

Critical aspects in dissolution testing of nanomaterials in the oro-gastrointestinal tract: the relevance of juice composition for hazard identification and grouping

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Supporting information

Type 1 formulation		NANoREG Juice [g/L]	DIN Juice [g/L]	
Saliva	NaCl	0.29	1.67	
	KSCN	0.20		
	NaSCN		0.50	
	Na ₂ SO ₄	0.57	1.83	
	NaHCO ₃	1.69	0.50	
	KCl	0.90	1.50	
	NaH ₂ PO ₄ *H ₂ O	1.02		
	KH ₂ PO ₄		2.00	
	CaCl ₂ *2H ₂ O		0.50	
	Uric Acid	0.015	0.33	
	Urea	0.20	0.03	
	Mucin	0.025	2.50	
	α-Amylase	0.29	0.83	
	Stomach	NaCl	2.63	4.14
KCl		0.82	1.00	
NaH ₂ PO ₄ *H ₂ O		0.31		
KH ₂ PO ₄			0.38	
CaCl ₂		0.31		
NH ₄ Cl		0.31		
Glucose		0.65		
Glucuronic acid		0.02		
Glucosamine- hydrochloride		0.33		
Urea		0.085		
Pepsin		2.50	1.43	
Mucin		3.00	4.28	
BSA		1.00		
Duodenum		KCl	0.56	0.30
	CaCl ₂	0.15		
	CaCl ₂ *2H ₂ O ₂		0.50	
	MgCl ₂ *6H ₂ O	0.05	0.20	
	NaHCO ₃	3.39	1.00	
	KH ₂ PO ₄	0.08		
	Urea	0.10	0.30	
	NaCl	7.01		
	Pancreatin	9.00	9.00	
	Trypsin		0.30	
	BSA	1.00		
	Lipase	1.50		
	Bile	NaCl	5.26	
		NaHCO ₃	5.78	
KCl		0.38		
CaCl ₂		0.17		
Urea		0.25		
BSA		1.80		
Bile		30.00	9.00	

Table SI1. Molecular composition of simulant saliva, stomach or intestinal juice - Type 1 formulation (NR and DIN 1 juices). The reported values are related to the concentration that each component has in the corresponding digestive fluid: The inorganic, organic/protein and enzyme components are identified by the colors blue, orange and green respectively.

NanoREG Juice (Type 1)		[g/L]	NanoREG Juice (Type 2)		[g/L]	
Saliva	NaCl	0.29	Saliva	NaCl	0.29	
	KSCN	0.20		KSCN	0.20	
	Na2SO4	0.57		Na2SO4	0.57	
	NaHCO3	1.69		NaHCO3	1.69	
	KCl	0.90		KCl	0.90	
	NaH2PO4*H2O	1.02		NaH2PO4*H2O	1.02	
	Uric Acid	0.015				
	Urea	0.20				
	Mucin	0.025				
	α -Amylase	0.29		α -Amylase	0.29	
Stomach	NaCl	2.63	Stomach	NaCl	2.63	
	KCl	0.82		KCl	0.82	
	NaH2PO4*H2O	0.31		NaH2PO4*H2O	0.31	
	CaCl2	0.31		CaCl2	0.31	
	NH4Cl	0.31		NH4Cl	0.31	
	Glucose	0.65				
	Glucuronic acid	0.02				
	Glucosaminehydrochloride	0.33				
	Urea	0.085				
	Pepsin	2.50		Pepsin	2.50	
Duodenum	Mucin	3.00	Duodenum			
	BSA	1.00				
	KCl	0.56		Duodenum	KCl	0.56
	CaCl2	0.15			CaCl2	0.15
	MgCl2*6H2O	0.05			MgCl2*6H2O	0.05
	NaHCO3	3.39			NaHCO3	3.39
	KH2PO4	0.08			KH2PO4	0.08
	Urea	0.10				
	NaCl	7.01			NaCl	7.01
	Pancreatin	9.00			Pancreatin	9.00
BSA	1.00					
Lipase	1.50	Lipase	1.50			
Bile	NaCl	5.26	Bile	NaCl	5.26	
	NaHCO3	5.78		NaHCO3	5.78	
	KCl	0.38		KCl	0.38	
	CaCl2	0.17		CaCl2	0.17	
	Urea	0.25				
	BSA	1.80				
	Bile	30.00				

NanoREG Juice (Type 3)		[g/L]	NanoREG Juice (Type 4)		[g/L]	
Saliva	NaCl	0.29	Saliva	NaCl	0.29	
	KSCN	0.20		KSCN	0.20	
	Na2SO4	0.57		Na2SO4	0.57	
	NaHCO3	1.69		NaHCO3	1.69	
	KCl	0.90		KCl	0.90	
	NaH2PO4*H2O	1.02		NaH2PO4*H2O	1.02	
	Uric Acid	0.015				
	Urea	0.20				
	Mucin	0.025				
	Stomach	NaCl		2.63	Stomach	NaCl
KCl		0.82	KCl	0.82		
NaH2PO4*H2O		0.31	NaH2PO4*H2O	0.31		
CaCl2		0.31	CaCl2	0.31		
NH4Cl		0.31	NH4Cl	0.31		
Glucose		0.65				
Glucuronic acid		0.02				
Glucosaminehydrochloride		0.33				
Urea		0.085				
Mucin		3.00				
Duodenum	BSA	1.00	Duodenum			
	KCl	0.56		Duodenum	KCl	0.56
	CaCl2	0.15			CaCl2	0.15
	MgCl2*6H2O	0.05			MgCl2*6H2O	0.05
	NaHCO3	3.39			NaHCO3	3.39
	KH2PO4	0.08			KH2PO4	0.08
	Urea	0.10				
	NaCl	7.01			NaCl	7.01
	BSA	1.00				
	Bile	NaCl			5.26	Bile
NaHCO3		5.78	NaHCO3		5.78	
KCl		0.38	KCl	0.38		
CaCl2		0.17	CaCl2	0.17		
Urea		0.25				
BSA		1.80				
Bile	30.00					

Table S12. Molecular composition of NanoREG (NR) juice of sub-group formulations - Type 1, 2, 3 and 4. The reported values are related to the concentration that each component has in the corresponding fluid. The inorganic, organic/protein and enzyme components are identified by the colors blue, orange and green respectively.

