical is known to work well with lipophilic (rather than hydrophilic) antioxidants in alcohol solvents [22]. The AC of RPC, WF, rapeseed oil, margarine, and coconut oil was determined by the modified DPPH method after conventional extraction and UAE, and the obtained results are listed in Table 1.

Table 1. Antioxidant capacity of biscuits' ingredients.

Ingredient	Antioxidant Capacity of Methanolic Extracts	
	Conventional Extraction	Ultrasound-Assisted Extraction
	$DPPH_{CE}$ * ± SD [μmol TE/100 g]	$DPPH_{UAE}$ * ± SD [μmol TE/100 g]
Rapeseed press cake	15,358 ± 412 ^{d,x}	15,630 ± 612 ^{d,x}
Wheat flour	126 ± 7 ^{b,x}	149 ± 5 ^{a,y}
Rapeseed oil	424 ± 10 ^{c,x}	456 ± 12 ^{c,x}
Margarine	171 ± 4 ^{b,x}	217 ± 3 ^{b,y}
Coconut oil	87 ± 2 ^{a,x}	118 ± 2 ^{a,y}

* n = 5; SD—standard deviation; Different letters within the same column (a–d) indicate significant differences between DPPH results of biscuits' ingredients. Different letters (x,y) within the same row indicate significant differences between DPPH of each ingredient extract prepared by the conventional extraction (CE) and the ultrasound-assisted extraction (UAE) (one-way ANOVA and Duncan test, $p < 0.05$).

It is noteworthy that the DPPH values of biscuits' ingredients differ significantly from each other (Duncan test). Moreover, DPPH results of WF, margarine, and coconut oil extracts prepared using the classical extraction method over 30 min were significantly lower than those obtained by the UAE for 5 min (Table 1). However, the Duncan test indicated that methanolic extracts from RPC and rapeseed oil after classical extraction, and sonication did not differ significantly in DPPH results.

The UAE permits higher extraction yields in a shorter time, thereby reducing the electrical energy input. An increase in the AC can be explained by the action of the cavitation bubbles generated during the propagation of the acoustic waves. The cavitation bubbles can disrupt the material cell wall, causing solvent diffusion and increasing the release of antioxidant compounds. On the other hand, ultra-sonication contributes to reducing the particle size of the raw material, which increases the surface area. Moreover, the ultrasound waves generate shear force during ultra-sonication thus, the mass transfer of the original material into an extract solution is enhanced [23].

It is evident that the RPC incorporated into the biscuits was the richest source of antioxidants. The methanolic extracts of RPC revealed the highest DPPH values (15,358 and 15,630 μmol TE/100 g for conventional extraction and UAE, respectively). In our previous report the DPPH ranged between 8770 and 33,980 μmol/100 g for ethanolic, methanolic, and aqueous RPC extracts [24]. These differences between the DPPH results for RPC samples can be explained by the influences of genetic, agronomic, environmental, and technological factors, as well as conditions of extract preparation, mainly polarity of the used solvent, which affect the total level of antioxidants.

However, among the evaluated fats, rapeseed oil had the highest DPPH results (424 and 456 μmol TE/100 g after conventional extraction and UAE, respectively), whereas the antioxidant potential of coconut oil determined by the same method was the lowest (87 and 118 μmol TE/100 g after conventional extraction and UAE, respectively) (Table 1).

Also, Casoni et al. [25] observed significantly higher radical scavenging capacity of rapeseed oil (RSC = 35.12%) to scavenge DPPH radical than coconut oils (RSC = 0.46–2.26%). This suggests that rapeseed oil is a rich source of antioxidants, mainly tocopherols (78.51 mg/100 g) and phenolic compounds (5.77 mg/100 g), while coconut oil contains a trace amount of tocopherols (2.90 mg/100 g) and phenolics (less than 0.01 mg/100 g) [26].

Nevertheless, margarine revealed approximately twofold lower DPPH results (171–217 μmol TE/100 g) in comparison with the refined rapeseed oil (DPPH = 424–456 μmol TE/100 g). Although, somewhat lower AC results (126–149 μmol TE/100 g) were determined for WF.

For comparison, WFs used for the preparation of functional shortbread cookies with fruit pomace, and biscuits incorporated with prickly pear peel, revealed significantly higher DPPH values (377–860,000 μmol TE/100 g) [6,14].

3.2. Antioxidant Capacity of Biscuits with Rapeseed Press Cake

The different fats such as rapeseed oil, margarine, and coconut oil with the declared amounts of SAFA at 2.3, 7.8, and 24.9 g, respectively, and increasing RPC powder levels from 0 to 40 g, were used for the preparation of functional biscuits. It can be noted that as the concentration of RPC in the baked products increased, the biscuits became darker in color (Figure 1).

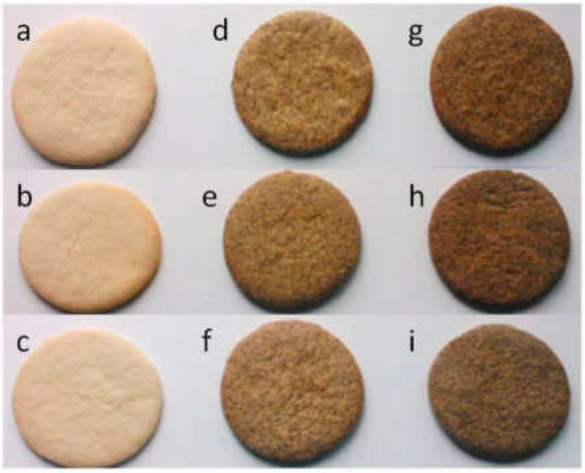

Figure 1. Photograph of biscuits prepared from dough: (**a**–**c**) without, (**d**–**f**) with 20 g and (**g**–**i**) with 40 g of rapeseed press cake using rapeseed oil, margarine, and coconut oil, respectively.

The biscuits fortified with RPC were found to be darker than the control sample without RPC flour. This suggests that RPC flour is a rich source of chlorophyll. Moreover, the darkening of biscuits enriched with RPC can be due to the browning of RPC carbohydrates during baking.

For comparison, with increasing concentrations of by-products, such as olive stone powder (5–15%), white grape pomace (10–30%), goji berry by-product (10–40%), defatted chia flour (5–20%), okara powder (20–40%), prickly pear peel (10–30%), defatted sunflower seed flour (18–36%) in doughs, the color of the enriched biscuits and cookies changed gradually from light brown to brown and finally to dark brown [1,8,10,12–15].

Furthermore, the DPPH values of the baked biscuits increased rapidly with the increasing content of the added RPC (Table 2).

Therefore, the highest DPPH results (8375–10,088 µmol TE/100 g and 4761–7395 µmol TE/100 g) were revealed for methanolic extracts of biscuits with the highest RPC level (40 g) obtained after classical extraction and UAE, respectively. However, the DPPH (535–843 µmol TE/100 g and 598–890 µmol TE/100 g) were the lowest for the studied samples without RPC.

The increase in DPPH values from 20.12 to 30.44%, 3.39–7.55 mmol TE/g, 198–236 mmol TE/g, and 1.5–2.9 mg TE/g was also observed when the biscuit doughs were fortified with increasing amounts of olive stone powder (5–15%), white grape pomace (10–30%), prickly pear peel (10–30%), and defatted sunflower seed flour (18–36%), respectively [1,8,14,15].

Table 2. Antioxidant capacity of the studied biscuits.

Exp.	Independent Variables (Coded Level)		Dependent Variables—Antioxidant Capacity of Methanolic Extracts			
			Conventional Extraction		Ultrasound-Assisted Extraction	
	RPC Content [g]	SAFA Content [g]	$DPPH_{CE}$ [μmol TE/100 g]		$DPPH_{UAE}$ [μmol TE/100 g]	
			Exp.* ± SD	Pred.	Exp.* ± SD	Pred.
1	0 (−1)	2.3 (−1)	843 ± 8 ^{b,x}	1413	890 ± 13 ^{b,x}	1220
2	0 (−1)	7.8 (0)	535 ± 5 ^{a,x}	8	598 ± 27 ^{a,x}	58
3	0 (−1)	24.9 (1)	575 ± 11 ^{a,x}	532	610 ± 5 ^{a,x}	820
4	20 (0)	2.3 (−1)	5588 ± 321 ^{d,x}	5055	3344 ± 49 ^{d,y}	3622
5	20 (0)	7.8 (0)	3600 ± 36 ^{c,x}	3653	2336 ± 16 ^{c,y}	2378
6	20 (0)	24.9 (1)	3904 ± 168 ^{c,x}	4185	3004 ± 53 ^{d,y}	2886
7	40 (1)	2.3 (−1)	10,088 ± 269 ^{g,x}	10,051	7395 ± 356 ^{g,y}	6787
8	40 (1)	7.8 (0)	8375 ± 334 ^{e,x}	8650	4761 ± 217 ^{e,y}	5461
9	40 (1)	24.9 (1)	9428 ± 421 ^{f,x}	9190	5807 ± 208 ^{f,y}	5715
10	20 (0)	7.8 (0)	3705 ± 54 ^{c,x}	3653	2630 ± 18 ^{c,y}	2378
11	20 (0)	7.8 (0)	3402 ± 80 ^{c,x}	3653	2328 ± 25 ^{c,y}	2378

* n = 5; SD—standard deviation; Exp.—experimental data; Pred.—predicted values; different letters within the same column (a–g) indicate significant differences between the DPPH of the studied biscuits; different letters (x,y) within the same row indicate significant differences between DPPH of biscuits extracts prepared by the conventional extraction (CE) and the ultrasound-assisted extraction (UAE) (one-way ANOVA and Duncan test, $p < 0.05$).

Interestingly, biscuits prepared by replacing coconut oil (lower DPPH = 87–118 μmol TE/100 g) with margarine (higher DPPH = 171–217 μmol TE/100 g) and the same content of RPC had a lower level of antioxidants capable of scavenging the free DPPH radical (Tables 1 and 2). Thus, replacement of fat with a richer in SAFA insignificantly increased the DPPH activity of the baked biscuits (Table 2, Duncan test). This can be explained by the fact that SAFA are strongly resistant to deterioration reactions during thermal oxidation, while antioxidants present in the dough are not consumed for the termination of oxidative processes by trapping free radicals.

Unexpectedly, the DPPH results of fortified biscuits' extracts decreased significantly after ultrasonic treatment (Table 2, Duncan test). A decrease in DPPH values for extracts obtained by UAE indicates that natural antioxidants present in RPC were degraded by free radicals generated upon the ultrasound irradiation [27]. On the other hand, the thickness of the boundary layer (the fortified biscuits–solvent interface) did not sufficiently decrease during mixing by means of ultrasound due to the ultrasonic power dissipation (180 W) used for too short treatment time (5 min × 3 times), and the transfer of antioxidants was limited.

3.3. Hedonic Scale Sensory Evaluation of Biscuits with Rapeseed Press Cake

It can be noted that there were significant differences in consumer acceptability between 11 biscuit samples containing 0, 20 and 40 g of RPC flour, respectively (Table 3, Duncan test).

Therefore, the samples studied can be divided into three groups according to the mean sensory scores for overall acceptability. The baked biscuits with the highest amount of RPC flour (40 g) had the lowest sensory score values for overall acceptance. The most preferred samples by Polish consumers were biscuits prepared without RPC (Table 3).

Table 3. Mean sensory scores for the color, odor, texture, flavor, overall acceptability, and purchase intent of the studied biscuits.

Exp.	Independent Variables (Coded Level)		Dependent Variables—Sensory Characteristics											
	RPC Content [g]	SAFA Content [g]	Color #		Odor #		Texture #		Flavor #		Overall Acceptability #		Purchase Intent ##	
			Exp.* ± SD	Pred.	Exp.* ± SD	Pred.	Exp.* ± SD	Pred.	Exp.* ± SD	Pred.	Exp.* ± SD	Pred.	Exp.* ± SD	Pred.
1	0 (−1)	2.3 (−1)	6.03 ± 0.56 ^{c}	6.12	5.77 ± 0.82 ^{c}	5.82	6.88 ± 0.42 ^{c}	6.51	6.89 ± 0.78 ^{c}	6.56	6.79 ± 0.87 ^{c}	6.60	3.32 ± 0.47 ^{c}	3.29
2	0 (−1)	7.8 (0)	6.10 ± 0.75 ^{c}	5.93	5.73 ± 0.53 ^{c}	5.64	5.52 ± 0.61 ^{a,b}	5.84	5.85 ± 0.82 ^{c}	6.14	6.07 ± 0.62 ^{c}	6.17	3.19 ± 0.79 ^{c}	3.21
3	0 (−1)	24.9 (1)	5.81 ± 1.14 ^{b,c}	5.89	6.10 ± 1.11 ^{c}	6.14	6.10 ± 0.97 ^{c}	6.15	6.42 ± 1.20 ^{c}	6.46	6.26 ± 0.45 ^{c}	6.34	3.33 ± 0.66 ^{c}	3.34
4	20 (0)	2.3 (−1)	5.56 ± 0.72 ^{b}	5.48	4.42 ± 0.96 ^{b}	4.45	6.30 ± 0.82 ^{c}	6.44	4.74 ± 0.46 ^{b}	4.90	4.68 ± 0.68 ^{b}	4.85	2.52 ± 0.68 ^{b}	2.52
5	20 (0)	7.8 (0)	5.29 ± 0.45 ^{b}	5.34	4.16 ± 0.25 ^{a,b}	4.20	5.62 ± 0.75 ^{a,b}	5.77	4.45 ± 0.84 ^{b}	4.45	4.44 ± 0.41 ^{b}	4.40	2.41 ± 0.41 ^{b}	2.45
6	20 (0)	24.9 (1)	5.56 ± 0.50 ^{b}	5.44	4.53 ± 0.71 ^{b}	4.49	6.36 ± 0.53 ^{c}	6.07	4.89 ± 0.77 ^{b}	4.65	4.74 ± 0.63 ^{b}	4.51	2.63 ± 0.57 ^{b}	2.60
7	40 (1)	2.3 (−1)	4.51 ± 0.71 ^{a}	4.50	3.93 ± 0.66 ^{a}	3.85	5.30 ± 0.91 ^{a}	5.53	3.48 ± 0.51 ^{a}	3.65	3.44 ± 0.79 ^{a}	3.46	1.78 ± 0.34 ^{a}	1.81
8	40 (1)	7.8 (0)	4.45 ± 0.33 ^{a}	4.41	3.45 ± 0.98 ^{a}	3.53	5.33 ± 0.35 ^{a}	4.86	3.52 ± 0.63 ^{a}	3.15	3.15 ± 0.52 ^{a}	2.99	1.81 ± 0.45 ^{a}	1.75
9	40 (1)	24.9 (1)	4.62 ± 0.81 ^{a}	4.66	3.62 ± 0.36 ^{a}	3.62	4.93 ± 0.49 ^{a}	5.17	3.03 ± 0.78 ^{a}	3.23	2.89 ± 0.64 ^{a}	3.04	1.90 ± 0.51 ^{a}	1.93
10	20 (0)	7.8 (0)	5.20 ± 0.52 ^{b}	5.34	4.23 ± 0.21 ^{b}	4.20	5.66 ± 0.62 ^{a,b}	5.77	4.32 ± 0.71 ^{b}	4.45	4.31 ± 0.41 ^{b}	4.40	2.47 ± 0.47 ^{b}	2.45
11	20 (0)	7.8 (0)	5.31 ± 0.43 ^{b}	5.34	4.19 ± 0.29 ^{a,b}	4.20	5.87 ± 0.77 ^{b}	5.77	4.50 ± 0.87 ^{b}	4.45	4.38 ± 0.46 ^{b}	4.40	2.43 ± 0.51 ^{b}	2.45

* n = 72; SD—standard deviation; # Color, odor, texture, flavor and overall acceptability are based on the 9 hedonic rating scale system, with anchoring point, 1—"disliked extremely" and 9—"liked extremely"; ## Purchase intent is based on the 5 scoring scale with anchoring point, 1—"certainly would not buy", 5—"certainly would buy"; Exp.—experimental data; Pred.—predicted values; different letters within the same column indicate significant differences between the studied biscuits' sensory characteristics (one-way ANOVA and Duncan test, $p < 0.05$).

The consumers' results revealed that the mean sensory scores for color, odor, texture, flavor, and overall acceptability of the non-supplemented biscuits prepared by using different types of fats ranged between 5.81 and 6.10, 5.73–6.10, 5.52–6.88, 5.85–6.89, and 6.07–6.79, respectively, and corresponded to the classifications "liked slightly" and "liked moderately" on the hedonic scale where the maximum score is 9. In the context of the proposed products' purchase intent frequency, the biscuits without RPC received the highest scores (3.19–3.33) on a 5-point scale and qualification as "would buy".

On the contrary, with increasing RPC content in the dough, the fortified biscuits' color changed gradually from light (biscuits without RPC) to dark for biscuits with the highest amount of RPC flour (40 g). Therefore, these samples scored the lowest for color (4.45–4.62), odor (3.45–3.93), flavor (3.03–3.52), and overall acceptability (2.89–3.44). The biscuits with the highest RPC level were characterized by a typical intensity of bitter and grassy flavor due to the high concentration of phenolic compounds present in RPC.

Nevertheless, insignificant differences in the mean texture scores were found between control samples (without RPC) and biscuits after the replacement of WF with 20 g of RPC flour (Table 3, Duncan test). However, biscuits incorporated with the highest RPC amount (40 g) scored the lowest for texture (4.93–5.33). For this reason, biscuits formulated with 40 g of RPC flour had the lowest purchase intent scores (1.78–1.90), being largely qualified with "would not buy". The biscuits enriched with 20 g of RPC flour were predominantly qualified as "would probably buy", but there were significant differences with the qualification "would buy" for the control samples without RPC (Table 3, Duncan test).

The Duncan test indicated insignificant differences in scores for color, odor, flavor, overall acceptability, and purchase intent between biscuits obtained from different types of fats with the same content of RPC (Table 3). Only biscuits without RPC and with 20 g of RPC flour prepared by using margarine had significantly lower texture scores than those baked from dough containing rapeseed and coconut oils.

The obtained results suggest that the replacement of WF with varying RPC levels in the recipe affects the overall acceptability of the functional biscuits due to atypical attributes such as bitter and grassy flavor and strange odor. A new component such as chocolate could probably mask the negative attributes and increase the overall acceptability of biscuits fortified with RPC.

3.4. Optimization Process

3.4.1. Fitting the Models

Experimental results of the responses, including the DPPH values and sensory characteristics of biscuits fortified with RPC, were fitted to the CCD, and the least-squares technique was used for calculation of the regression coefficients of the individual linear, quadratic, and interaction terms (Table 4).

Table 4. Regression coefficients of quadratic polynomial models for the studied response variables.

Response Variables	Model Coefficients					
	Intercept	RPC	SAFA	RPC^2	$SAFA^2$	RPC × SAFA
$DPPH_{CE}$ [μmol TE/100 g]	2227.6 **	148.3 **	−383.3 **	1.7 *	12.7 **	2.2×10^{-2}
$DPPH_{UAE}$ [μmol TE/100 g]	1909.4 *	102.7 *	−325.7 *	9.5×10^{-1}	11.3 *	7.4×10^{-1}
Color	6.2 ***	-2.5×10^{-2} *	-4.8×10^{-2}	-4.1×10^{-4} *	1.4×10^{-3}	4.4×10^{-4}
Odor	5.9 ***	-8.7×10^{-2} ***	-6.2×10^{-2} *	9.7×10^{-4} **	2.8×10^{-3} **	-6.1×10^{-4} *
Texture	6.9 ***	1.7×10^{-2}	-1.8×10^{-1} *	-1.0×10^{-3} *	6.2×10^{-3} *	4.6×10^{-6}
Flavor	6.8 ***	-9.2×10^{-2} **	-1.2×10^{-1} *	4.9×10^{-4}	4.2×10^{-3} *	-3.5×10^{-4}
Overall acceptability	6.9 ***	-9.6×10^{-2} **	-1.2×10^{-1} *	4.5×10^{-4} *	3.9×10^{-3} *	-1.7×10^{-4}
Purchase intent	3.3 ***	-4.1×10^{-2} **	-2.4×10^{-2}	8.5×10^{-5}	9.4×10^{-4} *	7.5×10^{-5}

Significant at the * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The second-order polynomial equations generated were used to predict the responses (Tables 2 and 3). The ANOVA results for the predicted response quadratic models are listed in Table 5. ANOVA test revealed that the quadratic polynomial models adequately represent responses of DPPH for extracts obtained by conventional extraction and UAE as well as sensory properties due to an insignificant lack-of-fit (F ranged between 2.89 and 18.83, $p > 0.05$) for each estimated response (Table 5).

Table 5. Analysis of variance (ANOVA) results for the studied responses of biscuits: $DPPH_{CE}$, $DPPH_{UAE}$, color, odor, texture, flavor, overall acceptability, and purchase intent.

Model Parameters	Degree of Freedom	Sum of Squares	Mean Square	F Value	Sum of Squares	Mean Square	F Value
			$DPPH_{CE}$			$DPPH_{UAE}$	
Regression	5	1.13×10^{8}	2.27×10^{7}	957.63 **	4.33×10^{7}	8.67×10^{6}	292.67 *
Residual	5	1.17×10^{6}	2.34×10^{5}		1.47×10^{6}	2.94×10^{5}	
Lack-of-fit	3	1.12×10^{6}	3.74×10^{5}	15.80	1.41×10^{6}	4.71×10^{5}	15.90
Pure error	2	4.73×10^{4}	2.37×10^{4}		5.92×10^{4}	2.96×10^{4}	
Total	10	1.15×10^{8}			4.48×10^{7}		
R^2			0.9902			0.9689	
Adjusted R^2			0.9804			0.9378	
			Color			Odor	
Regression	5	3.06	6.11×10^{-1}	178.04 *	7.90	1.58	1281.56 **
Residual	5	8.85×10^{-2}	1.77×10^{-2}		3.07×10^{-2}	6.15×10^{-3}	
Lack-of-fit	3	8.16×10^{-2}	2.72×10^{-2}	7.92	2.83×10^{-2}	9.42×10^{-3}	7.64
Pure error	2	6.87×10^{-3}	3.43×10^{-3}		2.47×10^{-3}	1.23×10^{-3}	
Total	10	3.14			7.93		
R^2			0.9737			0.9962	
Adjusted R^2			0.9477			0.9925	
			Texture			Flavor	
Regression	5	2.79	5.59×10^{-1}	154.89 *	14.17	2.83	328.31 *
Residual	5	7.16×10^{-1}	1.43×10^{-1}		5.05×10^{-1}	1.01×10^{-1}	
Lack-of-fit	3	6.89×10^{-1}	2.27×10^{-1}	12.56	4.88×10^{-1}	1.63×10^{-1}	18.83
Pure error	2	3.61×10^{-2}	1.80×10^{-2}		1.73×10^{-2}	8.63×10^{-3}	
Total	10	3.51			14.68		
R^2			0.7751			0.9664	
Adjusted R^2			0.5503			0.9328	
			Overall acceptability			Purchase intent	
Regression	5	15.59	3.12	736.33 *	3.04	6.08×10^{-1}	651.15 *
Residual	5	1.93×10^{-1}	3.85×10^{-2}		9.95×10^{-3}	1.99×10^{-3}	
Lack-of-fit	3	1.84×10^{-1}	6.14×10^{-2}	14.49	8.08×10^{-3}	2.69×10^{-3}	2.89
Pure error	2	8.47×10^{-3}	4.23×10^{-3}		1.87×10^{-3}	9.33×10^{-4}	
Total	10	15.78			3.05		
R^2			0.9882			0.9969	
Adjusted R^2			0.9763			0.9938	

* Significant at the $p < 0.05$ level; ** Significant at the $p < 0.001$ level; DPPH—2,2-diphenyl-1-picrylhydrazyl method; CE—methanolic extracts obtained by conventional extraction; UAE—methanolic extracts obtained by ultrasound-assisted extraction.

Moreover, high F-values ranged between 154.89 and 1281.56, and the probability values less than 0.05 and 0.001 indicated that the empirical models were significant (Table 5). For this reason, these proposed mathematical models are valid and convenient for predicting the antioxidant potential and sensory properties of functional biscuits prepared under any combination of RPC amount and fat type.

Additionally, determination coefficients (R^2) and adjusted R^2 were calculated to estimate the proposed models' goodness of fit (Table 5). The R^2 values (0.9664–0.9969) ensure a satisfactory fit of the proposed models to represent actual relationships between the responses ($DPPH_{CE}$, $DPPH_{UAE}$, color, odor, flavor, overall acceptability, and purchase intent) and the independent variables (RPC content and SAFA content). However, high values of adjusted R^2 = 0.9328–0.9938 indicated a close agreement between experimental and predicted results. On the contrary, the values of R^2 = 0.7751 and adjusted R^2 = 0.5503 for the response of biscuits' texture suggest that a high proportion of variability cannot be explained by the model, because R^2 should be at least 0.80 for a good fit.

All linear and quadratic parameters of the empirical models were highly significant (F = 19.73–4554.65, p = 0.00022–0.047) for the DPPH results of methanolic extracts obtained by conventional extraction and overall acceptability of the biscuits enriched with RPC, while the interaction between amounts of RPC and SAFA in functional biscuits produced a significant negative effect only on their odor (F = 66.85, p = 0.015). However, the quadratic parameter of the SAFA content and linear term of RPC of the models significantly influenced (F values ranged between 19.10 and 3221.88, $p < 0.05$) on flavor and purchase intent of the prepared biscuits. In addition, only two parameters (RPC and RPC^2) had significant effects on color of the novel biscuits (F = 19.64–846.11, p = 0.047–0.0012). Both linear (RPC, SAFA) and quadratic ($SAFA^2$) parameters of the models were highly significant (F = 27.44–1332.02 and p = 0.035–0.00075) for DPPH of methanolic extracts after UAE, whereas linear term of SAFA and interaction between independent variables (RPC × SAFA) caused insignificant effects (F = 0.00026–10.98, p = 0.080–0.99) on the texture of studied samples.

It is noteworthy that the variable with the largest positive effect on the antioxidant potential of the baked biscuits determined by DPPH assay was the linear term of RPC content (F = 1332.02–4554.65, $p < 0.001$). Nevertheless, this independent contributed variable the most negatively to all sensory characteristics of the fortified biscuits (F = 76.73–5902.97, $p < 0.05$).

3.4.2. Analysis of the Response Surfaces

The effects of the two independent variables (amounts of RPC and SAFA) on antioxidant potential of biscuits extracted by classical extraction and analyzed by the DPPH assay as well as their five sensory characteristics (color, odor, flavor, overall acceptability, and purchase intent) were illustrated using surface response and contour plots of the quadratic polynomial models (Figure 2).

As can be seen, the generated shapes of the response surfaces for AC results differ from those obtained for sensory scores. The DPPH of baked biscuits significantly increased with the increasing concentration of RPC in the dough (Figure 2a). The parabolic shape of the response surface for DPPH was caused by the positive values of the quadratic terms of RPC amount and content of SAFA in the fat used.

On the contrary, scores for color, odor, flavor, and overall acceptability and purchase intent of the biscuits analyzed were the highest when RPC was not added to WF and rapeseed oil or coconut oil were used for dough preparation (Figure 2b–f). The surface plots of odor, flavor, overall acceptability, and purchase intent indicate that the negative linear of RPC content and positive quadratic term of SAFA content were significant (Figure 2c–f). The elliptical contour of the odor plot confirms that there was an interaction between independent variables (Figure 2c), whereas the significant negative linear and quadratic effects of RPC amount resulted in a decrease of the score for biscuit color after the fortification with a higher RPC amount (Figure 2b).

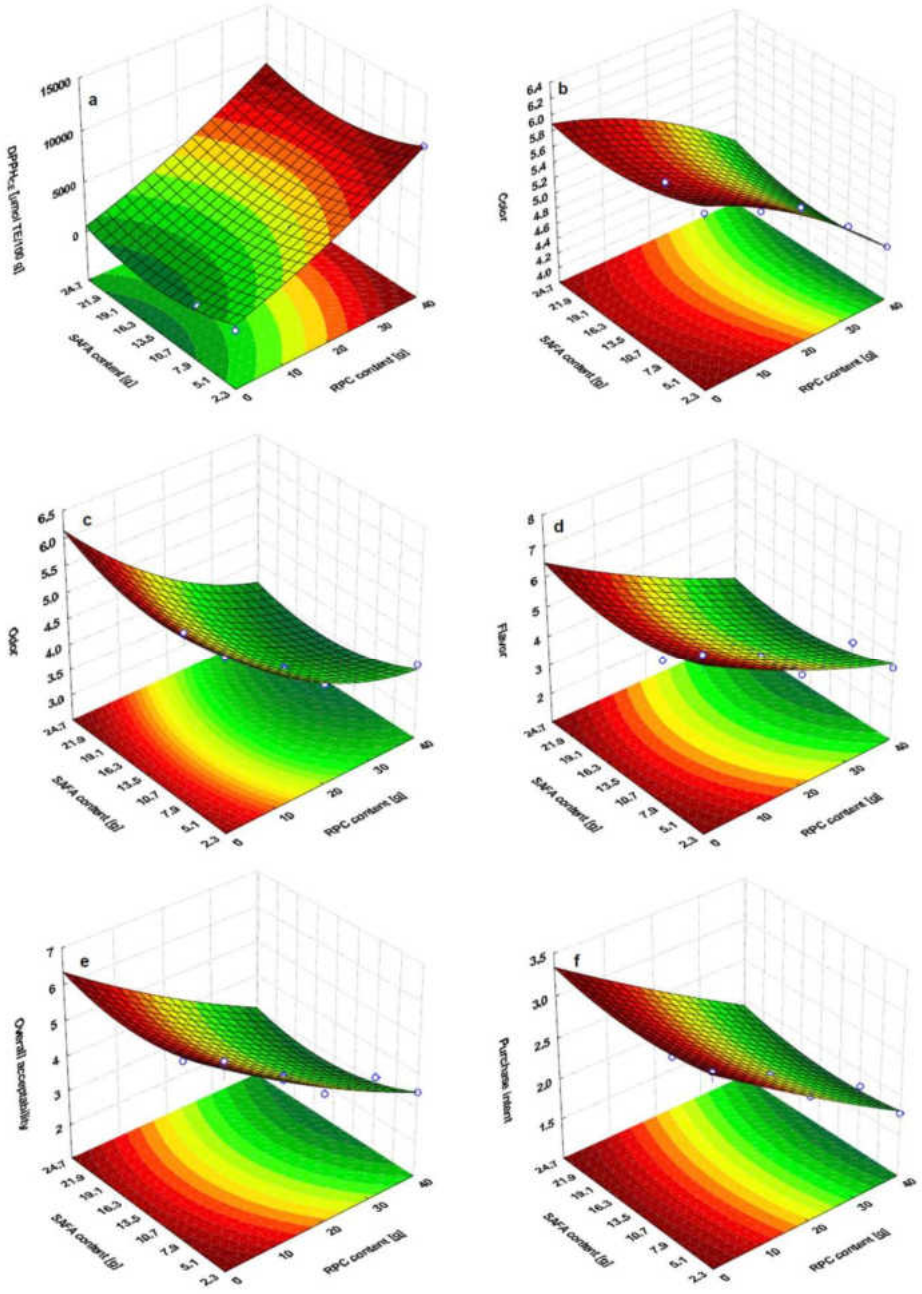

Figure 2. Response surfaces and contour plots for: (**a**) DPPH results of extracts obtained by conventional extraction ($DPPH_{CE}$), (**b**) color, (**c**) odor, (**d**) flavor, (**e**) overall acceptability, and (**f**) purchase intent of functional biscuits expressed as a function of the rapeseed press cake (RPC) content and saturated fatty acids (SAFA) content.

3.4.3. Verification of Predictive Models

Optimization of the prepared functional biscuits was performed to measure the optimum levels of independent variables (amounts of RPC and SAFA) to achieve the desired response goals. The antioxidant potential and sensory characteristics were specified as desired to be maximized. To evaluate the sufficiency of the proposed mathematical models, verification experiments were carried out at the predicted conditions derived from the RSM analysis. The predicted and experimental results are presented in Table 6.

Table 6. Predicted and experimental values of the studied responses for the optimum ingredients' amount.

Response Variables	Optimum Ingredients' Amount		Pred. Values	Exp. Values * ± SD	*p* Values
	RPC Content [g]	SAFA Content [g]			
$DPPH_{CE}$ [µmol TE/100 g]	40	2.3	10,050	10,151 ± 100	0.476
$DPPH_{UAE}$ [µmol TE/100 g]	40	2.3	6787	6875 ± 67	0.375
Color #	0	24.9	5.89	5.96 ± 0.11	0.632
Odor #	0.2	24.7	6.11	6.17 ± 0.13	0.736
Texture #	8.2	2.3	6.58	6.60 ± 0.14	0.913
Flavor #	0	24.9	6.46	6.52 ± 0.18	0.785
Overall acceptability #	0	24.9	6.34	6.39 ± 0.05	0.514
Purchase intent ##	0.1	24.8	3.33	3.48 ± 0.29	0.693

* n = 3; SD—standard deviation; # 9-point quality scale; ## 5-point quality scale.

Insignificant differences ($p > 0.05$) between the predicted and experimental response values confirmed that the proposed models were accurate and adequate for the optimization of amounts of two ingredients to baking functional biscuits with acceptable levels of RPC and sensory characteristics.

3.4.4. Physical Properties of Biscuits for Optimum Levels of Ingredients

The doughs were prepared by using RPC flour and SAFA at optimum levels for antioxidant potential (RPC = 40 g, SAFA = 2.3 g), and overall acceptability (RPC = 0 g, SAFA = 24.9 g) and physical properties of the baked biscuits were determined (Table 7).

Table 7. Physical properties of biscuits for the optimum ingredients' amount.

Optimum Levels	Diameter * ± SD [mm]	Thickness * ± SD [mm]	Spread Ratio * ± SD	Weight * ± SD [g]
RCP = 40 g SAFA = 2.3 g	67.00 ± 1.15 ^{a}	6.78 ± 0.26 ^{a}	9.90 ± 0.44 ^{a}	12.1644 ± 0.26 ^{a}
RCP = 0 g SAFA = 24.9 g	69.75 ± 1.50 ^{b}	7.75 ± 0.65 ^{b}	9.04 ± 0.65 ^{a}	15.1991 ± 0.65 ^{b}

* n = 6; SD—standard deviation; Different letters within the same column (a,b) indicate significant differences between physical parameters of biscuits.

The Duncan test indicated that RPC flour incorporation significantly decreased the diameter, thickness, and weight of the studied biscuits.

The decrease in diameter and thickness of cookies and biscuits with increasing amounts of added goji berry by-product (10–40%), okara powder (20–40%), prickly pear peel (10–30%), and sunflower seed flour (18–36%) was observed by other authors [10,13–15]. In contrast, the diameter value (52.36 mm) of control biscuit was lower than those (52.52–55.88 mm) measured for biscuits with 10–30% of white grape pomace, whereas the thickness of the studied samples decreased significantly (13.83–9.05 mm) after supplementation [8].

However, insignificant differences in the mean spread ratio were found between samples without RPC containing coconut oil and biscuits prepared with 40 g of RPC and rapeseed oil.

The insignificant increase in spread ratio and the significant contraction of diameter after addition of RPC flour can be attributed to the reduction of total gluten content as well as the increase in protein amount [10]. Probably, the weight of fortified biscuits was lower due to low oil absorption capacity of RPC flour [28]. For comparison, the spread ratio of biscuits before (48.9) and after (48.0–48.2) the addition of prickly pear peel did not differ significantly [14]. On the other hand, the changes in spread ratio of biscuits with white grape pomace (3.79–6.18) were significant at substitution levels between 10% and 30% [8].

4. Conclusions

The incorporation of RPC flour and the replacement of margarine by rapeseed oil into the formulation of WF-based biscuits resulted in a nutritionally enhanced product with a higher amount of antioxidants. Moreover, the UAE of ingredients used for dough preparation produced higher recoveries of total antioxidants in comparison with the conventional extraction.

A higher percentage of RPC in the formula increased the antioxidant potential of the proposed biscuits. However, these biscuits had lower sensory scores for color, odor, texture, flavor, overall acceptability, and purchase intent.

The estimated parameters, RPC content, and SAFA content affected the DPPH results, and sensory characteristics of the baked biscuits. However, the amount of RPC flour had a greater positive effect on the antioxidant potential of biscuits than the fat type used for dough preparation. Nevertheless, the added level of RPC was a more negatively effective independent variable on the sensory properties of the biscuits studied.

The good agreement between the predicted values and experimental results verified the validity of the proposed models and the optimal ingredient amounts for the baking of biscuits incorporating by-products of rapeseed oil industry.

Author Contributions: Conceptualization, A.S.-C., S.P. and M.M.; Data curation, A.S.-C., S.P. and M.M.; Formal analysis, S.P. and M.M.; Investigation, A.S.-C., S.P. and M.M.; Methodology, A.S.-C., S.P. and M.M.; Supervision, A.S.-C.; Visualization, A.S.-C., S.P. and M.M.; Writing—original draft, A.S.-C., S.P. and M.M.; Writing—review & editing, A.S.-C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors would like to thank Angelika Gawrońska for analysis of antioxidant capacity.

