SUPPLEMENTARY MATERIAL

Impact of the orange juice fruit matrix on postprandial glycemia: a crossover randomized trial in healthy young men with post hoc analysis of interindividual response variability

María-Teresa García-Conesa[*a], Rocío García Villalba[a], María-Dolores Frutos Lisón[a], Carlos Javier García[a], Javier Marhuenda[b], Pilar Zafrilla[b], Juan Antonio Tudela[c], Marta Ruiz Arráez[d], Gary Williamson[e], Carrie Ruxton[f] and Francisco Tomás-Barberán[a]

[a]Research Group on Quality, Safety and Bioactivity of Plant-Derived Foods, Centro de Edafología y Biología Aplicada del Segura- Consejo Superior de Investigaciones Científicas (CEBAS-CSIC), 30100 Murcia, Spain; [b]Faculty of Pharmacy and Nutrition, Campus de los Jerónimos, Guadalupe, Universidad Católica de Murcia (UCAM), 30107 Murcia, Spain; [c]Research Group on Microbiology and Quality of Fruit and Vegetables, Centro de Edafología y Biología Aplicada del Segura- Consejo Superior de Investigaciones Científicas (CEBAS-CSIC), 30100 Murcia, Spain; [d]Fruit & Tech., carretera Madrid-Cartagena 390, 30100 Murcia, Spain; [e]School of Biological Sciences, Queen's University Belfast, University Road, Belfast, BT7 1NN, UK; [f]Nutrition Communications, East Road, Cupar KY15 4HQ, UK

Supplementary materials and methods

Analysis of the nutritional composition of the test drinks. Total ashes were quantified using a gravimetric method based on the complete incineration of the organic matter at 550 °C to constant weight according to the AOAC (Association of Official Analytical Chemists) 945.46. [1] The quantitation of protein in the 100% juice was measured using the Kjeldahl digestion method and a nitrogen conversion factor of ×6.25 as in method AOAC 920.152. [2] The total crude fat content of the samples was determined by gravimetry using the reference Soxhlet extraction method described in AOAC 920.39. [3] Analysis of the water-soluble vitamin C was achieved by reversed-phase high-pressure liquid chromatography (HPLC) analysis followed by ultraviolet (UV) detection. [4,5] Total dietary fiber was analyzed by gravimetry using the reference AOAC-985.29. [6] Sugar composition (glucose, fructose, saccharose) was analyzed using ion chromatography with amperometric detection. [7,8] Total carbohydrate was estimated by subtracting the content of fiber, protein, fat, ashes and water. All the results were expressed in g/per bottle (total ingested quantity).

Minerals. Analyses of the minerals in the three drinks were carried out at the facilities in CEBAS-CSIC (http://www.cebas.csic.es/general_spain/ionomica.html). Digestion of the 100% OJ and 50% OJ was carried out in a microwave furnace equipment assisted with an aqueous mixture of HNO_3, and H_2O_2. The microwave heating digestion program started at 20 °C and 40 bar followed by a 10 bar increase per minute during 30 minutes and reached a maximum temperature of 220 °C. This temperature was maintained for 20 minutes. The cool digested solutions, as well as

the undigested 0% OJ and glucose solution were diluted and prepared for Inductively Coupled Plasma Optical Emission spectroscopy (ICP-OES) analysis.

Analyses of macro minerals (K, Mg, P, Ca, S, Si, Na), and micro minerals (Rb, Mn, B, Sr, Fe, Zn, Bi, Al, Cu, Ni, Mo, Ti) were achieved by ICP-OES using a Thermo ICAP 6500 DUO equipment (Thermo Fisher Scientific, Waltham, MA, USA). A multi mineral standard solution containing the 31 minerals analyzed (SCP Science, Quebec, Canada) was used to prepare calibration standards in high-purity deionized water. Each mineral determination was performed at specific wavelengths ranging from 167.1 to 670.8 nm. The results are shown as mg/per bottle (ingested quantity).