DIN Juice (Type 1)		[g/L]	DIN Juice (Type 3)		[g/L]
Saliva	NaCl	1.67	Saliva	NaCl	1.67
	NaSCN	0.50		NaSCN	0.50
	Na ₂ SO ₄	1.83		Na ₂ SO ₄	1.83
	NaHCO ₃	0.50		NaHCO ₃	0.50
	KCl	1.50		KCl	1.50
	KH ₂ PO ₄	2.00		KH ₂ PO ₄	2.00
	CaCl ₂ *2H ₂ O	0.50		CaCl ₂ *2H ₂ O	0.50
	Uric acid	0.33		Uric acid	0.33
	Urea	0.03		Urea	0.03
	Mucin	2.50		Mucin	2.50
α-Amylase	0.83				
Stomach	NaCl	4.14	Stomach	NaCl	4.14
	KCl	1.00		KCl	1.00
	KH ₂ PO ₄	0.38		KH ₂ PO ₄	0.38
	Mucin	4.28		Mucin	4.28
	Pepsin	1.43			
Duodenum	KCl	0.30	Duodenum	KCl	0.30
	CaCl ₂ *2H ₂ O	0.50		CaCl ₂ *2H ₂ O	0.50
	MgCl ₂ *6H ₂ O	0.20		MgCl ₂ *6H ₂ O	0.20
	NaHCO ₃	1.00		NaHCO ₃	1.00
	Urea	0.30		Urea	0.30
	Pancreatin	9.00			9.00
	Trypsin	0.30			
Bile	Bile	9.00	Bile	Bile	9.00

Table S13. Molecular composition of DIN juice sub-groups (Type 1 and 3). The reported values are related to the concentration that each component has in the corresponding fluid. The inorganic, organic/protein and enzyme components are identified by the colors blue, orange and green respectively.

NF concentration (mg/L)	M ₀ (mg/L)	M _{ion (s)} (mg/L)	M _{ion (st)} (mg/L)	M _{ion (int1)} (mg/L)	M _{ion (int2)} (mg/L)	t _{1/2} of int1 (hours)
25	19.095±0.98	0.76±0.16	0.81±0.04	0.45±0.04	0.53±0.02	0.09±0.03
50	39.00±0.64	0.77±0.10	1.78±0.81	0.82±0.15	0.91±0.12	0.56±0.28
100	67.93±1.85	0.87±0.06	2.53±0.42	1.29±0.09	1.33±0.29	0.95±0.23
250	172.90±23.38	1.74±0.40	8.35±0.67	3.20±0.19	3.53±0.23	0.66±0.24
500	314.28±46,93	2.03±0.47	16.21±2.45	6.19±0,65	6.44±0.80	0.77±0.03
1000	674.19±29.98	2.48±0.68	33.97±1.47	11.61±1.33	11.74±0.82	0.88±0.37

Table S14. Concentration (mg/L) of zinc ions as measured in the saliva (s) stomach (st) and intestine (int1 and int2) compartments. M₀ is the total ion concentration of the NF at time 0, supposing a 100% of dissolution (the calculated M₀ is diluted with the respect of digestive compartment dilution factor in which NFs undergo digestion, namely 1/7; 1/19; 1/39). The last column refers to the calculation of the dissolution half-life (t_{1/2}) measured after 155 min of OGI digestion, and it is expressed in hour accordingly to the formula as reported in Methods. Data is expressed as average ± SD (n ≥3).

DIN Type 1	Particles	Dissolved analyte (ion or organic molecule)					
	Concentration of analyte in saliva, mg/L	intestine, mg/L	stomach, mg/L	saliva, mg/L	intestine, %	stomach, %	saliva, %
TiO2 NM105 (nano, rutile/anatase)	398	0.025	0.039	0.052	0.042	0.032	0.013
TiO2 E171 (non-nano, anatase)	405	0.022	0.03	0.059	0.036	0.025	0.015
BaSO4 NM220	394	1.722	0.967	2.889	28.1	7.9	7.1
SiO2 NM200	313	5.237	2.451	1.843	11.15	2.61	0.59
Zn NM110	67	1.40	12.96	n.d.	17.34	80.10	n.d.
Zn NM110 *	67	2.37	11.14	n.d.	29.31	68.82	n.d.
Zn NM110 **	67	5.50	12.70	n.d.	68.00	78.00	n.d.
Zn NM110 ***	67	3.35	12.62	n.d.	41.39	77.97	n.d.
DIN Type 3							
CeO2 NM211	58	0	0.07	0.003	0.040	0.406	-0.001
CeO2 NM212	57	0.001	0.065	0.0004	0.005	0.383	0.002
TiO2 NM105 (nano, rutile/anatase)	404	0.005	0.026	0.058	0.008	0.022	0.014
TiO2 E171 (non-nano, anatase)	402	0.007	0.013	0.053	0.012	0.011	0.013
Pigment Red 254 nano (DPP)	6711	0.022	1.57E-05	0.00015	3.5E-03	1.1E-06	2.2E-06
Pigment Red 254 non-nano (DPP)	6695	0.143	9.60E-05	0.00016	2.4E-02	7.2E-06	2.4E-06
Pigment Yellow 110 nano (Isoindolinone)	6710	0.27	0.092	0.184	0.051	0.002	0.002
Pigment Yellow 110 non-nano (Isoindolinone)	6700	0.33	0.033	0.102	0.041	0.006	0.003
Pigment Yellow 62 (ML2A)	3717	1.15	0.78	1.28	0.179	0.053	0.019
Pigment Yellow 168 (ML2A)	3760	1.34	0.73	1.55	0.206	0.049	0.023
BaSO4 NM220	400	0.772	0.249	1.317	11.700	1.900	3.000
SiO2 DQ12 (quartz)	398	0.210	0.179	0.371	0.448	0.191	0.119
SiO2 NM200	314	3.96	0.7	0.71	8.404	0.744	0.227
ZnO NM110 (nano)	68	0.033	19.96	1.38	0.365	99.645	2.081
ZnO NM110 (nano) *	68	0.083	12.6	n.d.	1.018	77.053	n.d.
ZnO NM111 (nano, coated)	73	0.03	22.3	1.95	0.255	99.000	3.000
ZnO NM113 (non-nano)	65	0.035	19.6	1.57	0.351	94.000	2.000