Conflicts of Interest: The authors declare no conflict of interest.

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(D2) Poliński, S., Kowalska, S., Topka, P., & Szydłowska-Czerniak, A. (2021). Physicochemical, Antioxidant, Microstructural Properties and Bioaccessibility of Dark Chocolate with Plant Extracts. *Molecules*, *26*(18), 5523.

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Physicochemical, Antioxidant, Microstructural Properties and Bioaccessibility of Dark Chocolate with Plant Extracts

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Abstract: In this study, dark chocolates (DCh) containing zinc lactate (ZnL) were enriched with extracts from elderberries (EFrE), elderflowers (EFlE), and chokeberries (ChFrE) to improve their functional properties. Both dried plant extracts and chocolates were analyzed for antioxidant capacity (AC) using four different analytical methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), cupric ion-reducing antioxidant capacity (CUPRAC), and ferric-reducing antioxidant power (FRAP), while total phenolic content (TPC) was determined by Folin–Ciocalteu (F–C) assay. An increase in antioxidant properties of fortified chocolates was found, and the bioaccessibility of their antioxidants was evaluated. The highest AC and TPC were found in ChFrE and chocolate with chokeberries (DCh + ChFrE) before and after simulated in vitro digestion. Bioaccessibility studies indicated that during the simulated digestion the AC of all chocolates reduced significantly, whereas insignificant differences in TPC results were observed between chemical and physiological extracts. Moreover, the influence of plant extracts on physicochemical parameters such as moisture content (MC), fat content (FC), and viscosity of chocolates was estimated. Furthermore, scanning electron microscopy with dispersive energy spectroscopy (SEM-EDS) was used to analyze surface properties and differences in the chemical composition of chocolates without and with additives.

Keywords: dark chocolate; plant extracts; antioxidant capacity; phenolics content; in vitro digestion; scanning electron microscopy with dispersive energy spectroscopy

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Citation: Poliński, S.; Kowalska, S.; Topka, P.; Szydłowska-Czerniak, A. Physicochemical, Antioxidant, Microstructural Properties and Bioaccessibility of Dark Chocolate with Plant Extracts. *Molecules* **2021**, *26*, 5523. https://doi.org/10.3390/molecules26185523

Academic Editors: Alice Martins and Joaquina Pinheiro

Received: 15 August 2021
Accepted: 8 September 2021
Published: 11 September 2021

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1. Introduction

Dark chocolate (DCh) can be part of a diet that affects global public health and meets current dietary recommendations due to the antioxidant properties of cocoa mass, a principal ingredient containing high amounts of polyphenols, flavonoids, vitamins, and minerals [1].

On the other hand, the characteristics and manufacturing of chocolate potentially allow the addition of pro-health ingredients such as dried fruits and other parts of plants. Therefore, in recent years, the effects of dried fruits and plants, such as prunes, papaya, apricots, raisins, cranberries, lychee, longan, nettle, red raspberry leaves, Sakura green tea, turmeric powder, and yellow tea powdered extracts on the antioxidant capacity (AC) of white, milk, semisweet, and dark chocolates determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), ferric-reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC), as well as levels of dominant antioxidants have been investigated [2–7]. The incorporation of dried fruit and plant extracts into chocolates influenced their antioxidant and sensory properties and contributed to the dietary intake of polyphenolic antioxidants.

It is worth noting that there has been no reference to the changes in the antioxidant potential of chocolates fortified with elderberry and chokeberry extracts. However, black

chokeberry (*Aronia melanocarpa*) fruits are some of the richest sources of bioactive compounds, including flavonols, flavanols, phenolic acids, proanthocyanidins, and anthocyanins. Although these fruits are rarely used for direct consumption due to a tart and bitter taste caused by a high content of phenolic compounds, they can be widely utilized for the production of natural powders and dietary supplements with health-promoting properties [8]. Moreover, the black fruits, flowers, leaves, and bark of elderberry (*Sambucus nigra* L) contain high amounts of bioactive compounds such as phenolics, anthocyanins, and others possessing strong antioxidant, antibacterial, antiviral, antidepressant, antitumor, anti-inflammatory, antihypoglycemic, immune-modulating properties as well as the ability to reduce body fat and lipid concentration [9,10]. Nevertheless, gastrointestinal digestion affected elderberry antioxidants causing changes in their AC, chemical structure, and stability [11].

On the other hand, in vitro digestion models are becoming useful tools for studying the digestive properties of chocolates and cocoa-based products and for understanding the mechanisms of lipid absorption and the bioaccessibility of amino acids, bioactive amines, polyphenols, and other antioxidants [6,12,13].

Taking into account the health-promoting properties of DCh, elderberries, elderflowers, and chokeberries, it is possible to put forward the hypothesis that chocolates fortified with these dried plants and zinc lactate (ZnL) could be products for the enhancement of consumers' health.

Therefore, this work aimed to quantify for the first time the AC of DCh with ZnL after supplementation of powdered extracts from elderberries (EFrE), elderflowers (EFlE), and chokeberries (ChFrE). Moreover, the stability of their AC and total phenolic content (TPC) after in vitro gastrointestinal digestion using the DPPH, ABTS, cupric reducing antioxidant capacity (CUPRAC), FRAP, and Folin–Ciocalteu (F–C) methods, respectively, was estimated and discussed. The influence of these dried fruits and flowers on the physicochemical properties of DCh was determined. Finally, scanning electron microscopy (SEM) images of the supplemented chocolates and SEM with dispersive energy spectroscopy (EDS) techniques were used to study changes in particle shapes after fortification with nutritional additives rich in bioactive components, and to evaluate the chemical composition of the prepared chocolate samples.

2. Results and Discussion

2.1. Antioxidant Capacity of Plant Extracts

In order to receive reliable data on the overall antioxidant potential of EFrE, EFlE, and ChFrE, two radical scavenging assays (DPPH and ABTS) and two reducing methods (CUPRAC and FRAP) were applied. The obtained AC results (Table 1) revealed the same trend in the ability of the investigated extracts to act against free radicals ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) as the capability of compounds present in them to change the oxidation state of transition metals in complexes (copper(II)-neocuproine and iron(III)-2,4,6-tripyridyl-s-triazine).

Table 1. Antioxidant capacity and total phenolic content in plant extracts added to chocolates.

Sample	DPPH * ± SD (μmol TE/g)	ABTS * ± SD (μmol TE/g)	CUPRAC * ± SD (μmol TE/g)	FRAP * ± SD (μmol TE/g)	TPC * ± SD (mg GA/g)
EFrE	1911.6 ± 56.9 ^{a}	2337.1 ± 18.9 ^{a}	5710.4 ± 99.5 ^{a}	229.8 ± 4.5 ^{a}	32.5 ± 1.3 ^{a}
EFlE	4765.0 ± 26.6 ^{b}	6258.7 ± 131.2 ^{b}	6224.0 ±54.5 ^{b}	595.2 ± 3.4 ^{b}	81.9 ± 3.9 ^{b}
ChFrE	22,269.7 ± 161.4 ^{c}	27,194.2 ± 242.0 ^{c}	113,882.3 ± 303.1 ^{c}	2341.3 ± 10.3 ^{c}	3506 ± 2.3 ^{c}

* $n = 3$; SD—standard deviation; Mean values within the same column marked by different superscript small letters (a–c) are significantly different (one-way ANOVA and Duncan test, $p < 0.05$).

Results of AC and TPC illustrated that ChFrE was the richest source of hydrophilic and lipophilic antioxidants (Table 1). Therefore, the Duncan test indicated that AC and TPC in ChFrE were significantly higher than DPPH, ABTS, CUPRAC, FRAP, and TPC in EFrE and EFlE. On the other hand, differences in AC of the same extract determined by four modified analytical methods were observed. These discrepancies between the AC results may be attributed to the different mechanisms of the applied analytical methods,

including DPPH and ABTS mixed-mode methods involving both electron transfer (ET) and hydrogen atom transfer (HAT) mechanisms, as well as ET assays such as CUPRAC and FRAP. Simultaneous determination of lipophilic and hydrophilic antioxidants in various matrices can be achieved using CUPRAC and ABTS methods, while the DPPH test is suitable for evaluating the AC of lipophilic compounds. In contrast to the DPPH procedure, FRAP assay is specific for potential hydrophilic antioxidants, but it does not respond well to lipophilic antioxidants [14]. For this reason, AC results of water extracts from EFrE, EFlE, and ChFrE revealed higher reducing potencies for CUPRAC assay and antiradical activity determined by the ABTS method than scavenging capacity of DPPH radical and reducing abilities analyzed by the FRAP test (Table 1).

For comparison, water extract from chokeberries also possessed high antioxidant properties, and its AC when analyzed by the same antioxidant assays showed a similar tendency: ABTS (219.3 μmol TE/g) ≈ CUPRAC (212.9 μmol TE/g) > DPPH (87.2 μmol TE/g) > FRAP (57.4 μmol TE/g) [15].

It is noteworthy that the EFrE had significantly lower radical scavenging properties of DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ (11 times lower) as well as reducing abilities of Fe(III) (10 times lower) and Cu(II) (20 times lower) ions than ChFrE. However, DPPH, ABTS, FRAP, and TPC values in EFlE were above 2 times higher than those found for EFrE. Similarly, EFlE showed significantly higher reducing power determined by the CUPRAC method than EFrE (Duncan test, Table 1).

The AC and TPC data for ChFrE and EFrE are in agreement with those reported by other studies [16–18], showing that black chokeberries had much higher radical-scavenging activities evaluated by the DPPH and ABTS tests and higher amounts of phenolics than elderberries (Table 2).

Table 2. Antioxidant capacity and total phenolic content in elderberry, elderflower, and chokeberry extracts, as determined by other authors.

Analytical Methods	EFrE	EFlE	ChFrE
DPPH	100.16 μmol TE/g [16]		181.07 μmol TE/g [16]
	30–45% [18]		90–95% [18]
	50.25–67.69% [19]	91.95–94.15% [19]	
ABTS	37.91 μmol TE/g [16]		78.90 μmol TE/g [16]
	44.02 g/kg [17]		54.27 g/kg [17]
	3.20–36.50 mM TE/kg [20]	44.87–118.26 mM TE/kg [20]	
	397.5–581.3 μmol TE/g [21]	327.7–421.5 μmol TE/g [21]	
F–C	4415.33 mg/kg [16]		7194.40 mg/kg [16]
	80.71 g/kg [17]		115.15 g/kg [17]
	700–1050 mg/100 g [18]		3100–3600 mg GA/100 g [18]
	2687.6–6831.1 mg GA/kg [20]	7410–40,137 mg GA/kg [20]	
	5678.8–7087.3 mg ChA/100 g [21]	6164.4–7561.8 mg ChA/100 g [21]	

GA—gallic acid; ChA—chlorogenic acid; TE—Trolox

Additionally, the flower alcoholic extracts from elderberries exhibited stronger neutralizing activity of DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals and higher TPC in comparison with those results for berry extracts (Table 2 [19,20]). This suggests that cinnamic acids, flavonols, and anthocyanins are dominant in elderberry flowers. It is evident that the ABTS and TPC values obtained by Mikulic-Petkovsek et al. [20] were significantly lower than those determined for our EFrE and EFlE samples (Tables 1 and 2). On the contrary, Młynarczyk et al. [21] found a somewhat higher ABTS for elderberries than ABTS for elderflowers from cultivars grown in the wild and in an orchard. However, elderflowers were richer in TPC when compared with phenolic levels in fruits (Table 2).

2.2. Antioxidant Capacity and Total Phenol Content in Chocolates before and after In Vitro Simulated Digestion

Regarding the antioxidative characteristics of the investigated DCh enriched with EFrE, EFlE, and ChFrE, the AC determined by four different analytical methods and TPC results followed the same trend as for plant extracts.

It can be noted that the addition of plant extracts to DCh with ZnL caused a plant extract type-dependent statistically significant increase in DPPH, ABTS, CUPRAC, FRAP, and TPC results of fortified chocolate samples (Duncan test, Table 3).

Table 3. Antioxidant capacity and total phenolic content in the investigated chocolates before (Ac:H_2O:AA extract) and after (physiological extract) in vitro digestion.

Analytical Method	DCh		DCh + EFrE		DCh + EFlE		DCh + ChFrE	
	Ac:H_2O:AA Extract	Physiological Extract	Ac:H_2O:AA Extract	Physiological Extract	Ac:H_2O:AA Extract	Physiological Extract	Ac:H_2O:AA Extract	Physiological Extract
DPPH * ± SD (μmol TE/g)	144.2 ± 5.3^{b}	72.2 ± 5.1^{a}	149.3 ± 4.6^{b}	146.1 ± 1.9^{b}	364.3 ± 17.1^{c}	151.4 ± 12.5^{b}	942.7 ± 31.0^{e}	745.6 ± 12.7^{d}
ABTS * ± SD (μmol TE/g)	$433.9 \pm 11.5^{a,b}$	407.0 ± 1.1^{a}	672.6 ± 2.5^{d}	467.6 ± 1.6^{b}	1211.0 ± 8.92^{f}	575.8 ± 0.7^{c}	3592.0 ± 76.7^{g}	755.3 ± 6.4^{e}
CUPRAC * ± SD (μmol TE/g)	2985.4 ± 14.7^{e}	1346.3 ± 19.7^{c}	3118.5 ± 7.8^{f}	883.1 ± 36.0^{a}	3752.3 ± 27.3^{g}	1222.9 ± 46.6^{b}	$12,945.7 \pm 132.2^{h}$	2773.3 ± 53.9^{d}
FRAP * ± SD (μmol TE/g)	66.5 ± 0.7^{d}	29.9 ± 1.3^{a}	96.0 ± 1.1^{e}	44.3 ± 0.3^{b}	102.2 ± 0.8^{f}	57.8 ± 0.9^{c}	350.7 ± 2.3^{h}	133.7 ± 2.0^{g}
F–C * ± SD (mg GA/g)	$11.7 \pm 0.5^{a,b}$	9.8 ± 1.1^{a}	12.8 ± 0.4^{b}	$11.1 \pm 2.2^{a,b}$	17.9 ± 0.5^{c}	17.3 ± 1.1^{c}	70.9 ± 2.2^{e}	67.6 ± 2.8^{d}

* $n = 3$; SD—standard deviation; Mean values within the same row marked by different superscript small letters (a–h) are significantly different (one-way ANOVA and Duncan test, $p < 0.05$).

Therefore, DCh + ChFrE was the richest source of antioxidants and revealed the highest AC and TPC, whereas DCh without plant extracts had the lowest antioxidant properties (Table 3). It is evident that enrichment of DCh with EFrE containing the lowest amounts of antioxidants increased the ABTS and FRAP by about 50% and CUPRAC above 4%, but insignificant differences for DPPH and TPC values were observed between DCh and DCh + EFrE (Duncan test, Table 3). This fact confirms that cocoa and its derivatives as the main ingredients of DCh are renowned sources of natural phenolic compounds such as flavanols (epicatechin, catechin), proanthocyanidins, and anthocyanins, which have antioxidant properties. Additionally, DCh can contain other well-known antioxidants such as Maillard reaction products generated during high-temperature processes: drying, roasting, and conching [22].

For comparison, the addition of various plant extracts such as red raspberry leaves [5], yellow tea [7], Sakura green tea, turmeric powder [6], black carrot [12], dried cranberries, and prunes [2] to DCh samples and chocolate products caused an increase in AC and TPC analyzed by DPPH, ABTS, FRAP, and F–C assays (Table 4). Unexpectedly, the DPPH, ABTS, and TPC results [3] for all chocolate pralines produced with the addition of either longan or lychee were significantly lower than the antioxidant properties of control samples (Table 4). The authors explained that replacing chocolate corpus with a filling containing a lower level of antioxidants caused a decrease in the overall AC and TPC in fortified chocolate products.

The prepared DCh without and with plant extracts were exposed to a three-phased in vitro static digestion process simulating oral, gastric, and intestinal circumstances. The effect of simulated digestion on AC and TPC in all chocolates was estimated and presented in Table 3. As can be seen, TPC in DCh samples without and with EFrE and EFlE, DPPH of DCh + EFrE, and ABTS of DCh containing only ZnL did not change significantly after in vitro digestion (Duncan test, Table 3). This suggests that the studied chocolates might be a great source of bioaccessible phenolic compounds.

However, the reducing potencies of all studied physiological extracts determined by CUPRAC and FRAP assays were about 2–5 times lower than undigested samples. Interestingly, radical scavenging activity of enriched chocolates analyzed by the ABTS test decreased by 30%–79% after digestion, whereas this physiological process caused lower losses (20–58%) of total bioactive compounds present in DCh, DCh + EFlE and DCh + ChFrE, which were capable of scavenging the DPPH radical (Table 3). These discrepancies between AC values may be caused by differences in the matrix composition of the digested chocolates (without and with plant extracts), which contributed to the gradual release of antioxidants during in vitro digestion. The decrease in reducing activity and scavenging activity of the investigated chocolates after simulated digestion may be due to the loss of the bioactive compounds and/or chemical transformations.

Table 4. Antioxidant capacity and total phenolic content in plain and enriched chocolates, as determined by other authors.

Analytical Methods	Plain Chocolates	Enriched Chocolates
Chemical extracts		
DPPH	0.044 mmol TE/g for ChP [3]	0.022–0.031 mmol TE/g for ChP + longan [3]
		0.018–0.028 mmol TE/g for ChP + lychee [3]
	4012 mg TE/100 g for DCh [7]	4373 mg TE/100 g for DCh + yellow tea extract [7]
	0.08 mg TE/g for CCh [12]	0.16–0.40 mg TE/g for CCh + black carrot extract [12]
ABTS	1.91 mmol TE/L for DCh [2]	2.04 mmol TE/L for DCh + cranberries [2]
	0.060 mmol TE/g for ChP [3]	0.022–0.044 mmol TE/g for ChP + longan [3]
		0.028–0.039 mmol TE/g for ChP + lychee [3]
	9 mmol/g for DCh [5]	9–11.5 mmol/g for DCh + red raspberry leaves extract [5]
	11 mmol TE/100 g for DCh [6]	15.4 mmol TE/100 g for DCh + Sakura green tea leaves [6]
		12.2 mmol TE/100 g for DCh + turmeric powder [6]
FRAP	285 mg TE/100 g for DCh [7]	386 mg TE/100 g for DCh + yellow tea extract [7]
	8.06 mmol Fe(II)/L for DCh [2]	9.20 mmol Fe(II)/L for DCh + cranberries [2]
	13 mmol/g for DCh [5]	13–14.5 mmol/g for DCh + red raspberry leaves extract [5]
	10.1 mmol TE/100 g for DCh [6]	15.4 mmol TE/100 g for DCh + Sakura green tea leaves [6]
		10.3 mmol TE/100 g for DCh + turmeric powder [6]
F–C	4.8 mg GA/g for DCh [2]	6.2 mg GA/g for DCh + prunes [2]
	10 mg GA/g for ChP [3]	5–7 mg GA/g for ChP + longan [3]
		4.2–6.1 mg GA/g for ChP + lychee [3]
	15,425 µmol GA/100 g for DCh [6]	20,090 µmol GA/100 g for DCh + Sakura green tea leaves [6]
		17,887 µmol GA/100 g for DCh + turmeric powder [6]
	1760 mg C/100 g for DCh [7]	2400 mg C/100 g for DCh + yellow tea extract [7]
	56.0 mg GA/kg for CCh [12]	85.0–117.7 mg GA/kg for CCh + black carrot extract [12]
Physiological extracts		
DPPH	0.12 mg TE/g for CCh [12]	0.25–0.56 mg TE/g for CCh + black carrot extract [12]
ABTS	1.8–10 mmol TE/100 g for DCh [6]	2.2–11.4 mmol TE/100 g for DCh + Sakura green tea leaves [6]
		2.1–9.9 mmol TE/100 g for DCh + turmeric powder [6]
FRAP	0.9–3.9 mmol TE/100 g for DCh [6]	1–5.4 mmol TE/100 g for DCh + Sakura green tea leaves [6]
		0.8–4 mmol TE/100 g for DCh + turmeric powder [6]
F–C	1800–10,100 µmol GA/100 g for DCh [6]	1850–13,900 µmol GA/100 g for DCh + Sakura green tea leaves [6]
		1900–7800 µmol GA/100 g for DCh + turmeric powder [6]
	70.8 mg GA/kg for CCh [12]	106.6–287.7 mg GA/kg for CCh + black carrot extract [12]

DCh—dark chocolate; ChP—chocolate pralines; CCh—compound chocolate; C—catechin equivalent; TE—Trolox.

It is evident that the standardized static in vitro digestion of chocolates largely contributes to the structural modification and antioxidative activity alteration of their functional components such as polyphenols, hence a change in antioxidant properties of the consumed chocolates. However, compared with the control sample of DCh containing only ZnL, a significant increase in AC and TPC in all physiological extracts of enriched chocolates was observed (Table 3, Duncan test).

Additionally, Martini et al. [6] found that in vitro gastrointestinal digestion processes decreased the antioxidant properties of DCh without and with Sakura green tea and turmeric powder when analyzed by ABTS, FRAP, and F–C (Table 4). In contrast, DPPH and TPC values of compound chocolate samples fortified with black carrot extract significantly increased after in vitro digestion (Table 4, [12]). Thus, these supplemented confectionery products were advantageous for delivering and transporting phenolic and other antioxidant compounds.

2.3. Physicochemical Parameters of Chocolates

The moisture content (MC) of plain chocolate is related to the sequence of processes that uses a thermal treatment. Moisture is primarily decreased in the conching process. Therefore, the MC is a criterion for terminating this process [23]. In addition, water amount in chocolates contributed to their flow and sensory properties: color, appearance (mainly sugar bloom), grittiness, and hardness.

It can be noted that humidity of DCh containing ZnL without plant additives was significantly lower (MC = 0.30%) than DCh with plant extracts (MC = 0.37–0.73%) (Table 5).

Table 5. Physicochemical parameters of the investigated chocolates.

Sample	Moisture Content ± SD (%)	Fat Content ± SD (%)	Viscosity ± SD (mPa·s)
DCh	0.30 ± 0.01 [a]	32.91 ± 0.35 [d]	2979.24 ± 48.84 [a]
DCh + EFrE	0.73 ± 0.02 [d]	29.69 ± 0.28 [b]	4493.28 ± 48.94 [c]
DCh + EFlE	0.65 ± 0.02 [c]	30.78 ± 0.15 [c]	3239.72 ± 28.20 [b]
DCh + ChFrE	0.37 ± 0.02 [b]	29.12 ± 0.22 [a]	4509.56 ± 28.19 [c]

* $n = 3$; SD—standard deviation; Mean values within the same column are marked by different superscript small letters (a–d) are significantly different (one-way ANOVA and Duncan test, $p < 0.05$).

This can be explained by the presence of moisture in added plant powder extracts, whereas ZnL was the ingredient of DCh with the lowest initial MC. Although, the ZnL amount (0.0065%) in the prepared chocolates was 3 orders less than the concentration of plant extracts (5%). The moisture levels in the ingredients used and the processing method applied affected the final MC observed in chocolates. On the other hand, the water amount in chocolate formulations can be attributed to the hygroscopicity of additives. For this reason, ChFrE had relatively lower MC and hygroscopic properties than EFrE and EFlE. A low water binding capacity of ChFrE added to DCh led to moisture reduction during the conching process. Hence, an approximately 2 times lower moisture uptake was observed for DCh + ChFrE compared with MC in DCh + EFrE and DCh + EFlE (Table 5).

For comparison, DCh enriched with yellow tea extract had a higher MC (1.56 g/100 g) than the control sample (MC = 1.32 g/100 g) [7]. In contrast, the MC (1.78%) insignificantly decreased with increasing cinnamon bark oleoresin microcapsule content (c = 4, 6 and 8%) added to DCh bars (MC = 1.55–1.68%) [24].

Moreover, the viscosity of DCh (2979.24 mPa·s) increased after the incorporation of plant extracts (3239.72–4509.56 mPa·s) because moisture level has a severe thickening effect on chocolate. In the presence of water on the surface of the sugar particles, they start sticking together and form agglomerates, impeding the flow.

On the other hand, the increase in viscosity values for enriched chocolates was most likely to occur due to a decrease in the fat content (FC) in these samples (Table 5). Therefore, the plain chocolate without additives revealed the lowest MC and viscosity value, which was compensated by the highest free fat phase content.

Similarly, the addition of 2% yellow tea powdered extract caused a reduction in the fat phase in DCh (FC = 28.53 and 27.98 g/100 g for control and enriched chocolate samples, respectively) [7].

Insignificant differences in viscosity results for DCh + EFrE and DCh + ChFrE were likely caused by similar pectin content in these additives (Duncan test, Table 5). It is well known that pectins undergo gelatination during the heating process, and that the viscosity of enriched chocolate products increases.

The plastic viscosity of DCh (1.58 Pa·s) also increased (1.82–2.31 Pa·s) with an increase in concentrated raspberry leaf extract amounts in a sample from 1 to 3% [5].

2.4. Microstructural Properties of Chocolates

The SEM was used to image the effect of added fruits and flower extract powders on the surface morphology of DCh. The microstructural analysis of the chocolates without and with plant extracts revealed clear variations in crystalline network structure, inter-crystal connections, and particle distribution using 150×, 1000×, and 5000× magnification (Figure 1). The surface morphology of the chocolates was characterized by rough, sharp texture, and flaky surfaces formed by crystallized cocoa butter as the suspending medium. SEM pictures of all investigated DCh samples showed a heterogeneous and dry system made of clusters of crystals and agglomerated large structures with three-dimensional surfaces and irregular cavities. These large structures were made of sugar and cocoa crystals having irregular forms and sizes at the mass surface. The added ZnL can also be seen along with the cocoa mass as particles smaller than cocoa and sugar particles that adhered to

the larger and coarser cocoa particles. Therefore, some agglomerates with relatively small edges were found in control chocolate without plant extracts (Figure 1a–c), whereas a rough structure with relatively sharp edges can be observed in the fortified chocolate samples containing active compounds (Figure 1 d–l). This porous structure of DCh was the location for the entrapment of bioactive compounds present in added plant extracts.

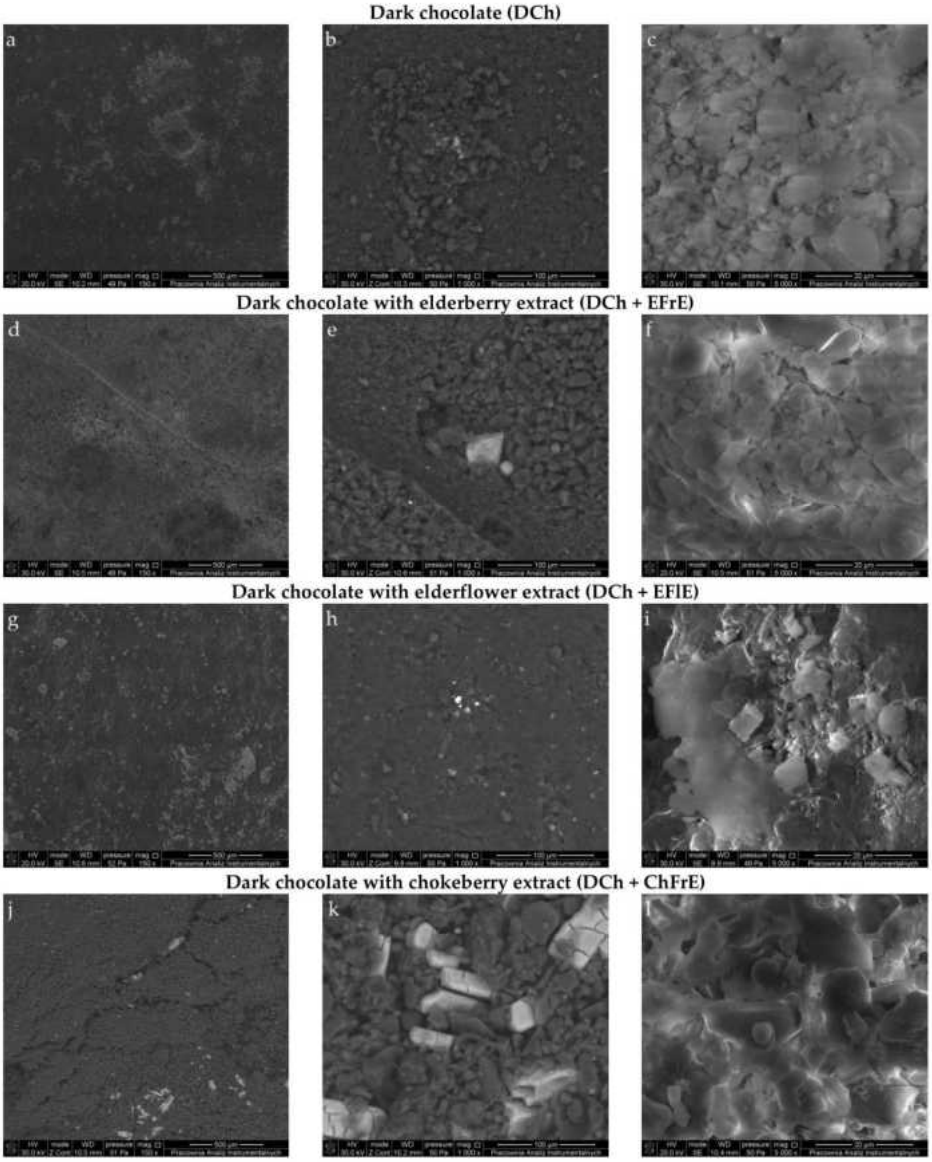

Figure 1. Scanning electron micrographs of dark chocolate containing ZnL (**a**,**b**,**c**), chocolate with EFrE (**d**,**e**,**f**), chocolate with EFlE (**g**,**h**,**i**), and chocolate with ChFrE (**j**,**k**,**l**) at 150, 1000 and 5000× magnification, respectively.

On the other hand, the chocolate with plant extracts contained higher humidity (Table 5) and could induce more amorphous sugar formation during chocolate production. Moreover, fortified chocolates showed a surface that was partly covered by protrusions and pores (Figure 1d–l). It is noteworthy that small particles filled the voids between big particles, which might increase packing fraction.

However, smaller particles of yoghurt powder added to probiotic milk chocolate in a concentration of 50% adhered to the larger and coarser cocoa and sugar particles. The cocoa particles were completely covered by smaller yoghurt powder particles in chocolate fortified with 100% yoghurt powder [25].

2.5. Energy Spectra Provided by SEM/EDS

The presence of chemical elements in the prepared chocolates and their relative compositions can also be determined using dispersive energy spectroscopy coupled with the SEM instrument (Figure 2).

EDS analysis through SEM confirmed the presence of Zn in all samples as indicated by peaks in EDS graphs (Figure 2a,d,g,j), but its concentration in enriched chocolates varied from 0.15% to 13.53%. This can be explained the possibility that Zn ion sorption had occurred on the surface of added plant extracts, and Zn aggregates were created. In contrast, an even distribution of Zn ions in the lowest amount (0.14–0.16%) was found for chocolate without plant extracts (Figure 2a–c).

The presence of carbon and oxygen can be attributed to the main organic ingredients of chocolates, such as cocoa butter and sugar. The same amounts of cocoa butter and sugar were used during the preparation of each chocolate. Thus, all EDS images contained carbon and oxygen at similar levels (C = 16.94–20.66%, O = 68.20–79.65%). High oxygen content on the surface of studied chocolates was associated with reactive oxygen-containing functional groups (carbonyl, hydroxyl, etc.).

Surprisingly, the atomic percentages of carbon (78.47–81.51%) and oxygen (18.49–21.53%) in the selected areas of untempered and bloomed chocolate samples with 38% cocoa butter estimated by SEM-EDS were exactly the opposite [26]. However, it is well known that EDS quantitative analysis of light elements may be considered only an estimate due to the intense absorption of X-ray emission inside the sample and the minimal excitation energy of the light elements (max. 1 keV) [27].

Moreover, signals from phosphorus, magnesium, and potassium were identified in the EDS spectra of analyzed chocolates (Figure 2a,d,g,j). This can be explained by the fact that cacao particles contain these elements [28]. However, the localization of phosphorus, magnesium, potassium, calcium, and sulfur indicated that they can also come from the added plant extracts. Interestingly, amongst these inorganic elements, K (0.53–1.64%) was the most abundant in all samples, and only DCh + EFlE had 2 times higher content of Ca (0.46%) than Mg (0.20%). The Ca and Mg levels in the remaining chocolates ranged between 0.12–0.19% and 0.28–0.46%, respectively. However, the appearance of Al in the spectra could be due to the aluminum stub on which adhesive tape with the powder sample was adhered.

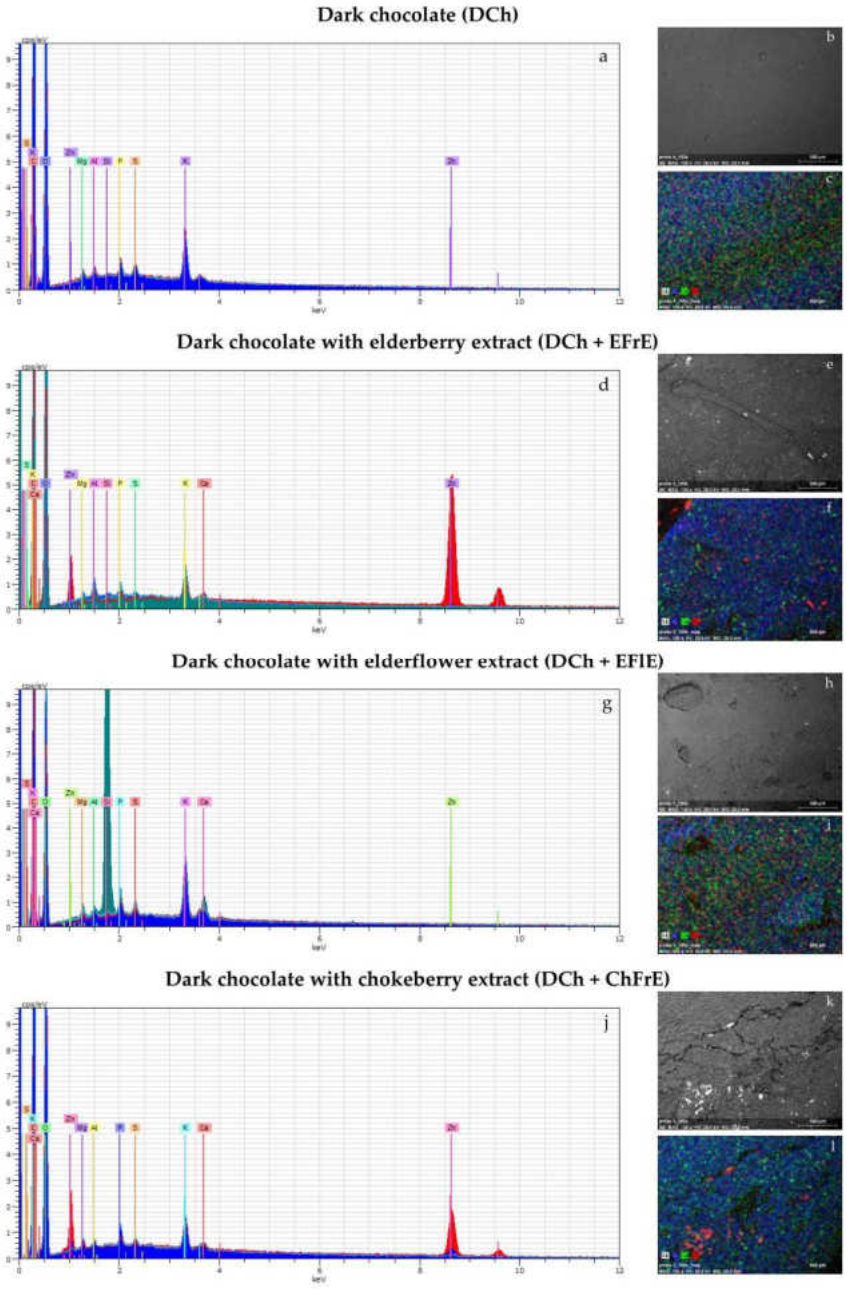

Figure 2. EDS spectra (**a**,**d**,**g**,**j**), SEM images (**b**,**e**,**h**,**k**) and mapping images by SEM–EDS (**c**,**f**,**i**,**l**) of dark chocolates without and with EFrE, EFlE and ChFrE.

3. Materials and Methods

3.1. Chemicals

All reagents were of analytical or HPLC grade and were purchased from Merck Life Science Sp. z o.o. (Poznań, Poland): 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ, 99%), neocuproine (98%), Folin–Ciocalteu's phenol reagent (F–C reagent, 2 N), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (TE, 97%), gallic acid (3,4,5-trihydroxybenzoic acid) (GA, 98%), iron(III) chloride hexahydrate, sodium acetate, hydrochloric acid, ammonium acetate, copper(II) chloride, potassium persulfate, pancreatin from porcine pancreas, α-amylase from human saliva (300–1500 units/mg), pepsin, bile salts, acetic acid, acetone, methanol (99.8%), ethanol (95.0%), sodium carbonate, and n-hexane. Redistilled water was used for preparation of solutions.