Anthropometric measurements. Measurements were performed under fasting conditions to ensure that recent food intake or hydration status did not confound the results. Body mass and body composition (% body fat and % muscle mass) were measured using the TANITABCV-454 Body Composition Analyzer (Tanita, Tokyo, Japan) with an accuracy of 100 g. Height was measured using a SECA 213 portable detachable stadiometer (SECA, Germany) with an accuracy of 1 mm. Body mass index (BMI) was calculated as the quotient between body weight in kilograms and the squared height in meters (kg/m2). Additional control measures—such as standardized participant positioning, consistent electrode placement, and regulated ambient conditions—were implemented to minimize variability and enhance the reliability of the data. Waist circumference (WC) was measured with a Lufkin Executive Thinline W606PM (Lufkin, United States) non-stretchable and retractable flexible steel metric tape measure, which has a blank space at the beginning of the tape of 8 cm and an accuracy of 1 mm.

Measurements were taken at the midpoint between the lower rib margin and the iliac crest.

Questionnaires. The Mediterranean Diet (MD) adherence was determined using the validated 14-MEDAS (Mediterranean Diet Adherence scoring) instrument. The adherence to the MD was categorized as follows: weak adherence, ≤5; moderate to fair adherence, 6–9; good or very good adherence ≥10. [9] The level of physical activity (PA) was estimated with the Global Physical Activity Questionnaire (GPAQ) which scores occupational, leisure-time and transport-related activity as well as sedentary behavior. [10,11] The PA was categorized into four levels (< 600 METs, 600-3999 METS, 4000-8000 METs and > 8000 METs) based on the WHO recommendations to attain some health benefits [12] and to reduce disease risk. [13] The quality of the participants' sleeping habits was examined with the Pittsburgh Sleep Quality Index (PSQI). The global score is obtained from the sum of the scores of seven components and ranges from 0 to 21 points, where the lower end represents good quality of sleep and the upper end represents poor quality of sleep. [14-16] The chronotypes of the participants were determined using the Morning-Evening Questionnaire (MEQ) that assesses whether a person is more active and alert at certain times of the day (morning or evening chronotype). [17,18]

Assessment of the main nutrients and (poly)phenol intake of the participants during the study. The main macronutrients (carbohydrates, sugars, fiber, protein, fat), macro minerals (K, Na, Ca, Mag, P), and vitamin C present in the diet of the participants were calculated using the freely available diet calculator (https://calcdieta.ienva.org/; Research Centre for Endocrinology and Clinical

Nutrition). (Poly)phenol intake was estimated from the main (poly)phenol containing food items (g/day) selected from the daily dietary records of the participants. The content of (poly)phenols (mg/100 g) was calculated using a simple Microsoft Excel spreadsheet and data extracted from Phenol Explorer database (www.phenol-explorer.eu accessed on February 2025). [19,20] Total daily (poly)phenol intake was estimated as the sum of the polyphenol intake from each food group. Results of the nutrients and (poly)phenols intake are the average from the 5-day dietary records of all the participants.

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Supplementary Table 1 Mediterranean diet adherence of the study participants. Responses to individual foods and drinks.

	Mean ± SD
14-MEDAS score	6.3 ± 1.6
14-MEDAS category	N (%)
Low (≤ 5)	6 (24%)
Moderate (6 – 9)	19 (76%)
High (≥ 10)	None
14-MEDAS food items	% of responders within MD recommendations
Olive oil as main culinary fat (yes)	80.0%
Olive oil (≥4 tbsp/day)	0.0%
Vegetables (≥2 portions/day)	32.0%
Fruits (≥3 portions/day)	8.0%
Red meat (<1 portion/day)	96.0%
Butter or cream (<1 portion/day)	92.0%
Legumes (≥3 portions/week)	16.0%
Fish/seafood (≥3 portions/week)	12.0%
Desserts (<3 portions/week)	88.0%
Nuts (≥3 portions/week)	20.0%
White meat preference (yes)	64.0%
"*Sofrito*" 5 (≥2 meals/week)	60.0%
Sweet drinks (<1 drink/day)	72.0%
Wine (7 to 14 glasses/week)	0.0%

Abbreviations: MEDAS, Mediterranean diet adherence score; tbsp, tablespoon; *"Sofrito"*: traditional Mediterranean sauce made of tomatoes, garlic, onion, or leeks sautéed in olive oil often used for cooking meals. Results are presented as the mean ± SD for the 14 MEDAS score (n=25). For the 14-MEDAS categories results are indicated as N (%): number and percentage of individuals within a category.

Supplementary Table 2 Lifestyle habits of the study participants.