Table S15. Dissolution results upon cascade *in vitro* digestion assay with DIN Juice (Type 1 and 3). The results are reported in the originally measured ion concentration (mg/L) and as dissolved fraction (% dissolved). * same incubation sample quantified by 1h ultracentrifugation at 40,000g (instead of 0.02µm filtration) and dilution in 1% HNO₃ for ICPMS; ** same incubation sample quantified by 1h ultracentrifugation at 40,000g, then microwave digestion of organics and analysis by ICPOES. *** same incubation sample quantified by 1h ultracentrifugation at 40,000g, and dilution in 1% HNO₃ for ICPMS, with matrix-matched calibration in diluted OGI fluid mix.

Sample	Crystallite size in nm	Uncertainty in nm	Difference significant?	Evaluated diffraction peak angle
TiO ₂ E171 after digestion	46	5	no	25.4 °
TiO ₂ E171 pristine	56	6		25.4 °
Pigment Yellow 110 nano (Isoindolinone) after digestion	12	1	borderline	25.1 °
Pigment Yellow 110 nano (Isoindolinone) Pristine	16	2		25.1 °
Pigment Yellow 110 non-nano (Isoindolinone) after digestion	26	3	no	25.1 °
Pigment Yellow 110 non-nano (Isoindolinone) Pristine	29	3		25.1 °
Pigment Yellow 62 (ML2A) after digestion	27	3	borderline	10.6 °
Pigment Yellow 62 (ML2A) Pristine	39	4		10.6 °
Pigment Yellow 168 (ML2A) after digestion	26	3	borderline	10.5 °
Pigment Yellow 168 (ML2A) Pristine	36	4		10.5 °
Pigment Red 254 nano (DPP) after digestion	16	2	no	25.7 °
Pigment Red 254 nano (DPP) Pristine	19	2		25.7 °
Pigment Red 254 non-nano (DPP) after digestion	22	2	borderline	25.7 °
Pigment Red 254 non-nano (DPP) Pristine	32	3		25.7 °
Background OGI fluids	/			/

Table S16. Crystallite size from XRD line broadening as an approach to the potential transformation of TiO₂ E171 as negative control and of organic pigments after the cascade *in vitro* digestion assay with DIN Type 1, compared to the pristine reference peaks and to the background of a blank run without pigments. The difference is considered as borderline significant when the difference exceeds the combined uncertainty, which is estimated at 10% of the measured crystallite size. None of the results exceeds twice the combined uncertainty, which would be considered as significant change.

NR Type 1	Particles	Dissolved analyte (ion or organic molecule)					
	M ₀ (mg/L)	Intestine, mg/L	stomach, mg/L	saliva, mg/L	intestine, %	stomach, %	saliva, %
TiO ₂ NM101	24.2±1.16	0.006±0.001	0.009±0.002	0.004±0.001	1.03±0.29	0.77±0.19	0.14±0.03
TiO ₂ E171	27.51±0.95	0.006±0.002	0.008±0.0009	0.004±0.003	0.95±0.33	0.58±0.04	0.10±0.08
TiO ₂ NM103	27.13±1.87	0.004±0.0005	0.004±0.001	0.018±0.003	0.71±0.11	0.35±0.13	0.50±0.09
ZnO NM110	39.00±0.64	0.91±0.12	1.78±0.81	0.77±0.10	85.92±7.79	89.68±22.58	13.28±0.92
ZnO NM111	40.78±3.44	0.88±0.05	2.28±0.54	0.23±0.04	76.7±4.81	83.81±7.22	3.88±0.39
SiO ₂ NM200	434.31±28.84	0.99±0.36	0.51±0.29	2.23±0.28	8.96±3.33	2.18±1.18	3.09±1.10
SiO ₂ NM203	448.10±24.31	1.04±0.28	0.46±0.35	2.25±0.10	11.48±4.42	2.05±1.71	3.58±0.08
SiO ₂ , Silica_Al	588.31±45.38	0.93±0.18	0.43±0.23	0.47±0.19	5.96±1.73	1.44±0.83	0.52±0.19
SiO ₂ , Silica_Silane	599.06±20.49	0.80±0.35	0.18±0.10	0.014±0.04	5.14±2.09	0.60±0.33	0.16±0.05
SiO ₂ , Silica_Std	569.20±38.58	1.17±0.18	1.04±0.29	2.76±0.93	8.08±1.73	3.45±1.14	3.23±1.08
SiO ₂ , MCM60	417.99±14.59	7.23±0.17	2.29±0.18	33.07±1.17	67.52±1.66	10.43±0.84	55.38±1.97
SiO ₂ , MCM170	712.14±22.72	10.24±1.10	3.08±0.29	12.15±1.39	56.11±6.07	8.21±0.79	11.94±1.36
Ag, NM300K	41.04±7.37	0.02±0.02	0.45±0.18	0.10±0.03	2.01±1.48	21.26±8.35	1.71±0.37