3.2. Materials

Plain dark chocolate (DCh) with 45% cocoa solids was produced through a classical technological process in the chocolate factory UNION CHOCOLATE Sp. z o.o. located in Żychlin (Poland).

Concentrated and vacuum-dried extracts from *Sambucus nigra* L. fruits (DER 4:1) (EFrE) and flowers (DER 4:1) (EFlE), as well as a standardized extract from *Aronia melanocarpa* (Michx.) Elliott fruits (ChFrE) separated by column chromatography were supplied by Greenvit Botanical Extracts Manufacturer in Zambrów (Poland). Zinc lactate (ZnL) (PURAMEX® ZN), a nutritional additive, was obtained from Corbion Group Netherlands B.V. (Amsterdam, The Netherlands).

3.3. Ultrasound-Assisted Extraction of Antioxidants from Plants

Extraction of antioxidants from EFrE, EFlE, and ChFrE was performed using an ultrasonic water bath (5200DTD, Chemland, Stargard Szczeciński, Poland) at power and frequency of 180 W and 40 kHz, respectively, equipped with a digital timer and temperature controller. Exactly, 0.5 g of each plant extract was mixed with hot redistilled water (25 mL) in Erlenmeyer flasks, stirred, and placed in an ultrasonic bath. Water in the ultrasonic bath was circulated and regulated at a constant temperature (25 ± 0.3 °C) to avoid water temperature increases as a result of exposure to ultrasound. Each sample was sonicated in duplicate for 10 min and centrifuged at 1880× *g* for 15 min (centrifuge MPW-54, Chemland, Stargard Szczeciński, Poland).

3.4. Preparation of Chocolates Fortified with Plant Extracts and Extraction Procedure

Dark chocolates incorporated with powder extracts from elderberries (DCh + EFrE), elderflowers (DCh + EFlE), and chokeberries (DCh + ChFrE), respectively, as functional ingredients were prepared in the Confectionery Factory (Kopernik S.A., Toruń, Poland) using a tempering machine with a mold filling device (Pomati T35, Codogno LO, Italy). The following procedure was used for the supplementation of chocolates: DCh was melted at 45 °C, and after the addition of ZnL (0.0065%) and each plant extract (5%), liquid chocolate was mixed in a mixer (Pomati S150, Codogno LO, Italy) and tempered at a working temperature of 31 °C. Then, polycarbonate molds were filled with the prepared chocolates. The weight of enriched chocolates in each mold was 95 g. The obtained chocolates were cooled in a cooling tunnel (Kreüter, Kühlkanal Universal K.K. 1050, Hamburg, Germany) at 10 °C for 30 min and taken out from the molds, after which they were stored at ambient temperature until the analyses.

Chocolate extracts were prepared using a conventional extraction technique as previously described by Adamson et al. [29] with some modifications. The ground chocolate samples (5.0 g) were defatted twice with 50 mL of n-hexane at room temperature for 30 min. The remaining defatted solids were air-dried for 24 h to evaporate the residual solvent. Antioxidants were extracted from each defatted chocolate sample (2.0 g) with 10 mL of an acetone–water–acetic acid mixture (Ac:H_2O:AA = 70:29.8:0.2, *v/v/v*) by using a shaker

SHKA 2508-1CE (Labo Plus, Warszawa, Poland) for 30 min. Extractions were carried out two times at room temperature. The combined extracts were filtered using polytetrafluorethylene syringe filters (PTFE, pore size 0.20 µm/diameter 13 mm, Merck Life Science Sp. z o.o., Poznań, Poland) and stored in a refrigerator until the AC and TPC analyses.

3.5. In Vitro Simulated Digestion of Chocolates

An in vitro digestion analysis mimicking the physiological situation in the digestive tract (simulated salivary fluid, simulated gastric fluid, and simulated intestinal fluid) was used to evaluate the bioaccessibility of antioxidant compounds in chocolates according to the procedure described by Dala-Paula et al. [13]. The simulated digestion procedures were as follows, 3 g of each tested chocolate was mixed with 3 mL simulated saliva (phosphate buffer solution: 0.04% NaCl and 0.004% $CaCl_2$, pH 6.9) containing 0.07 mg α-amylase in a centrifuge tube. Samples were subsequently incubated at 37 °C for 5 min in a shaking water bath type 357 (ELPAN, Lubawa, Poland). Then, the samples were mixed with 10 mL of simulated gastric fluid prepared by dissolving 1% pepsin, 3 g of NaCl, and 9 mL of HCl in 1 L of water (the pH was adjusted to 1.3 using 1 M HCl) and incubated at 37 °C for 120 min to simulate stomach conditions. The gastric digests were maintained on ice for 10 min to stop pepsin digestion. For the intestinal digestion stage, the pH of the gastric digests was raised to 6.5 by dropwise addition of 1 M $NaHCO_3$. Then an amount of freshly prepared pancreatin–bile salt solution sufficient to provide 0.005 g pancreatin and 0.03 g bile salt/g sample was added, and incubation was continued for an additional 120 min at the same temperature. To stop intestinal digestion, the sample was kept for 10 min in an ice bath. The pH was then adjusted to 7.2 by dropwise addition of 0.5 M NaOH. All samples were filtered (nylon filters of 0.45 µm, Merck Life Science Sp. z o.o., Poznań, Poland) and stored in opaque vials at 4 °C until analysis.

3.6. Physicochemical Analysis of Chocolates

3.6.1. Moisture and Fat Determination

The representative samples from each chocolate were analyzed for proximal composition (MC and FC) using an Instalab 600 Product Near Infrared Reflectance (NIR) Analyzer (Dickey-John Inc. Minneapolis, MI, USA). Analyzed chocolate was ground to uniform particle size (0.5 mm), then the sample cup was carefully filled with ground chocolate. Next, ground chocolate was gently tamped into the cup. The chocolate surface should be flat and flush with the rim of the cup. After closing, the sample cup was placed into the sample cell of the NIR product analyzer, and spectral data were recorded at room temperature as log(1/R), where R is the reflectance energy. Afterward, results of humidity and FC were read from the analyzer display. To minimize sampling error, triplicate samples were analyzed for all the samples of the calibration set. The average spectral data were used for NIR calibration.

3.6.2. Viscosity Determination

Chocolate viscosity was measured using a rotational viscometer RN 4.1 type produced by HAAKE Medingen GmbH (Ottendorf-Okrilla, Germany). Each chocolate sample (100 g) was placed in a canister closed with a lid. The sample was heated to 50 °C and held at 50 °C. The sample was mixed with a baguette to remove lumps and air bubbles every 15 min and controlled. The analysis was performed 2 h after the start of melting the chocolate, and the means of triplicate runs were recorded.

3.7. Antioxidant Capacity Determination

The AC and TPC in EFrE, EFlE, ChFrE, and chocolates enriched with them were determined by spectrophotometric DPPH, ABTS, CUPRAC, FRAP, and F–C methods according to procedures described in our previous article with some minor modifications [30]. The resulting absorbance of each obtained solution was measured in five repetitions using a Hitachi U-2900 spectrophotometer (Tokyo, Japan) in a 1-cm glass cell. The AC results

were expressed as μmol Trolox equivalents (TE) per 1 g of sample, while TPC values were expressed as mg gallic acid (GA) equivalents per 1 g of sample.

3.8. Scanning Electron Microscopy with Energy Dispersive X-ray Spectrometer

The morphology of chocolates was observed by scanning electron microscopy/focused ion beam (SEM/FIB) using Quanta 3D FEG microscope (Carl Zeiss, Göttingen, Germany). The micrographs were recorded under a low vacuum using a secondary electron detector (SE), and accelerating voltage ranging between 20.0 and 30.0 kV was chosen for SEM analysis. Samples were defatted and frozen in liquid nitrogen before measurements.

Additionally, the morphology and elemental composition of the defatted chocolates without and with plant extracts were analyzed with a scanning electron microscopy (SEM) LEO Electron Microscopy Ltd., 1430 VP (Cambridge, UK) equipped with detectors of backscattered electron (BSE), cathodoluminescence (CL), and an energy dispersive X-ray spectrometer (EDS) Quantax with an XFlash 4010 detector (Bruker AXS microanalysis GmbH, Berlin, Germany). EDS was used for the element-mapping analysis of chocolate samples. The elemental composition at different points of each sample was carried out at acceleration voltage, HV: 28.0 kV, live time 40 s, working distance, WD: 25.0 mm, and 100× magnification.

3.9. Statistical Analysis

The obtained results were expressed as mean ± standard deviation (SD). All data were statistically tested, and the means were compared by one-way analysis of variance (ANOVA) with subsequent comparisons by Duncan's test at a 0.05 significance level using Statistica 8.0 software (StatSoft, Tulsa, OK, USA).

4. Conclusions

In this study, DCh samples containing ZnL were successfully enriched with EFrE, EFlE, and ChFrE, which affected the physicochemical, antioxidant, and microstructural properties of new confectionery products. Moreover, the digestive stability of natural antioxidants from plant extracts present in fortified chocolates was estimated using the standardized static in vitro digestion model. Enrichment of DCh with plant extracts enhanced scavenging radical activity (DPPH and ABTS values), reducing ability (CUPRAC and FRAP values), and the level of total phenolics in undigested and digested chocolates. This increase is strictly associated with the botanical origin of extracts having high antioxidant potential as analyzed by the mentioned analytical methods. Changes in the composition of DCh by the addition of ZnL and plant extracts affected the extraction and release of bioactive compounds from the chocolate matrix, which in turn had an impact on their bioaccessibility and bioavailability. The addition of plant extracts to DCh resulted in higher MC, viscosity, and/or amorphous parts creating agglomeration of solid particles. Combining SEM imaging and EDS analysis revealed that unsupplemented and supplemented chocolates had noticeable differences in chemical composition and surface characteristics such as roughness, grain size, and presence of pores or protrusions.

With further improvements in the processing technology of cocoa-based products, EFrE, EFlE, and ChFrE can serve as healthy additives to plain chocolate, resulting in products with better health benefits for health-conscious consumers and the entire consuming populace.

Author Contributions: Conceptualization, A.S.-C., S.P., S.K. and P.T.; methodology, A.S.-C., S.P., S.K. and P.T.; software, A.S.-C.; validation, A.S.-C., S.P., S.K. and P.T.; formal analysis, S.P., S.K. and P.T.; investigation, A.S.-C., S.P., S.K. and P.T.; resources, A.S.-C. and S.P.; data curation, A.S.-C., S.P., S.K. and P.T.; writing—original draft preparation, S.P., S.K. and P.T.; writing—review and editing, A.S.-C.; visualization, A.S.-C., S.P., S.K. and P.T.; supervision, A.S.-C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are available from the authors.

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(D3) Poliński, S., Topka, P., Tańska, M., Kowalska, S., Czaplicki, S., & Szydłowska-Czerniak, A. (2022). Impact of Bioactive Compounds of Plant Leaf Powders in White Chocolate Production: Changes in Antioxidant Properties During the Technological Processes. *Antioxidants, 11*(4), 752.

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Impact of Bioactive Compounds of Plant Leaf Powders in White Chocolate Production: Changes in Antioxidant Properties during the Technological Processes

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Abstract: Bioactive compounds present in the powdered leaves of matcha green tea (*Camellia sinensis* L.) (MGTP) and moringa (*Moringa oleifera*) (MOLP) seem to be related to health benefits due to their antioxidant properties. The growing accessibility of these powders has led to their being more widely used in food production. The aim of this study was to evaluate the total phenolic content (TPC) and antioxidant capacity (AC) of white chocolate (WCh) supplemented with MGTP and MOLP. AC was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), cupric ion-reducing antioxidant capacity (CUPRAC), and ferric-reducing antioxidant power (FRAP) assays, whereas TPC was determined by the Folin–Ciocalteu (FC) method. Both additives were incorporated at four levels (1, 2, 3 and 4%) in two chocolate processing steps (conching and tempering). Additionally, the amounts of phenolic acids, tocopherols, and carotenoids in WCh samples enriched by MGTP and MOLP were determined to explain their influence on AC. The results showed that the chocolates supplemented with MGTP were characterized by higher antioxidant properties than those with MOLP. In turn, MOLP significantly increased the content of lipophilic antioxidants in chocolates, tocopherols and carotenoids, which also exhibit pro-health effects. Furthermore, the incorporation of these additives during the tempering process was more relevant to the improvement of the antioxidant properties of WCh.

Keywords: matcha; moringa; bioactive compounds; white chocolate; processing; antioxidant properties

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Citation: Poliński, S.; Topka, P.; Tańska, M.; Kowalska, S.; Czaplicki, S.; Szydłowska-Czerniak, A. Impact of Bioactive Compounds of Plant Leaf Powders in White Chocolate Production: Changes in Antioxidant Properties during the Technological Processes. *Antioxidants* **2022**, *11*, 752. https://doi.org/10.3390/antiox11040752

Academic Editors: Daniel Franco Ruiz, María López-Pedrouso and Jose Lorenzo Rodriguez

Received: 25 March 2022
Accepted: 7 April 2022
Published: 10 April 2022

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1. Introduction

Many nutritious plants are valuable sources of natural components that improve human health and well-being. In recent years, the nutritional and medicinal properties, such as antioxidant, antimicrobial, anti-inflammatory, antihypertensive, diuretic, antidiabetic, antihyperlipidemic, antineoplastic, antipyretic, antiulcer, cardioprotectant, and hepatoprotectant properties, of *Camellia sinensis* and *Moringa oleifera* plants have been widely reported [1,2].

Matcha (*Camellia sinensis* L.) is a powdered type of Japanese green tea, particularly rich in bioactive components, such as phenolic compounds, which constitute even up to 30% of dry mass (phenolic acids, flavonoids, especially flavans, also known as tea catechins, and tannins), alkaloids (methylxanthines, mainly caffeine) and amino acids (theanine, gamma amino butyric acid) [1]. Moringa (*Moringa oleifera*) is a medicinal Indian herb the leaves of which are a good source of phenolic acids, flavonoids, essential amino acids, minerals (calcium, potassium, magnesium) and fiber [2]. The bioactive compounds present in matcha

green tea powder (MGTP) and moringa leaf powder (MOLP) with antioxidant, chemopreventive and mood-enhancing activities play an important role in human health due to the prevention of chronic degenerative diseases. Furthermore, antioxidant compounds contained in MGTP and MOLP have become important in the food industry. Food products supplemented with them have better physicochemical and organoleptic characteristics and also have extended shelf lives. Natural antioxidants of MGTP and MOLP used as additives in various food products act with different functions (complexation with proteins and carbohydrates, inhibition of lipid oxidation and microbiological growth) through interaction/molecular modification mechanisms, enabling the development of innovative, healthy, nutritious and long shelf-life products with enhanced sensory acceptance [2].

Different studies have attracted great interest in the application of bioactive substances in the creation of functional foods. The effects of powdered leaves and extracts of *Camellia sinensis* L. and *Moringa oleifera* on the physicochemical and sensory quality characteristics of bakery [3,4], confectionery [5,6], dairy [7,8] and meat [9,10] products, beverages [11,12], noodles [13,14], oils and fat-based [15,16] products have been widely studied in recent years. Furthermore, incorporating bioactive compounds from tea and moringa powders and extracts into different types of chocolate has enhanced their antioxidant and antimicrobial activities, sensory acceptance, and shelf life through inhibition of lipid oxidation [17–24]. White chocolate (WCh) samples have also been supplemented with freeze-dried acai powder [25], cinnamon oleoresin [26] and cinnamon essential oil [27].

It is known that chocolate is one of the world's most popular sweets [28]. Different types of chocolate (e.g., dark, milk and white) can be found on the market, usually made by combining cocoa mass (cocoa powder, cocoa butter and/or cocoa liquor) with sugar and other additives in varying quantities. Among chocolate products, dark chocolate is the most desirable because it contains significantly more health-promoting polyphenols than milk chocolate. In turn, WCh has been regarded as an even unhealthier product than other types of chocolate because fat-free cocoa solids are omitted [18]. The production process of chocolate is unique and complicated and consists of six main stages, including mixing, refining, conching, tempering, molding and cooling. Among these, conching and tempering were identified as the key processes in chocolate manufacturing that affect the final characteristics of chocolate [29]. Conching consists of mixing, shearing and aerating the chocolate mass during heating at a specific temperature (>40 °C). This process contributes to the development of the viscosity, texture and flavor of chocolate [30]. Tempering is a controlled cocoa butter crystallization technique (at temperatures up to 32 °C) that helps stabilize the polymorphic transitions of cocoa butter crystals during storage. Tempering is a crucial phase in chocolate manufacture since it influences perception by consumers and provides the smooth and shiny appearance of chocolate [31].

However, the final content of bioactive compounds and the antioxidant potential of chocolate are a function of several variables, some related to the raw materials and others related to processing and formulation [32–34]. The increase in antioxidant properties during chocolate production, especially in white and milk chocolate types, has been investigated by many researchers. Although some researchers have successfully introduced matcha and moringa into WCh products [17,24,35], there are no results from testing of the supplementation stage on chocolate antioxidant properties. Literature data show that both additives mainly increase the content of phenolic compounds in chocolate products [17,18,24,35]. Their mechanism of antioxidant actions in food products is either by hydrogen atom transfer (HAT), single electron transfer (SET), sequential proton loss electron transfer (SPLET) or transition metal chelation (TMC) [36]. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay is the most extensively used to evaluate the antioxidant activity of foods rich in phenolic compounds, measuring their ability to act as free radical scavengers or hydrogen donors. A strong correlation between antioxidant activity measured by the DPPH assay and the content of phenolic compounds in chocolate has been confirmed by many researchers, e.g., [17,24,35,37]. However, MGTP and MOLP can provide lipophilic an-

tioxidants such as carotenoids and tocopherols which also affect the antioxidant properties of the enriched products [1,38–40].

The main objective of this study was to investigate for the first time the effect of the incorporation of MGTP and MOLP at four different concentrations into chocolate samples during two processes (conching and tempering) on the total phenolic content (TPC) and antioxidant capacity (AC) of the fortified products. Antioxidant properties of two plant leaf powders and enriched chocolate samples were determined spectrophotometrically by using four modified analytical methods: DPPH, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), cupric reducing antioxidant capacity (CUPRAC), and ferric reducing antioxidant power (FRAP), while TPC was analyzed by Folin–Ciocalteu (FC) assay. Additionally, the amounts of individual hydrophilic (phenolic acids) and lipophilic (tocopherols, carotenoids) antioxidants in leaf powders and chocolates supplemented with them were determined, and their impact on AC was examined.

2. Materials and Methods

2.1. Chemicals

Standard phenolic acids (purity > 97%), including caffeic, chlorogenic, *m*-coumaric, *p*-coumaric, ellagic, ferulic, gallic, gentistic, *p*-OH-benzoic, protocatechuic, salicylic, sinapic, syringic, vanillic acids and standard tocopherols (purity > 96%), including α-tocopherol, γ-tocopherol and δ-tocopherol, β-apo-8′-carotenal (purity > 96%), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, TE), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ, 99%), ammonium acetate, copper(II) chloride, dichloromethane, hydrochloric acid, iron(III) chloride hexahydrate, neocuproine and HPLC-grade solvents, such as acetonitrile, formic acid, iso-propanol, methanol, methyl tert-butyl ether and *n*-hexane, were purchased from Sigma Aldrich (Poznań, Poland). Analytical-grade reagents, such as acetic acid, acetone, diethyl ether, Folin–Ciocalteu (FC) reagent, methanol, potassium hydroxide, sodium carbonate and sodium sulfate were supplied by Chempur (Piekary Śląskie, Poland).

2.2. Materials

Plain white chocolate (WCh) with 33.6% total fat content was produced through a classical technological process in the chocolate factory BARRY CALLEBAUT BELGIUM NV–Aalstersestraat 122–9280 Lebbeke Wieze (Belgium).

WCh samples were supplemented with: matcha (*Camellia sinensis* L.), green tea powder (MGTP) produced by BioPlanet (batch number: 287-490520; use-by date: 31 December 2021; country of origin: China), and moringa (*Moringa oleifera*) leaf powder (MOLP) produced by Targoch (batch number: 100927; expiry date: 31 March 2021; country of origin: India).

2.3. Preparation of Chocolates Supplemented with MGTP and MOLP

Chocolate samples were produced under industrial conditions in the Confectionery Factory Kopernik S.A., Toruń, Poland. A chocolate-tempering machine, Pomati T35, with a mold-filling device (Codogno LO, Italy) was used to prepare WCh enriched with different amounts of MGTP and MOLP. The additives were introduced to the WCh during two processes, conching (Co) and tempering (Te).

First, 50 kg of WCh was melted at 45 °C in the chocolate heating and mixer tank Pomati 150 (Codogno LO, Italy). In the case of samples with supplementation during the Co process, the additives were added to the liquid chocolate. The WCh was homogenized with the addition of MGTP and MOLP for 5 h in the tank of the Pomati 150 (a simulation of the liquid phase of the Co process). The next step was the Te process. The chocolate mass was then poured into the Pomati T35 with a mold-filling device, and it was circulated between the sharpener tank and the coating belt for the dispensing of the chocolate directly into the molds. The Pomati T35 with a mold-filling device was switched to cooling mode,

and the temperature was set to 29 °C, while the temperature on the mold-filling device was 0.5 °C higher.

In the case of samples with supplementation during the Te process, the additives were mixed with the liquid chocolate for 10 min before it was poured into the molds. The pre-crystallized chocolate mass was then poured into 10 × 10 g plastic molds and cooled in a cooling tunnel (Kreüter, Kühlkanal Universal K.K. 1050, Hamburg, Germany) at 10 °C for 20 min. The samples were then packaged and stored in a dark and dry place at 18 °C until the analysis. The same procedure was carried out to produce chocolate samples enriched with MGTP and MOLP at four different concentrations (1, 2, 3 and 4%). The research material consisted of 17 chocolate samples (Figure 1). The control sample was plain white chocolate (WCh) without the addition of any leaf powder, and 16 supplemented chocolate samples were differentiated by additive type (MGTP, MOLP), amount (1, 2, 3, 4%) and supplementation stage (Co, Te).

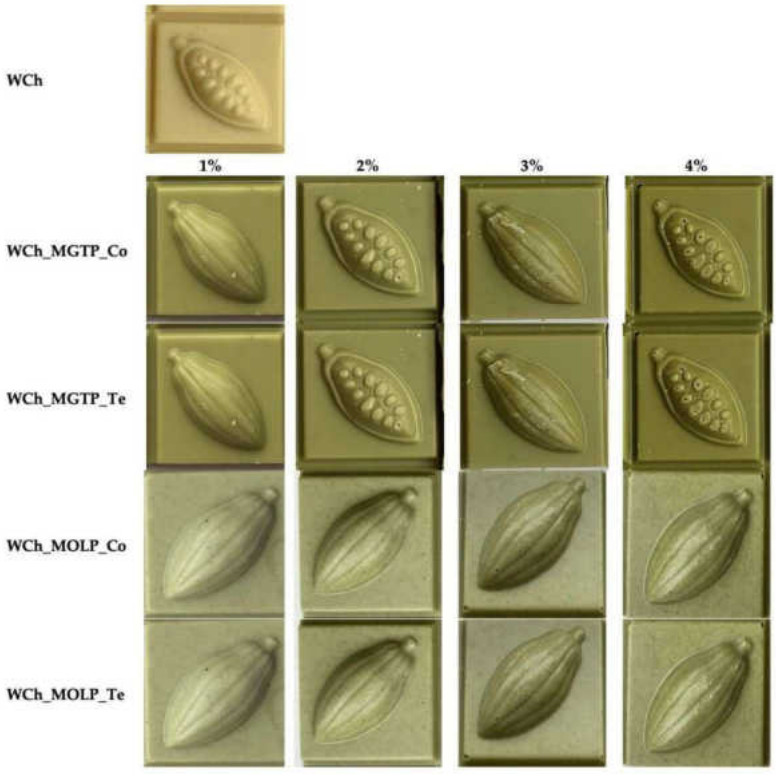

Figure 1. Photographs of white chocolate (WCh) samples without and with different amounts (1–4%) of matcha green tea powder (MGTP) and moringa leaf powder (MOLP) introduced during the conching (Co) and tempering (Te) processes.

2.4. Extraction of Phenolic Antioxidants from Leaf Powders

Extraction of antioxidants from MGTP and MOLP was performed using an ultrasonic water bath (5200DTD, Chemland, Stargard Szczeciński, Poland) equipped with a digital timer and temperature controller, at a power and frequency of 180 W and 40 kHz, respectively. Exactly 0.5 g of each powder was mixed with methanol (70%, *v*/*v*; 25 mL) in Erlenmeyer flasks, stirred and placed in an ultrasonic bath. Water in the ultrasonic

bath was circulated and regulated at a constant temperature (25 ± 0.3 °C) to avoid water temperature increases as a result of exposure to ultrasound. Each sample was sonicated in duplicate for 10 min and centrifuged at 1880× *g* for 15 min (centrifuge MPW-54, Chemland, Stargard Szczeciński, Poland).

2.5. Extraction of Phenolic Antioxidants from Chocolate Samples

Chocolate extracts were prepared using a conventional extraction technique as previously described by Adamson et al. [41] with some modifications. The ground chocolate samples (2.0 g) were weighed on an analytical balance and placed in a dry ground glass joint surface flask with a capacity of 100 mL. Then, 10 mL of methanol (70%, *v*/*v*) was added, and the mixture was shaken mechanically for 30 min. The procedure was repeated twice, and the obtained extracts were combined and filtered to give 20 mL of extract for analysis. The same procedure was used for each of the tested chocolates.

2.6. Determination of Total Phenolic Content in Leaf Powders and Chocolate Samples

The modified, previously described FC method [42] was used to determine TPC in extracts of phenolic compounds obtained from the used leaf powders and tested chocolates. Briefly, 0.25–0.5 mL of extracts were transferred into a 25 mL calibrated flasks, then 0.5 mL of FC reagent was added, and the mixtures were shaken for 3 min. Next, 1 mL of a saturated sodium carbonate solution was added and made up to the mark with distilled water. After 45 min, the solution was centrifuged at 1880× *g* for 5 min in a laboratory centrifuge MPW-54 and absorbance at 765 nm was measured against a reagent blank using a Hitachi U-2900 UV-VIS spectrophotometer (Tokyo, Japan). A calibration curve: A_{765} = (0.1034 ± 0.0025)c_{GA} + (0.0814 ± 0.0147) was prepared for the working solutions of gallic acid (GA) in the concentration range of 0.35–10.51 µg/mL. TPC values were expressed as mg GA equivalents per 100 g of sample.

2.7. Determination of Phenolic Acid Content in Leaf Powders and Chocolate Samples

The phenolic acid contents were determined by the RP-HPLC technique according to the method described by Skrajda-Brdak et al. [43]. Briefly, 5 mL of prepared extract of phenolic compounds was evaporated to dryness at temperatures below 50 °C in an R-210-type Büchi vacuum evaporator (Büchi Labortechnik, Flawil, Switzerland). The residue was dissolved in 20 mL of deionized water and acidified to pH 2. Then, phenolic acids were extracted 5 times with 20 mL of diethyl ether and the collected extracts were evaporated in a vacuum evaporator. The dry extract was re-dissolved in 1 mL of methanol and subjected to chromatographic separation on an Agilent Technologies (Santa Clara, CA, USA) 1200 series system fitted out with a photodiode detector with a Waters XBridge C18 column (Milford, MA, USA) (150 mm × 2.1 mm, 3.5 µm) at 30 °C. A gradient elution program was employed, using two elution solvents: solvent A (water/formic acid = 99.85/0.15, *v*/*v*) and solvent B (acetonitrile/formic acid = 99.85/0.15, *v*/*v*). The flow rate was 0.5 mL/min with a gradient elution program as follows: 0–3 min, 99% A; 3–15 min, 99–90% A; 15–25 min, 90–40% A; 25–27 min, 40–20% A; 27–30 min, 20% A; 30–33 min, 20–99% A, and was stable until 40 min. The detection was performed at wavelengths of 260, 280 and 320 nm. Phenolic acids were identified by comparison with the absorption spectra of the reference phenolic acids and their contents were determined from calibration curves of reference standards. The LOD and LOQ were 0.025–0.05 µg/mL and 0.08–0.17 µg/mL, respectively.

2.8. Determination of Lipophilic Antioxidants in Leaf Powders and Chocolate Samples

The content of tocopherols was determined according to the method described by Mikołajczak et al. [44]. Each sample of leaf powder (2 g) and chocolate (1 g) was extracted 3 times with *n*-hexane. The collected extracts were evaporated to dryness at temperatures below 50 °C in a R-210-type Büchi vacuum evaporator. Then, the residue was re-dissolved in 5 mL of *n*-hexane and subsequently centrifuged (10 min, 25,000× *g*) in a 5417R-type Eppendorf centrifuge (Eppendorf AG, Hamburg, Germany). The resultant solution was

analyzed using a HPLC Agilent Technologies 1200 chromatograph (Santa Clara, CA, USA) equipped with a fluorescence detector from the same company and a LiChrospher Si 60 column (250 mm × 4 mm × 5 µm, Merck, Darmstadt, Germany). A 0.7% solution of iso-propanol in *n*-hexane was used as the mobile phase at a flow rate of 1 mL/min. The fluorescence detector was set at excitation and emission wavelengths of 296 nm and 330 nm, respectively. Tocopherols were quantified using standards of α-, γ- and δ-tocopherols, and their contents were calculated using external calibration curves. The repeatability for α, γ- and δ-tocopherol determinations (expressed as a coefficient of variation) was 2.5%. LOQ was 0.2–0.45, µg/g, while linearity of the calibration curve was confirmed in the range of 0.02–16 µg/mL.

The content of carotenoids was determined according to the method described by Czaplicki et al. [45]. Each sample of leaf powder (1 g) and chocolate (2 g) was diluted in 10 mL *n*-hexane, and β-apo-8′-carotenal as an internal standard and a 40% potassium hydroxide solution in methanol were added. The mixture was shaken in a Multi Rotator RS-60 (Biosan, Riga, Latvia) in the dark at room temperature for 16 h. After saponification, 10% sodium sulfate was added, and carotenoids were extracted 5 times with *n*-hexane. Collected extracts were evaporated on a Büchi R-210 rotary evaporator at 45 °C. The residue was re-dissolved in methanol:dichloromethane mixture (45:55, *v*/*v*) analyzed using an Agilent Technologies 1200 RP-HPLC apparatus, equipped with a diode array detector (DAD), a YMC-C30 chromatography column (150 mm × 4.6 mm, 5 µm) and a YMC-C30 precolumn (10 mm × 4.6 mm, 3 µm) (YMC-Europe GmbH, Dinslaken, Germany). The column temperature was set at 30 °C and methanol (A) and methyl tert-butyl ether (B) gradient was programmed as follows: 0–5 min, 95% A; 5–25 min, 95–72% A; 25–33 min, 72–5% A; 33–60 min, 5–95% A. Carotenoids were identified at 450 nm based on retention times and absorption spectra of carotenoid standards. Their contents were calculated with reference to the internal standard. The repeatability for β-apo-8′-carotenal determination (expressed as a coefficient of variation) was 2.5%. LOQ was 0.05 µg/g of sample, while the linearity of the calibration curve was confirmed in the range of 1–150 mg/L. Calibration curves from 1 to 150 mg/L were obtained by plotting the peak area ratio of analyzed carotene standard to β-apo-8′-carotenal (internal standard) against the ratios of their concentration. The method linearity in the curve concentration range was shown by linear regression coefficients (R^2) which were above 0.998. Curves were prepared for standard lutein, zeaxanthin, α-carotene and β-carotene. For quantification of luteoxanthin and *cis*-lutein, the curve prepared for lutein was used as well as for quantification of 13-*cis*-β-carotene and the 9-*cis*-β-carotene curve prepared for β-carotene was used.

2.9. Determination of Antioxidant Capacity of Leaf Powders and Chocolate Samples

The AC was determined spectrophotometrically using DPPH, ABTS, CUPRAC and FRAP assays according to procedures described previously with some minor modifications [42]. AC values were expressed as millimoles of TE equivalent per 100 g of the studied sample.

2.9.1. DPPH Assay

The DPPH method was used to determine the AC of the extracts of MGTP, MOLP and chocolates. In brief, 0.2–0.5 mL of extracts were added to 1.8–1.5 mL of methanol and 0.5 mL of DPPH methanolic solution (304.0 µmol/L). The obtained mixtures were shaken vigorously and left in darkness for 15 min. The absorbance was measured at 517 nm against a reagent blank (2 mL of methanol and 0.5 mL of DPPH methanolic solution) using a Hitachi U-2900 UV-VIS spectrophotometer in a 1 cm glass cell. The scavenging of DPPH was calculated using Equation (1):

$$\%DPPH_{scavenging} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad (1)$$

where: $A_{control}$—absorbance of DPPH radical with methanol; A_{sample}—absorbance of DPPH radical with extract (or standard solution).

The calibration curve, $\%DPPH = (782.10 \pm 5.74)c_{TE} + (4.03 \pm 0.40)$, was prepared using working solutions of TE in methanol between 0.02 and 0.10 µmol/mL.

2.9.2. ABTS Assay

In this procedure, 0.1–0.3 mL of the studied extracts were added to 2.4–2.2 mL of $ABTS^{\bullet+}$ solution (7 mmol/L), and the mixtures were incubated at 30 °C for 5 min. The absorbance was measured at 734 nm against a reagent blank (2.5 mL of $ABTS^{\bullet+}$ solution).

The scavenging of ABTS was calculated using Equation (2):

$$\%ABTS_{scavenging} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad (2)$$

where: $A_{control}$—absorbance of ABTS radical cation with methanol; A_{sample}—absorbance of ABTS radical cation with extract (or standard solution).

The calibration curve, $\%ABTS = (405.39 \pm 3.40)c_{TE} + (10.38 \pm 0.30)$, was prepared using working solutions of TE between 0.01 and 0.15 µmol/mL.

2.9.3. CUPRAC Assay

In brief, 0.6 mL of each prepared extract, 2 mL of 0.01 mol Cu(II)/L, 2 mL of neocuproine solution (0.0075 mol/L) and 2 mL of ammonium acetate buffer (pH = 7) were transferred into 10 mL volumetric flasks and made up to volume with redistilled water. The obtained solutions were kept at room temperature for 30 min. The absorbance was measured at 450 nm against a reagent blank (2 mL of $CuCl_2$, 2 mL of neocuproine solution and 2 mL of ammonium acetate buffer made up to 10 mL with redistilled water).

The calibration curve, $A_{450} = (8.22 \pm 0.06)c_{TE} - (0.0097 \pm 0.0029)$, was prepared using working solutions of TE in methanol between 0.01 and 0.08 µmol/mL.

2.9.4. FRAP Assay

Briefly, 0.1–0.6 mL of extracts from leaf powders and chocolates and 2 mL of FRAP reagent (100 mL of a 10 mmol/L TPTZ solution in 40 mmol/L HCl, 10 mL of 20 mmol/L $FeCl_3$ and 25 mL of 0.1 mol/L acetate buffer, pH 3.6) were transferred into 10 mL volumetric flasks and made up to volume with redistilled water. After 20 min, the absorbance was measured at 593 nm against a reagent blank (2 mL of FRAP reagent made up to 10 mL with redistilled water).

The calibration curve, $A_{593} = (51.51 \pm 0.42)c_{TE} - (0.0023 \pm 0.0040)$, was prepared using working solutions of TE in methanol between 0.001 and 0.017 µmol/mL.

2.10. Chocolate Color Measurements

The color of chocolate samples was measured using equipment for digital image analysis (DIA), including a CCD (charge-coupled device) Nikon DXM-1200 color camera (Nikon Instruments, Melville, USA), a Kaiser RB 5004 HF—High Frequency Daylight Copy Light set (4 × 36 W fluorescent light tubes; color temperature about 5400 K) (Kaiser Fototechnik GmbH & Co. KG, Buchen, Germany) and LUCIA (Laboratory Universal Computer Image Analysis) G v. 4.8 software. The results were expressed in the parameters of the CIEL*a*b* model, and the total difference (ΔE) between a plain sample (*WCh*) and a supplemented white chocolate sample (*WChS*) was calculated using Equation (3):

$$\Delta E = \sqrt{(L^*_{WCh} - L^*_{WChS})^2 + (a^*_{WCh} - a^*_{WChS})^2 + (b^*_{WCh} - b^*_{WChS})^2} \quad (3)$$

The L^* parameter represented lightness, from the darkest black as $L^* = 0\%$ to the brightest white as $L^* = 100\%$. The a^* parameter represented green/red color (negative/positive values), while the b^* parameter represented blue/yellow color (negative/positive values).

The sample color was measured in triplicate on the chocolate surface immediately after production [46].

2.11. Sensory Acceptance Test

Experimental chocolates supplemented with 4% MGTP and MOLP during the tempering process were sensorily evaluated. The assessment was made by a trained panel consisting of 12 employees of the Confectionery Factory "Kopernik" S.A., including 9 women and 3 men, aged 24 to 53. All panelists were familiar with sensory analysis techniques and had prior experience of sensory evaluation of chocolate. The panelists were provided with water to rinse their mouths before and after testing each sample. The evaluation was carried out in natural sunlight at room temperature. Firstly, the attributes of each of the tested chocolates were identified. The assessment was discussed by the free-choice profile procedure, in which each individual was asked to describe, using their own terms, the appearance, flavor and texture of the chocolate samples. Then, the features were described according to a five-point rating scale, in which 5 represented extremely desirable quality, 4 desirable quality, 3 good quality, 2 reluctance and 1 product defectiveness.