	Mean ± SD
Physical activity score (METs min week)	7027 ± 4698
Physical activity category	N (%)
< 600 METs	None
600 – 3999 METs	6 (24%)
4000 – 8000 METs	15 (60%)
> 8000 METs	4 (16%)
Sleeping quality score	4.5 ± 2.5
Chronotype score	47.6 ± 10.0
Chronotype category	N (%)
Definitely morning (70-86)	None
"Lark", moderate morning (59-69)	3 (12%)
Intermediate (42-58)	15 (60%)
"Owl", moderate evening (31-41)	6 (24%)
Definitely evening (16-30)	1 (4%)

Abbreviations: METs, Metabolic Equivalent of Task. Results are presented as the mean ± SD (n=25) for continuous variables and as N (%): Number and percentage of responses within a category.

Supplementary Table 3 Estimated daily intake of macronutrients, vitamin C, and minerals of the participants (n=25) during the study period.

Nutrients (g/day)	Mean ± SD
Total CHOs	161.3 ± 54.9
Free sugars	44.6 ± 21.3
Starch	116.7 ± 44.8
Fiber	13.8 ± 7.7
Protein	88.9 ± 22.9
Total fat	77.3 ± 21.1
Micronutrients (mg/day)	
Vitamin C	53.0 ± 22.1
Na	2854.6 ± 811.7
K	2269.4 ± 758.1
P	1157.8 ± 408.2
Ca	612.2 ± 267.9
Mg	225.5 ± 95.5

Abbreviations: CHOs: Carbohydrates. Results are presented as the mean ± SD of the n=25 participants calculated from the dietary records collected from 5 separate days during the study.

Supplementary Table 4 Total daily dietary intake of (poly)phenols and distribution across the main food sources included in the analysis (data from 5-days dietary reports and analyzed by Phenol Explorer database).

	N (% of consumers)[1]	Mean ± SD[2] (mg/day)	% Contribution[3]
Total (poly)phenols	25 (100.0%)	1232.9 ± 921.7	-
Foods most consumed (>50% participants) and most contributing to the (poly)phenols intake			
Legumes	14 (56.0%)	1080.4 ± 1014.4	53.2%
Coffee, cocoa and derivatives	16 (64.0%)	291.9 ± 225.7	14.4%
Fruits and derivatives	21 (84.0%)	166.2 ± 119.3	8.2%
Cereals and derivatives	25 (100.0%)	111.9 ± 74.9	5.5%
Processed	19 (76.0%)	89.1 ± 124.8	4.4%
Vegetables	20 (80.0%)	56.9 ± 40.9	2.8%
Tubers	23 (92.0%)	26.5 ± 24.5	1.3%
Food items less consumed (<50% participants) and less contributing to the (poly)phenols intake			
Nuts	8 (32.0%)	111.6 ± 111.9	5.5%
Juices	5 (20.0%)	45.1 ± 24.1	2.2%
Condiments	2 (8.0%)	17.9 ± 21.6	0.9%
Alcoholic drinks	3 (12.0%)	15.4 ± 3.8	0.8%
Soy and derivatives	2 (8.0%)	7.9 ± 4.8	0.4%
Oils and olives	11 (44.0%)	6.7 ± 3.9	0.3%
Herbs	5 (20.0%)	1.2 ± 1.7	0.1%
Infusions	0 (0.0%)	-	0.0%

[1] Number of consumers of that food source (percentage of participants that consume that food source in comparison with the total sample included in the analysis (n=25));

[2] Intake of polyphenols (mg/day) presented as the mean ± SD of the n=25 participants.

[3] Percentage of contribution to the total intake of (poly)phenols.

Supplementary Table 5

Postprandial insulin response metrics, anthropometric characteristics and lifestyle profiles of the two clusters identified by the significant C_{max} differences between the test drinks.