Table S17. Dissolution results upon cascade in vitro digestion assay with NR Juice (Type 1). The results are reported in the originally measured ion concentration (mg/L) as measured by UF/ICP-OES and as dissolved fraction (% dissolved). NF concentration is 50 mg/L for TiO₂, ZnO and Ag NFs and 1000 mg/L for SiO₂ NFs.

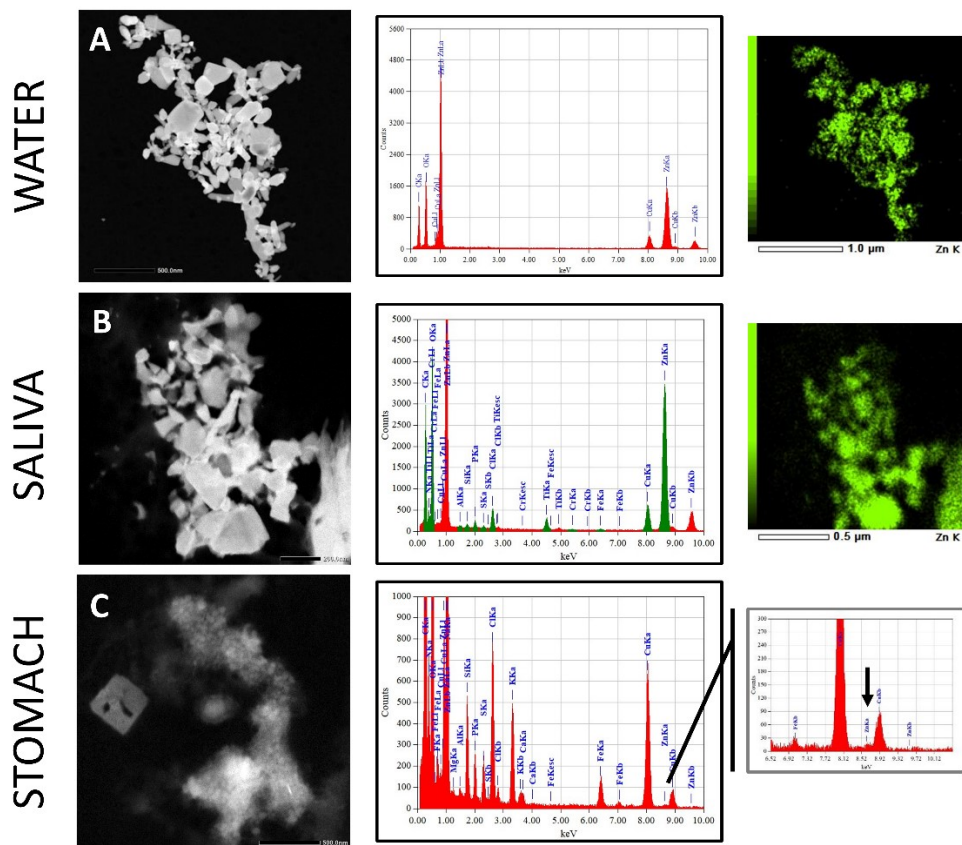


Figure S11. Biotransformation of NM110 (ZnO) upon the cascade *in vitro* digestion assay by using Type 1 Formulation (NR juices) followed by STEM-EDX analysis. Annular Dark Field STEM images of NM110 before digestion in water (50mg/L (A) and after digestion in saliva (B) and in stomach (C). Images are taken as soon as possible the addition of any juices. EDX spectra on central column show the elemental composition of the corresponding areas while EDX maps on the right column show the spatial distribution of Zn.

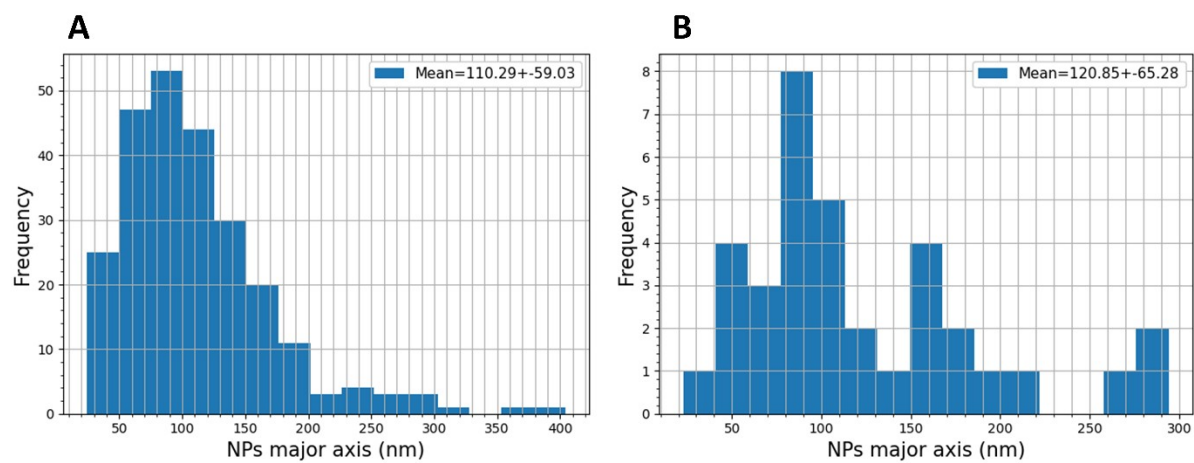


Figure S12. Size distribution by TEM of NM110 (ZnO) in water (A) and in saliva after 5 minutes of incubation (B).