2.12. Data Analysis

All chemical analyses were conducted in triplicate. The results were tested statistically using analysis of variance (ANOVA) followed by Tukey's test. The effects of the plant powder percentage and supplementation stage were determined using a three-way variance analysis with Wilk's test. Additionally, the Pearson's correlation coefficients were calculated for relationships between AC and the content of antioxidants. The analyses were performed using Statistica 13.1 PL software (StatSoft, Kraków, Poland) at $p \leq 0.05$ significance level.

3. Results

3.1. Characterization of Antioxidants and the Antioxidant Capacities of Leaf Powders Used for White Chocolate Supplementation

The TPC and HPLC results for leaf powders added to chocolate samples are presented in Table 1. As can be seen, the antioxidant profiles of MGTP and MOLP varied. Although both leaf powders, MGTP and MOLP, were characterized by a high TPC, MGTP was a significantly richer source of polyphenols than MOLP (over 5.5 times higher TPC).

Table 1. Chemical composition and antioxidant capacity of matcha green tea powder and moringa leaf powder.

Quality Parameter	Leaf Powder	
	MGTP	MOLP
	Hydrophilic antioxidants	
TPC (mg GA/100 g)	4556 ± 207	821 ± 40
	Phenolic acids (mg/100 g)	
Gallic acid	21.7 ± 0.8	0.93 ± 0.01
Protocatechuic acid	5.4 ± 0.7	5.01 ± 0.11
Gentistic acid	0.35 ± 0.01	<LOD
Chlorogenic acid	45.5 ± 1.6	3.71 ± 0.01
p-OH-benzoic acid	1.43 ± 0.04	9.1 ± 0.8
Caffeic acid	1.87 ± 0.24	2.79 ± 0.23
Salicylic acid	1.91 ± 0.07	0.47 ± 0.02
Syringic acid	<LOD	0.48 ± 0.01
Ferulic acid	<LOD	1.52 ± 0.25
m-Coumaric acid	1.51 ± 0.08	<LOD
Sinapic acid	2.04 ± 0.29	2.80 ± 0.11
Vanillic acid	1.02 ± 0.15	<LOD
p-Coumaric acid	0.51 ± 0.07	2.39 ± 0.10
Ellagic acid	0.01 ± 0.01	0.14 ± 0.01
Total phenolic acids	83.33 ± 0.08	29.3 ± 1.5

Table 1. *Cont.*

Quality Parameter	Leaf Powder	
	MGTP	MOLP
	Lipophilic antioxidants	
	Tocopherols (mg/100 g)	
α-tocopherol	0.04 ± 0.00	2.56 ± 0.02
γ-tocopherol	0.04 ± 0.00	0.62 ± 0.02
δ-tocopherol	0.05 ± 0.00	0.13 ± 0.01
Total tocopherols	0.13 ± 0.00	3.31 ± 0.01
	Carotenoids (mg/100 g)	
Luteoxanthin	4.61 ± 0.06	0.93 ± 0.01
Cis-lutein	2.98 ± 0.05	0.86 ± 0.01
Trans-lutein	64.0 ± 1.6	205.3 ± 0.7
Zeaxanthin	3.05 ± 0.01	38.57 ± 0.26
Cryptoxanthin	0.75 ± 0.01	3.37 ± 0.11
13-*cis*-β-carotene	n.d.	2.81 ± 0.02
α-carotene	0.59 ± 0.00	0.43 ± 0.01
Trans-β-carotene	1.57 ± 0.02	32.6 ± 0.4
9-*cis*-β-carotene	0.36 ± 0.01	4.13 ± 0.15
Others	0.77 ± 0.02	7.52 ± 0.26
Total carotenoids	78.7 ± 1.6	296.5 ± 1.0
	Antioxidant capacity (mmol TE/100 g)	
DPPH assay	260 ± 7	32.1 ± 1.1
ABTS assay	259.1 ± 1.3	33.5 ± 0.3
CUPRAC assay	153.5 ± 2.0	44.1 ± 0.6
FRAP assay	52.95 ± 0.16	9.65 ± 0.03

It was found that these plant raw materials contain a number of hydrophilic and lipophilic compounds with antioxidant activity. The hydrophilic antioxidants determined in the leaf powders were phenolic acids, and their higher content (83.33 mg/100 g) characterized MGTP. MOLP contained approximately 3 times fewer of these compounds than MGTP. The predominant phenolic acids in MGTP were chlorogenic acid (45.5 mg/100 g) and gallic acid (21.7 mg/100 g), which in total accounted for 81% of all phenolic acids. Other phenolic acids in this leaf powder were present in smaller amounts, not exceeding 5.5 mg/100 g. MOLP was characterized by a more differentiated phenolic acid profile. In this leaf powder, the contents of three phenolic acids accounted for 61%, including *p*-OH-benzoic acid (9.1 mg/100 g), protocatechuic acid (5.01 mg/100 g) and chlorogenic acid (3.71 mg/100 g). Caffeic, sinapic and *p*-coumaric acids were also present in greater concentrations in MOLP (2–3 mg/100 g).

In contrast, MOLP proved to be a significantly better source of lipophilic antioxidants such as carotenoids (296.5 mg/100 g) and tocopherols (3.31 mg/100 g). MGTP had a more than 3.5 times lower level of carotenoids than MOLP, and the content of tocopherols was only 0.13 mg/100 g of powder. Nine carotenoids in both leaf powders were identified, but the lutein fraction (both forms, *trans* and *cis*) was predominant and accounted for 70% in MOLP and 85% in MGTP. Zeaxanthin and β-carotene were present in a higher amount in MOLP, at 38.57 mg/100 g and 32.6 mg/100 g, respectively. The main tocopherol homologue in MOLP was α-tocopherol, which accounted for 77% of total tocopherols. The levels of γ- and δ-tocopherols were lower, at 0.62 and 0.13 mg/100 g, respectively. In MGTP, the percentage shares of all homologues in the total content of tocopherols were similar.

In order to evaluate the antioxidant properties of the MGTP and MOLP used in this study, two radical scavenging assays (DPPH and ABTS) and two reducing methods (CUPRAC and FRAP) were applied, and the results are presented in Table 1.

It can be noted that the MGTP extract indicated stronger DPPH and ABTS radical scavenging activities than the MOLP extract. The calculated DPPH and ABTS values for MOLP were 8-fold lower than those results for MGTP (Table 1). Furthermore, MOLP extract had 3 and 5 times lower CUPRAC (44.1 mmol TE/100 g) and FRAP (9.65 mmol/100 g)

values, respectively, in comparison with MGTP extract (CUPRAC = 153.5 mmol TE/100 g and FRAP = 52.95 mmol TE/100 g). These differences in the AC results, determined by the four analytical methods applied in this study, reflect a difference in the ability of antioxidant compounds in the extracts to quench radicals and reduce ferric and cupric ions. It is noteworthy that the simultaneous determination of lipophilic and hydrophilic antioxidants can be achieved by the CUPRAC and ABTS assays. In turn, the DPPH assay is known to work well with lipophilic antioxidants in alcohol solvents, while the FRAP method is especially appropriate for hydrophilic antioxidants but is not adequate for lipophilic antioxidants [36]. For this reason, high AC values for MGTP extract were related to a high content of phenolic antioxidants in this powder. However, similar DPPH, ABTS and CUPRAC results confirmed a dominant proportion of lipophilic antioxidants in MOLP extract (Table 1).

3.2. Phenolic Antioxidants Content in Supplemented White Chocolate

The total amount of polyphenols in the WCh without and with leaf powders added during the Co and Te processes are listed in Table 2.

Table 2. Total phenolic content in white chocolate with matcha green tea powder and moringa leaf powder added during conching and tempering.

Chocolate Sample	TPC	
	Content (mg GA/100 g)	Increase (Times) *
WCh	6.84 ± 0.05^{a}	-
WCh_1%MGTP_Co	50.9 ± 1.4^{e}	6.4
WCh_1%MGTP_Te	70.6 ± 1.2^{f}	9.3
WCh_2%MGTP_Co	90 ± 4^{g}	12.11
WCh_2%MGTP_Te	109.4 ± 1.7^{h}	15.0
WCh_3%MGTP_Co	97.2 ± 2.9^{i}	13.2
WCh_3%MGTP_Te	163.5 ± 0.8^{j}	22.9
WCh_4%MGTP_Co	134 ± 4^{k}	18.6
WCh_4%MGTP_Te	229 ± 7^{l}	32.5
WCh_1%MOLP_Co	11.36 ± 0.59^{a}	0.7
WCh_1%MOLP_Te	12.80 ± 0.49^{a}	0.9
WCh_2%MOLP_Co	19.95 ± 0.65^{b}	1.9
WCh_2%MOLP_Te	20.21 ± 0.09^{b}	1.9
WCh_3%MOLP_Co	29.21 ± 0.79^{c}	3.3
WCh_3%MOLP_Te	30.33 ± 0.82^{c}	3.4
WCh_4%MOLP_Co	39.96 ± 0.47^{d}	4.8
WCh_4%MOLP_Te	40.88 ± 0.58^{d}	5.0

* Increase calculated in relation to control sample (WCh). Values presented as mean ± standard deviation; means within the same column with superscripts having different letters (a–l) are significantly different (one-way ANOVA and Tukey's test, $p < 0.05$).

The control WCh used for the tests had a TPC of 6.84 mg GA/100 g, while supplemented chocolates were characterized by a higher concentration of these compounds. More favorable effects in this respect were observed for chocolates after the addition of MGTP. This additive incorporated during the Co process caused a 7 to 20 times increase in the total content of polyphenols. The same amounts of MOLP added during the chocolate conching resulted in an almost 2 to 6 times increase in the content of these compounds. However, the supplementation of WCh with MGTP and MOLP during the Te process increased TPC by 10–34 times and 2–6 times, respectively.

In this study, a destructive effect of the Co process on the content of polyphenols compared to the Te process was observed. In the case of chocolates supplemented with MGTP, depending on the degree of supplementation, the reduction in the presence of polyphenols was from about 30% to almost 50%. However, the reduction in TPC for chocolates enriched with MOLP was even and amounted to about 5%.

As seen in Table 3, the total content of phenolic acids in WCh was 363.1 μg/100 g.

Table 3. Phenolic acids content in white chocolate without and with 4% matcha green tea powder and moringa leaf powder added during conching and tempering.

Phenolic Acid	Content in WCh (μg/100 g)	Content in Supplemented WCh (μg/100 g)			
		4%MGTP_Co	4%MGTP_Te	4%MOLP_Co	4%MOLP_Te
Gallic acid	<LOD	744 ± 41 ^{b}	797 ± 28 ^{c}	37.3 ± 2.4 ^{a}	38.9 ± 1.2 ^{a}
Protocatechuic acid	21.42 ± 0.26 ^{a}	154 ± 4 ^{b}	156 ± 4 ^{b}	167 ± 14 ^{d}	162 ± 6 ^{c}
Gentistic acid	2.09 ± 0.48 ^{b}	8.32 ± 0.54 ^{c}	10.36 ± 0.73 ^{d}	1.78 ± 0.29 ^{a}	2.08 ± 0.15 ^{b}
Chlorogenic acid	7.00 ± 0.11 ^{a}	1693 ± 37 ^{e}	1684 ± 41 ^{d}	158.29 ± 0.05 ^{b}	160.00 ± 0.01 ^{c}
p-OH-benzoic acid	32.6 ± 1.1 ^{a}	49.3 ± 0.8 ^{b}	51.87 ± 0.91 ^{b}	266 ± 5 ^{d}	238 ± 6 ^{c}
Caffeic acid	4.05 ± 0.47 ^{a}	59 ± 2 ^{b}	72 ± 4 ^{c}	103.2 ± 2.9 ^{d}	107.6 ± 2.0 ^{e}
Salicylic acid	<LOD	76 ± 3 ^{b}	80.1 ± 1.1 ^{c}	14.4 ± 1.5 ^{a}	16.6 ± 1.8 ^{a}
Syringic acid	<LOD	<LOD	<LOD	19.0 ± 0.6 ^{a}	20.47 ± 0.28 ^{b}
Ferulic acid	11.38 ± 0.17 ^{b}	9.32 ± 0.86 ^{a}	10.3 ± 0.6 ^{a,b}	53.0 ± 2.8 ^{c}	71.2 ± 1.7 ^{d}
m-Coumaric acid	<LOD	56.6 ± 2.5 ^{a}	55.0 ±1.3 ^{a}	<LOD	<LOD
Sinapic acid	238 ± 1 ^{d}	180.3 ± 2.3 ^{b}	189.0 ± 1.2 ^{c}	177.44 ± 0.17 ^{a}	179 ± 6 ^{a,b}
Vanillic acid	45.35 ± 0.26 ^{b}	81.2 ± 2.5 ^{c}	80.6 ± 2.6 ^{c}	25.3 ± 0.7 ^{a}	26.3 ± 0.7 ^{a}
p-Coumaric acid	0.83 ± 0.25 ^{a}	12.7 ± 1.4 ^{b}	15.8 ± 0.4 ^{c}	52.72 ± 0.06 ^{d}	52 ± 5 ^{d}
Ellagic acid	< LOD	0.42 ± 0.00 ^{a}	0.42 ± 0.00 ^{a}	4.9 ± 1.3 ^{b}	4.64 ± 0.24 ^{b}
Total phenolic acids	363.1 ± 1.7 ^{a}	3124 ± 71 ^{b}	3202 ± 65 ^{c}	1080 ± 11 ^{d}	1079.6 ± 1.5 ^{d}

Values presented as mean ± standard deviation; means within the same row with superscripts having different letters (a–e) are significantly different (one-way ANOVA and Tukey's test, $p < 0.05$).

The predominant phenolic acid in this sample was sinapic acid, which accounted for approximately 66%. Furthermore, the plain WCh contained high amounts of protocatechuic, *p*-OH-benzoic and vanillic acids (21.42–45.35 μg/100 g). Gentistic, chlorogenic, caffeic, ferulic and *p*-coumaric acids were determined in the WCh, but at lower concentrations, not exceeding 12 μg/100 g. The samples of chocolates enriched with MGTP and MOLP powders were characterized by significantly higher contents of phenolic acids than that found in plain WCh. The addition of 4% MGTP increased the total content of phenolic acids in fortified chocolates by more than 8 times, while WCh samples with 4% of MOLP revealed only 3 times higher amounts of total phenolic acids. Chlorogenic and gallic acids predominated in MGTP (45.5 and 21.7 mg/100 g, respectively) as well as in chocolates enriched with this powder (1684–1693 and 797–744 μg/100 g, respectively), regardless of the supplementation stage. In turn, the WCh with 4% MOLP had the highest amounts of *p*-OH-benzoic, sinapic, protocatechuic and chlorogenic acids, ranging between 158.29 and 266 μg/100 g. Unexpectedly, a significantly lower amount of sinapic acid in fortified WCh than in the control sample probably can be explained by the possible chocolate matrix effect due to some macromolecules such as proteins and polysaccharides interacting with this compound present in natural additives and reducing its extractability.

In general, the Te process of WCh supplementation appeared to be more crucial for MGTP, as evidenced by the lesser reduction in phenolic acid content. In the case of MOLP addition, there were no significant differences in the contents of most phenolic acids and total phenolic acids in samples prepared during the Co and Te processes (Table 3, Tukey's test).

3.3. Lipophilic Antioxidants Content in Supplemented White Chocolate

As can be seen in Table 4, the main lipophilic compounds determined by the HPLC in the studied plain WCh were γ-tocopherol (627 μg/100 g), α-tocopherol (105.0 μg/100 g) and *trans*-β-carotene (52.49 μg/100 g).

Table 4. Contents of tocopherols and carotenoids in white chocolate without and with 4% matcha green tea powder and moringa leaf powder added during conching and tempering.

Compound	Content in WCh (μg/100 g)	Content in Supplemented WCh (μg/100 g)			
		4%MGTP_Co	4%MGTP_Te	4%MOLP_Co	4%MOLP_Te
		Tocopherols			
α-tocopherol	105.0 ± 1.7 ^{c}	77.1 ± 1.4 ^{b}	64.7 ± 2.4 ^{a}	114.2 ± 2.2 ^{d}	113 ± 5 ^{d}
γ-tocopherol	627 ± 7 ^{c}	486.1 ± 1.8 ^{a}	477 ± 5 ^{a}	536.6 ± 0.5 ^{b}	537 ± 7 ^{b}
δ-tocopherol	14.65 ± 0.23 ^{a}	14.13 ± 0.13 ^{a}	14.3 ± 1.2 ^{a}	16.34 ± 0.06 ^{b}	16.8 ± 0.6 ^{b}
Total tocopherols	746 ± 9 ^{e}	577.3 ± 0.6 ^{b}	556 ± 6 ^{a}	660 ± 8 ^{c}	667 ± 12 ^{d}
		Carotenoids			
Luteoxanthin	<LOD	140 ± 5 ^{b}	161 ± 9 ^{c}	38.3 ± 0.8 ^{a}	39.14 ± 0.11 ^{a}
Cis-lutein	<LOD	95.0 ± 2.1 ^{b}	124.2 ± 0.6 ^{c}	33.6 ± 0.4 ^{a}	34.6 ± 0.8 ^{a}
Trans-lutein	<LOD	2285 ± 18 ^{a}	2430 ± 11 ^{b}	8119 ± 31 ^{c}	8297± 74 ^{d}
Zeaxanthin	<LOD	73 ± 3 ^{a}	99 ± 10 ^{b}	1209 ± 18 ^{c}	1333 ± 36 ^{d}
13-*cis*-β-carotene	<LOD	<LOD	<LOD	97 ± 4 ^{a}	108 ± 15 ^{b}
α-carotene	<LOD	24 ± 3 ^{c}	22.46 ± 0.17 ^{c}	12.6 ± 1.0 ^{a}	16.0 ± 1.4 ^{b}
Trans-β-carotene	52.49 ± 0.18 ^{a}	93.7 ± 2.5 ^{b}	109 ± 9 ^{c}	1304 ± 45 ^{d}	1337 ± 49 ^{d}
9-*cis*-β-carotene	<LOD	13.0 ± 0.9 ^{b}	5.7 ± 1.0 ^{a}	172 ± 5 ^{c}	173 ± 4 ^{c}
Total carotenoids	52.49 ± 0.18 ^{a}	2724 ± 25 ^{b}	2951.8 ± 0.9 ^{c}	10986 ± 105 ^{d}	11339 ± 80 ^{e}

Values presented as mean ± standard deviation; means within the same row with superscripts having different letters (a–e) are significantly different (one-way ANOVA and Tukey's test, $p < 0.05$).

The addition of 4% MOLP to WCh during the Te process significantly (up to 11,339 μg/100 g) enriched the product with carotenoids. In the WCh_4%MGTP, the content of these compounds was about 4 times lower than in the WCh_4%MOLP, regardless of supplementation stage. However, the MGTP addition increased the content of carotenoids by over 50 times compared to plain WCh. The highest levels of lutein fractions (*cis*- and *trans*-isomers) were found in all enriched chocolates, accounting for 73–86% of all carotenoids. Both leaf powders increased the content of *trans*-β-carotene in WCh by 2 times for MGTP and 25 times for MOLP. It was also found that the addition of MOLP enriched WCh with zeaxanthin (up to 1333 μg/100 g) and *cis*-β-carotene (up to 282 μg/100 g), while the addition of MGTP enriched it with luteoxanthin (up to 161 μg/100 g) and zeaxanthin (up to 99 μg/100 g). In contrast, the total content of tocopherols in the supplemented WCh was lower compared to the plain WCh as a result of their lower levels in leaf powders (Table 1). A slightly higher concentration of α-tocopherol in WCh with MOLP (by about 8%) was noted compared to WCh.

Analyzing the impact of the supplementation stage on the lipophilic antioxidant profile, it was found that generally more carotenoids were retained in the product when both leaf powders were added during the Te process. In the case of tocopherols, varied effects were observed, but most of the differences were not statistically significant.

3.4. Antioxidant Capacity of Supplemented White Chocolate

Similarly, as in the case of the leaf powders, the AC of WCh without and with MGTP and MOLP added during the Co and Te processes was evaluated by two radical scavenging assays (DPPH and ABTS) and two reducing potential assays (CUPRAC and FRAP). The obtained DPPH, ABTS, CUPRAC and FRAP results are presented in Table 5.

Plain WCh was characterized by a relatively low AC, from 0.08 mmol TE/100 g (FRAP test) to 0.53 mmol TE/100 g (CUPRAC test). In the enriched chocolates, the addition of 1 to 4% MGTP to WCh during the Co process caused a significant increase in the AC values compared to WCh. The increase was from 5 times for WCh_1%MGTP_Co (CUPRAC assay) to 39 times for WCh_4%MGTP_Co (FRAP assay), whereas the MOLP increased the AC of chocolate samples by up to 6 times (the greatest enhancement was observed for WCh_4%MOLP_Co as determined by DPPH assay). The same amounts of MGTP added to WCh during the Te process increased AC by 5 times for WCh_1%MGTP_Te

(CUPRAC assay) up to 57 times for WCh_4%MGTP_Te (DPPH assay), but 4% of MOLP resulted in an AC increase of up to 7 times in the studied samples. A destructive effect of the Co process on the antioxidant properties of WCh incorporated with plant powders was also observed; in the case of chocolate supplemented with MGTP, depending on the degree of supplementation and the analytical method, the reduction in AC ranged from approximately 0% for WCh_1%MGTP analyzed by FRAP assay to almost 50% for WCh_4%MGTP tested by the DPPH method.

Table 5. Antioxidant capacity of white chocolate with matcha green tea powder and moringa leaf powder added during conching and tempering.

Chocolate Sample	AC Determined by Antioxidant Assays (mmol TE/100 g)			
	DPPH	ABTS	CUPRAC	FRAP
WCh	0.21 ± 0.01 ^{a}	0.43 ± 0.01 ^{a}	0.53 ± 0.01 ^{a}	0.08 ± 0.00 ^{a}
WCh_1%MGTP_Co	2.48 ± 0.03 ^{f}	3.77 ± 0.06 ^{f}	2.39 ± 0.10 ^{g,h}	0.92 ± 0.01 ^{g}
WCh_1%MGTP_Te	3.65 ± 0.6 ^{g}	4.13 ± 0.10 ^{f}	2.58 ± 0.09 ^{h}	0.92 ± 0.01 ^{g}
WCh_2%MGTP_Co	3.91 ± 0.15 ^{g}	3.77 ± 0.04 ^{f}	3.67 ± 0.12 ^{i}	1.65 ± 0.03 ^{h}
WCh_2%MGTP_Te	5.37 ± 0.14 ^{i}	7.49 ± 0.09 ^{g}	5.03 ± 0.09 ^{j}	1.69 ± 0.07 ^{h}
WCh_3%MGTP_Co	4.40 ± 0.13 ^{h}	9.07 ± 0.42 ^{h}	7.41 ± 0.18 ^{k}	2.10 ± 0.05 ^{i}
WCh_3%MGTP_Te	8.72 ± 0.33 ^{k}	10.82 ± 0.09 ^{i}	8.88 ± 0.18 ^{l}	2.24 ± 0.01 ^{j}
WCh_4%MGTP_Co	5.93 ± 0.16 ^{j}	12.52 ± 0.39 ^{j}	9.51 ± 0.14 ^{m}	3.13 ± 0.01 ^{k}
WCh_4%MGTP_Te	11.85 ± 0.39 ^{l}	13.88 ± 0.40 ^{k}	10.35 ± 0.15 ^{n}	3.18 ± 0.06 ^{k}
WCh_1%MOLP_Co	0.42 ± 0.01 ^{a,b}	0.51 ± 0.02 ^{a,b}	0.99 ± 0.01 ^{b}	0.16 ± 0.01 ^{b}
WCh_1%MOLP_Te	0.45 ± 0.01 ^{a,b}	0.57 ± 0.01 ^{a,b}	1.03 ± 0.01 ^{b}	0.20 ± 0.00 ^{b,c}
WCh_2%MOLP_Co	0.65 ± 0.02 ^{b,c}	0.84 ± 0.01 ^{a,b,c}	1.41 ± 0.02 ^{c}	0.26 ± 0.00 ^{c}
WCh_2%MOLP_Te	0.75 ± 0.01 ^{b,c}	0.93 ± 0.01 ^{a,b,c}	1.56 ± 0.01 ^{c,d}	0.27 ± 0.00 ^{c}
WCh_3%MOLP_Co	0.91 ± 0.01 ^{c,d}	0.99 ± 0.04 ^{b,c}	1.83 ± 0.01 ^{d,e}	0.37 ± 0.00 ^{d}
WCh_3%MOLP_Te	1.00 ± 0.03 ^{c,d}	1.58 ± 0.05 ^{d}	1.94 ± 0.01 ^{e,f}	0.38 ± 0.00 ^{d,e}
WCh_4%MOLP_Co	1.26 ± 0.02 ^{d,e}	1.30 ± 0.06 ^{c,d}	2.22 ± 0.01 ^{f,g}	0.46 ± 0.00 ^{e}
WCh_4%MOLP_Te	1.50 ± 0.03 ^{e}	2.15 ± 0.05 ^{e}	2.40 ± 0.01 ^{g,h}	0.59 ± 0.01 ^{f}

Values presented as mean ± standard deviation; means within the same column with superscripts having different letters (a–n) are significantly different (one-way ANOVA and Tukey's test, $p < 0.05$).

3.5. Effectiveness of Enrichment and Processing Stages in Increasing the Antioxidant Properties of Supplemented White Chocolate

In Table 6, the effect of different factors (plant powder percentage and the stage of its addition to the chocolate) on the TPC and AC of chocolate is presented. The highest effect of the powder percentage on both chocolate parameters, TPC and AC, was noted (57–100% of the explained variance). The MOLP percentage was especially highly decisive for the TPC and AC of supplemented chocolate (>74% of the explained variance). The MGTP percentage had a greater impact on AC as determined by ABTS, CUPRAC and FRAP assays (> 91% of the explained variance), while TPC and DPPH radical scavenging activity were less dependent on the powder percentage (68.60 and 56.78% of the explained variance, respectively).

The impact of the supplementation stage on the tested parameters was of greater importance for MGTP (22.09 and 31.07% of the explained variance for TPC and AC from the DPPH assay, respectively). However, the addition of MOLP at different processing stages generally had a relatively low or not statistically significant ($p > 0.05$) effect on the antioxidant properties of WCh (Table 6). The exception was only the AC determined by the ABTS assay because 15.02% of the explained variance was noted. It was found that the summed effect of two factors (powder type and supplementation stage) on TPC and DPPH of WCh with MGTP (9–12% of the explained variance) and ABTS of WCh with MOLP (10.48% of the explained variance) was moderate, while for other studied parameters, the influence of the interaction of the two factors was negligible.

Table 6. Effect of matcha green tea powder and moringa leaf powder percentage and the stage of their addition (percentage of explained variance) on total phenolic content and antioxidant capacity of white chocolate.

Factor	TPC	AC Determined by Antioxidant Assays			
		DPPH	ABTS	CUPRAC	FRAP
MGTP addition					
Powder percentage (PP)	68.60	56.78	91.49	96.59	99.63
Supplementation stage (SS)	22.09	31.07	5.62	2.56	0.12
PP x SS	9.05	11.81	2.59	0.72	0.10
Other factors	n.s.	n.s.	n.s.	n.s.	n.s.
MOLP addition					
Powder percentage (PP)	99.55	96.02	74.15	98.17	93.04
Supplementation stage (SS)	n.s.	2.69	15.02	1.49	3.03
PP x SS	0.20	1.11	10.48	0.30	3.88
Other factors	n.s.	n.s.	n.s.	n.s	n.s.

n.s. = effect not significant; three-way analysis with Wilks's test ($p \leq 0.05$).

3.6. Correlations between Antioxidant Capacity and Individual Antioxidants in Supplemented White Chocolate

Regression analysis was performed for correlations between the AC determined by four different analytical methods, TPC analyzed by FC assay and the contents of individual antioxidants in chocolates with MGTP and MOLP added during two technological processes (Table 7).

Table 7. Correlation coefficients (r) between antioxidant capacity determined by different analytical methods, total phenolics, phenolic acids, carotenoids and tocopherols in the investigated chocolates.

Compound	DPPH	ABTS	CUPRAC	FRAP
TPC	0.99 *	0.95 *	0.95 *	0.93 *
Gallic acid	−0.10	0.11	0.17	0.17
Protocatechuic acid	0.50	0.49	0.56	0.52
Gentistic acid	−0.19	−0.01	0.05	0.05
Chlorogenic acid	−0.06	0.15	0.21	0.22
p-OH-benzoic acid	0.56	0.35	0.35	0.31
Caffeic acid	0.49	0.37	0.42	0.38
Salicylic acid	−0.04	0.16	0.23	0.23
Syringic acid	0.44	0.24	0.24	0.21
Ferulic acid	0.29	0.11	0.11	0.08
m-Coumaric acid	−0.10	0.13	0.18	0.19
Sinapic acid	−0.55	−0.57	−0.63	−0.60
Vanillic acid	−0.26	−0.03	0.01	0.03
p-Coumaric acid	0.52	0.33	0.35	0.31
Ellagic acid	0.52	0.32	0.33	0.29
Total phenolic acids	0.01	0.21	0.27	0.27
Luteoxanthin	−0.02	0.16	0.22	0.22
Cis-lutein	−0.02	0.11	0.19	0.17
Trans-lutein	0.52	0.35	0.37	0.33
Zeaxanthin	0.44	0.24	0.25	0.21
13-*cis*-β-carotene	0.42	0.23	0.23	0.19
α-carotene	0.18	0.33	0.41	0.39
Trans-β-carotene	0.47	0.27	0.27	0.24
9-*cis*-β-carotene	0.49	0.30	0.31	0.27
Total carotenoids	0.50	0.33	0.35	0.31
α-tocopherol	0.27	0.09	0.04	0.03
γ-tocopherol	−0.24	−0.35	−0.43	−0.41
δ-tocopherol	0.30	0.09	0.09	0.06
Total tocopherols	−0.14	−0.28	−0.35	−0.33

* Statistically significant at $p \leq 0.05$.

It can be noted that the TPC results correlated significantly ($p < 0.05$) positively with a total antioxidant potential of studied chocolates determined by DPPH (r = 0.99), ABTS (r = 0.95), CUPRAC (r = 0.95) and FRAP (r = 0.93) methods.

However, moderate correlation coefficients (r = 0.29–0.56) for the relationships between some phenolic acids, such as protocatechuic, *p*-OH-benzoic, caffeic, *p*-coumaric and ellagic acids, and AC were observed. Unexpectedly, there were negative correlations between the contents of sinapic acid and all AC values (r varied from −0.55 to −0.63). This can be explained by the fact that the sinapic acid level in fortified chocolates with the highest AC values was significantly lower than in plain WCh without plant additives, having the lowest antioxidant potential analyzed by all analytical tests (Tables 3 and 5). It was also found that most phenolic acids and carotenoids in chocolates were more closely correlated with radical scavenging activity as analyzed by DPPH assay. Moreover, low positive correlation coefficients for the relationships between α- and δ-tocopherol and AC (r = 0.03–0.30) were calculated, while there were low negative correlations between γ-tocopherol and AC (r varied from −0.24 to −0.43) and total tocopherol and AC (r varied from −0.14 to −0.35). This fact indicated that tocopherol homologues in chocolate samples were not capable of scavenging DPPH radicals, ABTS radical cations and reducing oxidants (cupric and ferric ions).

3.7. Effect of Leaf Powders Addition on Color Parameters and Sensory Properties of White Chocolate

Color is one of the crucial attributes of a product because it attracts customers. It also influences acceptance of the sensory characteristics of a food product [47]. The color measurements confirmed that the composition of chocolate had a significant impact on the intensity of the green color (negative *a** values) and that it increased with increasing MGTP and MOLP concentrations in supplemented chocolates (Table 8).

Table 8. Color parameters and total color difference (ΔE) of white chocolate with matcha green tea powder and moringa leaf powder added during conching and tempering.

Chocolate Sample	Color parameters			ΔE (-) *
	*L** (%)	*a** (-)	*b** (-)	
WCh	96.9 ± 0.3^{h}	-3.28 ± 0.06^{j}	12.83 ± 0.10^{a}	-
WCh_1%MGTP_Co	88.53 ± 0.19^{g}	-5.7 ± 0.4^{i}	18.6 ± 0.4^{b}	10.4
WCh_1%MGTP_Te	89.47 ± 0.07^{g}	-5.8 ± 0.4^{i}	18.35 ± 0.23^{b}	9.6
WCh_2%MGTP_Co	84.5 ± 0.4^{f}	$-7.9 \pm 0.9^{g,h}$	$25.1 \pm 1.5^{d,e}$	18.0
WCh_2%MGTP_Te	85.23 ± 0.08^{f}	-7.4 ± 0.3^{h}	$22.9 \pm 0.5^{c,d}$	16.0
WCh_3%MGTP_Co	79.82 ± 0.24^{d}	$-11.6 \pm 0.5^{b,c,d}$	$34.8 \pm 0.3^{g,h}$	29.1
WCh_3%MGTP_Te	81.4 ± 0.4^{e}	$-9.4 \pm 0.4^{f,g}$	29.56 ± 0.14^{f}	23.6
WCh_4%MGTP_Co	76.04 ± 0.18^{a}	-13.4 ± 0.5^{a}	43.6 ± 1.3^{j}	38.5
WCh_4%MGTP_Te	$76.98 \pm 0.29^{a,b}$	$-13.1 \pm 0.5^{a,b}$	41.9 ± 0.5^{j}	36.6
WCh_1%MOLP_Co	88.25 ± 0.20^{g}	$-7.1 \pm 0.6^{h,i}$	$21.6 \pm 0.6^{b,c}$	12.9
WCh_1%MOLP_Te	88.83 ± 0.19^{g}	$-6.8 \pm 0.4^{h,i}$	$20.53 \pm 0.09^{b,c}$	11.7
WCh_2%MOLP_Co	84.25 ± 0.20^{f}	$-9.9 \pm 0.7^{e,f}$	28.7 ± 1.5^{f}	21.4
WCh_2%MOLP_Te	84.6 ± 0.5^{f}	-9.6 ± 0.6^{f}	$27.7 \pm 1.2^{e,f}$	20.3
WCh_3%MOLP_Co	80.17 ± 0.24^{d}	$-10.5 \pm 0.5^{d,e,f}$	33.0 ± 0.8^{g}	27.2
WCh_3%MOLP_Te	81.5 ± 0.5^{e}	$-11.3 \pm 0.7^{c,d,e}$	33.5 ± 1.5^{g}	27.0
WCh_4%MOLP_Co	77.73 ± 0.22^{c}	$-12.32 \pm 0.16^{a,b}$	38.7 ± 0.8^{i}	33.4
WCh_4%MOLP_Te	$77.21 \pm 0.29^{b,c}$	$-13.0 \pm 0.5^{a,b}$	39.5 ± 1.0^{i}	34.5

* Calculated in relation to control sample (WCh); values presented as mean ± standard deviation; means within the same column with superscripts having different letters (a–j) are significantly different (one-way ANOVA and Tukey's test, $p < 0.05$).

The highest greenness (*a** approximately -13) was found in all samples with 4% powdered leaves, regardless of their type and stage of supplementation. Yellowness (positive *b** values) was more dependent on the type of additive. It was found that the

values of the b^* parameter were more variable for MGTP additives (within a range of 18–44), and the highest value was determined in the case of WCh_4%MGTP_Co. Furthermore, all additives decreased the lightness (L^* values) from 7.6% (WCh_1%MGTP_Te) to 21.5% (WCh_4%MGTP_Co). In addition, the total color difference (ΔE) parameter which describes a difference between the color of white chocolate before and after supplementation was calculated. All values of ΔE were high and ranged from 9.6–38.5. It is assumed [48] that ΔE > 5 indicates that potential consumers would notice a different color in the case of each of the supplemented chocolates compared to WCh. Interestingly, unlike changes in TPC and AC, the supplementation stage generally did not have a significant effect on chocolate color parameters ($p > 0.05$). Therefore, for the sensory acceptance test, only two samples, WCh_4%MGTP_Te and WCh_4%MOLP_Te, were chosen. Average sensory assessments of individual attributes of chocolates with MGTP and MOLP are presented in Figure 2. In the chocolate supplemented with MGTP, the smell of sea mud, the smell of seaweed, the smell of wet straw and the taste of fish were distinguished, which were not recorded in the case of chocolate with MOLP.

The average score for WCh_4%MGTP_Te was 3.81, while WCh_4%MOLP_Te had a higher sensory score value of 4.74. Chocolate supplemented with MOLP was characterized by a more desirable sensory profile, resulting in the lack of negative discriminants present in chocolate with the addition of MGTP. Chocolate enriched with MOLP was frequently described as having an extremely desirable quality.