Variable	**Cluster 1** (n=12)	**Cluster 2** (n=11)	
Insulin	Mean ± SD	Mean ± SD	*p*-value
iAUC µU/mL 120 min (glucose control solution)	1030 ± 621	1369 ± 837	N.S.
iAUC µU/mL 120 min (0% OJ)	761 ± 500	1050 ± 280	0.10
iAUC µU/mL 120 min (50% OJ)	824 ± 576	1025 ± 594	N.S.
iAUC µU/mL 120 min (100% OJ)	900 ± 594	920 ± 492	N.S.
Mean C_{max} µU/mL (glucose control solution)	40.3 ± 16.6[2]	52.7 ± 24.7	N.S.
Mean C_{max} µU/mL (0% OJ)	31.0 ± 15.5	41.7 ± 11.4	0.08
Mean C_{max} µU/mL (50% OJ)	34.2 ± 18.3	33.4 ± 17.6	N.S.
Mean C_{max} µU/mL (100% OJ)	32.0 ± 16.4	31.2 ± 13.8	N.S.
Mean C_{15min} µU/mL (glucose control solution)	29.5 ± 18.0	22.8 ± 24.7	N.S.
Mean C_{15min} µU/mL (0% OJ)	26.7 ± 13.9	24.6 ± 16.6	N.S.
Mean C_{15min} µU/mL (50% OJ)	31.7 ± 19.3	16.1 ± 12.1	0.04
Mean C_{15min} µU/mL (100% OJ)	22.0 ± 15.1	15.4 ± 6.3	N.S.
Anthropometric and lifestyle	Mean ± SD	Mean ± SD	*p*-value
Age	22.7 ± 2.2	22.5 ± 3.4	N.S.
BMI (Kg/m^2)	24.7 ± 2.1	23.4 ± 2.3	N.S.
WC (cm)	83.7 ± 8.6	79.8 ± 9.1	N.S.
% Body muscle	49.7 ± 1.7	50.3 ± 2.2	N.S.
% Body fat	15.2 ± 1.2	14.8 ± 1.5	N.S.
14-MEDAS score	6.8 ± 1.6	5.8 ± 1.6	N.S.
Chronotype score	48.7 ± 9.2	47.0 ± 11.3	N.S.
Sleep quality score	4.1 ± 2.6	4.7 ± 2.4	N.S.
Physical activity (METs)	5625 ± 3057	8503 ± 5974	N.S.

Abbreviations: C_{max}, maximum (or peak) concentration; C_{15min}, concentration at time 15 min of the curve after ingestion of the test drink; iAUC, incremental area under the curve; OJ, orange juice; BMI, body mass index; WC, waist circumference; MEDAS, Mediterranean diet adherence; METs, Metabolic Equivalent of Task; N.S.: Not significant.

Results are presented as the mean ± SD of the total participants in each cluster. Clustering analysis was performed using the k-means clustering algorithm based on the C_{max} differences between responses to the four test drinks. Comparison between clusters was carried out using the Student's *t* test (level of significance *p*-value<0.05). Statistical analyses were performed in R 4.5.0 (R Core Team, 2025).

Supplementary Table 6

Discriminating compounds (VIP>1) tentatively identified as associated to the intake of 100% OJ, i.e. higher levels in the plasma samples 60 min after drinking the 100% OJ.