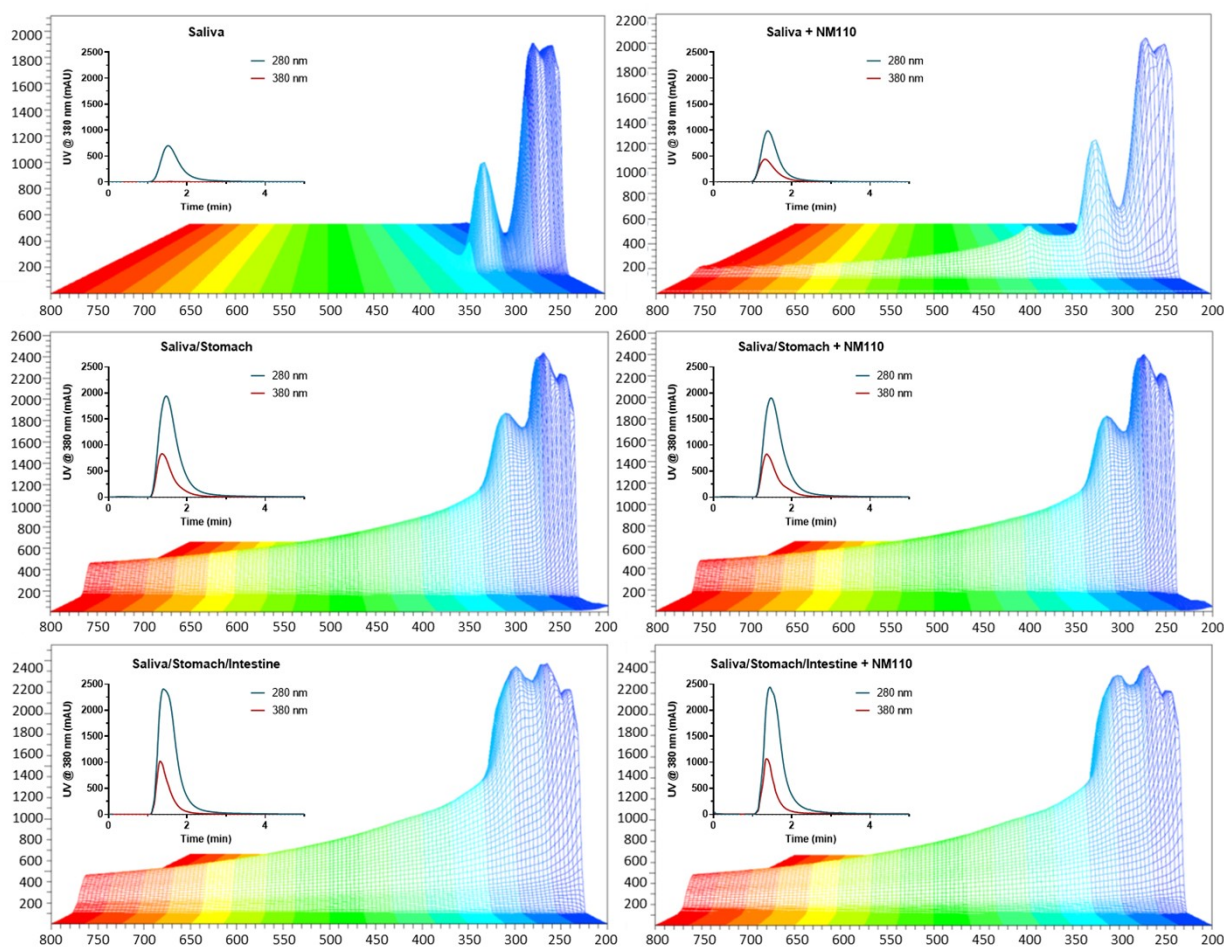


Figure S13. FIA UV signals and 3D absorption spectra acquired in flow during HF5 characterization of ZnO NPs along the cascade in vitro digestion assay (NM110 500mg/L). For 3D spectra, horizontal axis represents wavelength (nm), depth axis represents time (min), height axis represents absorption intensity (mAU). (A) Saliva compartment spectra without NM110. (B) Spectrum of saliva containing NM110. (C) Spectrum of the stomach compartment alone. (D) Spectrum of the stomach compartment containing NM110 from cascade digestion. (E) Spectrum of the intestine compartment alone. (F) Spectrum of the intestine compartment containing NM110 from cascade digestion (int2).