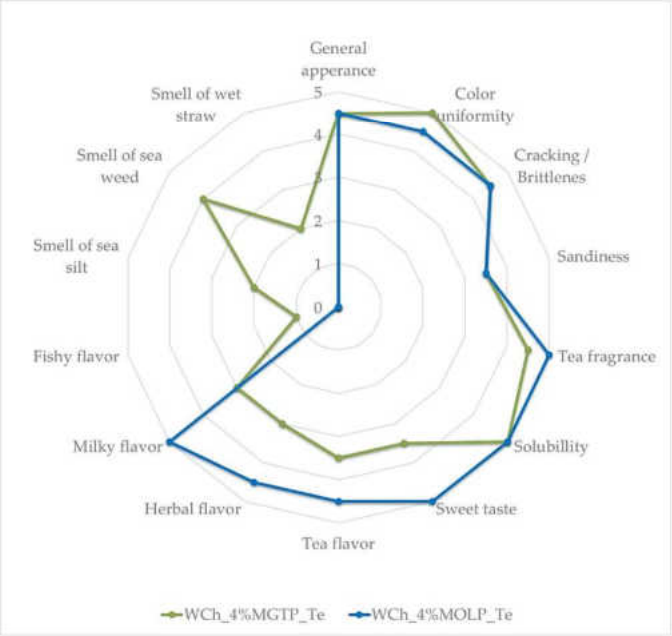

Figure 2. Average sensory ratings of individual attributes of white chocolates with 4% addition of matcha green tea powder and moringa leaf powder introduced during tempering process.

The evaluators paid particular attention to the wide range of flavor characteristics of chocolate supplemented with MGTP and unanimously agreed that it would be appreciated by connoisseurs. The sweet and milky taste of WCh was more noticeable in chocolate incorporated with MOLP than in chocolate with MGTP. This suggests that MGTP was more intense in flavor; thus, the flavor of the plain chocolate was masked. The smell of chocolate

with the addition of MOLP was assessed positively and the panelists did not notice any unusual aromas. Both chocolates, apart from the assessment of taste and smell, were subjected to an evaluation of physical characteristics, i.e., general appearance, where the focus was on the evaluation of gloss, the correct filling of the mold and even thickness of the chocolate bar, cracking/brittleness, sandiness, color uniformity and spreadability. The quality of the chocolates did not differ from each other; only the uniformity of the color of the chocolate supplemented with MGTP was rated higher than that of the sample with the addition of MOLP. This was probably due to the less powdery quality of moringa leaves in comparison with MGTP. The spreadability of both chocolates was unanimously and approvingly assessed and was due to the same WCh type used to produce the final products.

4. Discussion

Vegetable raw materials, cocoa and chocolate are products rich in phenolic compounds; therefore, they have become the subject of many publications concerning the determination of antioxidant properties. Todorowic et al. [49] analyzed several types of chocolate, differing mainly in their cocoa contents. Based on the results obtained by them, it can be concluded that the content of cocoa in chocolate has a relevant impact on the amount of polyphenol compounds and the antioxidant potential resulting from their presence. As the cocoa content increases, the antioxidant potential of chocolate also increases. The total content of polyphenols in plain WCh analyzed in our study was at a low level, not exceeding 10 mg GA/100 g of chocolate. The data available in the literature indicate a higher content of these compounds in this type of chocolate. For example, Genovese and Lannes [50] reported that the phenolic content in WCh commercially purchased in Brazil was 96 mg GA/100 g of chocolate. On the other hand, WCh available on the market in Malaysia, used in the research of Meng et al. [34], contained 126.39 mg of catechin/100 g of chocolate. In our study, by adding powdered matcha green tea and moringa leaves, a significant increase in the concentration of polyphenols was achieved. In particular, the addition of MGTP made it possible to produce chocolate with a high polyphenol content (up to 229 mg GA/100 g), similar to the level of polyphenols in dark chocolates available on the market. Mikołajczak and Tańska [51] stated that the content of polyphenols in dark chocolates available on the Polish market was in the range of 252.38–703.13 mg of catechin/100 g. Nevertheless, dark chocolates available in Malaysia and Brazil were characterized by a higher concentration (up to 700 mg/100 g) of these compounds [34,50].

Unfortunately, the characterization of the phenolic profile of chocolates is very limited. Some reports described the phenolic composition of dark chocolate, focusing on flavan-3-ols as the major class in chocolate phenolic profiles, but only an accurate and comprehensive characterization of the phenolic profile of dark chocolate and the impact of Sakura green tea leaves and turmeric powder were investigated by Martini et al. [19]. Individual phenolic compounds in dark chocolate were identified (158, including 67 for the first time) using liquid chromatography-electrospray ionization mass spectrometry. Therefore, our study revealed for the first time that the addition of MGTP and MOLP as well as technological processes (Co and Te) influenced and modified the hydrophilic and lipophilic antioxidant profiles of WCh, increasing individual antioxidant concentrations and total antioxidant potential.

On the other hand, WCh without additives revealed the weak antioxidant properties determined by analytical tests with different mechanisms, including electron transfer (ET) and mixed-mode (ET/hydrogen atom transfer—HAT) assays. However, the addition of powdered plants to WCh samples resulted in a linear increase in their antioxidant potential. It is noteworthy that MGTP containing high amounts of hydrophilic antioxidants with high total antioxidant potential more effectively increased the antioxidant properties of fortified WCh chocolates than the addition of MOLP richer in lipophilic antioxidants. This diversity was caused by different contents of compounds with antioxidant properties, especially polyphenols, carotenoids and tocopherols, in plant powders added to chocolates. Moreover, the variability of AC values for investigated leaf powders and chocolates fortified with

them can be explained by the influences of genetic, environmental and technological factors, which affect antioxidant contents. Undoubtedly, the different mechanisms of the analytical methods applied caused discrepancies between the AC results of natural additives and final products.

For comparison, Afifah and Niwat [52] reported a lower antioxidant potential of moringa compared to green tea. It is worth mentioning that powdered moringa leaves enriched WCh samples mainly with lipophilic antioxidants (carotenoids and tocopherols). Our study also confirmed the enhancement of hydrophilic antioxidants and the total AC of chocolates after MOLP addition. Similarly, TPC (10.20–13.83 mg GA/g), DPPH (EC_{50} = 13.53–8.83 mg/mL) and FRAP (9.32–14.86 mM TE/g) values for dark chocolates increased with increasing the concentrations of added moringa leaf powder (1, 3 and 5%) [24].

In contrast, phenolic acids, rutin, vitamin C, tocopherols, epigallocatechin gallate, epigallocatechin, epicatechin gallate and epicatechin were the most abundant antioxidants present in matcha green tea [53]. Lončarević et al. [18] reported that supplementation of WCh with green tea from 60 to 100 g/kg resulted in a significant increase in total phenolics, antioxidant potential and epigallocatechin-3-gallate content (TPC = 0.41 and 2.20–2.73 g GA/kg, DPPH = 1.22 and 12.55–16.12 mmol TE/kg and EGCG = 0.09 and 0.51–0.62 g/kg for control and fortified chocolates, respectively). Moreover, WCh samples with matcha tea revealed approximately 4–15 times higher TPC, DPPH, ABTS and reducing power values than the plain control chocolate [17].

There are a limited number of studies related to the bioactive compounds changed during chocolate processing. In our study, WCh with leaf powders added during the Co process had lower AC and TPC and total phenolic acids, tocopherols and carotenoids contents. Sulistyowati and Misnawi [54] linked these changes to the elevated temperature during conching. Furthermore, Di Mattia et al. [55] showed that changes in the composition of phenolic compounds were dependent on processing conditions. Furthermore, the oxidation and enzymatic mechanisms during the Co process may also influence the content of polyphenols. As a result of these reactions, complexes between polyphenols, amino acids, peptides and proteins are formed [32]. Nevertheless, the loss of polyphenols during the Te process has not been observed by researchers, but this stage in chocolate processing does not involve high temperatures [56].

5. Conclusions

The study confirms that chocolates supplemented with powdered matcha green tea and moringa leaves are products with sophisticated sensory characteristics and positive health properties due to significantly higher concentrations of bioactive polyphenolic compounds than are found in plain WCh. Due to the presence of plant powders, innovative chocolates can be classified as confectionery products having high antioxidant properties.

The supplementation of plain WCh at different stages of the production process caused a significant increase in AC and TPC determined by the modified DPPH, ABTS, CUPRAC, FRAP and FC methods, respectively. It was demonstrated that WCh samples fortified with MGTP had a much higher polyphenol content and, at the same time, much more excellent antioxidant properties than WCh enriched with MOLP. Supplementation of WCh samples with colored and flavored additives also resulted in changes in the color and organoleptic properties of the products. Generally, MOLP had a more beneficial effect on the sensory profile of WCh and a richer flavor bouquet.

In this study, the effect of incorporating two leaf powders during two processes (Co and Te) on phenolic and lipophilic antioxidant contents in WCh was observed for the first time. Although MOLP additives provided a lower amount of polyphenols than MGTP, it provided significantly higher levels of lipophilic antioxidants, mainly carotenoids, which also had strong antioxidant properties. Additionally, it was found that there were relationships between the amounts of leaf powders added and the AC and TPC in supplemented chocolates. Nevertheless, significant positive correlations were calculated between the four

analytical methods used to determine AC and TPC. In contrast, the results for AC and concentrations of individual hydrophilic and lipophilic antioxidants did not correlate well.

Furthermore, it is worth noting that the stage of supplementation with leaf powders could also be an important factor in determining chocolate's pro-health effects. The results obtained in the study showed that the incorporation of powdered plant additives during the Co process had a negative effect on the content of polyphenols and carotenoids and the AC of the finished product.

This work highlights that there is a need for further studies to obtain results useful for chocolate manufacturers not only in terms of obtaining the organoleptic properties preferred by consumers but also in developing products having beneficial effects on consumer health.

Author Contributions: Conceptualization, S.P., P.T., A.S.-C. and M.T.; methodology, S.P., P.T., S.K., S.C., A.S.-C. and M.T.; software, A.S.-C. and M.T.; validation, A.S.-C., M.T., S.P., P.T., S.C. and S.K.; formal analysis, P.T., M.T., S.P., S.K. and S.C.; investigation, S.P., P.T., S.K., A.S.-C. and M.T.; resources, S.P., P.T., S.K., A.S.-C. and M.T.; data curation, A.S.-C., M.T., S.C., S.P., P.T. and S.K.; writing—original draft preparation, S.P. and P.T.; writing—review and editing, A.S.-C. and M.T.; visualization, S.P., P.T., S.K., S.C., A.S.-C. and M.T.; supervision, A.S.-C. and M.T.; project administration, S.P., P.T., A.S.-C. and M.T.; funding acquisition, S.P. and A.S.-C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All of the data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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(D4) Poliński, S., Topka, P., Tańska, M., Kowalska, S., Czaplicki, S., & Szydłowska-Czerniak, A. (2022). Effect of Grinding Process Parameters and Storage Time on Extraction of Antioxidants from Ginger and Nutmeg. *Molecules, 27*(21), 7395.

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Effect of Grinding Process Parameters and Storage Time on Extraction of Antioxidants from Ginger and Nutmeg

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Abstract: The aim of this study was to optimize the grinding process parameters (mesh size of grinder sieve (X_1), the peripheral velocity of the grinding wheels (X_2)), and the storage time (X_3) of ground ginger rhizome and nutmeg to obtain ethanol and ethanol-water extracts with improved antioxidant properties. The optimal conditions were estimated using response surface methodology (RSM) based on a three-variable Box–Behnken design (BBD) in order to maximize the antioxidant capacity (AC) determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) methods, and the total phenolic content (TPC) was determined by the Folin–Ciocalteu (F–C) method in spice extracts. Additionally, the phenolic acid profiles in extracts from optimized conditions were analyzed using ultra-performance liquid chromatography (UPLC). It was found that the optimal preparation conditions for antioxidant extraction were dependent on the spice source and solvent type. The best antioxidant properties in nutmeg extracts were achieved for X_1 = 1.0 mm, X_2 = 40–41 Hz and X_3 = 7 days, whereas the optimized parameters for ginger extracts were more varied (1.0–2.0 mm, 43–50 Hz and 1–9 days, respectively). The ginger extracts contained 1.5–1.8 times more phenolic acids, and vanillic, ferulic, gallic, and *p*-OH-benzoic acids were dominant. In contrast, the nutmeg extracts were rich in protocatechuic, vanillic, and ferulic acids.

Keywords: vegetal spices; grinding process; antioxidant capacity; phenolic acids; Box–Behnken design; response surface methodology

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Citation: Poliński, S.; Topka, P.; Tańska, M.; Kowalska, S.; Czaplicki, S.; Szydłowska-Czerniak, A. Effect of Grinding Process Parameters and Storage Time on Extraction of Antioxidants from Ginger and Nutmeg. *Molecules* **2022**, *27*, 7395. https://doi.org/10.3390/molecules27217395

Academic Editor: Maria Atanassova

Received: 5 October 2022
Accepted: 25 October 2022
Published: 31 October 2022

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1. Introduction

The first evidence of using spices for culinary purposes comes from the Neolithic era [1]. In ancient times, spices were used for culinary rituals, perfumery, and medicine. During the Middle Ages, the culinary role of spices increased significantly, retaining their importance in medicinal applications [2]. Geographical discoveries and trade development contributed to increased spice popularity beginning in the 16th century, and the number of uses for spices in food, medicine, and perfumery multiplied during this time period [3]. Nowadays, spices are an inherent part of all known cuisines, but it is obvious that spices are far more important in some cuisines than others [4]. "Spices" is a culinary term, not one of the botanical categories, and does not refer to a specific part of a plant or plant species [5]. Spices can be sourced from different parts of plants, such as leaves, buds, bark, roots, berries, seeds, and flowers.

One of the best-known spices can be counted as ginger (*Zingiber officinale* Roscoe) rhizome, which is a perennial plant native to Southern Asia. Spice produced from ginger rhizome is widely used as a spice due to its characteristic pungency and piquant flavor. However, ginger rhizome also plays a considerable role in medicine. This well-known

spice has a long history of use in Chinese and Ayurvedic medicine as an antiemetic, antipyretic, and anti-inflammatory agent [6]. Nowadays, there are many proven medical properties of ginger rhizome, such as anticarcinogenic, immune-modulatory, antibacterial, antifungal, anti-hyperglycaemic, antiviral, antipyretic, analgesic, and antiatherosclerotic activity. Furthermore, ginger is known to increase the motility of the gastrointestinal tract [7–10]. Ginger rhizome also indicated high antioxidant properties and considerable amounts of polyphenols, which are positively correlated [11–13].

Another widely used and highly interesting spice from a medical point of view is nutmeg. Nutmeg is a spice obtained from the dried seeds of the nutmeg tree (*Myristica fragrans*), which originally came from the Maluku Province of Indonesia [14]. It is often used as a spice in cuisines because of its sweet, spicy, and nutty taste, but due to its healing properties, it has also found application in natural medicine. Nutmeg has a variety of important health benefits, including brain stimulation, heart function stimulation, detoxification properties, insomnia treatment, toothache treatment, and anti-inflammatory properties [15]. There is also a scientific rationale for the traditional use of nutmeg in the management of male sexual disorders [16]. However, the consumption of nutmeg in certain doses causes toxic and narcotic effects [17–19]. Moreover, the prolonged abuse of nutmeg can lead to chronic psychosis, which can be identified by impaired thinking and emotions [20]. The toxic properties of nutmeg follow from the myristicin contained in this spice. Given that no holistic treatment for nutmeg intoxication has been developed, higher doses of nutmeg should be avoided [21]. As in the case of ginger, nutmeg indicates antibacterial, antifungal, and antioxidant properties [22,23].

Most spices are usually added to savory dishes, but ginger rhizome and nutmeg also appear as ingredients in recipes for sweet pastries and desserts. One of the well-known applications of both spices is in gingerbread production. These spices need to be subjected to a grinding process before their use. It is known that grinding processes alter the physical, chemical, functional, structural, and biological properties of raw materials. Moreover, obtained powders with smaller particle sizes promote the release of bioactive compounds and increase their antioxidant activity [24]. Archana et al. [25] reported that the grinding changed the crystal structure, internal cohesion, and interplanar distance of the nanocrystalline ginger powder. Moreover, the phenolic amounts, antioxidant properties, and superoxide radical scavenging of ginger powder increased due to changes in the crystalline structure, surface morphology, and large surface-to-volume ratio. Furthermore, volatile oil and oleoresin content (non-volatile resinous fraction comprising heat components, fixative, natural antioxidants, and pigments) were substantially different for nutmeg ground by ambient, chilled, and liquid nitrogen methods. Moreover, grinding equipment and differences in the cracking degree of the original nutmeg samples influenced the particle size of ground spice. At the same time, the temperature did not affect the particle size and uniformity of the ground nutmeg [26]. Spice grinding is one of the key processes in gingerbread production. Nevertheless, due to the grinding process improving the physicochemical and functional characteristics of the prepared spice powders, they can be suitable in the food, pharmaceutical, and medicine industries, and other related sectors for the development of new formulas of functional foods rich in bioactive compounds and new composite or functional materials [24]. For instance, ginger powders obtained by biological and conventional agricultural practices were used to design emulsions by employing Pickering particles that act both as physical emulsion stabilizers and as interfacial reservoirs of bioactive compounds. The proposed ginger powder-based Pickering emulsions had high stability to oxidation and promising antioxidant and α-amylase inhibitory activity [27]. Moreover, the addition of ginger powder to wheat bread and cooked pork burgers markedly increased the antioxidant properties and functionality of these enriched products [28,29]. Consequently, ginger powder as an ingredient in the formulation of the burgers reduced lipid oxidation and the total saturated fatty acids in burgers, increasing the nutritional values of these ready-to-cook products [29].

Often, spices are converted to powders by the traditional mechanical process of grinding, which leads to an increase in temperature as high as 43–95 °C [30]. The study of Makanjuola [31] suggests that particle size influences the extraction of antioxidants. Furthermore, the optimum powder size that would maximize antioxidant extraction may be dependent on the solvent used and the antioxidant property being measured.

Although the antioxidant activity and phenolic compounds contents of herbs and spices have been extensively studied, a study on the effect of time after grinding on antioxidant properties is still lacking. Taking into account the significance of the grinding process for ginger and nutmeg antioxidant properties, it is expected that optimization of this process can affect the health-promoting properties of these spices and thus gingerbreads that contain them. Therefore, this work is focused for the first time on the optimization of grinding process parameters and the storage time of ground ginger rhizome and nutmeg to obtain their ethanol and ethanol-water extracts with high antioxidant properties. Variables such as the mesh size of the grinder sieve (X_1), the peripheral velocity of the grinding wheels (X_2), and the storage time (X_3) were optimized to enhance the antioxidant potential of the ground spices by using response surface methodology (RSM) with a Box–Behnken design (BBD). Moreover, modified spectrophotometric methods, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and the Folin–Ciocalteu (F–C) test were used to determine the antioxidant capacity (AC) and total phenolic content (TPC) in ethanol and ethanol-water extracts of the prepared spice powders stored in air for different periods. However, ultra-performance liquid chromatography (UPLC) coupled with a triple quadrupole mass spectrometer was applied to profile the phenolic acids in ginger rhizome and nutmeg extracts obtained under optimal conditions.

2. Results and Discussion

2.1. Effect of Grinding Process and Storage Time on Antioxidant Properties of Ginger Rhizome and Nutmeg

The AC and TPC (experimental and predicted) results of ethanol and ethanol-water extracts of ground ginger rhizome and nutmeg are listed in Tables 1 and 2, respectively.

Significant differences in the mean values of AC and TPC in the ethanol and ethanol-water extracts of ginger rhizome and nutmeg were observed (Tukey's test, Tables 1 and 2).

As can be seen in Table 1, the grinding process of ginger rhizome influenced the highest DPPH method result for ethanol-water extraction (DPPH = 189.9 mmol TE/100 g) when a 1.4 mm mesh size grinder sieve was used, the highest peripheral velocity of grinding wheels (50 Hz) was set, and the storage time was 14 days. However, the lowest results of the DPPH method for extraction with ethanol (DPPH = 22.5 mmol TE/100 g) were achieved for a 2.0 mm mesh size, 40 Hz peripheral velocity, and a 0-day storage time, while for extraction with ethanol-water (DPPH = 26.4 mmol TE/100 g) for a 2.0 mm mesh size, 30 Hz peripheral velocity, and a 7-day storage time.

Unexpectedly, the ethanol-water extraction of freshly ground ginger rhizome using 30 Hz peripheral velocity and a 1.4 mm mesh size caused the lowest ABTS method result (ABTS = 80.4 mmol TE/100 g). It is worth noting that the highest ABTS results for this spice were obtained using a 1.0 mm mesh size grinder, 30 Hz peripheral velocity, and a 7-day storage time (454.0 and 440.0 mmol TE/100 g for the ethanol and ethanol-water extracts, respectively). This suggests that the results of the antioxidant properties are related to the degree of spice sample granulation.

It can be noted that the highest TPC result analyzed by the F–C method (507.4 mg GA/100 g) was obtained for the ethanol extraction of phenolic compounds from ginger rhizome ground by a 1.0 mm mesh size grinder, using the highest peripheral velocity of grinding wheels (50 Hz) and stored for 7 days. Replacing the extraction solution with ethanol-water in the case of ginger rhizome ground in a grinder with a larger mesh (1.4 mm) and slower peripheral velocity of the grinding wheels (30 Hz) resulted in the lowest amount of polyphenols in the fresh sample (TPC = 81.0 mg GA/100 g).

On the other hand, the highest DPPH result (230.0 mmol TE/100 g) was obtained for the ethanol extract of nutmeg ground at the lowest peripheral velocity of grinding wheels (30 Hz), sieved by a 1.0 mm mesh size grinder sieve, and stored for 7 days. However, the lowest DPPH results revealed ethanol and ethanol-water extracts (29.8 and 28.3 mmol TE/100 g, respectively), prepared from fresh ground nutmeg using a 40 Hz peripheral velocity of the grinding wheels and sieved to the largest particle size (2.0 mm).

Compared with the DPPH results, only an increase in the peripheral velocity of the grinder to 50 Hz caused the highest ABTS values for ethanol (634.0 mmol TE/100 g) and ethanol-water (657.4 mmol TE/100 g) extracts of nutmeg. However, a decrease in the peripheral velocity of grinding wheels to 30 Hz and using the largest sieve mesh (2.0 mm) resulted in the lowest ABTS results (62.3 and 147.5 mmol TE/100 g) in both nutmeg extracts (Table 2).

Table 1. Three-level Box–Behnken design with experimental ($\overline{x} \pm SD$) and predicted results for the antioxidant properties of ginger rhizome.

Exp.	Independent Variables			Dependent Variables					
	X_1 (mm)	X_2 (Hz)	X_3 (Days)	DPPH Method (mmol TE/100 g)		ABTS Method (mmol TE/100 g)		F–C Method (mg GA/100 g)	
				Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
Extraction with Ethanol									
1	2.0 (1)	40 (0)	0 (−1)	22.5 ± 0.1^{a}	22.3	95.3 ± 1.9^{a}	92.4	$153.9 \pm 3.2^{b,c}$	149.6
2	2.0 (1)	40 (0)	14 (1)	29.6 ± 0.3^{c}	29.4	157.3 ± 2.8^{b}	154.4	$151.5 \pm 2.4^{a,b}$	147.2
3	2.0 (1)	50 (1)	7 (0)	42.2 ± 0.9^{f}	42.5	229.1 ± 0.9^{g}	232.0	$162.7 \pm 1.6^{c,d}$	167.0
4	2.0 (1)	30 (−1)	7 (0)	38.5 ± 0.2^{e}	38.8	$198.2 \pm 0.9^{d,e}$	201.1	202.7 ± 1.9^{f}	207.0
5	1.4 (0)	50 (1)	0 (−1)	23.1 ± 0.5^{a}	23.1	96.9 ± 2.3^{a}	96.9	278.5 ± 1.5^{g}	278.5
6	1.4 (0)	50 (1)	14 (1)	37.6 ± 0.6^{e}	37.6	184.0 ± 4.0^{c}	184.0	178.5 ± 2.6^{e}	178.5
7	1.4 (0)	30 (−1)	0 (−1)	26.9 ± 0.3^{b}	26.9	196.0 ± 2.0^{d}	196.0	165.3 ± 2.8^{d}	165.3
8	1.4 (0)	30 (−1)	14 (1)	33.1 ± 0.3^{d}	33.1	262.0 ± 2.0^{h}	262.0	$152.9 \pm 2.6^{a,b}$	152.9
9	1.4 (0)	40 (0)	7 (0)	31.9 ± 0.6^{d}	28.8	209.5 ± 1.6^{f}	197.9	$355.9 \pm 4.8^{h,i}$	355.8
10	1.0 (−1)	40 (0)	0 (−1)	86.4 ± 0.7^{g}	86.7	378.0 ± 8.0^{j}	380.9	383.3 ± 4.1^{i}	387.6
11	1.0 (−1)	40 (0)	14 (1)	95.0 ± 1.2^{h}	95.3	308.0 ± 5.0^{i}	310.9	464.1 ± 4.0^{l}	468.4
12	1.0 (−1)	50 (1)	7 (0)	99.0 ± 1.2^{i}	98.8	430.0 ± 5.0^{k}	427.1	507.4 ± 11.5^{m}	503.1
13	1.0 (−1)	30 (−1)	7 (0)	113.0 ± 2.0^{j}	112.8	454.0 ± 9.0^{l}	451.1	434.5 ± 2.7^{k}	430.2
14	1.4 (0)	40 (0)	7 (0)	29.4 ± 0.5^{c}	28.8	$206.1 \pm 1.9^{e,f}$	197.9	348.0 ± 3.2^{h}	355.8
15	1.4 (0)	40 (0)	7 (0)	25.2 ± 0.2^{b}	28.8	178.0 ± 4.0^{c}	197.9	363.4 ± 4.4^{i}	355.8
Extraction with Ethanol-Water									
1	2.0 (1)	40 (0)	0 (−1)	52.9 ± 1.3^{c}	45.6	99.3 ± 1.0^{b}	79.3	146.5 ± 2.0^{b}	131.0
2	2.0 (1)	40 (0)	14 (1)	67.1 ± 0.9^{d}	59.8	108.6 ± 1.7^{b}	88.6	158.6 ± 2.9^{c}	143.1
3	2.0 (1)	50 (1)	7 (0)	43.6 ± 0.9^{b}	50.9	127.0 ± 2.8^{c}	147.0	228.0 ± 2.4^{g}	243.6
4	2.0 (1)	30 (−1)	7 (0)	26.4 ± 0.6^{a}	33.7	101.9 ± 2.5^{b}	121.9	211.6 ± 1.8^{f}	227.2
5	1.4 (0)	50 (1)	0 (−1)	85.9 ± 1.8^{f}	85.9	171.0 ± 4.1^{e}	171.0	199.8 ± 2.8^{e}	199.8
6	1.4 (0)	50 (1)	14 (1)	189.9 ± 2.6^{m}	189.9	284.8 ± 2.7^{f}	284.8	181.1 ± 3.0^{d}	181.1
7	1.4 (0)	30 (−1)	0 (−1)	54.0 ± 0.2^{c}	54.0	80.4 ± 1.3^{a}	80.4	81.0 ± 1.2^{a}	81.0
8	1.4 (0)	30 (−1)	14 (1)	112.6 ± 4.7^{h}	112.6	125.3 ± 2.3^{c}	125.3	185.1 ± 2.2^{d}	185.1
9	1.4 (0)	40 (0)	7 (0)	120.4 ± 0.9^{i}	122.4	145.7 ± 1.8^{d}	130.4	215.1 ± 1.6^{f}	225.8
10	1.0 (−1)	40 (0)	0 (−1)	171.2 ± 3.7^{l}	178.5	321.3 ± 6.6^{g}	341.3	332.6 ± 2.8^{i}	348.2
11	1.0 (−1)	40 (0)	14 (1)	78.7 ± 0.9^{e}	86.0	367.0 ± 5.6^{h}	387.0	189.0 ± 1.3^{d}	204.6
12	1.0 (−1)	50 (1)	7 (0)	166.0 ± 0.7^{k}	158.7	429.3 ± 7.7^{i}	409.3	390.2 ± 8.1^{j}	374.7
13	1.0 (−1)	30 (−1)	7 (0)	92.6 ± 1.8^{g}	85.3	440.0 ± 9.2^{j}	420.0	390.3 ± 8.7^{j}	374.8
14	1.4 (0)	40 (0)	7 (0)	118.8 ± 1.8^{i}	122.4	120.9 ± 2.2^{c}	130.4	210.2 ± 4.0^{f}	225.8
15	1.4 (0)	40 (0)	7 (0)	128.0 ± 2.1^{j}	122.4	124.5 ± 3.0^{c}	130.4	252.2 ± 4.4^{h}	225.8

$n = 5$; $\overline{x} \pm SD$—mean value ± standard deviation; different letters ($^{a–m}$) within the same column indicate significant differences (one-way ANOVA and Tukey's test, $p \leq 0.05$); coded levels are given in parentheses; X_1—mesh size of grinder sieve, X_2—peripheral velocity of grinding wheels, X_3—storage time of ground spices.

Similarly, the lowest TPC results in the nutmeg ethanol and ethanol-water extracts (125.9 and 148.2 mg GA/100 g, respectively) were observed for samples freshly ground at a lower peripheral velocity of the grinding wheels (30–40 Hz) and sieved through larger mesh sizes (1.4–2.0 mm). Nevertheless, the highest TPC result (511.1 mg GA/100 g) in the ethanol extract of nutmeg was found at the central point of BBD, whereas the ethanol-water extract of fresh nutmeg ground using a medium peripheral velocity of grinder

(40 Hz) and sieved by the lowest mesh size (1.0 mm) was the richest source of polyphenols (TPC = 459.6 mg GA/100 g).

Table 2. Three-level Box–Behnken design with experimental ($\bar{x} \pm SD$) and predicted results for the antioxidant properties of nutmeg.

Exp.	Independent Variables			Dependent Variables					
	X_1 (mm)	X_2 (Hz)	X_3 (Days)	DPPH Method (mmol TE/100 g)		ABTS Method (mmol TE/100 g)		F–C Method (mg GA/100 g)	
				Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
Extraction with Ethanol									
1	2.0 (1)	40 (0)	0 (−1)	29.8 ± 0.6^{a}	7.4	71.9 ± 1.2^{a}	66.7	125.9 ± 2.4^{a}	163.8
2	2.0 (1)	40 (0)	14 (1)	42.1 ± 1.1^{c}	19.7	163.0 ± 4.0^{c}	157.8	191.1 ± 3.7^{c}	229.0
3	2.0 (1)	50 (1)	7 (0)	60.7 ± 0.7^{e}	83.1	206.0 ± 3.0^{d}	211.2	246.3 ± 2.4^{e}	208.4
4	2.0 (1)	30 (−1)	7 (0)	31.1 ± 0.6^{a}	53.5	62.3 ± 1.5^{a}	67.5	187.1 ± 3.2^{c}	149.2
5	1.4 (0)	50 (1)	0 (−1)	35.3 ± 0.4^{b}	35.3	143.7 ± 1.1^{b}	143.7	243.1 ± 3.0^{e}	243.1
6	1.4 (0)	50 (1)	14 (1)	41.2 ± 0.7^{c}	41.2	327.0 ± 6.0^{f}	327.0	320.5 ± 3.0^{g}	320.5
7	1.4 (0)	30 (−1)	0 (−1)	53.7 ± 0.9^{d}	53.7	$154.0 \pm 3.0^{b,c}$	154.0	144.6 ± 2.8^{b}	144.6
8	1.4 (0)	30 (−1)	14 (1)	77.6 ± 0.7^{f}	77.6	223.0 ± 3.0^{e}	223.0	232.7 ± 1.5^{d}	232.7
9	1.4 (0)	40 (0)	7 (0)	173.4 ± 1.4^{i}	174.3	446.9 ± 10.2^{i}	449.2	445.7 ± 2.9^{i}	487.9
10	1.0 (−1)	40 (0)	0 (−1)	52.4 ± 0.6^{d}	74.8	430.0 ± 10.0^{h}	435.2	495.5 ± 6.6^{j}	457.6
11	1.0 (−1)	40 (0)	14 (1)	140.5 ± 1.2^{g}	162.9	506.0 ± 6.0^{k}	511.2	315.9 ± 3.7^{g}	278.0
12	1.0 (−1)	50 (1)	7 (0)	162.2 ± 2.6^{h}	139.8	634.0 ± 14.0^{l}	628.8	362.8 ± 5.7^{h}	400.7
13	1.0 (−1)	30 (−1)	7 (0)	230.0 ± 5.0^{k}	207.6	377.0 ± 7.0^{g}	371.8	261.7 ± 4.7^{f}	299.6
14	1.4 (0)	40 (0)	7 (0)	189.5 ± 1.9^{l}	174.3	$437.0 \pm 9.0^{h,i}$	449.2	506.8 ± 7.0^{k}	487.9
15	1.4 (0)	40 (0)	7 (0)	159.9 ± 1.4^{h}	174.3	463.8 ± 2.4^{j}	449.2	511.1 ± 9.7^{k}	487.9
Extraction with Ethanol-Water									
1	2.0 (1)	40 (0)	0 (−1)	28.3 ± 0.9^{a}	21.5	165.5 ± 3.8^{b}	140.5	169.2 ± 3.3^{b}	186.7
2	2.0 (1)	40 (0)	14 (1)	41.8 ± 2.0^{b}	35.0	170.3 ± 3.1^{b}	145.3	198.6 ± 2.3^{c}	216.1
3	2.0 (1)	50 (1)	7 (0)	39.1 ± 0.6^{b}	45.9	191.8 ± 4.2^{c}	216.8	279.9 ± 4.4^{e}	262.4
4	2.0 (1)	30 (−1)	7 (0)	$33.4 \pm 0.4^{a,b}$	40.2	147.5 ± 3.4^{a}	172.5	233.3 ± 4.6^{d}	215.8
5	1.4 (0)	50 (1)	0 (−1)	64.0 ± 0.7^{d}	64.0	261.1 ± 5.8^{e}	261.1	348.5 ± 2.8^{g}	348.5
6	1.4 (0)	50 (1)	14 (1)	89.4 ± 1.4^{e}	89.4	345.9 ± 7.9^{f}	345.9	281.2 ± 4.9^{e}	281.2
7	1.4 (0)	30 (−1)	0 (−1)	$36.1 \pm 0.3^{a,b}$	36.1	204.5 ± 4.4^{c}	204.5	232.7 ± 5.1^{d}	232.7
8	1.4 (0)	30 (−1)	14 (1)	53.1 ± 0.6^{c}	53.1	238.1 ± 4.4^{d}	238.1	148.2 ± 1.3^{a}	148.2
9	1.4 (0)	40 (0)	7 (0)	143.5 ± 1.7^{h}	149.9	458.5 ± 10.9^{g}	460.8	443.7 ± 5.0^{i}	427.5
10	1.0 (−1)	40 (0)	0 (−1)	108.7 ± 4.7^{f}	115.5	354.2 ± 5.0^{f}	379.2	459.6 ± 6.6^{j}	442.1
11	1.0 (−1)	40 (0)	14 (1)	154.1 ± 3.0^{i}	160.9	620.1 ± 12.1^{j}	645.1	272.7 ± 2.6^{e}	255.2
12	1.0 (−1)	50 (1)	7 (0)	186.9 ± 1.9^{j}	180.1	657.4 ± 0.7^{k}	632.4	312.9 ± 7.6^{f}	330.4
13	1.0 (−1)	30 (−1)	7 (0)	132.9 ± 4.1^{g}	126.1	520.2 ± 19.4^{i}	495.2	424.8 ± 8.6^{h}	442.3
14	1.4 (0)	40 (0)	7 (0)	163.8 ± 5.3^{i}	149.9	481.6 ± 3.0^{h}	460.8	419.1 ± 7.1^{h}	427.5
15	1.4 (0)	40 (0)	7 (0)	$142.4 \pm 2.5^{g,h}$	149.9	442.3 ± 8.4^{g}	460.8	419.6 ± 4.4^{h}	427.5

$n = 5$; $\bar{x} \pm SD$—mean value ± standard deviation; different letters ($^{a-l}$) within the same column indicate significant differences (one-way ANOVA and Tukey's test, $p \leq 0.05$); coded levels are given in parentheses; X_1—mesh size of grinder sieve, X_2—peripheral velocity of grinding wheels, X_3—storage time of ground spices.

The obtained AC and TPC results indicate that the size of the spice powders significantly impacted the antioxidant properties of the prepared extracts. The extracts obtained from the spice powder sieved by a smaller mesh size generally had higher levels of antioxidants. This suggests that antioxidant compounds in finer particles are exhibited to be more extractable by two commonly used solvents. The similar efficiency of antioxidant extraction for ethanol and an ethanol-water mixture can be explained by the theory that ethanol may weaken the bonds between polyphenolics-protein and polyphenolics-cellulose, releasing antioxidants from the studied spices [32]. Moreover, the higher peripheral velocity of the grinder and the longer exposure time of the ground spices appeared to be sufficient to recover more of the bioactive compounds from them in the prepared extracts. This supports the hypothesis that a higher peripheral velocity of the grinder could produce free radicals during the grinding process, which may act as stress signals and trigger stress responses in spices, causing the greater synthesis of antioxidants at more extended storage in contact with air, thus increasing their antioxidant potential [33]. In contrast, Ghasemzadeh et al. [34] demonstrated the negative effect of storage time on antioxidant properties.

