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Free fatty acid profiling of Greek yogurt by liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis

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ABSTRACT

Yogurt is a fermented dairy product of high nutritional value, very popular in many parts of the world. Free fatty acids (FFAs), which are formed during fermentation, may cause changes in organoleptic properties of yogurt, and thus, the determination of FFAs is of importance. We present a liquid chromatography–high resolution mass spectrometry (LC-HRMS) method, which allows the simultaneous determination of a large set of common and uncommon FFAs in yogurt samples, avoiding any derivatization step. Twenty-five common saturated and unsaturated FAs, together with 21 saturated hydroxy fatty acids (SHFAs) and 17 saturated oxo fatty acids (SOFAs), were analyzed in 26 cow and 7 sheep Greek yogurt samples. A detailed analysis of bioactive SHFAs and SOFAs was carried out in yogurt samples for the first time. Differences at the concentrations of six common FAs and five oxidized FAs between the cow and sheep samples were observed. Based on these FAs, Principal Component Analysis (PCA) allows the discrimination of cow from sheep yogurt samples.

1. Introduction

Yogurt is one of the most important fermented dairy products, highly popular and consumed in many parts of the world, particularly in Europe, North America and Middle East (Chandan et al., 2017; Fisberg & Machado, 2015). It is a product of high nutritional value, being a rich source not only of nutrients such as proteins, vitamins and minerals, but also of beneficial microbes. Yogurt intake is increasingly attracting special attention, because consumption of yogurt has been associated with human health benefits, more than other types of dairy products. Systematic reviews and *meta*-analyses of cohort studies have suggested association between dairy products intake and a decreased risk of type 2 diabetes (T2D) (Gijsbers et al., 2016). Recent review articles highlight the positive role of yogurt consumption in the management of type II diabetes, although the results from clinical trials studying the effects of yogurt and other dairy products on T2D risk factors remain controversial (O'Connor et al., 2019; Salas-Salvadó et al., 2017; Yanni et al., 2020). The impact of each class of yogurt's ingredients and the mechanisms of their action have not been fully clarified. However, it is believed that yogurt's fatty acids (FAs) play a role and they contribute to health beneficial effects.

The majority of FAs in yogurt are found in their esterified form, as triglycerides. However, lipases of lactic acid bacteria hydrolyze milk fat and they are responsible for the subsequent production of free fatty acids (FFAs) (Alm, 1982; Deeth, 1976; Guler, 2008). Although all strains of lactic starters share lipolytic activity as a basic property, the degree of fat lipolysis varies from strain to strain. During the fermentation process, generation of FFAs takes place. Elevated levels of FFAs can cause changes in organoleptic properties, including flavor changes and defects of the product for the consumer and as a consequence, the determination of the levels of FFAs is of importance.

The beneficial or detrimental effect of each one of the diverse FFAs to human health and the physiological contribution of each particular FFA, particularly in metabolic or cardiovascular diseases, remain still under investigation. However, both the chemical properties, for example, saturation, mono-unsaturation, poly-unsaturation, and length of the chain (short, medium or long) and the particular biological functions of each distinct FFA are important parameters for its overall effect on

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Table 1
Contents of common FFAs in cow and sheep yogurt samples (μg/g yogurt).

Free Fatty Acid	Cow Yogurt ($n = 26$), triplicates			α	Sheep Yogurt ($n = 7$), triplicates			α
	Minimum Value (μg/g)	Maximum Value (μg/g)	Mean Value ± SD (μg/g)		Minimum Value (μg/g)	Maximum Value (μg/g)	Mean Value ± SD (μg/g)	
C6:0	0.1	0.6	0.3 ± 0.1	***	0.2	0.5	0.4 ± 0.1	***
C8:0	0.4	1.2	0.7 ± 0.2	***	0.5	6.1	2.3 ± 1.1	**
C9:0	0.1	0.4	0.2 ± 0.1	***	0.2	0.4	0.3 ± 0.1	***
C10:0	0.6	4.9	2.0 ± 0.8	***	4.2	9.2	6.6 ± 1.2	***
C12:0	1.6	15.8	6.9 ± 4.0	***	4.8	10.5	7.6 ± 1.1	***
C14:0	2.4	31.4	12.7 ± 4.1	***	5.7	14.6	9.9 ± 3.4	***
C14:1	0.1	6.7	2.0 ± 1.1	***	0.2	0.6	0.4 ± 0.1	***
C15:0	1.0	4.0	2.1 ± 0.5	***	1.5	2.3	1.9 ± 0.2	***
C16:0	4.4	14.3	8.9 ± 2.2	***	5.9	12.0	9.3 ± 2.1	***
C16:1	0.2	8.2	3.4 ± 2.1	***	0.7	6.3	2.6 ± 1.1	**
C17:0	0.5	2.6	1.3 ± 0.5	***	0.6	3.1	1.7 ± 0.5	**
C17:1	<LOQ [a]	0.7	0.2 ± 0.1[b]	***	0.1	0.5	0.2 ± 0.1	***
C18:0	3.2	8.8	5.9 ± 1.1	***	4.1	7.0	5.9 ± 1.1	***
C18:1	9.2	80.4	42.4 ± 12.1	***	15.4	62.5	41.3 ± 10.2	***
C18:2	0.5	8.6	3.8 ± 2.4	***	0.8	5.9	3.6 ± 1.5	**
C18:3	<LOQ [c]	1.5	0.4 ± 0.3[b]	***	0.1	2.0	0.8 ± 0.5	***
C20:0	0.2	0.6	0.4 ± 0.1	***	0.2	0.4	0.3 ± 0.1	***
C20:3	0.2	2.8	1.0 ± 0.5	***	0.1	0.7	0.3 ± 0.1	**
C20:4	0.2	5.4	1.7 ± 1.1	***	0.3	6.0	2.1 ± 1.1	**
C20:5	0.2	1.1	0.5 ± 0.2	***	0.2	1.2	0.7 ± 0.2	**
C22:0	0.1	0.5	0.3 ± 0.1	***	0.1	0.6	0.3 ± 0.1	**
C22:4	<LOQ [d]	0.7	0.2 ± 0.2[b]	***	<LOQ [d]	0.8	0.2 ± 0.1[b]	**
C22:5	0.2	2.8	1.0 ± 0.5	***	0.3	10.8	2.8 ± 1.1	**
C22:6	0.1	1.1	0.4 ± 0.1	***	0.8	12.7	4.1 ± 2.1	*
C24:0	<LOQ [e]	6.9	1.1 ± 1.1[b]	**	<LOQ [d]	3.4	1.2 ± 0.5[b]	*

Content lower than LOQ in [a]1, [c]3, [d]2, [e]10 samples; [b] the mean value was determined using medium-bound approach; <LOQ: lower of limit of quantification; SD: standard deviation; α: level of significance; NS: $p > 0.05$. * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$.

human health. Ideally, an analytical method able to quantify each one of a large set of common and uncommon (trace) FAs in foods such as yogurt is highly desirable.