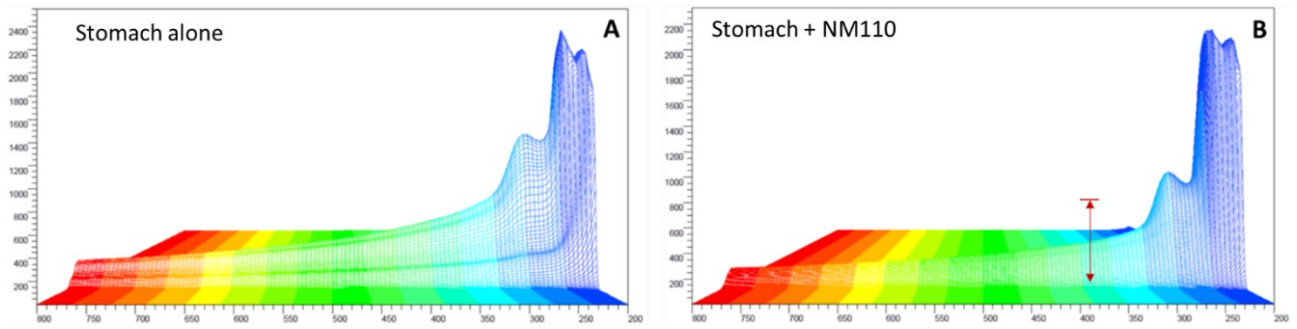
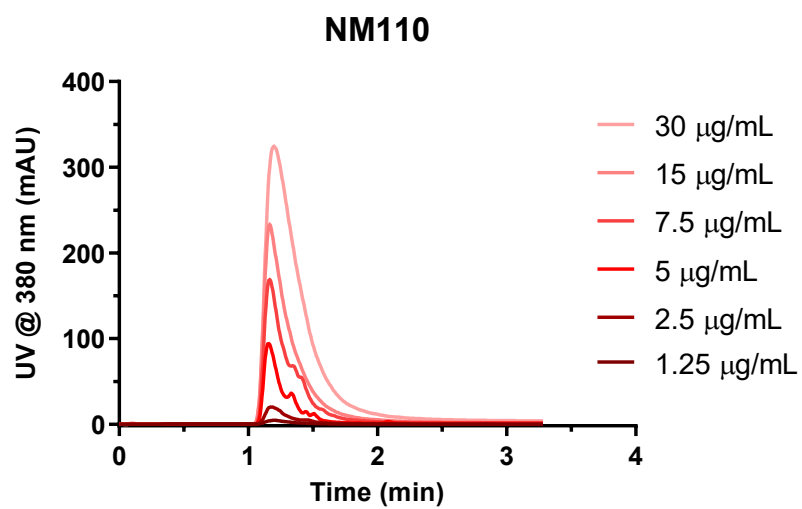


Figure S14. 3D absorption spectra acquired in flow during HF5 characterization of ZnO NFs spiked in the stomach compartment (final concentration of NM110 of 250 mg/L). For 3D spectra, horizontal axis represents wavelength (nm), depth axis represents time (min), height axis represents absorption intensity (mAU). (A) Stomach compartment spectra alone. (B) Stomach spectra after addition of NM110 (ratio 1:1) (time 0). Red arrow: expected signal due to NM110 NMs in case of no dissolution.



FigureS15. FIA UV absorption profile of increasing amounts of NM110 in water.

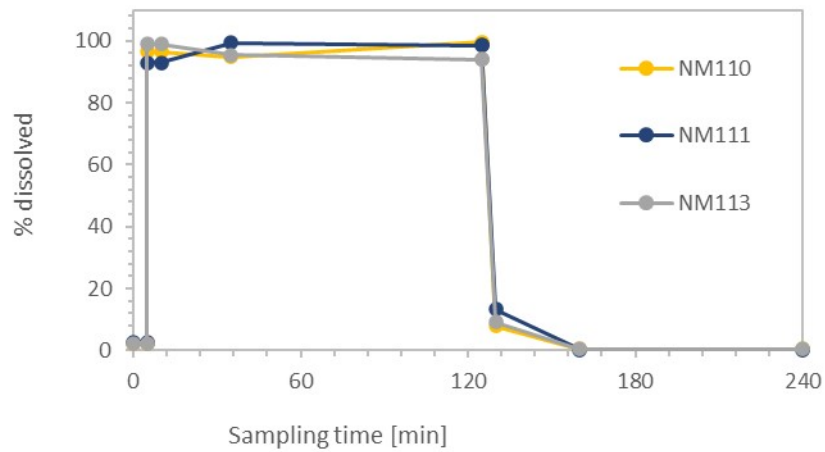


Figure S16. Dissolution kinetics of ZnO NFs (NM110 and NM111) and non-nanoform (NM113) upon the application of the cascade *in vitro* digestion assay using Type 3 formulation (DIN juices). 67mg/L in saliva is the tested concentration. Figure 4 of the main text shows the dissolved percentage at the respective final time points of the three compartments. Saliva phase 0 to 5 min., stomach phase 5 to 125 min., intestine phase 125 to 240 min.