The TPC results of the ground ginger rhizome and nutmeg extracts were in agreement with some previously published data (TPC = 101.6–2314.0 mg GA/100 g and 49.82–268.0 mg GA/100 g

for ginger and nutmeg, respectively) [35–40]. However, these authors [36,37,39] found significantly lower radical scavenging capacities of ginger and nutmeg against DPPH radical (0.922 mmol TE/100 g and 0.844 mmol TE/100 g, respectively) and ABTS radical cations (0.348 mmol TE/100 g and 0.455–19.1 mmol TE/100 g, respectively) compared with DPPH and ABTS values for the studied extracts of the two spice powders. Discrepancies in the antioxidant properties of spices can be explained by the influences of genetic, agronomic, and environmental factors, as well as different solvents and techniques used in the preparation of spice samples, which would affect the antioxidant contents.

This study, similar to other works [35,36], demonstrated that AC and TPC in ginger rhizome and nutmeg have a significant and positive correlation. Therefore, regression analysis was performed for the correlations among the antioxidant properties of the ethanol and ethanol-water extracts of both spices, as determined by the proposed DPPH, ABTS, and F–C methods. The calculated correlation coefficients (r = 0.5122–0.7905, p = 0.0000001–0.0038) suggest that there are significant relationships among the antioxidant potential results for extracts obtained using two different solvents. The obtained results of AC indicated that there are significant, positive correlations between the DPPH and ABTS values for the ethanol (r = 0.7905, p = 0.0000001) and ethanol-water (r = 0.6720, p = 0.00005) extracts of the investigated spices. These high r values demonstrated that the antioxidant compounds present in spice extracts were capable of scavenging DPPH radical and ABTS radical cations. However, lower correlation coefficients were calculated between DPPH–F–C methods for ethanol (r = 0.5593, p = 0.0013) and ethanol-water (r = 0.5122, p = 0.0038) extracts of spices. This suggests that the DPPH method brings an important limitation to the determination of hydrophilic antioxidants such as polyphenols [41]. However, significant linear correlations between the ABTS and F–C methods for the studied spice extracts were found (r = 0.7274, p = 0.000005 and r = 0.7077, p = 0.000012 for the ethanol and ethanol-water extracts, respectively). This can be explained by the fact that the ABTS radical cation enables the simultaneous determination of hydrophilic and lipophilic antioxidants present in the prepared extracts of spices [41].

2.2. *Model Fitting and Data Analysis Using Response Surface Methodology*

The RSM was applied to obtain the optimal parameters of the grinding process and storage time of ground ginger rhizome and nutmeg. The regression coefficients of the polynomial Equation (1) were calculated using experimental values, including DPPH, ABTS, and TPC in the ethanol and ethanol-water extracts of ginger rhizome and nutmeg (Tables 1 and 2), and the generated equations were used in the prediction of the response values of the antioxidant properties of the investigated extracts of spices. The final partial cubic model (PCM) for the preparation of ginger rhizome and nutmeg before the extraction of antioxidants could be represented by the polynomial equations suggested using ANOVA as listed in Table 3.

The ANOVA results (Table 4) for the developed partial cubic model (PCM, Equation (1)) of all the three dependent variables (DPPH, ABTS, and TPC in the ethanol and ethanol-water extracts of ginger rhizome and nutmeg) revealed that the models were adequate with a desirable coefficient of multiple determination (R^2) and adjusted R^2.

As can be seen, the R^2 values for these response variables were higher than 0.8, indicating that the proposed regression models were of goodness of fit [42]. However, low values of adjusted R^2 = 0.6868 and 0.7267 for the responses of DPPH and TPC determined by the F–C method for the nutmeg ethanol extract did not indicate a close agreement between the experimental and predicted results.

The PCM model adequacy was tested using the lack-of-fit Fisher's test (F-test), which was insignificant for $p > 0.05$ (Table 4). The ANOVA results of DPPH, ABTS, and TPC for the ginger rhizome and nutmeg ethanol and ethanol-water extracts revealed an insignificant lack of fit (F values = 0.044–18.3, $p > 0.05$). Therefore, these models were adequate for prediction within the range of variables employed. In addition, high F-values (53.7–361.1 for the DPPH and ABTS of both extracts and TPC in the ethanol extract of ginger rhizome,

as well as 68.5–218.9 for the ABTS of the ethanol and ethanol-water extracts and TPC in the ethanol-water extract of nutmeg) demonstrated that the empirical models were significant with low probability values ($p < 0.05$). This suggests that the proposed mathematical models are valid and convenient for predicting the antioxidant properties of ginger rhizome (except TPC in ethanol-water extract) and nutmeg (except DPPH for both studied extracts and TPC in ethanol extract) as determined by the DPPH, ABTS, and F–C methods under any combinations of variables (the preparation conditions of spices for extraction process). Obviously, the low F-values (16.1 and 18.3 for TPC in the ethanol extract of nutmeg and the ethanol-water extract of ginger rhizome, respectively, as well as 26.2 and 26.9 for the DPPH of both extracts of nutmeg) and p-values > 0.05 suggest that the models' predictions of the total amounts of polyphenols and antioxidants able to quench the DPPH radical in the prepared extracts of nutmeg were insignificant.

Table 3. Regression coefficients of the partial cubic model for the antioxidant properties of ginger rhizome and nutmeg extracts.

Term	Coefficient					
	DPPH Method (mmol TE/100 g)	**ABTS Method (mmol TE/100 g)**	**F–C Method (mg GA/100 g)**	**DPPH Method (mmol TE/100 g)**	**ABTS Method (mmol TE/100 g)**	**F–C Method (mg GA/100 g)**
	Ginger Rhizome Ethanol Extract			**Ginger Rhizome Ethanol-Water Extract**		
β_0	839.4 *	1372.3	−52.4	351.5	4752.7 **	2748.2
β_1	−831.1 *	−223.9	−1164.1	−666.8	−5288.8 **	−3247.7
β_2	−11.3	−0.44	43.5 *	12.7	−88.9 *	−32.7
β_3	0.06	−58.1	114.6 **	−98.6 **	−30.5	−54.5
β_{11}	243.8 *	−58.1	384.0	215.4	1651.7 *	999.2
β_{22}	0.08 *	0.40 *	−0.62 **	−0.11 *	0.43 *	0.17
β_{33}	−0.14	−1.1 *	−2.1 **	−0.02	−0.16	−1.7 *
β_{12}	5.4	−51.3	20.4	−0.51	77.7 *	32.6
β_{13}	2.0	93.1	−98.6 *	124.7 **	40.4	121.9
β_{23}	0.03	0.08	−0.31 *	0.16 *	0.25	−0.44
β_{112}	−1.5	18.0	−8.7	−0.77	−25.3 *	−10.6
β_{113}	−0.70	−27.9	30.9 *	−39.0 **	−14.4	−36.9
	Nutmeg Ethanol Extract			Nutmeg Ethanol−Water Extract		
β_0	−314.9	−3969.4 **	−1468.2	−702.7	−1449.1	2880.1 *
β_1	81.9	3555.8 *	−568.9	183.9	673.6	−4654.7 *
β_2	18.6	168.4 **	111.9	35.7	76.3 *	−31.1
β_3	65.1 *	−0.46	−93.1	26.0	126.9 *	−15.5
β_{11}	−114.7	−1232.6 *	119.6	−60.4	−242.2	1360.8 *
β_{22}	−0.34 *	−1.1 **	−1.4 *	−0.37 *	−0.73 *	−1.7 *
β_{33}	−1.8 **	−2.7 **	−2.4 *	−1.1 *	−2.6 **	−2.2 **
β_{12}	5.8	−108.7 *	3.4	−4.0	−15.8	116.0 *
β_{13}	−43.7	40.5	164.3	−12.5	−110.0	37.3
β_{23}	−0.06	0.41	−0.04	0.03	0.18	0.06
β_{112}	−0.30	34.3 *	−1.8	0.54	3.7	−36.0 *
β_{113}	12.8	−13.1	−48.9	3.4	30.4	−7.3

Significant at the * $p < 0.05$; ** $p < 0.01$.

Therefore, only the linear parameter of the mesh size of the grinder sieve (X_1) and two quadratic terms of the peripheral velocity of the grinding wheels (X_2^2) and the storage time of the ground spices (X_3^2) had significant effects on DPPH values of the studied extracts of nutmeg (F = 19.2–166.7, p = 0.0059–0.048) and TPC in the ethanol (F = 38.1–50.4, p = 0.019–0.025) and ethanol-water (F = 39.7–73.6, p = 0.013–0.024) extracts of nutmeg and ginger rhizome, respectively. Unexpectedly, the same parameters showed significance on the DPPH of the ethanol extract of ginger rhizome. However, a high corresponding determination coefficient, R^2 = 0.9983, adjusted as R^2 = 0.9923 and a significant F value = 125.3 of this model indicate a good relationship between experimental and predicted values (Table 4). Moreover, one linear term (X_1) and all quadratic terms (X_1^2, X_2^2, X_3^2) showed significance (F = 19.8–331.3,

p = 0.0030–0.047) on the ABTS of ethanol ginger rhizome extract. However, all linear terms (X_1, X_2, X_3), two quadratic parameters (X_1^2 and X_2^2), and interactions between these independent variables ($X_1^2 \times X_2$) exhibited significant effects (F = 18.9–874.4, p = 0.0011–0.049) on the ABTS of the ethanol-water ginger rhizome extract. On the contrary, this interaction ($X_1^2 \times X_2$) and storage time of ground ginger rhizome (X_3 and X_3^2) caused insignificant effects on the DPPH of the ethanol-water extract (F = 0.083–1.53, p = 0.341–0.800) and TPC in ethanol extract (F = 0.645–14.3, p = 0.063–0.506).

Table 4. Analysis of variance (ANOVA) results for the responses: antioxidant properties of ginger rhizome and nutmeg extracts determined by DPPH, ABTS, and F–C methods.

Model Parameters	df	SS	MS	F Value	SS	MS	F Value
		Ginger Rhizome Ethanol Extract			Ginger Rhizome Ethanol-Water Extract		
				DPPH Method			
Regression	11	15,804.6	1436.8	125.3 *	31,255.0	2841.4	117.6 *
Residual	3	23.4	7.8		479.0	159.7	
Lack-of-fit	1	0.5	0.5	0.044	430.7	430.7	17.8
Pure error	2	22.9	11.5		48.3	24.2	
Total	14	15,828.0			31,734.0		
R^2, Adjusted R^2		0.9983, 0.9923				0.9859, 0.9341	
				ABTS Method			
Regression	11	176,690.3	16,062.8	53.7 *	242,400.4	22,036.4	122.7 *
Residual	3	665.7	221.9		3559.1	1186.4	
Lack-of-fit	1	67.9	67.9	0.23	3200.0	3200.0	17.8
Pure error	2	597.8	298.9		359.1	179.6	
Total	14	177,356.0			245,959.5		
R^2, Adjusted R^2		0.9960, 0.9814				0.9845, 0.9274	
				F–C Method			
Regression	11	235,560.5	21,414.6	361.1 **	106,111.1	9646.5	18.3
Residual	3	267.4	89.1		2989.2	996.4	
Lack-of-fit	1	148.8	148.8	2.5	1934.4	1934.4	3.7
Pure error	2	118.6	59.3		1054.8	527.4	
Total	14	235,827.9			109,100.3		
R^2, Adjusted R^2		0.9988, 0.9945				0.9713, 0.8661	
		Nutmeg Ethanol Extract			Nutmeg Ethanol-Water Extract		
				DPPH Method			
Regression	11	63,367.1	5760.6	26.2	42,957.5	3905.2	26.9
Residual	3	4462.3	1487.4		663.1	221.0	
Lack-of-fit	1	4023.1	4023.1	18.3	372.7	372.7	2.6
Pure error	2	439.2	219.6		290.4	145.2	
Total	14	67,829.4			43,620.6		
R^2, Adjusted R^2		0.9329, 0.6868				0.9847, 0.9286	
				ABTS Method			
Regression	11	44,2194.8	40,199.5	218.9 **	409,498.0	37227.1	95.4 *
Residual	3	583.6	194.5		5770.2	1923.4	
Lack-of-fit	1	216.3	216.3	1.2	4990.0	4990.0	12.8
Pure error	2	367.3	183.7		780.2	390.1	
Total	14	44,2778.4			415,268.2		
R^2, Adjusted R^2		0.9987, 0.9937				0.9859, 0.9340	
				F–C Method			
Regression	11	236,564.6	21,505.9	16.1	149,008.4	13,546.2	68.5 *
Residual	3	14,175.2	4725.1		2845.4	948.5	
Lack-of-fit	1	11,498.9	11498.9	8.6	2450.0	2450.0	12.4
Pure error	2	2676.3	1338.2		395.4	197.7	
Total	14	250,739.8			151,853.8		
R^2, Adjusted R^2		0.9414, 0.7267				0.9813, 0.9128	

df—degrees of freedom; SS—sum of square; MS—mean of square; R^2—coefficient of determination; F value—F-statistic; * Significant at the probability value, $p < 0.05$ level; ** Significant at the $p < 0.01$ level.

In addition, the linear parameter of the peripheral velocity of the grinding wheels (X_2), the quadratic term of the mesh size of the grinder sieve (X_1^2), and the interactions between $X_2 \times X_3$ and $X_1^2 \times X_3$ had no significant effects on the TPC in the prepared ethanol-water extract of nutmeg (F = 0.037–18.1, p = 0.051–0.603). The similar terms, all linear (X_1, X_2, X_3), two quadratic parameters $\left(X_2^2, X_3^2\right)$, and interaction ($X_1 \times X_3$) or ($X_1^2 \times X_2$) indicated notable significant effects (F = 32.9–1418.9, p = 0.0007–0.029) on the ABTS of both investigated extracts of nutmeg.

2.3. Analysis of Response Surfaces

The regression equations, predicting the effects of the mesh size of the grinder sieve (X_1), the peripheral velocity of grinding wheels (X_2), and the storage time of the ground spices (X_3), were explained by 3D response surface plots. The selected response surface plots were generated by plotting two factors over their respective ranges, while the third factor was kept at a constant value: 1.4 mm, 40 Hz, and 7 days for the X_1, X_2 and X_3 independent variables, respectively (Figure 1).

It can be noted that the shapes of the response surfaces for DPPH, ABTS and TPC determined by the F–C method for the ethanol and ethanol-water extracts of the two studied spices were different from each other (Figure 1). However, the parabolic shapes of these surfaces indicate that the quadratic terms of independent variables were significant. The mesh size of the grinder sieve displayed negative linear (X_1) and quadratic (X_1^2) effects on the antioxidant potential of the ethanol and ethanol-water extracts of ginger rhizome. Therefore, the response surfaces and contour plots of the DPPH and ABTS (Figure 1a,b) of the ethanol extract and TPC in the ethanol-water extract (Figure 1f) of this spice depicted a maximum at the lowest X_1 (1.0 mm) and X_2 (30 Hz) or an intermediate X_3 (7 days). However, using a higher peripheral velocity of the grinding wheels, after 7 days of storage, the ethanol extract of ground ginger rhizome revealed the highest total polyphenols content (Figure 1c). The negative value of the linear term of the mesh size of the grinder sieve (X_1) increased the DPPH of the ginger rhizome ethanol-water extract along with a decrease in the X_1 independent variable (Figure 1d). However, the significant positive interaction between the corresponding variables ($X_1 \times X_3$) and X_1^2 increased the DPPH of this extract prepared by ginger rhizome after using an intermediate mesh size of the grinder sieve size (X_1 = 1.4 mm) for the longest storage time (X_3 = 14 days). Moreover, the significant positive linear effect of X_2 and X_3 on the ABTS of the ginger rhizome ethanol-water extract resulted in the enhancement of its ability to scavenge the ABTS radical cation along with an increase in the peripheral velocity of the grinding wheels and the storage time of this spice (Figure 1e).

The parabolic shape of the response surface for the DPPH of the prepared nutmeg extracts (Figure 1g,j) was caused by the positive values of the quadratic terms of the peripheral velocity of the grinding wheels (X_2^2) and the storage time of the ground nutmeg (X_3^2). Therefore, the response surface and contour plot of the DPPH of the ethanol extract of nutmeg (Figure 1g) was the maximum at an intermediate peripheral velocity of the grinding wheels (40 Hz) after 7 days of storage. On the other hand, the negative linear effect of the mesh size of the grinder sieve (X_1) with the quadratic term of the peripheral velocity of the grinding wheels used grinder (X_2^2) showed that an increase in X_1 decreased the DPPH results of the ethanol-water nutmeg extract, which is related to a consistently better extraction efficiency of antioxidants from samples ground with smaller mesh sizes of grinder sieve and higher peripheral velocity of grinding wheels (Figure 1j). The elliptical contours of the ABTS plots of the two nutmeg extracts confirm that there was an interaction between the independent variables (Figure 1h,k). It was observed that the ABTS results of both nutmeg extracts were raised with an increase in the peripheral velocity of the grinding wheels (X_2) and the storage time (X_3). Thus, the highest ABTS had extracts prepared from nutmeg ground using a higher peripheral velocity of the grinder (40–50 Hz), sieved through the lowest mesh (1.0 mm) after storage for a longer time (7–14 days) (Tables 1 and 2). Moreover, the significant negative linear effect of X_1 and the positive quadratic impact of X_3 on the

TPC in the ethanol extract of nutmeg resulted in an enhancement of the polyphenols concentration with the prolongation of the storage time of the nutmeg sieved using a smaller mesh size of the grinder sieve (Figure 1i). At a fixed mesh size (X_1), the positive quadratic effects of the peripheral velocity of the grinding wheels (X_2^2) and the storage time (X_3^2) became more significant as their gradual increase enhanced the TPC in the ethanol-water extract of nutmeg (Figure 1l).

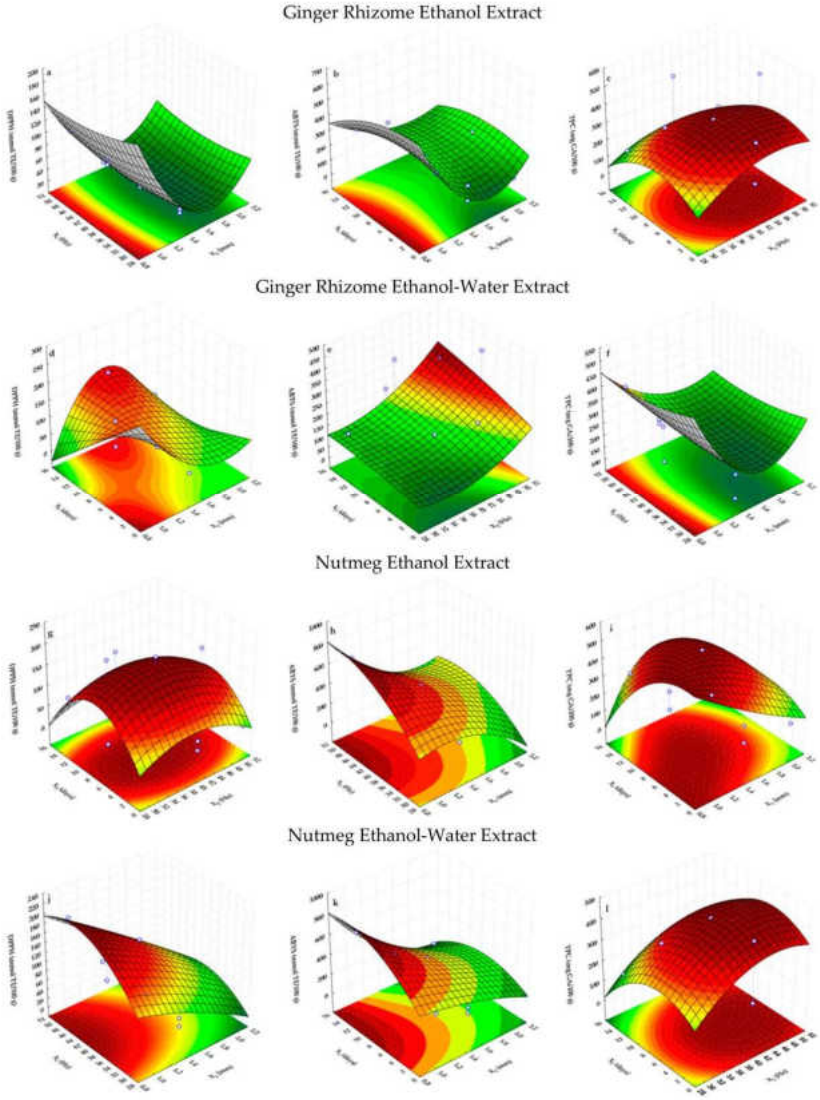

Figure 1. Response surfaces and contour plots showing the interactive influence of the mesh size of the grinder sieve (X_1), the peripheral velocity of grinding wheels (X_2) and the storage time of the ground spices (X_3) on their antioxidant capacity determined by the DPPH (**a**,**d**,**g**,**j**), ABTS (**b**,**e**,**h**,**k**), and F–C (**c**,**f**,**i**,**l**) methods.

2.4. Verification of the Optimal Extraction Models

This study investigated the optimal conditions of spice preparation (the mesh size of the grinder sieve, the peripheral velocity of the grinding wheels, and the storage time) for producing the suitable antioxidant properties of the ethanol and ethanol-water extracts of ginger rhizome and nutmeg as determined by the DPPH, ABTS, and F–C methods. The optimal conditions to maximize the antioxidant potential of the two spices predicted by the response surface methodology are summarized in Table 5.

Table 5. Predicted and experimental ($\overline{x} \pm SD$) values of the studied responses for the optimum conditions of spices preparation.

Response Variable	Optimum Conditions			Predicted Values	Experimental Values
	X_1 [mm]	X_2 [Hz]	X_3 [Days]		
Ginger Rhizome Ethanol Extract					
DPPH (mmol TE/100 g)	2.0	43	9	30.0	34.5 ± 0.1
ABTS (mmol TE/100 g)				179.5	176.2 ± 2.5
TPC (mg GA/100 g)				222.6	216.0 ± 9.3
Ginger Rhizome Ethanol-Water Extract					
DPPH (mmol TE/100 g)	1.0	50	1	189.9	180.8 ± 4.3
ABTS (mmol TE/100 g)				365.5	361.4 ± 2.6
TPC (mg GA/100 g)				399.8	392.3 ± 8.6
Nutmeg Ethanol Extract					
DPPH (mmol TE/100 g)	1.0	41	7	204.4	209.8 ± 0.3
ABTS (mmol TE/100 g)				620.3	619.55 ± 2.8
TPC (mg GA/100 g)				486.1	482.9 ± 3.9
Nutmeg Ethanol-Water Extract					
DPPH (mmol TE/100 g)	1.0	40	7	188.3	185.3 ± 0.2
ABTS (mmol TE/100 g)				630.6	659.4 ± 2.3
TPC (mg GA/100 g)				459.5	453.2 ± 7.4

$n = 5$; $\overline{x} \pm SD$—mean value ± standard deviation.

The optimal sample preparation conditions for antioxidant extraction were found to be dependent on the spice source and a solvent. As can be seen for the two spices, the best AC results were achieved for samples ground at 40 to 50 Hz of the peripheral velocity of the grinding wheels, sieved by 1.0 to 2.0 mm of the mesh size of the grinder sieve, and stored for a variable time of 1–9 days.

The predicted DPPH, ABTS, and TPC values for each extract of both spices at the optimal conditions were experimentally validated to verify the reliability of the optimization results. The experimental values of the antioxidant properties of the ethanol and ethanol-water extracts of ginger rhizome and nutmeg obtained under these optimized conditions were found to be close to the predicted values (Table 5). These results indicate that the Box–Behnken models successfully optimized the conditions of spice preparation for the increased yield of antioxidant extraction and the enhancement of antioxidant potential with an accurate and reliable prediction.

2.5. Composition of Phenolic Acids in Ginger Rhizome and Nutmeg Extracts Obtained Using Optimal Grinding Parameters and Storage Time

The spice extracts that were obtained by the optimum grinding process and storage time were analyzed to identify and quantify phenolic acids, and the results are presented in Table 6. In general, significantly ($p \leq 0.05$) higher contents of phenolic acids were extracted from both ginger rhizome and nutmeg using pure ethanol (26 and 30% more in total, respectively, compared to ethanol-water extraction). Furthermore, the ginger rhizome extracts had a higher phenolic acid content (460.42 and 364.79 µg/100 mL in the

ethanol and ethanol-water extracts, respectively) than the nutmeg extracts (300.52 and 199.86 μg/100 mL total in the ethanol and ethanol-water extracts, respectively).

Table 6. Content of phenolic acids ($\overline{x} \pm SD$) in ginger rhizome and nutmeg extracts obtained with the optimal grinding process and storage time.

Phenolic Compounds (μg/100 mL)	Ginger Rhizome Extract		Nutmeg Extract	
	Ethanol	Ethanol-Water	Ethanol	Ethanol-Water
Caffeic acid	6.71 ± 0.45	<LOD	<LOD	<LOD
Ellagic acid	62.58 ± 3.09 ^{a}	26.90 ± 0.38 ^{b}	<LOD	<LOD
Ferulic acid	59.99 ± 1.30 ^{a}	49.45 ± 1.28 ^{b}	34.64 ± 2.05 ^{c}	22.47 ± 0.16 ^{d}
Gallic acid	53.60 ± 1.86 ^{a}	43.08 ± 4.43 ^{b}	<LOD	<LOD
p-Coumaric acid	23.10 ± 1.15 ^{b}	35.99 ± 0.14 ^{a}	19.36 ± 0.63 ^{c}	12.94 ± 0.15 ^{d}
p-OH-Benzoic acid	65.08 ± 2.75 ^{a}	39.91 ± 0.13 ^{b}	38.21 ± 0.89 ^{b}	12.38 ± 0.40 ^{c}
Protocatechuic acid	19.70 ± 1.93 ^{d}	26.44 ± 0.72 ^{c}	100.87 ± 1.40 ^{a}	75.91 ± 0.55 ^{b}
Salicylic acid	26.83 ± 2.67 ^{b}	35.29 ± 2.01 ^{a}	12.58 ± 1.43 ^{c}	11.11 ± 0.75 ^{c}
Sinapic acid	13.66 ± 1.45 ^{b,c}	12.58 ± 0.92 ^{c}	25.14 ± 1.74 ^{a}	15.24 ± 0.16 ^{b}
Syringic acid	13.98 ± 1.58 ^{b}	11.72 ± 0.18 ^{c}	18.12 ± 0.73 ^{a}	11.55 ± 0.39 ^{c}
Vanillic acid	115.19 ± 5.18 ^{a}	83.43 ± 0.70 ^{b}	51.60 ± 0.19 ^{c}	38.26 ± 0.71 ^{d}

LOD—limit of detection; $n = 3$; $\overline{x} \pm SD$—mean value ± standard deviation; different letters ($^{a-d}$) within the same line indicate significant differences (one-way ANOVA and Tukey's test, $p \leq 0.05$).

Eleven phenolic acids were identified in the studied extracts. Regardless of the extractant used, vanillic acid was the main phenolic acid in the ginger rhizome extracts (up to 25%), while protocatechuic acid was the most abundant in the nutmeg extracts (up to 38%). The ethanol extract of ginger rhizome also contained higher amounts of *p*-OH-benzoic, ellagic, ferulic, and gallic acids, and lower amounts of salicylic, *p*-coumaric, protocatechuic, syringic, sinapic, and caffeic acids. In contrast, in the ethanol-water extract obtained from this spice, caffeic acid was not detected. The ethanol-water extract of ginger rhizome was also found to be richer in *p*-coumaric, protocatechuic, and sinapic acids compared to the ethanol extract. In nutmeg extracts, caffeic and ellagic acids were below detectable levels. In addition to protocatechuic acid, vanillic, ferulic, sinapic, and *p*-OH-benzoic (in ethanol-water extract) acids were found in higher amounts in these extracts, while the other acids were minor phenolic compounds in these extracts (up to 6.5% of total phenolic acids).

Variation in the composition of phenolic compounds in extracts obtained using different solvents has also been demonstrated in other studies. For example, Ghafoor et al. [11] showed that gallic acid, protocatechuic acid, catechin, and 1,2-dihydroxybenzene were the key phenolic constituents in the methanol-water extracts from ginger rhizome. In turn, Tohma et al. [43] detected pyrogallol, *p*-OH-benzoic acid, ferulic acid, vanillin, *p*-coumaric acid, gallic acid, and caffeic acid in ethanol extract obtained from this spice.

These various levels of phenolic acids from the same spice as a result of different solvents used for the extraction might be due to their properties, mainly hydrophobicity. Muzolf-Panek and Stuper-Szablewska [44] observed that spices richer in phenolic compounds with higher hydrophobicity (e.g., rosemary, clove) were characterized by the highest level of TPC in ethanol extract, while spices with a higher content of phenolic compounds with a relatively low hydrophobicity (e.g., caraway) showed the highest TPC values in water and aqueous extracts. Similarly, in our work, ethanol extracts contained a generally higher amount of more hydrophobic phenolic acids (e.g., *p*-OH-benzoic, vanillic, ferulic, sinapic acids) compared to ethanol-water extracts.

3. Materials and Methods

3.1. Materials

Fresh ginger (*Zingiber officinale* Roscoe) rhizome originating from Niger was purchased from Medium Company (Kalisz, Poland). The material was cleaned from the roots and outer cork layer, washed, dried, and sliced. Dried ginger root served as a research material.

Nutmeg (*Myristica fragrans*) originating from Indonesia and was purchased from Medium Company. The formed dried, sorted nuclei of the nutmeg were used in the study. The materials were kept in a cool and dry place before being ground in a paper bag with a polyethylene (PE) liner.

3.2. Grinding Process and Storage Conditions

The ginger rhizome and nutmeg were initially shredded (universal shredder RU/S, Coffee Service Sp. z o.o., Warsaw, Poland), transferred to a grinder (universal grinder MUCS/S 800 DC, Coffee Service Sp. z o.o.), and ground in it according to the assumed parameters. The grinding process was carried out in the Confectionery Factory (Fabryka Cukiernicza Kopernik S.A., Toruń, Poland). Both spices were ground using grinder sieves with diameters of 1.0, 1.4, and 2.0 mm. The grinding proceeded at three peripheral velocities of grinding represented by the grinding motor inverter setting, at 30, 40, and 50 Hz. Furthermore, three different time frames between grinding and sample extraction were applied immediately after grinding (0 days) and 7 and 14 days after grinding. The ground spices were stored in a PE zip bag at 20 °C without light prior to analysis.

3.3. Preparation of Ethanol and Ethanol-Water Extracts

The ground spices (0.1000 ± 0.0001 g), prepared according to the Box–Behnken plan, were weighed into the test tubes with the screw caps, and then 5 mL of ethanol (Merck Life Science Sp. z o.o., Poznań, Poland) or a mixture of ethanol and water (1:1) was added. A suspension was formed by vigorously shaking the test tube. The suspension was extracted at 80 °C for 30 min in an ultrasonic bath (5200DTD; Chemland, Stargard Szczeciński, Poland). After extraction, the samples were centrifuged for 10 min in a laboratory centrifuge at 4500 rpm (MPW-54, Chemland), the extracts were separated from the spices, and the extractions were repeated by adding 5 mL of ethanol or ethanol-water mixture to the same sample.

3.4. Antioxidant Properties Determination

The AC and TPC in ethanol and ethanol-water extracts of two ground spices were determined by using the spectrophotometric DPPH, ABTS, and F–C methods, respectively, according to previously reported protocols [45]. The AC results were expressed as mmol Trolox equivalents (TE) per 100 g of sample, while the TPC values were expressed as mg gallic acid (GA) equivalents per 100 g of sample. All the reagents used in these methods were of analytical grade and were purchased from Merck Life Science Sp. z o.o. (Poznań, Poland): 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2′ azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), Folin–Ciocalteu's phenol reagent (F–C reagent, 2 N), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (TE, 97%), and gallic acid (3,4,5-trihydroxybenzoic acid) (GA, 98%). The resulting absorbance of each obtained solution was measured in five repetitions using a Hitachi U-2900 UV-VIS spectrophotometer (Tokyo, Japan) in a 1 cm glass cell. The AC and TPC results were calculated based on the standard curves: %DPPH = (782.10 ± 5.74)c_{TE} + (4.03 ± 0.40), %ABTS = (405.39 ± 3.40)c_{TE} + (10.38 ± 0.30), and TPC = (0.1034 ± 0.0025)c_{GA} + (0.0814 ± 0.0147) prepared for the working solutions in the concentration ranges of 0.02 and 0.10 µmol TE/mL, 0.01 and 0.15 µmol TE/mL, and 0.35–10.51 µg GA/mL, respectively.

3.5. Experimental Design and Mathematical Model

An experimental design based on the chemometric approach was preferred to reduce the number of experiments and to consider the interaction between the variables. The Box–Behnken experimental design based on 15 runs in RSM was selected for numerical optimization to achieve the best response values. A three-factor, three-level response surface test was designed with the mesh size of the grinder sieve (X_1, mm), the peripheral velocity of the grinding wheels (X_2, Hz), and the storage time of the ground spices (X_3, days) as independent variables, and the antioxidant properties of ginger rhizome and

nutmeg, determined by three analytical methods, DPPH, ABTS, and F–C, as response values. The independent variables for the grinding process and storage time of examined spices are listed in Tables 1 and 2, where the experimental range and levels are specified (low, medium, and high denoted as −1, 0, and 1, respectively). The factor levels were fixed based on the preliminary experiment trials.

The effects of these three independent variables on the antioxidant properties of both studied spices can be approximated using the following partial cubic model (PCM) as shown in Equation (1):

$$Y_n = \beta_0 + \beta_1 \times X_1 + \beta_2 \times X_2 + \beta_3 \times X_3 + \beta_{11} \times X_1^2 + \beta_{22} \times X_2^2 + \beta_{33} \times X_3^2 + \beta_{12} \times X_1 \times X_2 + \beta_{13} \times X_1 \times X_3 + \beta_{23} \times X_2 \times X_3 + \beta_{112} \times X_1^2 \times X_2 + \beta_{113} \times X_1^2 \times X_3 \quad (1)$$

where: Y_n is one of the three predicted responses, X_1, X_2 and X_3 represent the independent variables, β_0 is the constant, β_1, β_2, and β_3 are the linear-term coefficients, β_{11}, β_{22}, and β_{33} are the quadratic-term coefficients, and β_{12}, β_{13}, β_{23}, β_{112}, and β_{113} are the cross-term coefficients.

The polynomial Equation (1) visualizes the relationship between the response and the experimental levels of each factor and deduces the optimum conditions from the response surface and contour plots. The coefficient of determination (R^2) and adjusted coefficient of determination (adjusted R^2) were used to evaluate the accuracy and general ability of the polynomial regression models. The significance of the independent variables, their interactions, and regression coefficients was tested by an analysis of variance (ANOVA) for each response. A lack-of-fit analysis was applied to determine the variance and adequacy of the model's results that were fitted.

3.6. Determination of Phenolic Acids

The phenolic acids were determined chromatographically. Briefly, 20 mL of extract was evaporated to dryness at temperatures below 50 °C in an R-210-type Büchi vacuum evaporator (Büchi Labortechnik, Flawil, Switzerland). The residue was dissolved in 20 mL of deionized water (HLP 5 deionizer, Hydrolab, Gdańsk, Poland), acidified to pH 2 with 6 M hydrochloric acid (POCH, Gliwice, Poland), and 0.2 mL of 3,5-dichloro-2-hydroxybenzoic solution (1 mg/1 mL) in diethyl ether (Chempur, Piekary Śląskie, Poland) as an internal standard was added. Then, phenolic acids were extracted 5 times with 20 mL of diethyl ether, and the collected extracts were evaporated in a vacuum evaporator (Büchi Labortechnik, type R-210). The dry extract was re-dissolved in 2 mL of methanol and subjected to chromatographic separation. Phenolic acid UPLC analysis was performed on an Agilent 1290 Infinity system coupled with a 6470 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with an electrospray ionization (ESI) source. Compounds were separated using a Synergi Fusion-RP column (100 × 2 mm, 2.8 µm, Phenomenex, Torrance, CA, USA) with the temperature set at 20 °C. A gradient elution program was employed, using two elution solvents: solvent A (water/formic acid; 99.9/0.1, *v*/*v*) and solvent B (acetonitrile/formic acid; 99.9/0.1, *v*/*v*). Chromatography-grade acetonitrile was purchased from Sigma-Aldrich (St. Louis, MO, USA, supplier Poznań, Poland), and analytical-grade formic acid was purchased from Chempur, Karlsruhe, Germany. The flow rate was 0.4 mL/min with a gradient elution program as follows: 0–1 min 97% A; 1–8 min, 97–60% A; 8–10 min, 60–40% A; 10–11 min, 40–97% A and was stable until 15 min. Mass spectrometry data were obtained by an electrospray ionization (ESI) source in negative ionization mode. Source conditions were: drying gas temperature of 350 °C, drying gas flow of 10 L/min, nebulizer pressure of 30 psi, sheath gas temperature of 300 °C, sheath gas flow of 11 L/min, and capillary voltage of 3500 V. Specific MRM mode parameters for the targeted compounds were optimized through the Agilent optimizer software (Mass Hunter Optimizer), including MRM transitions, collision energy, fragmentor voltage, dwell time, and cell accelerator voltage. The selected parameters for phenolic acids are scheduled in Table 7. All standards of phenolic acids (declared purity of >97%) were purchased from Sigma-Aldrich.