During the last two years our group has intensively studied the existence of uncommon oxidized saturated FAs in milk (Batsika et al., 2021; Kokotou et al., 2021; Kokotou et al., 2020a; Kokotou et al., 2020b). Adopting both suspect and targeted lipidomics approaches, we have identified in milk previously unrecognized families of saturated hydroxy fatty acids (SHFAs) and saturated oxo fatty acids (SOFAs) (Kokotou et al., 2021; Kokotou et al., 2020b). We have synthesized libraries of SHFAs and SOFAs and we have demonstrated interesting biological activities for these classes of unusual FAs. Hydroxystearic acids (HSAs) and hydroxypalmitic acids (HPAs) exhibit anti-proliferation activity, while particular regio-isomers, 7HSAs and 9HSAs, protect β-cells from cytokine-induced apoptosis (Kokotou et al., 2020a). SOFAs, in particular 6OSA and 7OSA, were found to present anti-proliferation activity suppressing the expression of both STAT3 and c-myc (Batsika et al., 2021). Collectively, these previously unrecognized classes of uncommon FAs may play a role in protection and promotion of human health.

As developed more than forty years ago, the most common method for the determination of FFAs in yogurt and other dairy products has been the gas chromatography combined with either flame ionization detection (GC-FID) or mass spectrometry detection (GC–MS) (Deeth et al., 1983). This method requires the conversion of FFAs into the corresponding methyl esters (FAMEs). Güler et al. employed GC–MS to study volatile compounds and FFAs in set types yogurts made of ewes' and goats' milk using different starter cultures (Güler & Gürsoy-Balci, 2011; Güler & Park, 2011). Most recently, FFAs have been studied in yogurts fermented with different starter and it was found that storage of yogurt resulted in an increase of short-chain FAs content and a decrease of saturated FAs and medium-chain FAs contents (Gu et al., 2021).

The aim of our work was the development of an analytical method able to detect and quantify a large set of FFAs in yogurt, avoiding tedious preparation of sample and a derivatization step. We present herein a liquid chromatography–high resolution mass spectrometry (LC-HRMS) method, allowing the determination of a big variety of common and uncommon FFAs in yogurt samples. Special attention is given to SHFAs and SOFAs, whose presence in yogurt is studied in detail for the first time.

2. Materials and methods

2.1. Chemicals and reagents

All the solvents used were of LC-MS analytical grade. Acetonitrile was purchased from Carlo Erba (Val De Reuil, France), isopropanol and methanol from Fisher Scientific (Laughborough, UK), and formic acid 98–100 % from Chem-Lab (Zedelgem, Belgium). Caproic acid (C6:0) was purchased from Alfa Aesar (>98 %, Lancashire, UK), caprylic acid (C8:0) from Sigma Aldrich (>99.5 %, Steinheim, Germany), nonanoic acid (C9:0) from Cayman Chemical Company (>98 %, Ann Arbor, MI, USA), capric acid (C10:0) from Sigma Aldrich (>99 %, Steinheim, Germany), lauric acid (C12:0) from Acros Organics (>99 %, Geel, Belgium), myristic acid (C14:0) from Sigma Aldrich (>99.5 %, Steinheim, Germany), myristoleic acid (C14:1) from Sigma Aldrich (>99 %, Steinheim, Germany), pentadecanoic acid (C15:0) from Sigma Aldrich (>99 %, Steinheim, Germany), palmitic acid (C16:0) from Fluka (analytical standard, Karlsruhe, Germany), *cis*-9-palmitoleic acid (C16:1) from Fluka (analytical standard, Karlsruhe, Germany), margaric acid (C17:0) from Sigma Aldrich (>98 %, Steinheim, Germany), 10-*Z*-heptadecenoic acid (C17:1) from Cayman Chemical Company (>98 %, Ann Arbor, MI, USA), stearic acid (C18:0) from Fluka (analytical standard, Karlsruhe, Germany), oleic acid (C18:1) from Fluka (analytical standard, Karlsruhe, Germany), linoleic acid (C18:2) from Sigma Aldrich (>99 %, Steinheim, Germany), linolenic acid (C18:3) from Sigma Aldrich (>99 %,Steinheim, Germany), arachidic acid (C20:0) from Cayman Chemical Company (>98 %, Ann Arbor, MI, USA), dihomo-γ-linolenic acid (C20:3)

Table 2
Contents of SHFAs and SOFAs in cow and sheep yogurt samples (ng/g yogurt).

Fatty Acid	Cow Yogurt ($n = 26$), triplicates				Sheep Yogurt ($n = 7$), triplicates			
	Minimum Value (ng/g)	Maximum Value (ng/g)	Mean Value ± SD (ng/g)	α	Minimum Value (ng/g)	Maximum Value (ng/g)	Mean Value ± SD (ng/g)	α
3HCA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
3HLA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
3HMA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
16HPA	7.8	22.9	14.6 ± 0.1	***	19.7	53.2	36.7 ± 0.2	***
11HPA	9.2	39.0	21.3 ± 0.2	***	8.1	73.0	31.2 ± 0.2	**
10HPA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
9HPA	5.0	47.8	25.5 ± 0.1	***	7.2	22.6	15.8 ± 0.1	***
8HPA	9.4	27.7	15.0 ± 0.1	***	n.d.	52.6	22.2 ± 0.1	**
7HPA	12.5	82.8	42.4 ± 0.3	***	15.2	48.6	32.6 ± 0.1	***
6HPA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
3HPA	24.8	85.1	52.2 ± 0.1	***	60.7	81.1	71.0 ± 0.3	***
2HPA	5.0	65.0	11.6 ± 0.2	***	5.2	17.4	12.8 ± 0.2	***
12HSA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
11HSA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
10HSA	39.1	301.8	94.1 ± 0.3	***	n.d.	466.2	127.1 ± 0.3	*
9HSA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
8HSA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
7HSA	30.0	191.0	71.9 ± 0.2	***	17.2	103.8	53.8 ± 0.1	*
6HSA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
3HSA	<LOQ[a]	32.2	8.1 ± 0.1[b]	***	<LOQ[c]	25.5	4.0 ± 0.1[b]	NS
2HSA	<LOQ [a]	23.5	4.0 ± 0.1[b]	**	n.d.	n.d.	n.d.	–
14OPA	<LOQ [c]	45.4	21.8 ± 0.1[b]	***	<LOQ [c]	57.5	13.2 ± 0.1[b]	*
10OPA	n.d.	n.d.	n.d.	–	<LOQ [c]	9.5	4.0 ± 0.1	NS
9OPA	<LOQ [c]	50.4	24.2 ± 0.1[b]	***	10.9	34.1	21.9 ± 0.2	***
8OPA	<LOQ [d]	20.3	10.0 ± 0.2[b]	***	<LOQ [c]	38.5	5.5 ± 0.1[b]	NS
7OPA	<LOQ [d]	35.3	16.7 ± 0.1[b]	***	<LOQ [c]	26.4	11.9 ± 0.1[b]	*
6OPA	<LOQ [d]	34.1	4.2 ± 0.1[b]	**	<LOQ [d]	16.8	7.7 ± 0.1[b]	**
5OPA	<LOQ [d]	201.5	28.6 ± 0.1[b]	***	<LOQ [d]	33.6	17.3 ± 0.1[b]	**
16OSA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
12OSA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
10OSA	4.9	25.2	13.1 ± 0.1	***	10.7	142.2	41.2 ± 0.2	**
9OSA	<LOQ [c]	42.1	22.5 ± 0.2[b]	***	<LOQ [d]	33.2	18.6 ± 0.1[b]	**
8OSA	<LOQ [e]	30.6	15.8 ± 0.1[b]	***	<LOQ [d]	31.7	16.0 ± 0.1[b]	*
7OSA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
6OSA	<LOQ [f]	5.7	4.0 ± 0.1	*	n.d.	n.d.	n.d.	–
5OSA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–
4OSA	<LOQ [e]	18.9	4.0 ± 0.1[b]	**	<LOQ [c]	21.5	5.1 ± 0.1[b]	*
3OSA	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	–