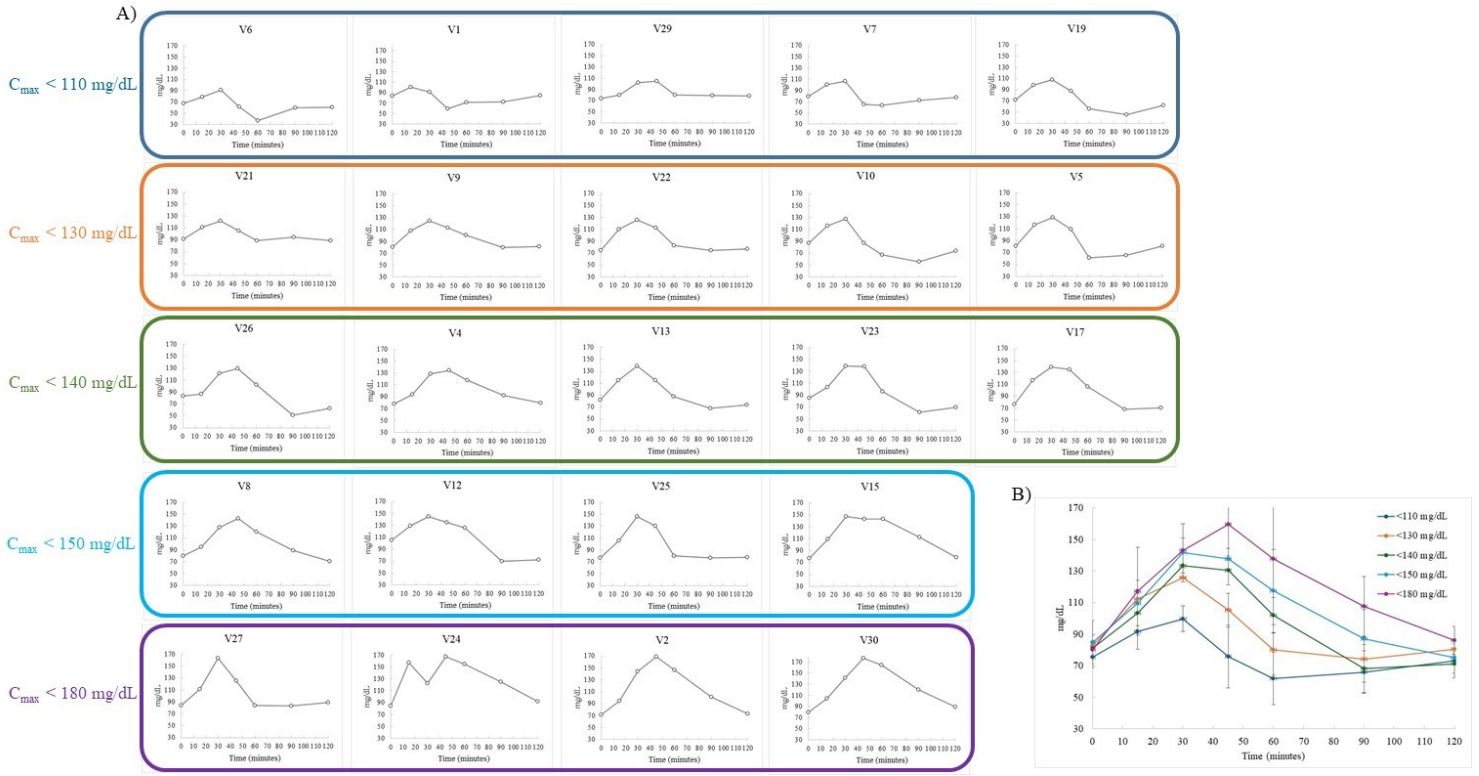
Compound	Formula	*m/z*	ppm	Rt
Terpenoids				
Limonene-8,9, diol-glucuronide (uroterpenol-glucuronide)	$C_{16}H_{26}O_8$	345.1562	2.07	10.52
Perillic acid-8,9-diol glucuronide[1]	$C_{16}H_{24}O_{10}$	375.1289	-1.85	4.6
Perillic acid-8,9-diol glucuronide	$C_{16}H_{24}O_{10}$	375.1297	-0.12	5.97
Urodiolenone glucuronide (nootkatone glucuronide)	$C_{21}H_{32}O_9$	427.1971	-0.44	8.76
Limonene-8,9-diol	$C_{12}H_{10}O$	169.0661	0.089	12.9
Perillyl-beta-D-glucuronide	$C_{16}H_{24}O_7$	327.1449	0.09	12.36
Perillic acid	$C_{10}H_{14}O_2$	165.0927	3.67	12.93
Perillic acid glucuronide	$C_{16}H_{22}O_8$	341.1244	0.46	12.94
Dihydroperillic acid	$C_{10}H1_6O_2$	167.1080	2.35	13.35
Phenolic compounds				
Sinapic acid-O-sulfate	$C_{11}H_{12}O_8S$	303.0182	0.64	6.15
Ferulic acid sulfate	$C_{10}H_{10}O_7S$	273.0076	0.6	6.58
Dihydroferulic acid glucuronide[1]	$C_{16}H_{20}O_{10}$	371.0978	-1.47	7.86
Polymethoxyflavones				
Hydroxy-trimethoxy flavone sulfate	$C_{18}H_{16}O_9S$	407.0440	-0.44	11.99
Hydroxy-pentamethoxyflavone sulfate 1	$C_{20}H_{20}O_{11}S$	467.0646	-1.3	12.46
Hydroxy-pentamethoxyflavone sulfate 2	$C_{20}H_{20}O_{11}S$	467.0653	0.66	12.69
Hydroxy-tetramethoxyflavone sulfate	$C_{19}H_{18}O_{10}S$	437.0537	-1.44	12.82
Hydroxy-pentamethoxyflavone sulfate 3	$C_{20}H_{20}O11S$	467.0651	-0.82	12.91

Abbreviations: OJ, orange juice; Rt, retention time; PLS-DA, Partial Least Squares Discriminant Analyses; VIP, Variable Importance in Projection.