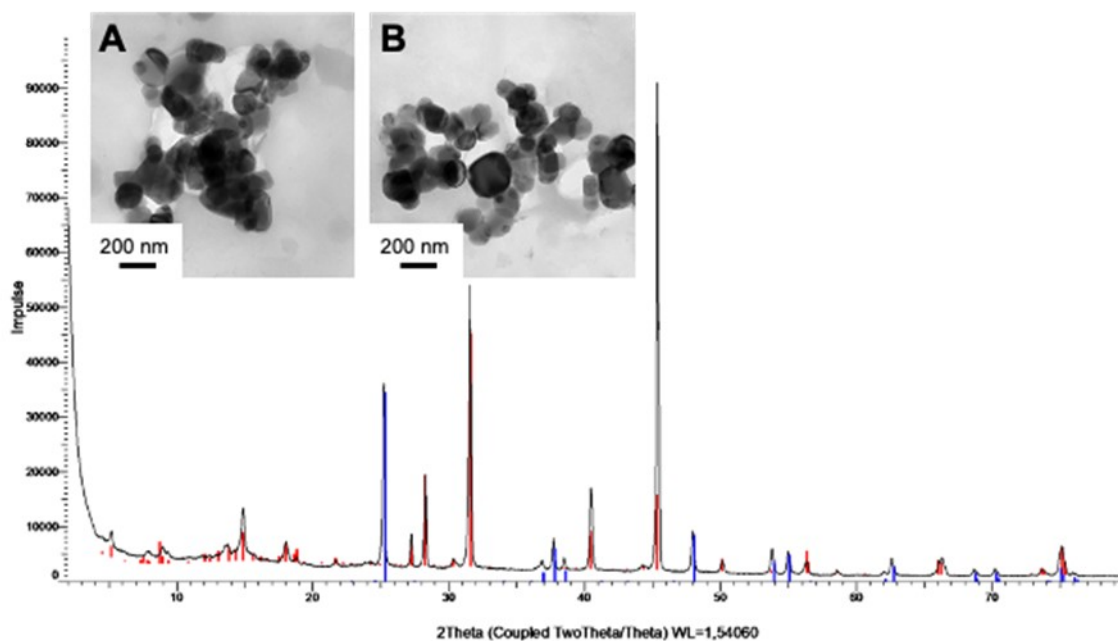
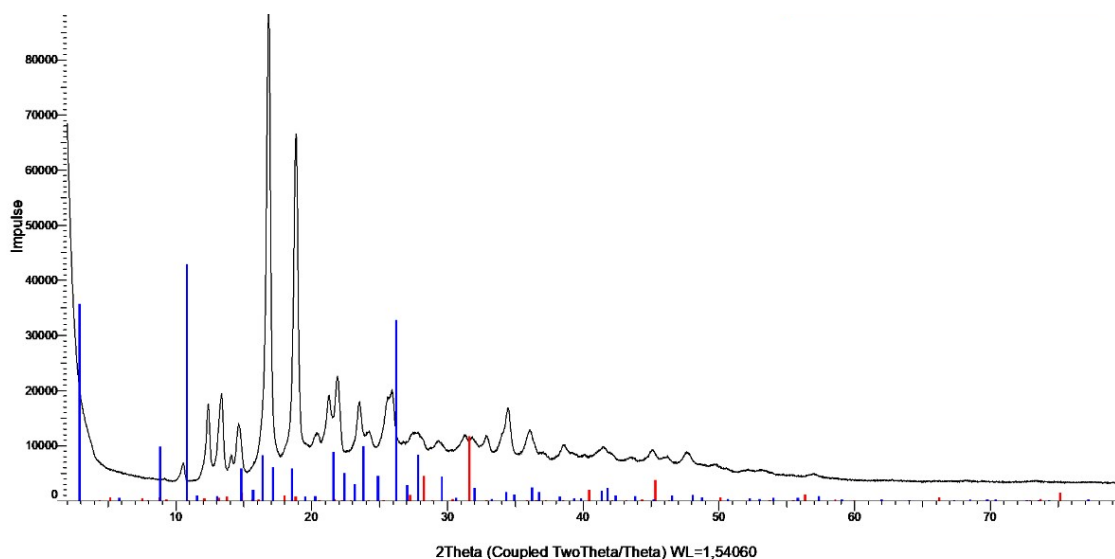


Figure S17: TiO₂ E171 dissolution upon the application of the cascade *in vitro* digestion assay analyzed by XRD in DIN juices. TiO₂ E171 pristine particles (blue), TiO₂ E171 after dissolution in cascaded OGI fluids (black) and the juice background (red). TEM images of E171 after incubation in (A) DIN Type 1 juices and (B) DIN Type 3 juices.

a)



b)