Content lower than LOQ in [a]18, [c]5, [d]3, [e]8, [f]24 samples; [b] the mean value was determined using medium-bound approach; <LOQ: lower of limit of quantification; n.d.: not detected; SD: standard deviation; α: level of significance; NS: $p > 0.05$. * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$.

from Cayman Chemical Company (>98 %, Ann Arbor, MI, USA), arachidonic acid (C20:4) from Sigma Aldrich (>99, Steinheim, Germany), 5,8,11,14,17-*Z*-eicosapentaenoic acid (C20:5) from Fluka (analytical standard, Karlsruhe, Germany), behenic acid (C22:0) from Sigma Aldrich (>99 %, Steinheim, Germany), *cis*-7,10,13,16-docosatetraenoic acid (C22:4) from Sigma Aldrich (>98 %, Steinheim, Germany), 7,10,13,16,19-*cis*-docosapentaenoic acid (C22:5) from Cayman Chemical Company (>98 %, Ann Arbor, MI, USA), 4,7,10,13,16,19-*cis*-docosahexaenoic acid (C22:6) from Sigma Aldrich (>98 %, Steinheim, Germany), lignoceric acid (C24:0) from Cayman Chemical Company (>98 %, Ann Arbor, MI, USA). 2-Hydroxypalmitic acid (2HPA) and 2-hydroxystearic acid (2HSA) were commercially available from Cayman Chemical (Michigan, USA), and 16-hydroxypalmitic acid (16HPA) from Sigma-Aldrich (Darmstadt, Germany). 3-Hydroxycapric acid (3HCA), 3-hydroxylauric acid (3HLA), 3-hydroxymyristic acid (3HMA), 11-hydroxypalmitic acid (11HPA), 10-hydroxypalmitic acid (10HPA), 9-hydroxypalmitic acid (9HPA), 8-hydroxypalmitic acid (8HPA), 7-hydroxypalmitic acid (7HPA), 6-hydroxypalmitic acid (6HPA), 3-hydroxypalmitic acid (3HPA), 12-hydroxystearic acid (12HSA), 11-hydroxystearic acid (11HSA), 10-hydroxystearic acid (10HSA), 9-hydroxystearic acid (9HSA), 8-hydroxystearic acid (8HSA), 7-hydroxystearic acid (7HSA), 6-hydroxystearic acid (6HSA) and 3-hydroxystearic acid (3HSA) were synthesized following the general method previously described by us (Kokotou et al., 2020a). 14-Oxopalmitic acid (14OPA), 10-oxopalmitic acid (10OPA), 9-oxopalmitic acid (9OPA), 8-oxopalmitic acid (8OPA), 7-oxopalmitic acid (7OPA), 6-oxopalmitic acid (6OPA), 5-oxopalmitic acid (5OPA), 16-oxostearic acid (16OSA), 12-oxostearic acid (12OSA), 10-oxostearic acid (10OSA), 9-oxostearic acid (9OSA), 8-oxostearic acid (8OSA), 7-oxostearic acid (7OSA), 6-oxostearic acid (6OSA), 5-oxostearic acid (5OSA), 4-oxostearic acid (4OSA) and 3-oxostearic acid (3OSA) were synthesized at the Laboratory of Organic Chemistry, National and Kapodistrian University of Athens (Batsika et al., 2021). The full list of analytes is presented in Table 1S (Supplementary Material).

2.2. Stock and working solutions

Stock solutions of the standard compounds (1000 mg/L in methanol) were prepared and stored at 4 °C. Working solutions (500 and 1000 ng/mL) were prepared daily by appropriate dilution.

2.3. Instrumentation

An ABSciex Triple TOF 4600 (ABSciex, Darmstadt, Germany) combined with a micro-LC Eksigent (Eksigent, Darmstadt, Germany) and an autosampler set at 5 °C and a thermostated column compartment were used to perform the LC-MS/MS measurements. Electrospray ionization (ESI) in negative mode was used for all the MS experiments. The data

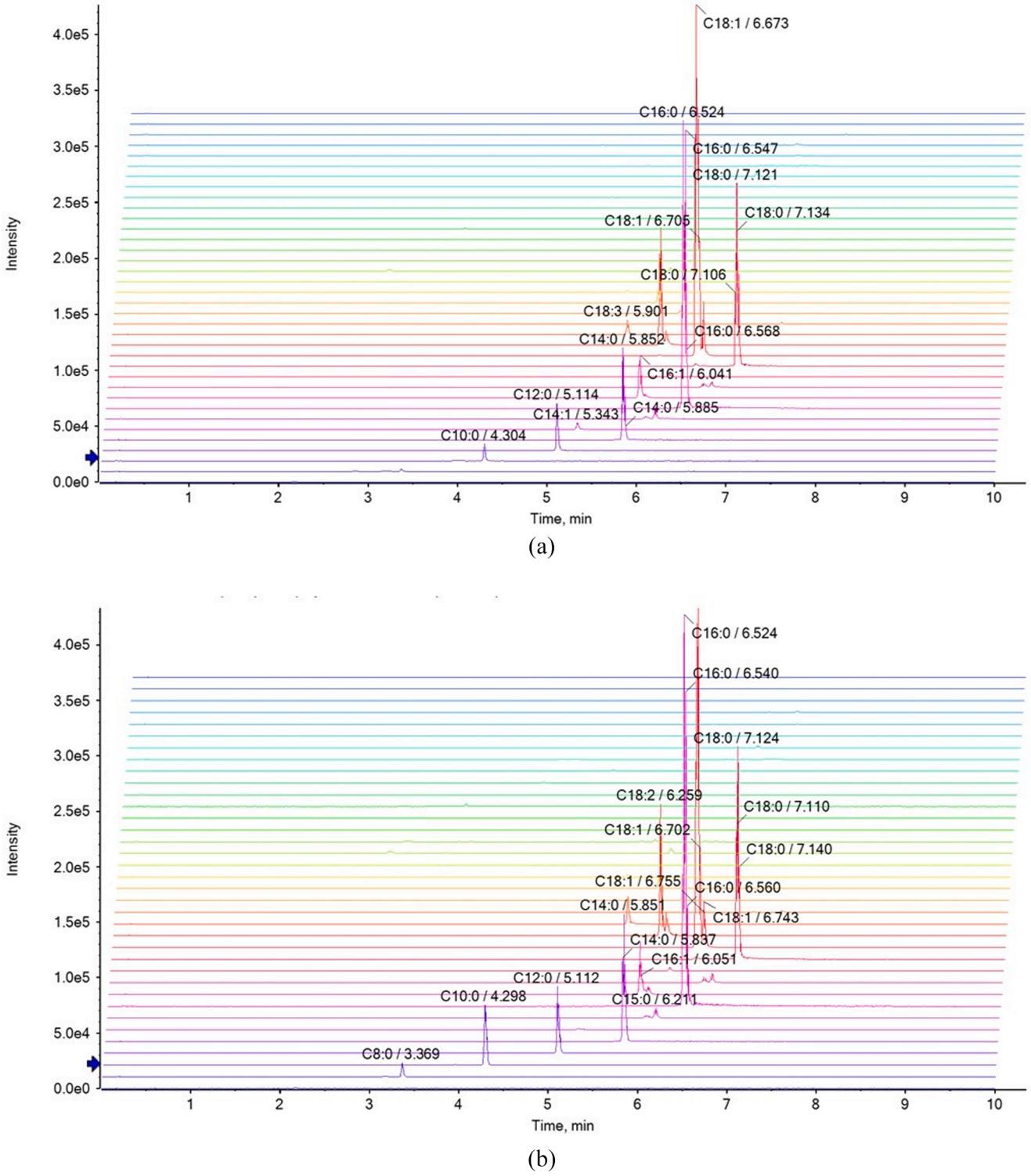


Fig. 1. Extracted ion chromatograms (EICs) of common FFAs in a cow yogurt (A) and a sheep yogurt (B) sample.