[1] Metabolites with a statistically significant (p<0.05) higher level in cluster 1 than in cluster 2. Comparison between clusters was carried out using the Student's *t* test.

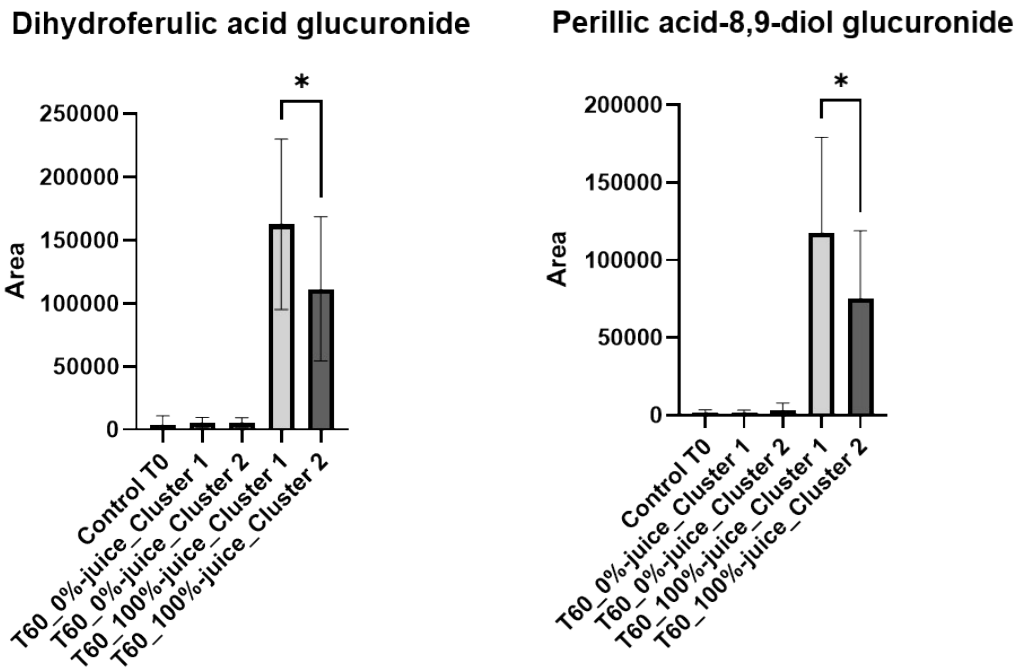
Analyses were performed with the MassHunter Qualitative Analysis software (version B.10.0, Agilent Technologies, Waldbronn, Germany) and by multivariate analysis with MetaboAnalyst 6.0 (metaboanalyst.ca, Xia Lab) applying PLS-DA and the VIP score plot of the PLS-DA.

Supplementary Figure 1 Individual glycemic responses of the volunteers to the glucose control solution. A) Glucose response per volunteer ranked from lowest to highest C_{max}. B) Average glucose responses of the 5 groups marked in different color (values are shown as mean ± SD).



Abbreviations: C_{max}, maximum (or peak) concentration; V, volunteer.

Supplementary Figure 2 Intensity difference for the discriminant metabolites and biomarkers of orange juice intake, dihydroferulic acid glucuronide and perillic acid-8,9-diol glucuronide, between the time-point 0 min and the time-point 60 min of the glucose curve for 100% OJ and 0% OJ, and for cluster 1 and cluster 2.



Abbreviations: OJ, orange juice; T0, time-point 0 min of the glucose curve; T60, time-point 60 min of the glucose curve.

Results are shown as the mean ± SD. * Metabolites with a statistically significant (p<0.05) higher level in cluster 1 than in cluster 2. Comparison between clusters was carried out using the Student's *t* test. Statistical analyses were carried out by multivariate analysis with MetaboAnalyst 6.0 (metaboanalyst.ca, Xia Lab) applying PLS-DA and the VIP score plot of the PLS-DA.