Table 7. Related MS data of investigated phenolic acids in the UPLC analysis.

Phenolic Compounds	[M-H]- (*m*/*z*)	Product Ion (*m*/*z*)	Dwell	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)
3,5-Dichloro-2-OH-benzoic acid	207	163	40	104	16	7
Caffeic acid	179	135.1	40	104	16	7
Chlorogenic acid	353.1	191.1	40	104	12	7
Ellagic acid	301	300.1	40	168	36	7
Ferulic acid	193.1	134.1	40	104	16	7
Gallic acid	169	125.1	40	104	12	7
p-Coumaric acid	163	119.1	40	72	16	7
Protocatechuic acid	153.1	109.1	40	104	12	7
p-OH-Benzoic acid	137	93.1	40	72	16	7
Salicylic acid	137.1	93.1	40	72	16	7
Sinapic acid	223.1	208.1	40	104	12	7
Syringic acid	197	182.1	40	104	12	7
Vanillic acid	167	152.1	40	72	12	7

The contents of phenolic acids were determined from the calibration curves of reference standards. The internal standard used aided in better quantification to decide whether the compound recovery is complete. The calibration parameters of phenolic acids with their standard deviations, regression coefficient (R^2), limit of detection (LOD), and limit of quantification (LOQ) values are given in Table 8.

Table 8. The calibration parameters of phenolic acids using rate of peak normalizations, with their determination coefficient, LOD, and LOQ values.

Phenolic Compounds	Equation of Linear Regression	R^2	LOD	LOQ
3,5-Dichloro-2-OH-benzoic acid	y = 3061x + 40,191	0.959	0.118	0.392
Caffeic acid	y = 15,829x + 56,299	0.995	0.038	0.127
Chlorogenic acid	y = 12,320x + 25492	0.998	0.171	0.570
Ellagic acid	y = 1143x + 2849	0.995	0.215	0.717
Ferulic acid	y = 2442x + 2696	0.999	0.159	0.528
Gallic acid	y = 6702x + 61,152	0.978	0.549	1.830
p-Coumaric acid	y = 11,203x + 86,477	0.983	0.138	0.460
Protocatechuic acid	y = 17,995x + 92,122	0.992	0.416	1.388
p-OH-Benzoic acid	y = 16,541x + 145,092	0.976	0.302	1.007
Salicylic acid	y = 32,481x + 226,324	0.986	0.002	0.006
Sinapic acid	y = 3165x + 2785	0.999	0.373	1.245
Syringic acid	y = 1590x − 5036	0.995	0.038	0.126
Vanillic acid	y = 826x − 1108	1.000	0.065	0.218

R^2—coefficient of determination; LOD—limit of detection (mg/L); LOQ—limit of quantification (mg/L).

3.7. Statistical Analysis

All experimental runs were conducted five or three times and are presented as the mean ± standard deviation (SD). Tukey's test was performed to analyze significant differences ($p \leq 0.05$) between the obtained results of the DPPH, ABTS, TPC, and individual phenolic compounds in both investigated extracts of ginger rhizome and nutmeg.

The Pearson correlation analysis was performed to establish the correlations between the three analytical methods used for the determination of the antioxidant properties of spice extracts prepared using different conditions of the grinding process and two solvents.

The Statistica 8.0 software (StatSoft, Tulsa, OK, USA) was utilized for the statistical analysis, the design of the experiment, the construction of the response surface contour plots, and the calculation of the optimum conditions.

4. Conclusions

In the current study, the grinding parameters and storage time of the ground species influenced the antioxidant properties of ethanol and ethanol-water extracts. It was observed that the mesh size had the most significant negative effect on the antioxidant properties of all studied extracts of ginger rhizome and ethanol-water extracts of nutmeg. However, the storage time of ground nutmeg and the peripheral velocity of grinding were more effective independent variables on the DPPH and TPC results of its ethanol extracts, respectively. Furthermore, the experimental results agreed well with the predicted values, indicating that the Box–Behnken model can be successfully used to optimize the conditions of spice preparation before the extraction of antioxidants for food applications. Besides the spice-preparing parameters, the polarity of the solvent used as an extractant is also important. A comparison of the antioxidant properties of the extracts prepared with two commonly used solvents indicated that the ethanol-water mixture increased the extraction of the total phenols from ginger rhizome, while for the extraction of total phenols from nutmeg, ethanol is more suitable. However, ethanol, regardless of the specie type, proved to be a more efficient solvent for the extraction of phenolic acids. Furthermore, the ginger rhizome extracts had a higher phenolic acid content than the nutmeg extracts. These findings could help the food industry to produce cost-effective products containing spices rich in antioxidant compounds. Both types of spices are important ingredients in gingerbread recipes, so especially this way of using optimization in their preparation before adding them to the dough seems advisable.

Author Contributions: Conceptualization, S.P., P.T., A.S.-C. and M.T.; methodology, S.P., P.T., S.K., A.S.-C. and S.C.; software, A.S.-C. and M.T.; validation, A.S.-C., M.T., S.P., P.T., S.K. and S.C.; formal analysis, P.T., M.T., S.P. and S.K.; investigation, S.P., P.T., S.K., A.S.-C., M.T. and S.C.; resources, S.P., P.T., A.S.-C. and M.T.; data curation, A.S.-C., M.T., S.P., P.T., S.K. and S.C.; writing—original draft preparation, S.P. and P.T.; writing—review and editing, A.S.-C. and M.T.; visualization, S.P., P.T., A.S.-C. and M.T.; supervision, A.S.-C. and M.T.; project administration, S.P., P.T., A.S.-C. and M.T.; funding acquisition, S.P. and A.S.-C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The data presented in this study are available on request from the corresponding author.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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(D5) Topka, P., **Poliński, S**., Sawicki, T., Szydłowska-Czerniak, A., & Tańska, M. (2023). Effect of Enriching Gingerbread Cookies with Elder (*Sambucus nigra* L.) Products on Their Phenolic Composition, Antioxidant and Anti-Glycation Properties, and Sensory Acceptance. *International Journal of Molecular Sciences*, *24*(2), 1493.

International Journal of
Molecular Sciences

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Effect of Enriching Gingerbread Cookies with Elder (*Sambucus nigra* L.) Products on Their Phenolic Composition, Antioxidant and Anti-Glycation Properties, and Sensory Acceptance

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Abstract: Elder products are still underutilized sources of phytochemicals, mainly polyphenols, with extensive pharmacological effects on the human body. In this study, gingerbread cookies covered in chocolate (GC) were enriched with elderflower dry extract (EF) and juice concentrate (EB). The cookies (GC, GCEF, and GCEFEB) and the additives (EF and EB) were analyzed for total phenolic content (TPC), phenolic compound profile, antioxidant capacity (AC), and advanced glycation end products' (AGEs) formation in both the free and bound phenolic fractions. Sensory analysis of the cookies was performed using an effective acceptance test (9-point hedonic scale), and purchase intent was evaluated using a 5-point scale. It was found that the flavonoid content was significantly increased (20–60%) when EF and EB were added to the cookies. Moreover, the EF addition to chocolate-covered GCs enhanced the content of phenolic acids (up to 28%) in the bound phenolic fraction. An increase in the AC values of enriched cookies was found, and the free phenolic fraction differed significantly in this regard. However, inhibition of AGEs by elder products was only observed in the bound phenolic fraction. In addition, EF and EB improved the overall acceptance of the cookies, mostly their taste and texture. Thus, elder products appear to be valuable additives to gingerbread cookies, providing good sensory quality and functional food characteristics.

Keywords: functional food; antioxidant capacity; phenolic acids; flavonoids; advanced glycation end products; sensory analysis

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Citation: Topka, P.; Poliński, S.; Sawicki, T.; Szydłowska-Czerniak, A.; Tańska, M. Effect of Enriching Gingerbread Cookies with Elder (*Sambucus nigra* L.) Products on Their Phenolic Composition, Antioxidant and Anti-Glycation Properties, and Sensory Acceptance. *Int. J. Mol. Sci.* **2023**, *24*, 1493. https://doi.org/10.3390/ijms24021493

Academic Editor: Shian-Jang Yan

Received: 18 December 2022
Revised: 5 January 2023
Accepted: 10 January 2023
Published: 12 January 2023

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1. Introduction

The production of gingerbread in Poland has a long tradition, with the first mention dating back to the 14th century. The Polish city of Toruń is particularly related to the tradition of its baking; it was there that the first gingerbread factory in Poland was established in 1751. Gingerbread is a sweet bakery product that can take the form of a cake or a cookie, in which the key ingredients are specified spices. The formula for its dough may vary depending on local custom, but there are some characteristic ingredients used for cake baking, such as wheat flour, rye flour, honey, cinnamon, and ginger. In mass production, honey is increasingly being replaced by invert sugar or glucose-fructose syrup. This confectionery product belongs to the category of bakery products with a long shelf life [1–3].

The presence of a relatively large amount of spices in gingerbread makes it a value-added product with health-promoting properties. Recent studies confirmed that the consumption of spices typically used in gingerbread production, such as cinnamon, ginger, clove, nutmeg, and coriander, has protective effects for disease management, including cardiovascular ailments, diabetes, hypertension, dyslipidemia, stroke, cataracts, macular degeneration, impaired cognition, inflammation, and cancer [4–9]. These diseases belong to the group of aging diseases, as their incidence increases significantly with age, and these diseases contribute to high rates of morbidity and mortality in the elderly. Many bioactive compounds with pharmacological effects on the human body have been identified in gingerbread spices, primarily phenolic compounds and essential oils. For example, cinnamaldehyde, cinnamyl acetate, cineole, coumarin, ethyl cinnamate, linalool, humulene, β-caryophyllene, and τ-cadinol were determined in cinnamon bark; 6-gingerol, 6-paradol, 6-gingerdiol, gingerdione, shogoal, zingiberene, citral, bisabolene, cineol, α-farnesene, β-phellandrene, and zingerone in ginger rhizome; carvacrol, thymol, eugenol, cinnamaldehyde, and eugenyl acetate in clove; eugenol, myristicin, elemicin, sabinene, safrole, methyl eugenol, α-pinene, β-pinene, myristic acid, and 4-terpineol in nutmeg; linalool, geraniol, geranyl acetate, and camphor in coriander [10–14].

Due to consumer preferences, a significant portion of gingerbread products available on the market are coated in dark chocolate, which is a source of flavanols, including epicatechin, catechins, and procyanidins [15–18]. Additionally, the gingerbread can be enriched with other pro-health ingredients, e.g., rose hip pulp powder [19], camel thorn and peppermint powders [20], sea buckthorn flour [21], blended roasted pumpkin, blended roasted beetroots, and baked tomatoes [22]. Nevertheless, there are no published studies that consider the incorporation of black elder (*Sambucus nigra* L.) products in gingerbread. Literature data showed that elderberries (the fruits of the elder tree) are rich in many bioactive compounds (mainly anthocyanins, including cyanidin 3-glucoside and cyanidin 3-sambubioside, phenolic acids, flavonols and flavonol esters, lectins, and vitamin C) that exhibit diverse health functions, including antioxidant, anti-inflammatory, anticancer, anti-influenza, antimicrobial, and antidiabetic, as well as having neuroprotective activities and being cardiovascular protective [23–25]. In addition, elderflowers (the flowers of the elder tree) are a rich source of flavonoids such as kaempferol, astragalin, quercetin, quercetin-3-*O*-glucoside, rutin, isoquercitrin, and hyperoside. Moreover, pectins, tannins, and phenolic acids have also been found in elderflowers [23,26]. Elderflowers can be used both for the prevention and therapy of a wide array of diseases due to their immunomodulatory, anti-inflammatory, antimicrobial, antioxidant, and antiviral activities [27,28].

Although the phytochemicals in elder products have complex polypharmacological actions, the most popular of these is their antioxidant effect. A recent study showed that the antioxidant activity of elderflower and elderberry extracts resulted from the presence of phenolic compounds, mainly flavonols, phenolic acids, and anthocyanins [29–31]. Furthermore, more and more researchers highlight the potential anti-aging mechanisms of polyphenols, including antioxidant signaling, preventing cellular senescence, targeting microRNA, influencing NO bioavailability, and promoting mitochondrial function [32–36]. Huang et al. [37] also reported the anti-glycation activity of elderberry phenolics. Advanced glycation end products (AGEs), also known as glycotoxins, are created in products through a nonenzymatic reaction between reducing sugars and free amino groups of proteins, lipids, or nucleic acids, especially after exposure to higher temperatures and lower moisture. Excessively high levels of AGEs have been linked to the development of diabetes and several other chronic diseases [38,39]. Although the use of single polyphenols as pharmaceuticals in the prevention of age-related disorders is gaining popularity, the consumption of polyphenol-rich foods appears to be more physiologically relevant in daily life and can probably provide more benefits due to the cumulative effect of multiple polyphenols.

Taking into account the health-promoting properties of spices, dark chocolate, and elder products, it is possible to put forward the hypothesis that gingerbread cookies coated

in dark chocolate and incorporated with elderflower extract and elderberry juice could be a product that positively affects consumers' health.

Therefore, this work aimed to quantify, for the first time, the antioxidant and anti-glycation properties of gingerbread cookies with the commercially available elder products, elderflower dry extract (EF) and elderberry juice concentrate (EB). In the study, the free (extracted with 80% methanol) and bound (hydrolyzed with 2 M sodium hydroxide) phenolic fractions were analyzed separately to show the effect of elder products on the bioavailability of phenolic compounds for the human body. It is widely recognized that the sensory experience is important for the acceptance of a new product; therefore, a sensory analysis of the studied cookies was also performed.

2. Results and Discussion

2.1. Phenolic Composition and Antioxidant Properties of Elderflower Dry Extract and Elderberry Juice Concentrate Used for Gingerbread Cookie Fortification

The characteristics of free and bound phenolic fractions in elderflower dry extract (EF) and elderberry juice concentrate (EB) used for gingerbread cookie fortification are shown in Table 1.

Table 1. Content of free and bound phenolic compounds and their antioxidant capacity ($\bar{x} \pm SD$) for elderflower dry extract (EF) and elderberry juice concentrate (EB).

	EF		EB	
	Free Phenolic Fraction	**Bound Phenolic Fraction**	**Free Phenolic Fraction**	**Bound Phenolic Fraction**
TPC (μg CE/g FW)	831.14 ± 12.23 ^{a}	850.94 ± 13.21 ^{a}	437.69 ± 16.69 ^{b}	90.58 ± 7.92 ^{c}
Content of Phenolic Acids (μg/g FW)				
Benzoic Acid	26.84 ± 0.74 ^{a}	20.26 ± 1.02 ^{b}	24.14 ± 0.24 ^{a}	<LOD
Caffeic Acid	<LOD	4.80 ± 0.29 ^{a}	2.14 ± 0.06 ^{b}	<LOD
p-Coumaric Acid	<LOD	3.43 ± 0.12 ^{b}	<LOD	18.75 ± 0.58 ^{a}
Ferulic Acid	10.27 ± 0.24 ^{c}	442.79 ± 26.17 ^{a}	148.53 ± 0.65 ^{b}	11.03 ± 0.56 ^{c}
m-Hydroxybenzoic Acid	2.65 ± 0.07 ^{a}	1.86 ± 0.06 ^{b}	<LOD	<LOD
p-Hydroxybenzoic Acid	15.08 ± 1.03 ^{a}	11.34 ± 0.42 ^{b}	2.08 ± 0.08 ^{c}	<LOD
Sum of Phenolic Acids	54.84 ± 0.87 ^{c}	484.50 ± 26.96 ^{a}	176.89 ± 0.56 ^{b}	39.78 ± 0.93 ^{d}
Content of Flavonoids (μg/g FW)				
Apigenin	<LOD	0.62 ± 0.01 ^{b}	<LOD	6.66 ± 0.41 ^{a}
Epicatechin	<LOD	13.99 ± 0.49 ^{b}	15.80 ± 1.29 ^{b}	32.06 ± 1.78 ^{a}
Isorhamnetin-3-*O*-glucoside	23.28 ± 0.70 ^{a}	7.67 ± 0.72 ^{b}	<LOD	<LOD
Isorhamnetin-3-*O*-rutinoside	1.62 ± 0.04 ^{a}	<LOD	<LOD	<LOD
Kaempferol-3-*O*-rutinoside	35.29 ± 2.01 ^{a}	14.23 ± 0.27 ^{b}	11.67 ± 0.48 ^{c}	<LOD
Myricetin-3-*O*-glucoside	4.57 ± 0.27	<LOD	<LOD	<LOD
Myricetin-3-*O*-rutionoside	2.27 ± 0.16 ^{b}	3.64 ± 0.14 ^{a}	<LOD	<LOD
Naringenin	4.40 ± 0.05 ^{b}	4.26 ± 0.01 ^{b}	<LOD	5.45 ± 0.08 ^{a}
Quercetin	1.31 ± 0.01 ^{b}	<LOD	22.71 ± 0.74 ^{a}	<LOD
Quercetin-3-*O*-glucoside	131.17 ± 7.66 ^{a}	60.41 ± 3.95 ^{c}	98.02 ± 2.94 ^{b}	0.95 ± 0.05 ^{d}
Quercetin-3-*O*-vicianoside	<LOD	0.90 ± 0.02	<LOD	<LOD
Quercetin-*O*-hexosyl-*O*-hexoside	6.17 ± 0.07 ^{a}	2.18 ± 0.14 ^{b}	<LOD	<LOD
Quercetin-*O*-pentosyl-hexoside	<LOD	1.75 ± 0.05	<LOD	<LOD
Quercetin-dihexoside	4.41 ± 0.13	<LOD	<LOD	<LOD
Sum of Flavonoids	219.07 ± 9.52 ^{a}	109.63 ± 4.36 ^{c}	148.20 ± 3.78 ^{b}	45.12 ± 2.04 ^{d}
Antioxidant Capacity (μM TE/g FW)				
DPPH Assay	10.74 ± 0.33 ^{b}	11.31 ± 1.05 ^{b}	9.19 ± 0.38 ^{a}	8.38 ± 0.86 ^{c}
ABTS Assay	72.33 ± 1.74 ^{b}	58.07 ± 1.87 ^{c}	187.73 ± 4.77 ^{a}	10.53 ± 0.74 ^{d}

n = 3; $\bar{x} \pm SD$—mean value ± standard deviation; LOD—limit of detection; DPPH—2,2-diphenyl-1-picrylhydrazyl; ABTS—2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); FW—fresh weight; different letters ($^{a–d}$) within the same line indicate significant differences (one-way ANOVA and Tukey's test, $p \leq 0.05$).

There were significant differences in the total content of phenolic compounds (TPC) between EF and EB for both free and bound phenolic fractions. It is noteworthy that the free phenolic fraction content in EF (831.14 μg/g) was nearly two times higher than that in

EB (437.69 µg/g). Even greater differences were observed in the content of the bound phenolic fraction between EF (850.94 µg/g) and EB (90.58 µg/g). These results confirm that EF was a richer source of free and bound phenolics than EB. In our previous study, a water extract from elderflowers also revealed approximately 2.5 times higher TPC (81.9 mg gallic (GA) acid/g) than a water extract from elderberries (TPC = 32.5 mg GA/g) [40]. Additionally, flowers of different elderberry species or interspecific hybrids (TPC = 7410–40137 µg GA/g) contained significantly higher levels of phenolic compounds compared to berries (TPC = 2687.6–6831.1 µg GA/g) [41].

Six phenolic acids were identified in the studied extracts (Table 1). Benzoic acid was the main phenolic acid in the free phenolic fraction of EF extract (49%), while ferulic acid was the most abundant in the bound phenolic fraction of EF (91%). Ferulic acid was also the main phenolic acid in the free phenolic fraction of EB extracts (84%), while *p*-coumaric acid was the main phenolic acid in the bound phenolic fraction of EB extract (47%). The sum of phenolic acids in the free phenolic fraction of EF (54.84 µg/g) was 8.8 times lower than the sum of phenolic acids in the bound phenolic fraction of EF (484.50 µg/g). In contrast, the sum of phenolic acids in the free phenolic fraction of EB (176.89 µg/g) was 4.5 times higher than the sum of phenolic acids in the bound phenolic fraction of EB (39.78 µg/g). Furthermore, fourteen flavonoids were identified in the studied extracts. In both the free and bound phenolic fractions of EF and also in the free phenolic fraction of EB, the main identified flavonoid was quercetin-3-*O*-glucoside (which accounted for 60%, 55%, and 66%, respectively), while epicatechin was the most abundant in the bound phenolic fraction of EB (which accounted for 71%). It should be emphasized that the sum of flavonoids in EF, both in free (219.07 µg/g) and bound (109.63 µg/g) phenolic fractions, was significantly higher than in EB, in which the content of these compounds was 148.20 µg/g and 45.12 µg/g, respectively.

Comparing the two raw materials used as gingerbread ingredients, it can be seen that EF can be a better source of phenolic compounds than EB. However, in the case of EB, larger amounts of free phenolic acids may be incorporated into the cookies. Furthermore, a large proportion of the flavonoids in EF were bound. Although both elder products were high in ferulic acid and quercetin-3-*O*-glucoside, EF also contained other phenolic acids (caffeic, benzoic and hydroxybenzoic acids) and flavonoids (isorhamnetin, kaempferol, myricetin and quercetin derivatives), while EB was high in benzoic and *p*-coumaric acids, as well as epicatechin and quercetin. Other studies also showed that hydroxycinnamic acids represented the major share of phenolics in elderberries and elderflowers, whereas from the group of flavonoids, different quercetin glycosides, kaempferol glycosides, isorhamnetin glycosides, and epicatechin were mainly quantified [27,42]. The anti-aging, anti-inflammatory, antiproliferative, anticancer, antibacterial, and antioxidant properties of phenolic compounds were reported by Albuquerque et al. [43] and Rahman et al. [44]. Among these compounds, ferulic acid was widely applied, mainly in skin care formulations, as a delayer of skin photoaging processes [45]. Additionally, its effect was verified against acute and chronic pathologies, e.g., intestinal ischemia, cancer, cardiovascular and skin diseases, diabetes, cochlear oxidative damage due to repeated noise exposure, and oxidative cellular stress in human dermal fibroblasts, as well as against neurodegenerative pathologies, especially Alzheimer's disease [46]. Importantly, Rondini et al. [47] discovered that consuming ferulic acid through food had a greater impact on the human body than supplementing it with pure ferulic acid. In turn, quercetin is one of the most well-known flavonoids, which may reduce cellular aging by improving cell proliferation and the repair of the heterochromatin structure [48]. It is rapidly metabolized and excreted without accumulating in the body. Furthermore, it easily crosses the blood-brain barrier and exhibits neuroprotective activity, as well as playing a crucial role as an anti-inflammatory molecule [49].

Analyzing the antioxidant properties, it was found that the antioxidant capacity (AC) determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was higher in EF (10.74 µM TE/g and 11.31 µM TE/g for free and bound phenolic fractions, respectively) than in EB (9.19 µM TE/g and 8.38 µM TE/g, respectively) (Table 1). These results were consis-

tent with TPC results. Unexpectedly, there was no correlation between the AC analyzed by the two analytical assays. The AC determined by the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay in EF was 72.33 μM TE/g in the free phenolic fraction and 58.07 μM TE/g in the bound phenolic fraction, while in EB the AC results were 187.73 μM TE/g and 10.53 μM TE/g, respectively.

On the contrary, a water extract from elderberries had significantly lower radical scavenging properties of DPPH$^{\bullet}$ (1911.6 μM TE/g) and ABTS$^{\bullet+}$ (2337.1 μM TE/g) than elderflower extract (DPPH = 4756.0 μM TE/g and ABTS = 6258.7 μM TE/g) [40]. Moreover, the ABTS values (3.20–39.59 μM TE/g) of various elderberry species and hybrids were considerably lower compared to the ABTS results (44.87–118.26 μM TE/g) of elderberry flowers [41].

However, the ABTS results for free and bound phenolic fractions of EF and EB were higher than the DPPH values. This suggests that the ABTS$^{\bullet+}$ radical cation is reactive towards most antioxidants (mainly free phenolics), including both hydrophilic and lipophilic compounds, whereas the DPPH$^{\bullet}$ radical can only be dissolved in organic media, especially in alcoholic media, which is an important limitation for the determination of hydrophilic antioxidants.

These findings are in line with the results of earlier studies, in which the antioxidant properties of elderflower and elderberry extracts are linked to the content of phenolic compounds [40–42].

2.2. *Effect of Elderflower Dry Extract and Elderberry Juice Concentrate Additions on the Phenolic Composition of Gingerbread Cookies*

The results of the chemical analysis of the gingerbread cookies showed that the addition of commercially available elder products had a generally positive effect on the content of phenolic compounds; however, the combined use of flower and juice products in the formulation was proven to be more favorable (Table 2).

Table 2. Content of free and bound phenolic compounds and their antioxidant capacity ($\overline{x} \pm SD$) for gingerbread cookies covered in chocolate without additives (GC), and with elderflower dry extract (GCEF), as well as enriched with elderberry juice concentrate (GCEFEB).

Phenolic Compounds	GC		GCEF		GCEFEB	
	Free Phenolic Fraction	Bound Phenolic Fraction	Free Phenolic Fraction	Bound Phenolic Fraction	Free Phenolic Fraction	Bound Phenolic Fraction
TPC (μg CE/g FW)	236.34 ± 5.87 ^{c}	67.68 ± 3.62 ^{d}	277.44 ± 8.50 ^{a}	67.77 ± 9.03 ^{d}	256.16 ± 9.55 ^{b}	68.79 ± 6.44 ^{d}
Content of Phenolic Acids (μg/g FW)						
Benzoic Acid	6.41 ± 0.00 ^{a}	<LOD	6.57 ± 0.13 ^{a}	<LOD	6.01 ± 0.09 ^{a}	<LOD
Caffeic Acid	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
p-Coumaric Acid	<LOD	2.27 ± 0.08 ^{c}	<LOD	4.27 ± 0.10 ^{b}	<LOD	4.48 ± 0.02 ^{a}
Ferulic Acid	9.69 ± 0.76 ^{a}	3.89 ± 0.16 ^{d}	9.99 ± 0. 48 ^{a}	4.45 ± 0.28 ^{c}	7.62 ± 0.46 ^{b}	3.63 ± 0.16 ^{d}
m-Hydroxybenzoic Acid	0.64 ± 0.05 ^{c}	1.98 ± 0.09 ^{a}	0.58 ± 0.03 ^{c}	1.41 ± 0.09 ^{b}	<LOD	1.53 ± 0.09 ^{b}
p-Hydroxybenzoic Acid	1.34 ± 0.07 ^{b}	1.11 ± 0.09 ^{c,d}	1.26 ± 0.02 ^{c}	1.70 ± 0.12 ^{a}	0.99 ± 0.03 ^{d}	1.72 ± 0.07 ^{a}
Sum of Phenolic Acids	18.08 ± 0.77 ^{a}	13.24 ± 0.10 ^{d}	18.40 ± 0.63 ^{a}	15.82 ± 0.40 ^{b}	14.62 ± 0.50 ^{c}	15.36 ± 0.19 ^{b}
Content of Flavonoids (μg/g FW)						
Apigenin	<LOD	0.79 ± 0.04 ^{b}	<LOD	1.00 ± 0.06 ^{a}	<LOD	0.88 ± 0.05 ^{b}
Epicatechin	<LOD	14.43 ± 1.16 ^{c}	4.23 ± 0.18 ^{d}	23.33 ± 0.33 ^{b}	<LOD	24.65 ± 0.13 ^{a}
Isorhamnetin-3-*O*-glucoside	5.26 ± 0.16 ^{b}	<LOD	6.04 ± 0.34 ^{a}	<LOD	6.50 ± 0.07 ^{a}	<LOD
Isorhamnetin-3-*O*-rutinoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Kaempferol-3-*O*-rutinoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Myricetin-3-*O*-glucoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Myricetin-3-*O*-rutionoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Naringenin	<LOD	1.81 ± 0.01 ^{a}	<LOD	1.85 ± 0.06 ^{a}	<LOD	1.85 ± 0.06 ^{a}
Quercetin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Quercetin-3-*O*-glucoside	0.97 ± 0.05 ^{c}	<LOD	2.42 ± 0.02 ^{b}	<LOD	3.04 ± 0.06 ^{a}	<LOD
Quercetin-3-*O*-vicianoside	1.87 ± 0.13 ^{b}	<LOD	2.19 ± 0.12 ^{a}	<LOD	1.78 ± 0.09 ^{b}	<LOD
Quercetin-*O*-hexosyl-*O*-hexoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Quercetin-*O*-pentosyl-hexoside	1.52 ± 0.07 ^{a}	<LOD	1.26 ± 0.04 ^{b}	<LOD	1.15 ± 0.05 ^{c}	<LOD
Quercetin-dihexoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Sum of Flavonoids	9.63 ± 0.38 ^{e}	17.03 ± 1.19 ^{c}	16.13 ± 0.36 ^{c}	26.19 ± 0.35 ^{b}	12.47 ± 0.09 ^{d}	27.39 ± 0.11 ^{a}

$n = 3$; $\overline{x} \pm SD$—mean value ± standard deviation; LOD—limit of detection; different letters ($^{a-e}$) within the same line indicate significant differences (one-way ANOVA and Tukey's test, $p \leq 0.05$).

There were clear differences in TPC between studied gingerbread cookies covered in chocolate: without additives (GC), enriched with elderflower dry extract (GCEF), and enriched with elderflower dry extract and elderberry juice concentrate (GCEFEB). The highest TPC result in the free phenolic fraction was determined in GCEF (277.44 μg/g), while the lowest was in GC (236.34 μg/g). The lower results of TPC in bound phenolic fraction than in free phenolic fraction were noted: 67.68 μg/g in GC, 67.77 μg/g in GCEF, and 68.79 μg/g in GCEFEB, respectively.

The addition of elderberry and elderflower extracts to dark chocolate also caused a plant extract type-dependent statistically significant increase in TPC results of fortified chocolate samples (11.7, 12.8, and 17.9 mg GA/g for chocolate without and with elderberry and elderflower extracts, respectively) [40]. Moreover, the enrichment of short crust cookies with elderflower, and wheat flour cookies with freeze-dried elderberries, increased the TPC from 1.01 mg GA/g and 91.26 mg/100 g in the control samples to 2.22 mg GA/g and 144.69 mg/100 g in the supplemented cookies [50,51]. Przybylski et al. [22] also found a high content of total polyphenols in gingerbread cakes after supplementation with tomato, beetroot, and pumpkin purée (39.02, 33.88, and 29.85 mg GA/100 g, respectively). On the contrary, similar TPC results were observed in gingerbread without (215.59 mg GA/100 g) and fortified with 3% chicken eggshell powder (214.01 mg GA/100 g) [3].

Six phenolic acids were identified in the studied phenolic fractions from gingerbread cookies, and ferulic acid was the main phenolic acid in all free phenolic fractions, with 54% in the GC, and GCEF, and 52% in the GCEFEB (Table 2). Ferulic acid was also the main phenolic acid in bound phenolic fractions in the GC (29%) and GCEF (28%), while *p*-coumaric acid was the most abundant in the GCEFEB (29%). Total phenolic acid contents ranged from 13.24 μg/g in the bound phenolic fraction of the GC to 18.40 μg/g in the free phenolic fraction of the GCEF. Furthermore, fourteen flavonoids were identified in phenolic fractions of the studied gingerbread cookies (Table 2). The highest content of flavonoids was observed in the bound phenolic fraction of the GCEFEB (27.39 μg/g), whereas the lowest flavonoid content was observed in the free phenolic fraction of the GC (9.63 μg/g). Isorhamnetin-3-*O*-glucoside was the main flavonoid in all free phenolic fractions, respectively, 55% in GC, 37% in GCEF, and 52% in GCEFEB, whereas epicatechin was the most abundant in the bound phenolic fraction, respectively, 85% in GC, 89% in GCEF, and 90% in GCEFEB.

When the effect of the elder products on the content of phenolic compounds in gingerbread cookies was examined, it was discovered that EF and EB increased the amounts of bound phenolic acids and both forms of flavonoids. In turn, the content of free phenolic acids was similar in the GCEF or lower in the GCEFEB compared to conventional gingerbread cookies (GC). This is probably a result of their binding by carbohydrates and proteins, and especially, ferulic acid and hydroxybenzoic acids seem to be more sensitive to heat treatment, which is consistent with the results of Liazid et al. [52].

The significant increase in the content of bound *p*-coumaric (up to 97%) and *p*-hydroxybenzoic acids (up to 55%) was noted after the addition of EF and EB (Table 2). In the case of flavonoids, these additives increased the content of free quercetin-3-*O*-glucoside (by 149% for EF and by 212% for EF + EB) and bound forms of epicatechin (by 26% and 11%, respectively) and apigenin (by 62% and 71%, respectively).

2.3. Effect of Elderflower Dry Extract and Elderberry Juice Concentrate Additions on the Antioxidant Properties of Gingerbread Cookies

Two radical scavenging assays (DPPH and ABTS) were used to receive reliable data on the effect of the addition of elderflower dry extract (EF) and elderberry juice concentrate (EB) to gingerbread cookies, and how this impacted their antioxidant properties (Figure 1).

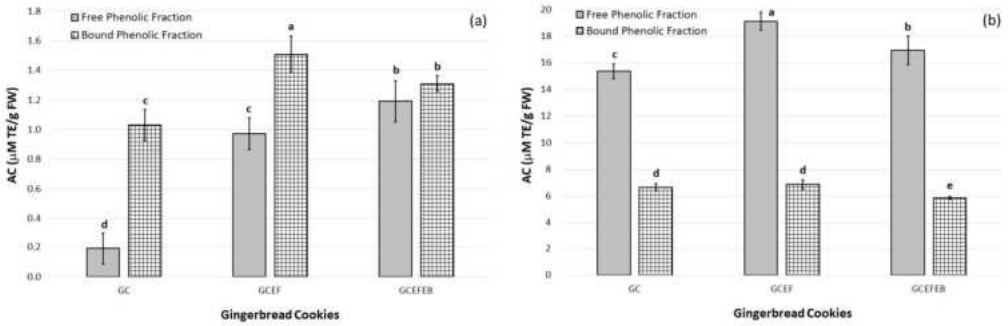

Figure 1. Antioxidant capacity (AC) results ($\overline{x} \pm SD$, mean value ± standard deviation) of gingerbread cookies covered in chocolate without additives (GC), and with elderflower dry extract (GCEF), as well as enriched with elderberry juice concentrate (GCEFEB), determined by DPPH (**a**) and ABTS (**b**) assays. Different letters (a–e), separately for each assay, indicate significant differences (one–way ANOVA and Tukey's test, $p \leq 0.05$).

There were significant differences in the AC results of the same phenolic fraction determined by two different analytical assays. In the DPPH assay, the AC in the bound phenolic fraction was higher than that in the free phenolic fraction (1.03–1.51 μM TE/g vs. 0.19–1.19 μM TE/g, respectively), while the opposite was true in the ABTS assay (5.88–6.89 μM TE/g vs. 15.37–19.12 μM TE/g, respectively). It is noteworthy that the ABTS results were more than 14 times higher than those obtained by DPPH for the free phenolic fraction and 4 times higher for the bound phenolic fraction.

Similarly, the ABTS values (433.9–1211.0 μM TE/g) of dark chocolates without and with elderberry and elderflower extracts were significantly higher than those of the DPPH (144.2–364.3 μM TE/g) [40]. This variability between the DPPH and ABTS results may be due to different affinities of the applied analytical methods toward hydrophobic and hydrophilic antioxidants. The ABTS assay is applicable for both lipophilic and hydrophilic antioxidants, while the hydrophobic nature of the DPPH$^{\bullet}$ radical limits the determination of hydrophilic antioxidants using the DPPH method. Moreover, ABTS$^{\bullet+}$ radical cations are more reactive than DPPH$^{\bullet}$ radicals due to the reactions of potential antioxidants with ABTS$^{\bullet+}$ involving both hydrogen atom transfer (HAT) and single electron transfer (SET), unlike the reactions with DPPH$^{\bullet}$ radicals, which mainly involve the HAT mechanism [40]. On the other hand, the structures of aromatic compounds provide a chromophoric system, which leads to interference in DPPH$^{\bullet}$ radicals. Therefore, the differences in antioxidant properties may be related to the chemical structure and type of antioxidants detected in gingerbread cookies.

The AC results confirmed that the bound phenolic fractions of gingerbread cookies without and with EF and EB were identified as more potent DPPH$^{\bullet}$ radical scavengers. In contrast, their free phenolic fractions more effectively scavenged the ABTS$^{\bullet+}$ radical cation (Figure 1). The AC increase in enriched gingerbread cookies can be explained by the fact that added plant extracts were a good source of bioactive phenolic compounds, including flavonoids (Table 1). Moreover, heat treatment during baking gingerbread cookies can enhance their antioxidant properties due to the formation of Maillard reaction products.