acquisition method consisted of a TOF-MS full scan *m/z* 50–850 and several information dependent acquisition (IDA)-TOF-MS/MS product ion scans using 40 V collision energy (CE) with 15 V collision energy spread (CES) used for each candidate ion in each data acquisition cycle (1091). This workflow allows quantitation (primarily using TOF-MS) and confirmation (using IDA-TOF-MS/MS) in a single run. The MS resolution working conditions were: ion energy 1 (IE1) −2.3, vertical steering (VS1) −0.65, horizontal steering (HST) 1.15 and vertical steering 2 (VS2) 0.00. A Halo C18 2.7 μm, 90 Å, 0.5 × 50 mm^2 column from Eksigent was used for the present study. The mobile phase consisted of a gradient (phase A: acetonitrile (0.01 % formic acid) / isopropanol 80/20 v/v; phase B: H_2O (0.01 % formic acid)) and the elution gradient adopted started with 5 % of phase B for 0.5 min, gradually increasing to 98 % in the next 7.5 min. These conditions were kept constant for 0.5 min, and then the initial conditions (95 % solvent B, 5 % solvent A) were restored within 0.1 min to re-equilibrate the column for 1.5 min for the next injection (flow rate: 55 μL/min).

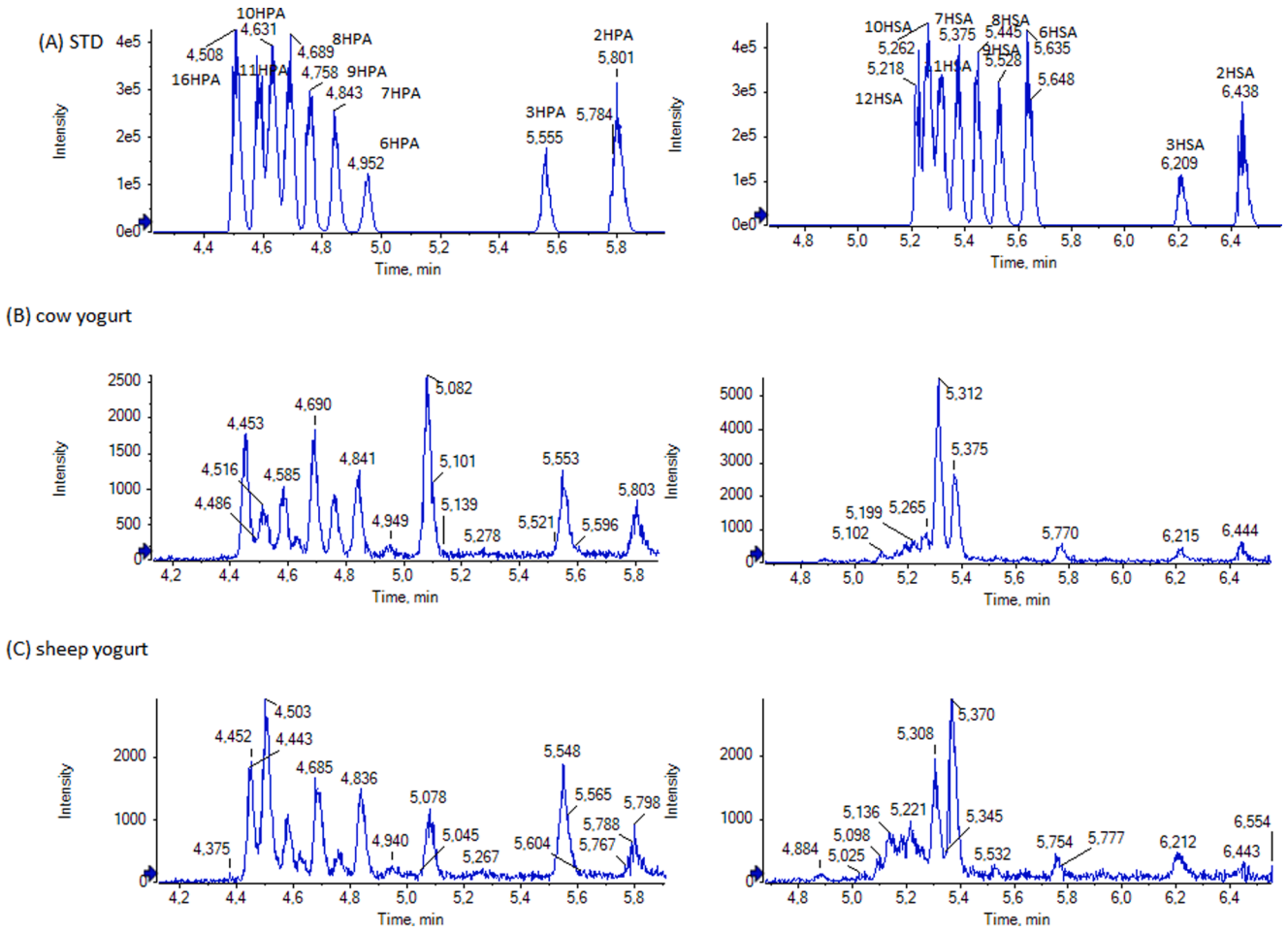


Fig. 2. Extracted ion chromatograms (EICs) of HPAs and HSAs in a standard solution (500 ng/mL) (A), and in a representative cow yogurt sample (B) and a sheep yogurt sample (C).

2.4. Data processing and quantification

The data acquisition was carried out with MultiQuant 3.0.2 and PeakView 2.1 from ABSciex (Darmstadt, Germany). EICs were obtained with the use of MultiQuant 3.0.2, which created the base peak chromatograms for the masses that achieve a 0.01 Da mass accuracy width. The relative tolerance of the retention time was set within a margin of ± 2.5 %. The integration of the peak areas was performed manually using MultiQuant 3.0.2, as previously described (Kokotou et al., 2021; Kokotou et al., 2020b), and in all cases, the same integration parameters were used.

2.5. Sample preparation

Methanol (4 mL) was added to the fluid-like yogurt sample (1 g) in a screw cap glass centrifuge tube. The mixture was vortexed for about 30 s and the suspension was centrifuged at 4000 × g for 10 min. The proteins were precipitated and 500 μL of the clear supernatant was then mixed with 500 μL of water in a vial and this mixture was used for the LC-MS/MS analysis. A similar extraction protocol of FFAs has been successfully applied in milk samples, with satisfactory recoveries for all analytes (Kokotou et al., 2021; Kokotou et al., 2020b; Kokotou et al., 2020c).

2.6. Method validation

Cow yogurt samples were spiked with a mixed standard solution of all analytes at three different concentration levels to estimate the recovery and the intra-day variations. The recovery was used for the quantification of the selected compounds in yogurt.

2.7. Yogurt samples

Thirty-three brand fresh Greek yogurts products were collected from the local market in Athens, Greece. 26 of them were cow yogurt products and 7 of them sheep yogurt samples. The yogurt samples were natural, without added sugars, colorings, fruit preserves, etc.

2.8. Statistical analysis

Level of significance was estimated using Excel t-Test: two-sample assuming unequal variances. Principal Component Analysis (PCA) was performed using XLSTAT (2018).