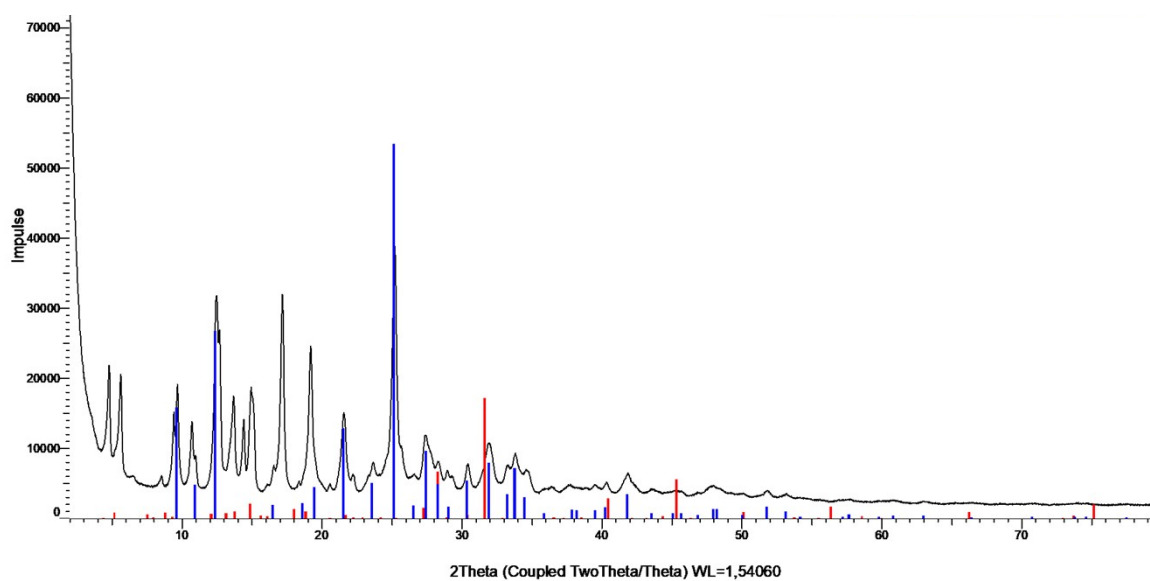


Figure S18. XRD analysis of the potential transformation of organic pigments. Diffractogram (black line) after the cascaded *in vitro* digestion assay (DIN Type 1), compared to the pristine reference peaks (blue bars) and to the background of a blank run without pigments (red bars). a) Pigment Yellow 168 (ML2A), b) Pigment Yellow 110 non-nano (Isoindolinone). Apart from the characteristic diffraction angles, also the line broadening is evaluated to obtain the crystallite size (Table S16), and both parameters identify the changes to the diffractogram of Pigment Yellow 168 (ML2A) as borderline significant, and the changes to the diffractogram of Pigment Yellow 110 non-nano (Isoindolinone) as not significant.

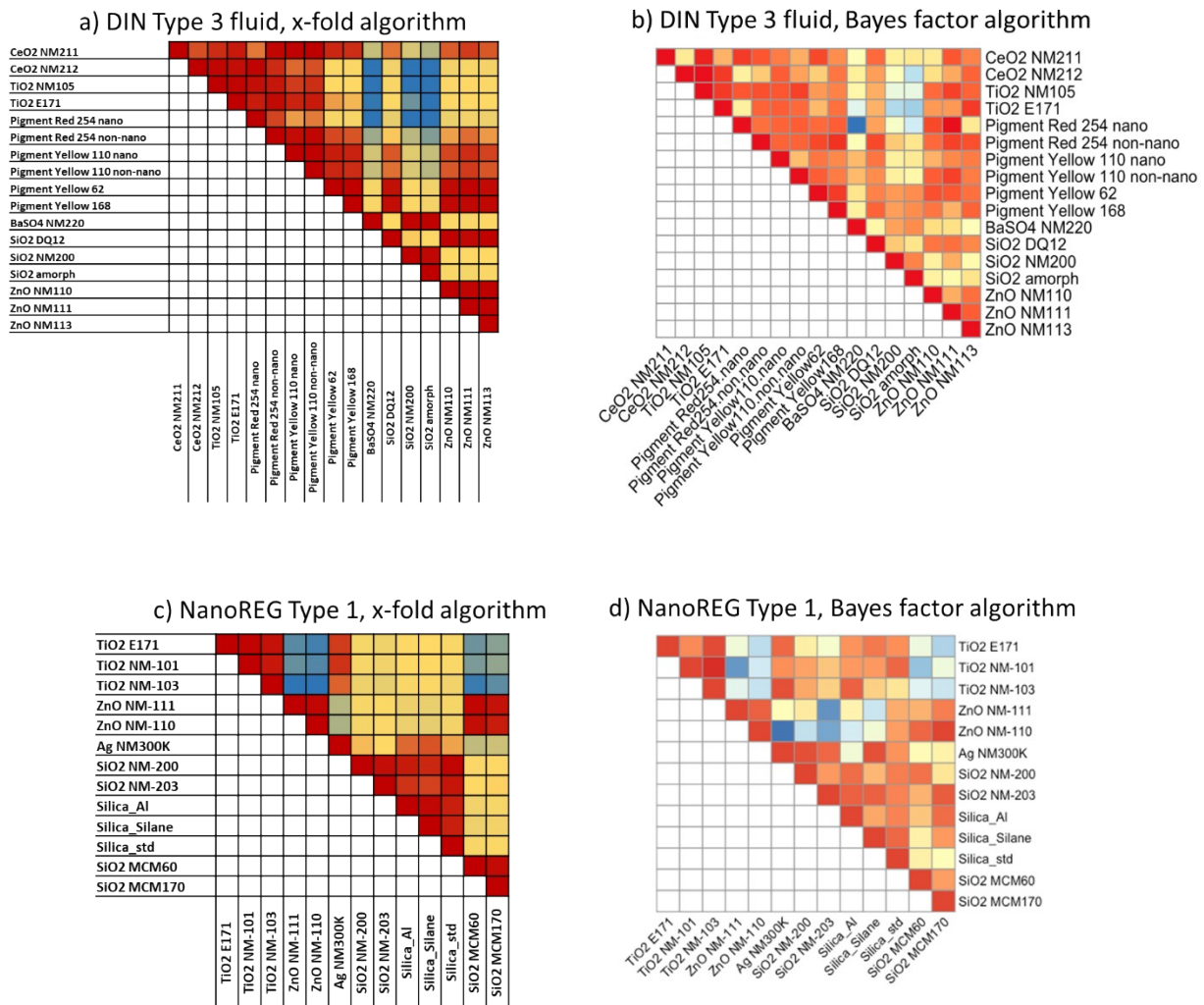


Figure S19. Cascaded incubation in simulants without (a,b) or with (c,d) digestive enzymes applied as grouping criterion on different NFs. The identical data of % dissolved in the intestine phase (int2) is represented by two different similarity algorithms, namely a,c) by x-fold algorithm; b,d) by Bayes factor algorithm. See Figure 5 for analogous evaluations of the stomach phases. Color codes were adjusted to each dataset with red indicating similarity, and blue indicating difference: X-fold: Type 3 DIN: red=1, yellow=20, blue=1,000; Type 1 NR: red=1, yellow=5, blue=100. Bayes: Type 3 DIN: red=+2.5, yellow=-2, blue=-6.5; Type 1 NR: red=+2, yellow=0, blue=-3.