Previous research also reported that the DPPH and ABTS of the short crust cookies supplemented with elderflower significantly increased from 0.47 mg TE/g and 1.25 mg TE/g in the control sample to 2.10 mg TE/g and 3.45 mg TE/g in the enriched sample, respectively [50]. In addition, the DPPH (9.25 μM/g) and ABTS values (9.42 μM/g) of shortbread and wheat flour cookies fortified with elderberries were higher than scavenging activity of control samples (DPPH = 2.60 μM/g and ABTS = 1.11 μM/g) [51,53]. Unexpectedly, the addition of 3% eggshell powder to the gingerbread samples caused a decrease in their

antioxidant properties (DPPH = 388.13 and 370.44 mg TE/100 g, and ABTS = 453.79 and 448.82 mg TE/100 g for control and enriched samples, respectively) [3].

The differences between the AC of free and bound phenolic fractions in the three types of gingerbread cookies were stable in the ABTS assay but unstable in the DPPH assay (Figure 1). It was found that the GCEFEB had the lowest differences between the AC of free and bound phenolic fractions in the DPPH assay (Figure 1a). Furthermore, the GCEF was characterized by the highest AC of free and bound phenolic fractions measured by the ABTS assay (Figure 1b), in contrast to the results obtained by the DPPH assay, where the GCEF had a lower AC of free phenolic fraction than the GCEFEB (Figure 1a).

2.4. Relationships between Phenolic Composition and Antioxidant Properties of Gingerbread Cookies

It can be noted that there were moderate relationships (r = 0.44–0.82) between the phenolic compound content and antioxidant properties of gingerbread cookies (Table 3).

Table 3. Pearson's correlation coefficients (r) between antioxidant properties and the content of phenolic compounds of studied gingerbread cookies.

Phenolic Compounds Content	Antioxidant Properties	
	DPPH Assay	ABTS Assay
Total Phenolic Compounds	0.77 *	0.57
Total Phenolic Acids	0.66 *	0.44
Total Flavonoids	0.82 *	0.68 *

*—statistically significant at $p \leq 0.05$.

The highest correlation coefficient (r = 0.82) was between the DPPH assay and total flavonoids, while the lowest correlation coefficient (r = 0.44) was calculated between the ABTS assay and total phenolic acids. Moreover, similar correlations were found between the ABTS values and total flavonoids (r = 0.68), DPPH results, and the total content of phenolic acids (r = 0.66). This data suggests that the content of polyphenols can be used as an indicator of the strength of antioxidant activity.

For comparison, higher correlation coefficients (r = 0.9993 and 0.9899) were calculated for relationships between radical scavenging activities determined by the DPPH and ABTS assays and TPC in short crust cookies supplemented with edible flowers [50]. Moreover, the correlations exhibited that the epicatechin and epigallocatechin contents in these cookies showed strong positive correlations with the DPPH (r = 0.8547 and 0.8762) and ABTS (r = 0.8740 and 0.8903) scavenging activities, even if their amount in the cookies was not very high.

2.5. Effect of Elderflower Dry Extract and Elderberry Juice Concentrate Additions on Advanced Glycation End Products' Formation in Gingerbread Cookies

Dietary advanced glycation end products (AGEs) are formed in thermally treated foods as a result of the Maillard reaction. These products are essential to the total pool of AGEs produced in the living organism [54]. The inhibitory effects of the elder products and gingerbread cookies evaluated by the BSA-glucose model are shown in Figure 2.

It was found that the inhibitory activity of the EB samples (free and bound fractions) revealed a higher value of AGEs' inhibition (93.0% and 89.6%, respectively), while the inhibitory effect of aminoguanidine solution (AG) was 92.4%. The enrichment of gingerbread cookies with dry elderflower extract (GCEF) and elderberry juice concentrate (GCEFEB) did not significantly increase the inhibitory activity against AGEs' formation in free phenolic fractions compared to the control cookies. However, bound phenolic fraction samples were characterized by higher inhibitory activity values, and the highest value was observed in the bound fraction obtained from cookies enriched with EF and EB (Figure 2). This phenomenon may be related to hydrolysis, which resulted in the release of more

compounds with an inhibitory activity against AGEs. The increased contents of epicatechin and/or naringenin present were observed in the bound fraction. Previously published studies also showed that epicatechin and naringenin, as major dietary flavonoids, could inhibit the formation of AGEs [55,56]. In addition, other phenolic compounds derived from plant extracts also inhibited the formation of AGEs. Their main mechanism is inhibiting the production of free radicals in the glycation process [57,58].

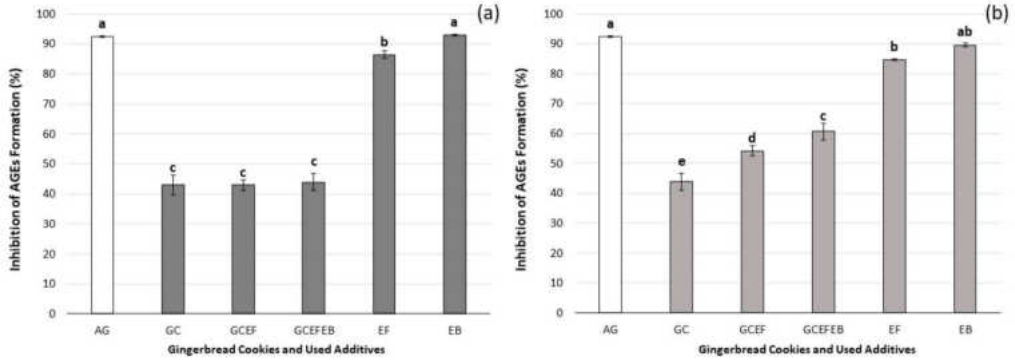

Figure 2. The inhibitory effects of gingerbread cookies covered in chocolate without additives (GC) and with elderflower dry extract (GCEF), as well as enriched with elderberry juice concentrate (GCEFEB); elderflower dry extract (EF); elderberry juice concentrate (EB); aminoguanidine solution (AG, as a positive control; 1 mM/L) against AGEs' formation determined for free (**a**) and bound (**b**) phenolic fractions. Different letters (a–e), separately for each phenolic fraction, indicate significant differences (one-way ANOVA and Tukey's test, $p \leq 0.05$).

The correlation studies demonstrated that the inhibitory effects of analyzed samples against the formation of AGEs were correlated with their bioactive compound contents (for free fractions: TPC/AGEs inhibition, r = 0.77; the sum of phenolic acid/AGEs inhibition, r = 0.83; the sum of flavonoids/AGEs inhibition, r = 0.94; and for bound fractions: TPC/AGEs inhibition, r = 0.53; the sum of phenolic acid/AGEs inhibition, r = 0.55; the sum of flavonoids/AGEs inhibition, r = 0.72) and AC (ABTS assay/AGEs inhibition, r = 0.60; DPPH assay/AGEs inhibition, r = 0.95) determined in the samples. Our data on the anti-glycation effect are in agreement with the previously published research [54,59].

2.6. Effect of Elderflower Dry Extract and Elderberry Juice Concentrate Additions on the Physical Characteristics and Sensory Acceptance of Gingerbread Cookies

Physical measurements indicated that the addition of EF and EB did not affect the height and diameter of the gingerbread cookies (Table 4). Moreover, as was to be expected, the addition of EB caused an increase in the weight of the gingerbread cookies.

Similarly, the addition of elderberry and other fruit pomaces had a negligible effect on the geometric features of shortbread cookies. All baked cookies preserved their round shape and diameter (57.22 and 56.75–57.54 mm for cookies without and with fruit pomace, respectively) [53].

On the other hand, the presence of EF and EB additives in the GCs had no significant impact on surface color, defined using lightness (L*) and chromaticity parameters, redness (a*) and yellowness (b*) (Table 4). However, the incorporation of gingerbread cookies with EF and EB significantly affected the cross-section color by decreasing L* value and increasing a* and b* values (except GCEFEB). Therefore, the lightness of the GCEFEB was reduced from 50.50 and 75.82 to 49.29 and 68.24 for the surface and cross-section color, respectively (Table 4). Moreover, the a* (−1.10 and −1.40) and b* (27.89 and 50.20) values for the surface and cross-section of the GCEF were higher compared with the control GC

(a* = −1.26 and −1.96, b* = 26.94 and 48.46, respectively). On the contrary, a decrease in the cross-section values of a* (−2.62) and b* (42.94) was observed after the incorporation of EF and EB (Table 4).

Table 4. Physical parameters ($\overline{x} \pm SD$) of gingerbread cookies covered in chocolate without additives (GC), and with elderflower dry extract (GCEF), as well as enriched with elderberry juice concentrate (GCEFEB).

Parameter	GC	GCEF	GCEFEB
Weight (g)	14.03 ± 0.34 ^{b}	14.07 ± 0.59 ^{b}	19.06 ± 0.86 ^{a}
Height (mm)	23.06 ± 0.59 ^{a}	23.00 ± 0.30 ^{a}	22.36 ± 0.16 ^{a}
Diameter (mm)	54.55 ± 0.82 ^{a}	54.66 ± 0.75 ^{a}	54.50 ± 0.56 ^{a}
Surface Color			
L*	50.50 ± 0.61 ^{a}	49.88 ± 0.54 ^{a}	49.29 ± 0.78 ^{a}
a*	−1.26 ± 0.23 ^{a}	−1.10 ± 0.34 ^{a}	−1.07 ± 0.37 ^{b}
b*	26.94 ± 0.74 ^{a}	27.89 ± 0.70 ^{a}	27.58 ± 0.36 ^{a}
Cross-section Color			
L*	75.82 ± 0.45 ^{a}	72.48 ± 0.44 ^{b}	68.24 ± 3.24 ^{c}
a*	−1.96 ± 0.94 ^{ab}	−1.40 ± 0.29 ^{a}	−2.62 ± 0.34 ^{b}
b*	48.46 ± 0.13 ^{b}	50.20 ± 0.60 ^{a}	42.94 ± 0.53 ^{c}

n = 10; $\overline{x} \pm SD$—mean value ± standard deviation; different letters ($^{a–c}$) within the same column indicate significant differences (one-way ANOVA and Tukey's test, $p \leq 0.05$).

In a previous article, the lightness (L* = 57.22) and redness (a* = −2.39) of cookies with elderberry pomace were also lower than the L* (94.82) and a* (−1.76) values for the control cookies [53]. However, the yellowness (b*) of these enriched cookies increased from 18.32 to 22.19. Nevertheless, all color parameters L*, a* and b* decreased with the addition of freeze-dried elderberry to gluten-free wafer batter and wafer sheets [60]. Although elderberry flower powder contained color pigments, the low differences in the color parameters of the fortified gingerbread cookies were affected by their dark color compared with the shortbread cookies [53] and wafers [60].

It is well known that color parameters clearly affect consumers' acceptance of the visual appearance of final food products. As can be seen in Table 5, there were differences in the sensory characteristics between each type of the studied gingerbread cookies, resulting from the raw materials used.

Table 5. Mean sensory scores ($\overline{x} \pm SD$) for the color, odor, texture, flavor, and overall acceptability of gingerbread cookies covered in chocolate without additives (GC), and with elderflower dry extract (GCEF), as well as enriched with elderberry juice concentrate (GCEFEB).

Sample	Color	Odor	Texture	Flavor	Overall Acceptance
GC	7.38 ± 1.31 ^{a}	7.64 ± 0.90 ^{b}	3.38 ± 1.14 ^{c}	2.88 ± 1.08 ^{b}	3.17 ± 0.91 ^{b}
GCEF	7.24 ± 0.94 ^{a}	8.38 ± 0.62 ^{a}	5.10 ± 0.80 ^{b}	6.98 ± 0.69 ^{a}	7.16 ± 0.67 ^{a}
GCEFEB	7.22 ± 0.93 ^{a}	8.39 ± 0.67 ^{a}	7.10 ± 1.00 ^{a}	7.71 ± 1.03 ^{a}	7.97 ± 0.95 ^{a}

n = 112; $\overline{x} \pm SD$—mean value ± standard deviation; color, odor, texture, flavor and overall acceptability are based on the 9-point hedonic rating scale system, with anchoring point, 1—"disliked extremely" and 9—"liked extremely"; different letters ($^{a–c}$) within the same column indicate significant differences (one-way ANOVA and Tukey's test, $p \leq 0.05$).

It is noteworthy that for such attributes as flavor, and texture, significant differences between the GCEFEB, GCEF, and GC were demonstrated. The highest results for these attributes were obtained for the GCEFEB: 7.71 for flavor and 7.10 for texture on a 9-point scale. A slightly lower rating for flavor was noted in the GCEF (6.98), whereas a very low score was obtained for the GC (2.88). The texture attributes for the GCEF and GC were also significantly lower than the GCEFEB and were 5.10 and 3.38, respectively. In opposition to flavor and texture attributes, the color and odor scores obtained for the GCEFEB, GCEF, and GC were comparable, and they were assessed, respectively, at 7.22, 7.24, and 7.38

for the color attribute and 8.39, 8.38, and 7.64 for the odor attribute. Regardless of the above ratings, the overall acceptability was assessed for each type of gingerbread cookie (Table 5). The highest score (7.97), which corresponded to the classification "liked very much", was obtained by the GCEFEB. A slightly lower score (7.16) was had for the GCEF, and a significantly lower score (3.17), which corresponded to the classification "disliked moderately", was found for the GC.

The evaluators paid particular attention to the wide range of flavor characteristics, which varied depending on the type of gingerbread cookie. Gingerbread cookies covered in chocolate (GC) scored the lowest in terms of texture and flavor, at a level of approximately "disliked moderately". These properties in the enriched gingerbread cookies were more acceptable, and they were described as "liked moderately" for the GCEFEB, while they scored as "neither liked nor disliked" (texture) or "liked slightly" (flavor) for the GCEF.

Other studies also showed that supplementing gingerbread with plant powders positively affected its sensory characteristics. For example, Ghendov-Mosanu et al. [19] introduced 2% and 4% of rose hip pulp powder to gingerbread and observed that these additives improved its general characteristics, but a lower concentration was preferred due to the specific smell and taste of rose hips. In addition, Sanokulovich et al. [20] established that the addition of powders from medicinal plants in dosages ranging from 0.5% to 2.0% was more recommended for gingerbread. They found that increasing the concentration of peppermint and camel thorn powders led to the appearance of bitterness in the taste, darkening of the crumb, and a deterioration of its porosity. Furthermore, the sensory evaluation of gingerbread fortified with 3% eggshell powder [3] also exhibited moderate–high (7.00–7.80 on a 9-point structured hedonic scale) consumer acceptability. There were no significant differences between the sensory profiling (appearance, aroma, texture, and taste) of gingerbread with 3% eggshell powder and the control sample. Consequently, the addition of pumpkin, tomato, and beetroot to gingerbread at a level of up to 25% provided an adequate effect on the sensory quality of the enriched products while still being acceptable to consumers [22]. The tested attributes in terms of appearance liking, aroma liking, texture liking, taste/flavor liking, and the overall liking of gingerbreads enriched with tomato and pumpkin were rated highly (above a 6.00 score). In turn, Tańska et al. [53] showed that the addition of elderberry pomace to shortbread cookies caused a decrease in sensory acceptance. The cookies were characterized by a more perceptible taste and aroma and were sourer.

On the contrary, our results of the hedonic evaluation of the GC, GCEF, and GCEFEB by untrained panelists demonstrated that the GCEF and GCEFEB stood out in sensory acceptance. The GCEFEB and GCEF were pleasant and well-accepted by panelists. Unexpectedly, the enrichment of the GC with EF and EB rich in free and bound phenolic compounds (Table 2) improved the sensory quality, making the new products more accepted. Phenolic compounds are closely associated with sensory quality. Oxidative changes during processing can decrease the sensory evaluation of the final products and reduce the willingness to purchase them [61]. However, this study confirmed that the production of GCs enriched with EF and EB, having high nutritional and antioxidant properties and good sensory acceptance, can be an appropriate strategy from a commercial point of view.

The desire to buy locally produced gingerbread cookies was directly proportional to sensory assessment. In the context of the proposed products' purchase intent frequency, the GCEFEB received the highest scores (4.39 ± 0.06 on a 5-point scale) and was qualified as "certainly would buy" (Figure 3).

As can be seen, both EF and EB had a positive effect on the purchase intent of the new gingerbread cookies. It is noteworthy that the results of overall acceptability (Table 5) were correlated with purchase intent, which is very valuable information from a commercial point of view. The GCEFEB received 4.39 points, which is a result indicating great potential for this product on the market. In the same study, the GCEF had moderate purchase intent scores of 3.54 points, being largely qualified with "probably would buy". The lowest purchase intention (2.64 points) was declared for the GC, which suggests that the

product in this form should not be launched on the market. This phenomenon is probably related to the unusual aroma of cakes with elderberry products, which may be attractive to consumers. In addition, elderberries have a low sugar content compared to other fruit species [41]. Therefore, they are an ideal additive that does not increase the caloric content of a new product.

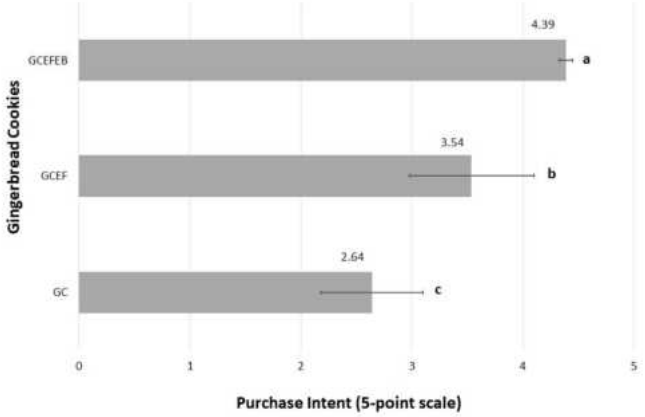

Figure 3. Average ratings of purchase intent of gingerbread cookies covered in chocolate without additives (GC), and with elderflower dry extract (GCEF), as well as enriched with elderberry juice concentrate (GCEFEB). Purchase intent was based on the 5-point scale (1 = certainly would not buy, 2 = probably would not buy, 3 = might or might not buy, 4 = probably would buy, and 5 = certainly would buy). Different letters (a–c) indicate significant differences (one-way ANOVA and Tukey's test, $p \leq 0.05$).

3. Materials and Methods

3.1. Chemicals

Standard phenolic compounds (purity > 97%), including phenolic acids (benzoic, caffeic, ferulic, *p*-coumaric, *m*-hydroxybenzoic, *p*-hydroxybenzoic) and flavonoids (apigenin, D-catechin, epicatechin, kaempferol, naringenin, quercetin), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), and HPLC-grade solvents and reagents such as acetonitrile, ammonium formate, bovine serum, formic acid, and glucose were purchased from Sigma Aldrich (Poznań, Poland). Analytical-grade reagents such as acetic acid, Folin–Ciocalteau (F-C) reagent, methanol, phosphate buffer, potassium dithionite, sodium carbonate, and sodium hydroxide were supplied by Chempur (Piekary Śląskie, Poland).

3.2. Materials

Three types of gingerbread cookies were produced through a traditional technological process in the Confectionery Factory (Fabryka Cukiernicza Kopernik S.A., Toruń, Poland). Ingredients and photos of the samples are presented in Table 6.

Samples of gingerbread cookies (GC) were supplemented with powdered elderberry flower extract (EF) supplied by Greenvit Botanical Extracts Manufacturer in Zambrów (Poland), and concentrated elderberry fruit juice (EB) supplied by DÖHLER Natural Food & Beverage Ingredients (Darmstadt, Germany). The GCs covered in chocolate but without elder products were used as a control sample. The materials were kept in a polyethylene (PE) bag in a cool and dry place until the research was performed.

Table 6. Photos and ingredients of the three types of gingerbread cookies produced.

	Gingerbread Covered in Chocolate	Gingerbread Covered in Chocolate Fortified by Elderflower Dry Extract	Gingerbread Covered in Chocolate Fortified by Elderflower Dry Extract and Filled with Elderberry Juice Concentrate
Code	GC	GCEF	GCEFEB
Photo			
Ingredients	wheat flour, chocolate 31% [sugar, cocoa mass, cocoa butter, emulsifier (soy lecithin), flavor], sugar, rye flour, color (caramel), spices, raising agent (ammonium carbonates), aroma, salt, acidity regulator (citric acid)	wheat flour, chocolate 31% [sugar, cocoa mass, cocoa butter, emulsifier (soy lecithin), flavor], sugar, rye flour, color (caramel), spices, raising agent (ammonium carbonates), powdered elderberry flower extract 0.31%, aroma, salt, acidity regulator (citric acid)	wheat flour, chocolate 25% [sugar, cocoa mass, cocoa butter, emulsifier (soy lecithin), flavor], filling 19% [sugar, concentrated apple puree, concentrated elderberry juice 0.4%, acidity regulator (citric acid), aroma, preservative (potassium sorbate), gelling agent (pectins)], sugar, rye flour, color (caramel), spices, raising agent (ammonium carbonates), powdered elderberry flower extract 0.25%, aroma, salt, acidity regulator (citric acid)

3.3. Extraction of Free and Bound Phenolic Fractions

Free and bound phenolic compounds were extracted using the method by Šťastná et al. [50] with some modifications. The ground gingerbread samples (laboratory mill type A 10; IKA Labortechnik, Staufen, Germany) were weighed (10 ± 0.001 g) into dark flasks, and 40 mL of 80% methanol was added to each flask. In turn, the samples of EF and EB were lower in weight (2 ± 0.001 g), and 20 mL of 80% methanol was added into each flask. The mixtures were sonicated for 1 h in an ultrasonic bath (InterSonic, Olsztyn, Poland) and then they were centrifuged at 13,000× *g* for 15 min (type 5810R; Eppendorf AG centrifuge, Hamburg, Germany). Supernatants were used as free phenolic fractions.

The precipitates after free phenolic extraction were used for the extraction of bound phenolics. Briefly, 25 mL of 0.1 M NaOH was added to each precipitate and left in an ultrasonic bath (InterSonic) for 1 h. The mixtures were centrifuged at 13,000× *g* for 15 min (type 5810R; Eppendorf AG centrifuge). Then, the pH of the supernatants was adjusted to the range of pH 3–5 using 6 M HCl and centrifuged again at 13,000× *g* for 15 min (type 5810R; Eppendorf AG centrifuge). These supernatants were used as bound phenolic fractions.

3.4. Determination of Total Phenolic Content

The total content of phenolic compounds (TPC) was determined spectrophotometrically with the Folin–Ciocalteau reagent, according to Zakrzewski et al. [62], with some modifications. The color reaction was carried out by adding the Folin–Ciocalteau reagent (0.25 mL), 14% sodium carbonate (1.5 mL), and distilled water (3.15 mL) to the polyphenol extract (0.1 mL). After mixing, the solution was left for 60 min, and absorbance was measured against the reagent sample (without the phenolic extract) at a wavelength of 720 nm using a FLUOstar Omega microplate reader (BMG LABTECH, Offenburg, Germany). The TPC analysis was performed in triplicate for each sample, and the results were expressed as the µg catechin equivalent (CE) per 1 g of sample fresh weight (FW). The following equation was used for calculating the TPC: Absorbance at 720 nm = 2.9023x + 0.0521 (R^2 = 0.9986).

3.5. HPLC Determination of Phenolic Compound Content

The analysis of individual phenolic compounds was performed according to the methodology described by Zakrzewski et al. [62] with some modifications. The qualitative and quantitative analyses of the polyphenols were carried out using an ultra-high performance liquid chromatography (UHPLC) system (Nexera XR, Shimadzu, Japan) coupled with a diode area detector (DAD) and mass spectrometer (LCMS-2020, Shimadzu, Japan). The measurement parameters were as follows: 0.01% formic acid in water with 1 mM ammonium formate (eluent A) and 0.01% formic acid in 95% acetonitrile solution with 1 mM ammonium formate (eluent B); flow rate 0.37 mL/min; scanning in negative ionization; column Kinetex (2.6 µm particle size; 100 mm × 4.6 mm) (Phenomenex, Torrance, CA, USA); oven temperature was 40 °C; sample injection volume 10 µL. An analysis was conducted in the selected ion monitoring mode (SIM). Analyzed compounds were identified according to their qualitative ions, retention times, and λ_{max}, as summarized in Table S1. The quantities of polyphenols were calculated from the UHPLC-DAD-MS peak area against commercially available standards (*p*-coumaric, *m*-hydroxybenzoic, *p*-hydroxybenzoic, caffeic, ferulic and benzoic acids, epicatechin, quercetin, apigenin, and naringenin), while the glycosidic forms of quercetin, kaempferol, myricetin, and isorhamnetin were expressed as quercetin or kaempferol equivalents.

The least squares method was used to obtain the equations of the calibration curves ($y = ax + b$). A goodness of fit was given by the coefficient of determination (R^2), which is evidence of linearity for all analyzed phenolic compounds in the concentration range from 0.01 to 150 µg/mL (Table S1). The limit of detection (LOD) and limit of quantification (LOQ) values were calculated based on the signal-to-noise (S/N) ratio. The level of noise was measured from the chromatograms obtained for the standard solutions at the lowest concentration level. The LOD was calculated as being three times higher than the level of noise, and the LOQ was equal to ten times the noise level. The phenolic compounds were determined in triplicate for each sample and expressed as µg per 1 g of sample FW.

3.6. Determination of Antioxidant Properties

The antioxidant capacity (AC) of elder products and gingerbread cookies was studied using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays, according to Horszwald and Andlauer [63] with some modifications. Both antioxidant tests were performed in triplicate for each sample.

In the case of the DPPH assay, each extract (50 µL) was added to a DPPH solution (450 µL, 0.2 mmol/L in methanol), and the mixture was shaken and incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm against methanol using a FLUOstar Omega microplate reader. The AC was determined based on a curve of the % DPPH radical scavenging activity of different Trolox concentrations (within the range of 0.05 to 2 mmol/L) in methanol and expressed as µM TE (Trolox equivalent) per 1 g of sample FW. The following calibration equation was used: $\%DPPH = 242.66x + 7.9544$ ($R^2 = 0.9973$).

According to the ABTS assay, the 7 mmol/L aqueous solution of ABTS (10 mL) and the 51.4 mmol/L aqueous solution of potassium dithionite ($K_2S_2O_4$) (0.5 mL) were mixed in order to obtain an $ABTS^{\bullet+}$ radical cation solution with an absorbance value of 0.7 at $\lambda = 734$ nm. Then, 20 µL of each extract was added to 1480 µL of $ABTS^{\bullet+}$ solution. The reaction was performed at 30 °C in the dark for 6 min. After this time, the values of absorbance were recorded using a microplate reader (FLUOstar Omega). The Trolox solution (stock solution, 1 mmol/L) was used for calibration, and the AC was expressed as the µM TE per 1 g of sample FW. The following calibration equation was used: $\%ABTS = 104.03x + 1.1905$ ($R^2 = 0.9999$).

3.7. Anti-Glycation Assay

To determine the anti-glycation properties of elder products and gingerbread cookies, the bovine serum (BSA)-glucose model was used to describe the AGEs' formation. In the

first step, the obtained extracts were dried under nitrogen. After drying, samples were dissolved in this same amount of phosphate buffer (0.1 M, pH 7.4) and used directly for the anti-glycation assay as described by Przygodzka and Zieliński [54]. Fluorescence intensity (excitation wave 330 nm and emission wave 410 nm) was measured using a microplate reader (FLUOstar Omega). The percent inhibition of AGEs' formation by a sample, or the aminoguanidine (AG) solution (1 mM) used as a positive control, was calculated. The analysis was performed in triplicate for each sample.

3.8. Measurements of Physical Parameters

The gingerbread cookies were characterized by weight, size (diameter and height) and color parameters. The measurements were performed 2 h after baking for 10 cookies in each sample.

The weight of the cookies was determined with an electronic weighing balance (type 125A, Precisa Gravimetrics AG, Dietikon, Switzerland). The height and diameter of the cookies were measured using a vernier caliper.

The color was determined on the cookie's surface and its cross-section. A digital image analysis (DIA) was used for these measurements. The equipment consisted of a charge-coupled device (CCD) color camera (DXM-1200, Nikon Instruments, Melville, NY, USA), a Kaiser RB 5004 HF–High Frequency Daylight Copy Light set with 4 × 36 W fluorescent light tubes (color temperature about 5400 K) (Kaiser Fototechnik GmbH and Co., KG, Buchen, Germany), and Laboratory Universal Computer Image Analysis (LUCIA) G v. 4.8 software (Laboratory Imaging, Prague, Czech Republic). The results were expressed in the CIEL*a*b* color model, where the L* parameter represented lightness (in the range of 0–100, from the darkest black to the brightest white, respectively), the a* parameter represented green/red color (negative/positive values), and the b* parameter represented blue/yellow color (negative/positive values) [53].

3.9. Sensory Acceptance Test

Sensory analysis of the gingerbread cookies was performed using an effective acceptance test with 112 untrained panelists (54 males and 58 females) in the age range of 18–63 recruited among customers and employees of the Confectionery Factory (Fabryka Cukiernicza Kopernik S.A., Toruń, Poland). The sensory test was conducted two days after the baking trials using a 9-point hedonic scale (1 = disliked extremely, 2 = disliked very much, 3 = disliked moderately, 4 = disliked slightly, 5 = neither liked nor disliked, 6 = liked slightly, 7 = liked moderately, 8 = liked very much, and 9 = liked extremely), according to Wichchukit and O'Mahony [64]. The participants were asked to assess the following attributes: liking of color, liking of odor, liking of texture, liking of flavor, and overall acceptability. Additionally, the purchase intent was evaluated using a 5-point scale (1 = certainly would not buy, 2 = probably would not buy, 3 = might or might not buy, 4 = probably would buy, and 5 = certainly would buy). For this reason, there was a question: "How likely is it that you will buy this product if it will be available in stores?" at the end of the questionnaire card. Each untrained panelist evaluated a total of three types of gingerbread cookies in an odor-free plastic container with a lid labeled with a 3-digit code in a randomized order to avoid an order effect [65]. The panelists used warm dark tea to rinse their mouths between samples testing.

3.10. Data Analysis

All obtained results were analyzed using Statistica 13.0 PL software (StatSoft, Kraków, Poland) at a significance level of $p \leq 0.05$. They were checked for normal distribution (Shapiro–Wilk's test) and homogeneity of variances (Levene's test). The differences between samples were determined using a one-way ANOVA with a Tukey's test. Additionally, Pearson's correlation coefficients (r) were calculated to determine the relationships between the antioxidant properties and the contents of phenolic compounds.

4. Conclusions

The present study has generated important information related to the increase in phenolic compounds as well as the in vitro antioxidant properties of gingerbread cookies (GC) by introducing elderflower dry extract (EF) and elderberry juice concentrate (EB) into the formulation. Gingerbread cookies with the addition of both elder products were characterized by the highest content of flavonoids. Regardless of the enrichment applied, the bound flavonoids were dominant. In contrast, these additives did not increase the content of free phenolic acids. Furthermore, the content of bound phenolic acids was higher in the enriched GC, with a slightly greater concentration when only EF was incorporated into the chocolate coating. The higher content of flavonoids was strongly correlated with the AC. The anti-glycation properties of the elder products were confirmed for the bound phenolic fraction, which is probably a result of the higher content of flavonoids in this phenolic fraction, especially epicatechin and naringenin. In addition, the positive influence of complex elder products on the formation of the sensory properties of the gingerbread cookies was revealed. It was noted that such attributes as flavor and texture were significantly improved by these additives.

It can be concluded that it is possible to produce gingerbread that maintains good sensory qualities and exhibits the characteristics of a health-promoting functional food, due to its antioxidant and anti-glycation properties, thanks to the addition of polyphenols from elder products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24021493/s1.

Author Contributions: Conceptualization, S.P., P.T., A.S.-C. and M.T.; methodology, S.P., P.T., A.S.-C., M.T. and T.S.; software, A.S.-C. and M.T.; validation, P.T., S.P., M.T. and T.S.; formal analysis, P.T., M.T. and T.S.; investigation, S.P., P.T., A.S.-C., M.T. and T.S.; resources, S.P., P.T.; data curation, S.P., P.T., T.S., M.T. and A.S.-C.; writing—original draft preparation, S.P., P.T., MT., A.S.-C.; writing—review and editing, A.S.-C., M.T. and T.S.; visualization, S.P., P.T., A.S.-C., M.T., T.S. and supervision, A.S.-C. and M.T.; project administration, S.P. and P.T.; funding acquisition, S.P. and A.S.-C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The data presented in this study are available on request from the corresponding author.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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6. Oświadczenia współautorów

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Oświadczenie

Niniejszym oświadczam, że jako Współautor poniższych publikacji:

(D1) Szydłowska-Czerniak, A., Poliński, S., & Momot, M. (2021). Optimization of ingredients for biscuits enriched with rapeseed press cake—Changes in their antioxidant and sensory properties. Applied Sciences, 11(4), 1558.

mój wkład polegał na wspólnym opracowaniu koncepcji merytorycznej, analizie i współinterpretacji wyników oraz pisaniu wstępnej wersji manuskryptu.

(D2) Poliński, S., Kowalska, S., Topka, P., & Szydłowska-Czerniak, A. (2021). Physicochemical, antioxidant, microstructural properties and bioaccessibility of dark chocolate with plant extracts. Molecules, 26(18), 5523.

(D3) Poliński, S., Topka, P., Tańska, M., Kowalska, S., Czaplicki, S., & Szydłowska-Czerniak, A. (2022). Impact of bioactive compounds of plant leaf powders in white chocolate production: Changes in antioxidant properties during the technological processes. Antioxidants, 11(4), 752.

(D4) Poliński, S., Topka, P., Tańska, M., Kowalska, S., Czaplicki, S., & Szydłowska-Czerniak, A. (2022). Effect of Grinding Process Parameters and Storage Time on Extraction of Antioxidants from Ginger and Nutmeg. Molecules, 27(21), 7395.

mój wkład polegał na wspólnym opracowaniu koncepcji merytorycznej, przygotowaniu próbek do badań, przeprowadzeniu badań, analizie i współinterpretacji wyników oraz pisaniu wstępnej wersji manuskryptu.

(D5) Topka, P., Poliński, S., Sawicki, T., Szydłowska-Czerniak, A., & Tańska, M. (2023). Effect of Enriching Gingerbread Cookies with Elder (Sambucus nigra L.) Products on Their Phenolic Composition, Antioxidant and Anti-Glycation Properties, and Sensory Acceptance. *International Journal of Molecular Sciences*, *24*(2), 1493.

mój wkład polegał na wspólnym opracowaniu koncepcji merytorycznej, przygotowaniu próbek do badań, analizie i współinterpretacji wyników oraz pisaniu wstępnej wersji manuskryptu.

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Oświadczenie w sprawie publikacji stanowiących rozprawę doktorską
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Niniejszym oświadczam, że mój udział w każdej z poniższych publikacji polegał na ustalaniu koncepcji pracy, projektowaniu doświadczeń, obejmował dyskusję naukową wyników i redakcję tekstu publikacji.

1. Szydłowska-Czerniak, A., Poliński, S., & Momot, M. (2021). Optimization of ingredients for biscuits enriched with rapeseed press cake—Changes in their antioxidant and sensory properties. *Applied Sciences, 11*(4), 1558.
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Oświadczenie

Niniejszym oświadczam, że jako Współautor poniższych publikacji:

(D3) Poliński, S., Topka, P., Tańska, M., Kowalska, S., Czaplicki, S., & Szydłowska-Czerniak, A. (2022). Impact of bioactive compounds of plant leaf powders in white chocolate production: Changes in antioxidant properties during the technological processes. Antioxidants, 11(4), 752.

(D4) Poliński, S., Topka, P., Tańska, M., Kowalska, S., Czaplicki, S., & Szydłowska-Czerniak, A. (2022). Effect of grinding process parameters and storage time on extraction of antioxidants from ginger and nutmeg. Molecules, 27(21), 7395.

(D5) Topka, P., Poliński, S., Sawicki, T., Szydłowska-Czerniak, A., & Tańska, M. (2023). Effect of enriching gingerbread cookies with elder (*Sambucus nigra* L.) products on their phenolic composition, antioxidant and anti-glycation properties, and sensory acceptance. *International Journal of Molecular Sciences*, *24*(2), 1493.

mój wkład polegał na uczestniczeniu w prowadzeniu badań, analizie i współinterpretacji wyników, pisaniu wstępnej wersji manuskryptu, przygotowanie ostatecznej wersji manuskryptu oraz odpowiedzi na recenzje.

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mój wkład polegał na przygotowaniu próbek do badań, przeprowadzeniu badań, analizie i współinterpretacji wyników

(D5) Topka, P., Poliński, S., Sawicki, T., Szydłowska-Czerniak, A., & Tańska, M. (2023). Effect of Enriching Gingerbread Cookies with Elder (Sambucus nigra L.) Products on Their Phenolic Composition, Antioxidant and Anti-Glycation Properties, and Sensory Acceptance. *International Journal of Molecular Sciences*, *24*(2), 1493.

mój wkład polegał na wspólnym opracowaniu koncepcji merytorycznej, przygotowaniu próbek do badań, przeprowadzeniu badań, analizie i współinterpretacji wyników oraz pisaniu wstępnej wersji manuskryptu.

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mój wkład polegał na przeprowadzeniu analiz chromatograficznych oraz współinterpretacji uzyskanych wyników.

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mój wkład polegał na przeprowadzeniu analizy chromatograficznej i analizy właściwości antyglikacyjnych, oraz współinterpretacji uzyskanych wyników.

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