3. Results and discussion

3.1. Sample preparation and method validation

A simple sample preparation procedure was followed, involving the addition of methanol for protein precipitation. After centrifugation, the supernatant was used for the analysis. For the verification of the accuracy and precision, the guidelines of the EU Commission decision 202/657/EC were followed. The yogurt samples were spiked with a mixed standard solution of all analytes, at three different concentration levels with three replicates for each fortification level. Satisfactory recoveries indicate the accuracy of the proposed method. The precision was investigated by means of the relative standard deviation (%RSD)

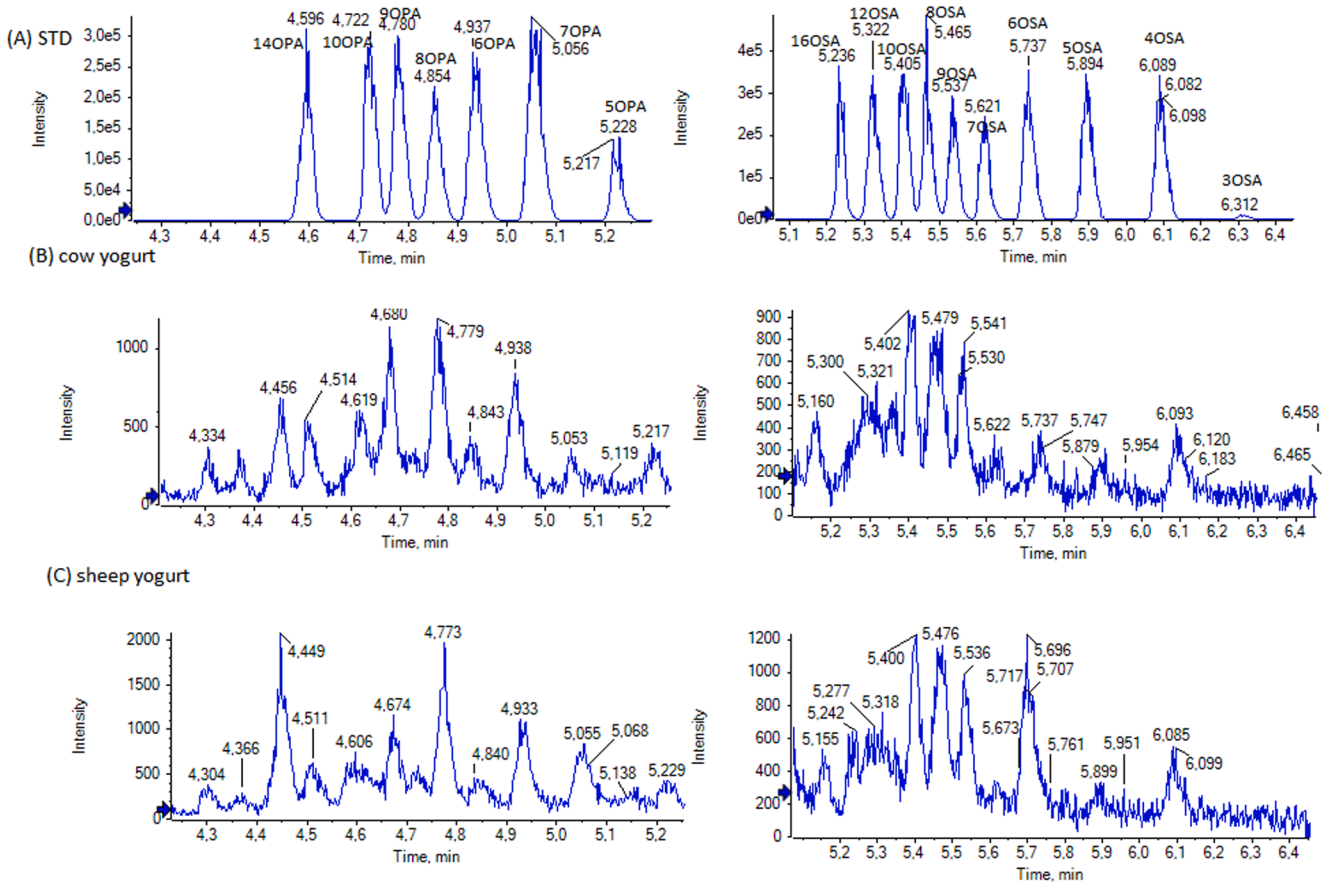


Fig. 3. Extracted ion chromatograms (EICs) of OPAs and OSAs in a standard solution (500 ng/mL) (A), and in a representative cow yogurt sample (B) and a sheep yogurt sample (C).

(Table 2S, Supplementary Material). For common FAs, the recoveries ranged from 71 to 107, 75 to 106 and 70 to 103 for the low, medium and high spike level, respectively, while the %RSD values ranged from 0.01 to 17.73 (Table 2S). For SHFAs, the recoveries ranged from 78 to 116 for the low spike level, from 79 to 96 for the medium spike level and from 89 to 100 for the high spike level (Table 2S). The %RSD values ranged from 0.29 to 10.49 (Table 2S). For SOFAs, the recoveries ranged from 74 to 109, 72 to 97 and 76 to 99 for the low, medium and high spike level, respectively, while the %RSD values ranged from 0.06 to 17.63 (Table 2S).

3.2. Analysis of samples

We have developed a rapid LC-HRMS method, which allows the simultaneous determination of a variety of FFAs (sixty-three) in yogurt samples in a 10-min run. More specifically, the common FAs C6:0, C8:0, C9:0, C10:0, C12:0, C14:0, C14:1, C15:0, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:3, C20:4, C20:5, C22:0, C22:4, C22:5, C22:6, C24:0 were analyzed. In addition, nine regio-isomers of hydroxypalmitic acid (2HPA, 3HPA, 6HPA, 7HPA, 8HPA, 9HPA, 10HPA, 11HPA, 16HPA), nine regio-isomers of hydroxystearic acid (2HSA, 3HSA, HSA, 7HSA, 8HSA, 9HSA, 10HSA, 11HSA, 12HSA), three 3-hydroxy FAs (3HCA, 3HLA and 3HMA), seven regio-isomers of oxo-palmitic acid (14OPA, 10OPA, 9OPA, 8OPA, 7OPA, 6OPA, 5OPA) and ten regio-isomers of oxostearic acid (16OSA, 12OSA, 10OSA, 9OSA, 8OSA, 7OSA, 6OSA, 5OSA, 4OSA, 3OSA) were included in the study. The exact masses $[M-H]^-$ of all analytes together with their chromatographic retention times R_t are summarized in Table 1S (Supplementary Material). Limits of detection (LOD) and quantification (LOQ), including some data of our previous reports (Kokotou et al., 2021; Kokotou et al., 2020b; Kokotou et al., 2020c), are summarized in Table 1S. The extracted ion chromatograms (EICs) of common FFAs in a cow yogurt sample (A) and a sheep yogurt sample (B) are presented in Fig. 1 and in Supplementary Material (Fig. 1S and 2S). The EICs of HPAs and HSAs in a standard solution are presented in Fig. 2A. The reference isobaric HPAs and HSAs were distinctly separable with the present chromatographic technique. EICs of HPAs and HSAs in a representative cow sample and a sheep sample are shown in Fig. 2B and 2C, respectively. Fig. 3A shows EICs of OPAs and OSAs in a standard solution, while EICs of OPAs and OSAs in a representative cow sample and a sheep sample are shown in Fig. 3B and 3C, respectively.

26 cow yogurt samples and 7 sheep yogurt samples have been analyzed in the present study. The contents of FFAs, SHFAs and SOFAs in cow and sheep yogurt samples (μg/g yogurt) are summarized in Tables 1 and Table 2.

In cow yogurt, C18:1 was found to be most abundant FFA (42.4 ± 12.1 μg/g), followed by C14:0 (12.7 ± 4.1 μg/g) (Table 1). Thirteen FFAs were estimated at concentrations between 10.0 and 1.0 μg/g (C10:0, C12:0, C14:1, C15:0, C16:0, C16:1, C17:0, C18:0, C18:2, C20:3, C20:4, C22:5, C24:0), while ten FFAs were found at concentrations lower than 1.0 μg/g (C6:0, C8:0, C9:0, C17:1, C18:3, C20:0, C20:5, C22:0, C22:4, C22:6).

C18:1 was found to be the most abudant FFA (41.3 ± 10.2 μg/g) in sheep yogurt, too (Table 1), at similar levels with those in cow yogurt. Fourteen FFAs were estimated in the range of 10.0 to 1.0 μg/g (C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C16:1, C17:0, C18:0, C18:2, C20:4, C22:5, C22:6, C24:0), while ten FFAs at lower concentrations than 1.0 μg/g (C6:0, C9:0, C14:1, C17:1, C18:3, C20:0, C20:3, C20:5, C22:0, C22:4).

The difference in the contents of seven FFAs in cow and sheep yogurt

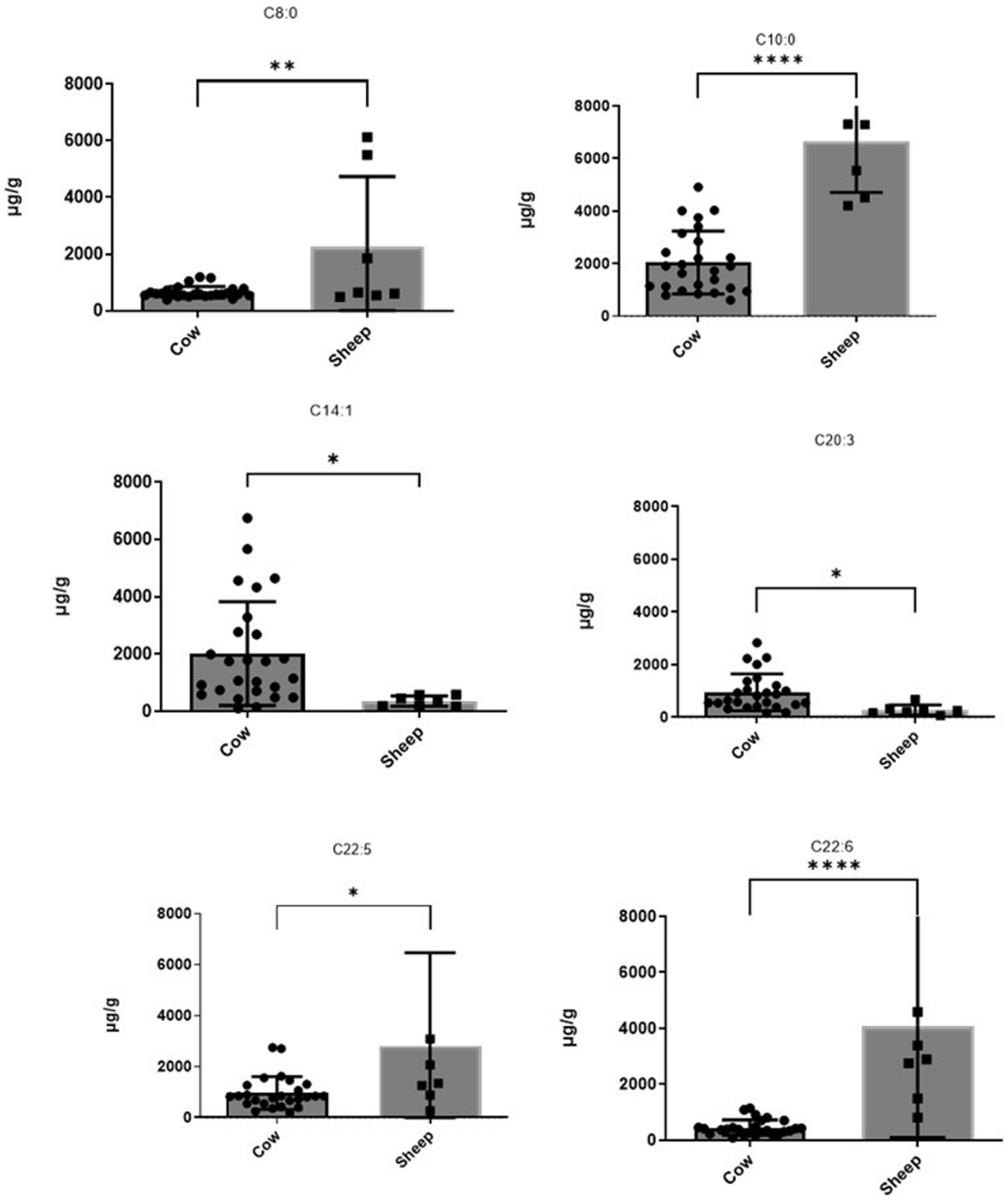


Fig. 4. Comparison of C8:0, C10:0, C14:1, C20:3, C22:5 and C22:6 concentrations (μg/g) in cow and sheep yogurt. Graphs were created using GraphPad Prism 9.2.0. One-way ANOVA statistical analysis was performed for each separate set comparing to control. ns: $p > 0.05$. * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$, **** $p < 0.0001$.

samples stood out as notable. C8:0, C10:0, C18:3, C22:5 and C22:6 were found to be at higher concentrations in sheep yogurt in comparison to cow yogurt, while C14:1 and C20:3 at higher concentrations in cow yogurt in comparison to sheep yogurt (Table 1). The alterations of the levels of these FFAs in cow and sheep yogurt samples are better demonstrated in Fig. 4. Only most recently, medium-chain FAs have attracted the interest as bioactive ingredients. C10:0 has shown to stimulate autophagy (Warren et al., 2021), while both C8:0 and C10:0 are involved in the promotion of GABA synthesis in neurons (Andersen et al., 2021).

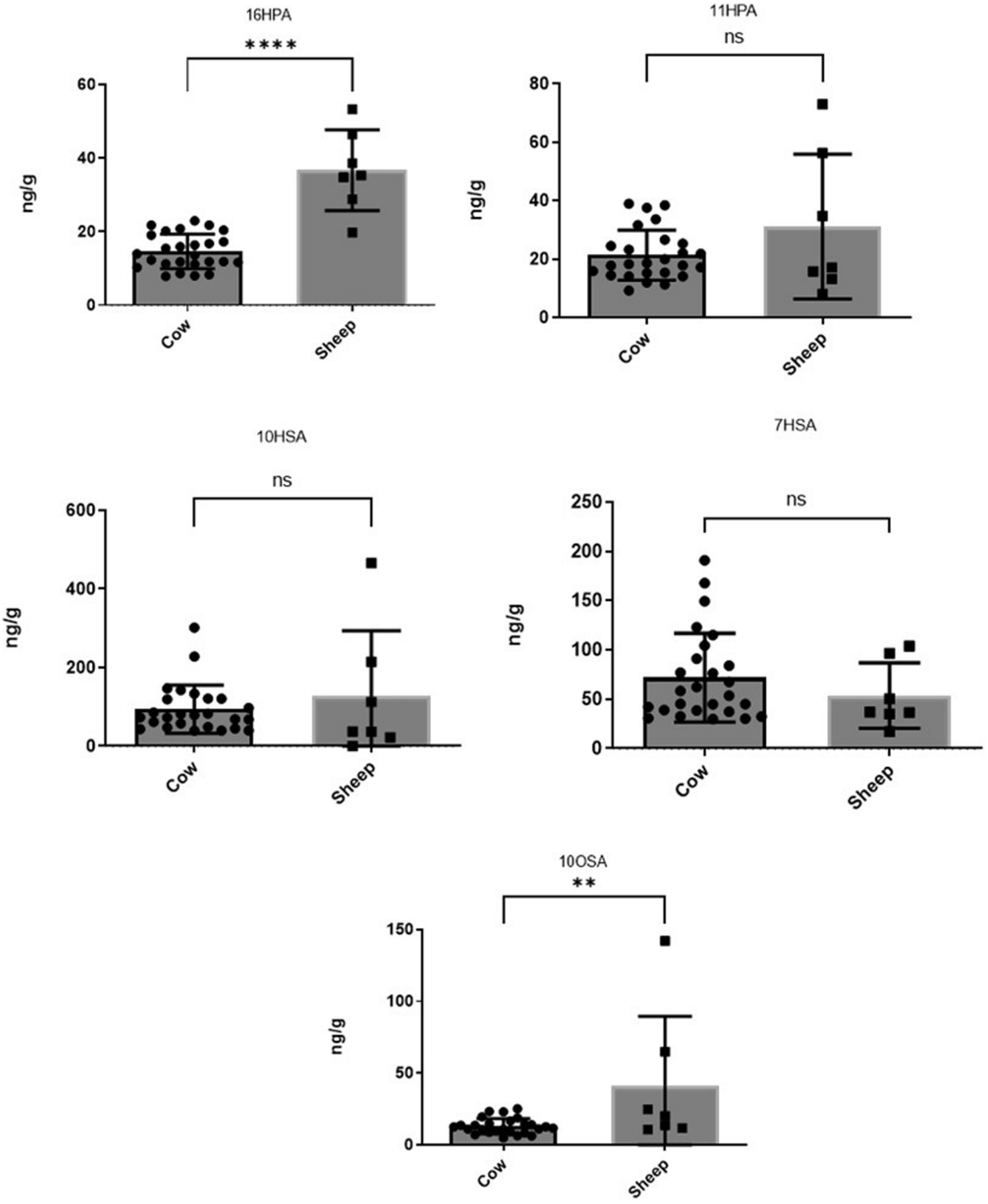
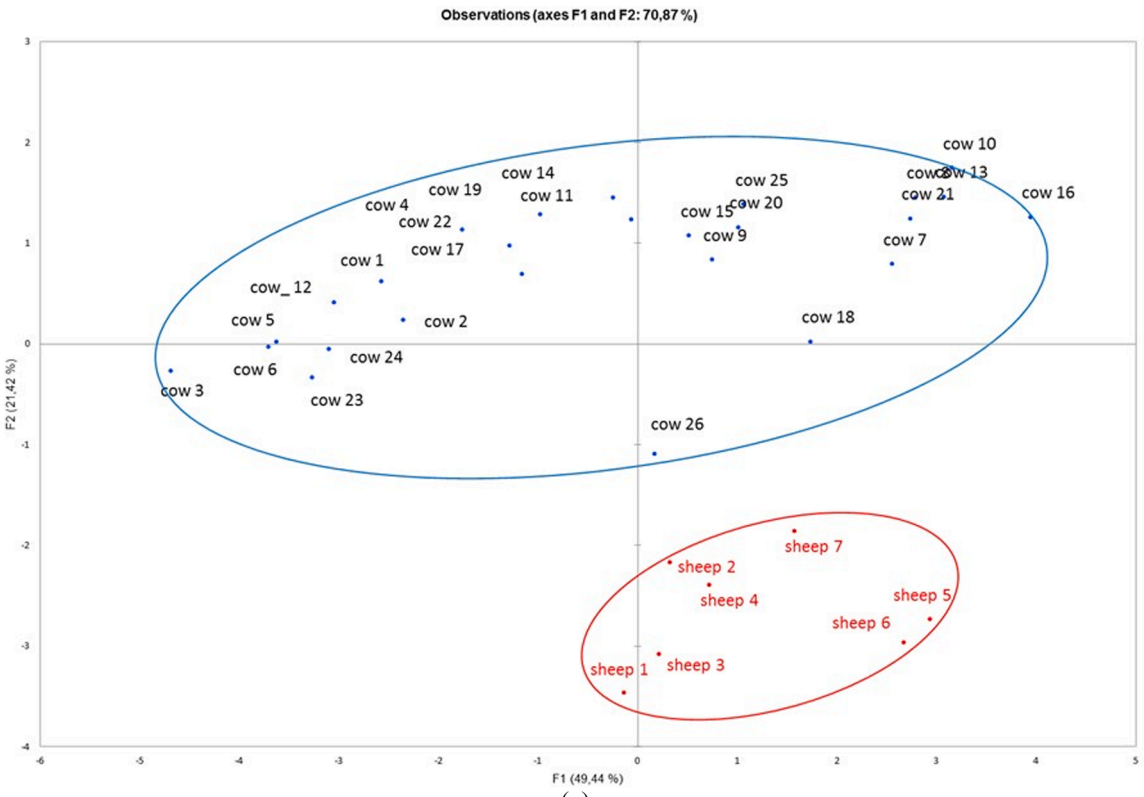


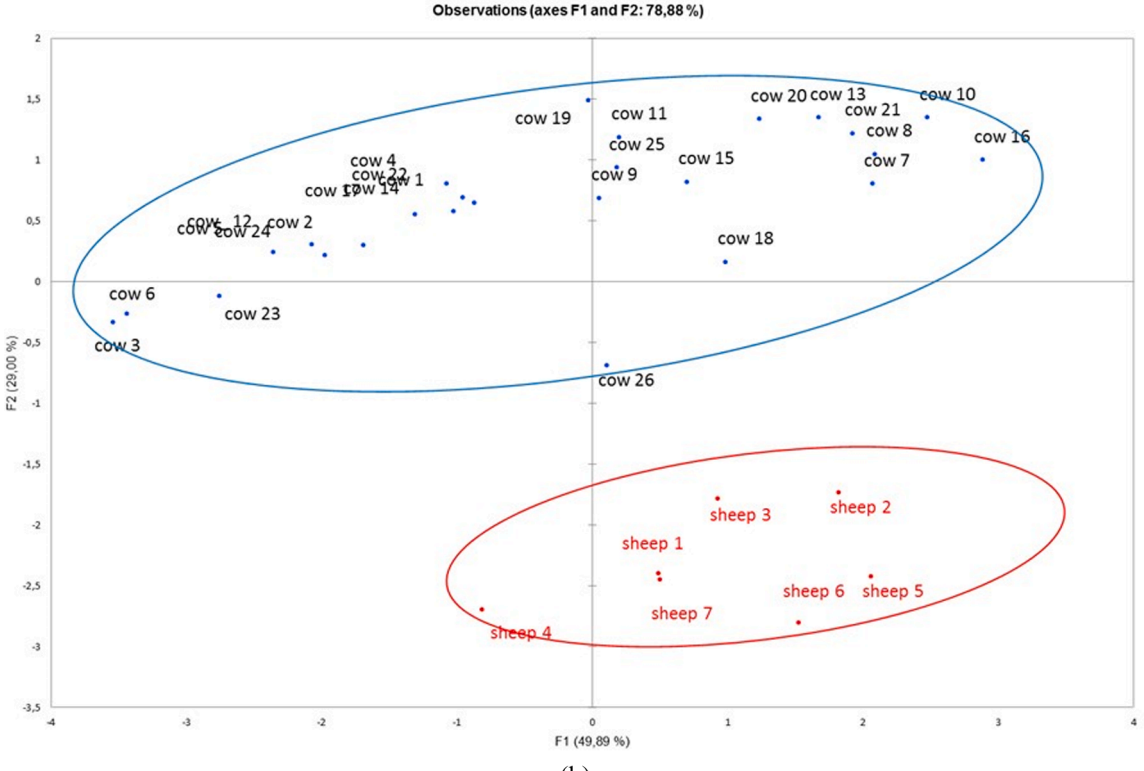
Fig. 5. Comparison of 16HPA, 11HPA, 10HSA, 7HSA and 10OSA concentrations (ng/g) in cow and sheep yogurt. Graphs were created using GraphPad Prism 9.2.0. One-way ANOVA statistical analysis was performed for each separate set comparing to control. ns: $p > 0.05$. * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$, **** $p < 0.0001$.

A comparison of the levels of each particular FFA estimated in the present work with those reported in previous studies (Gu et al., 2021; Güler & Gürsoy-Balci, 2011; Güler & Park, 2011) reveals differences. However, we have to take into account that the composition of milk or yogurt FAs depends on various parameters, including milk origin (cow, sheep, goat etc), animal feeding, and fermentation and storage conditions. It has been shown that dietary supplementation of cows with dried olive pomace modifies FAs composition in milk (Castellani et al., 2017). It has been also demonstrated that dietary grape pomace supplementation in lactating dairy cows affects the quality of both milk and the derived dairy products, causing a general increase in the concentration of polyunsaturated FAs (Ianni & Martino, 2020). In particular for yogurt samples, olive leaves supplementation was shown to induce an increase in FFAs, which could be attributed to an increased lipolysis by microbial and endogenous milk enzymes (Bennato et al., 2020). Thus, a straightforward comparison of the level of each particular FFA in different yogurt samples is not easy, because a number of parameters affect it.

In cow yogurt, seven HPAs (16HPA, 11HPA, 9HPA, 8HPA, 7HPA, 3HPA and 2HPA) were detected and quantified. 3HPA was determined as the most abundant (52.2 ± 0.1 ng/g), followed by 7HPA (42.4 ± 0.3 ng/g) (Table 2). 4HSAs (10HSA, 7HSA, 3HSA and 2HSA) were also quantified, with 10HSA being the most abundant (94.1 ± 0.3 ng/g), followed by 7HSA (71.9 ± 0.2 ng/g). In sheep yogurt, the same seven HPAs (16HPA, 11HPA, 9HPA, 8HPA, 7HPA, 3HPA and 2HPA) were also



(a)



(b)

Fig. 6. Principal component analysis (PCA) biplot graph of FFAs from cow and sheep yogurt samples using 11 variables (A) and 6 variables (B).

quantified. The most abundant was found to be again 3HPA (71.0 ± 0.3 ng/g), followed by 16HPA (36.7 ± 0.2 ng/g). 3HSAs (10HSA, 7HSA, 3HSA) were also determined, and 10HSA was found at the highest concentration (127.1 ± 0.3 ng/g), followed by 7HSA (53.8 ± 0.1 ng/g). None of 3HCA, 3HLA, 3HMA were detected in either cow or sheep yogurt samples.

Both OPAs and OSAs were found at lower concentrations than their corresponding HPAs and HSAs in either cow or sheep yogurt (Table 2). Six and seven OPAs were found in cow and sheep yogurt, respectively, at concentrations ranging from 29 ng/g to 4 ng/g. Five and six OSAs were detected and quantified in cow and sheep yogurt, respectively. 10OSA, 9OSA and 8OSA were found as the most abundant of this family in both cases. In Fig. 5, the alterations of the levels of these SHFAs and SOFAs in cow and yogurt samples are depicted.

We have previously demonstrated that SHFAs and SOFAs are minor components of milk (Kokotou et al., 2020b; Kokotou et al., 2021). Thus, we believe that SHFAs and SOFAs found in yogurt originate from raw milk.

3.3. Principal component analysis (PCA)

Multivariate statistical analysis was used to map the distribution of yogurt samples and identify cow and sheep samples. The FFAs data were analyzed by PCA to establish any "clustering" with respect to two groups. A PCA model was constructed using 11 variables, six FAs (C8:0, C10:0, C14:1, C20:3, C22:5 and C22:6) and five oxidized saturated FAs (16HPA, 11HPA, 10HSA, 7HSA and 10OSA). As illustrated in Fig. 6A, the first two components of the model (PC1 and PC2) explained 70.87 % of the variance and the score plot of PC1 (49.44 %) versus PC2 (21.42 %) indicates a perfect discrimination of the two groups of yogurt samples. The sheep yogurt samples are located at the lower right part of the plot, while the majority of the cow yogurt samples are located at the upper part.

Reduction of the number of variables, limiting them to six FAs (C8:0, C10:0, C14:1, C20:3, C22:5 and C22:6), led to similarly nice discrimination. As illustrated in Fig. 6B, the first two components of the model (PC1 and PC2) explained 78.88 % of the variance. The biplot graph of PC1 (49.89 %) versus PC2 (29.00 %) depicts a perfect discrimination of cow and sheep groups of yogurt samples.

Metabolomics in dairy science is increasingly attracting high interest, because it allows evaluation of milk and milk product quality (Jia et al., 2022; Suh, 2022). A very recent metabolomics study has shown that fermentation temperature affects the quality of yogurt (Yang et al., 2021), while metabolic changes in yogurt during storage at a refrigerated storage condition have been most recently studied (Sharma & Ramanathan, 2021). The latter study showed that FA metabolism in yogurt altered during 14–28 days of storage (Sharma & Ramanathan, 2021). The method developed in the present work extends our knowledge on FFA profiling of yogurt and may find various applications in studying the alterations of FFAs levels during fermentation or varying storage conditions. In addition, the differences in the composition of particular FAs found in this study might help in adulteration studies (Teixeira et al., 2021).

4. Conclusion

In the present work, we describe a LC-HRMS method, which allows the simultaneous determination of a large variety of common and uncommon FFAs in yogurt samples. The method employs a simple sample preparation and does not require any derivatization step. Twenty-five saturated and unsaturated FAs, together with twenty-one SHFAs and seventeen SOFAs, were analyzed in samples of Greek cow and sheep yogurt, extending our knowledge on FFAs in yogurt. For the first time, the levels of SHFAs and SOFAs were studied in detail in yogurt. 10HSA, 7HSA and 16HPA were found at concentrations higher than 50 ng/g. 9OPA, 5OPA, 10OSA, 9OSA and 8OSA were the most abundant SOFAs, found at concentrations lower than their corresponding SHFAs. Based on FFAs data, PCA analysis permits the discrimination of cow from sheep yogurt samples.

CRediT authorship contribution statement

Christiana Mantzourani: Methodology, Software. **Charikleia S. Batsika:** Methodology. **Maroula G. Kokotou:** Conceptualization, Methodology, Software, Writing – review & editing. **George Kokotos:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data supporting this study are included in the article and supplemental data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111751.

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