Circulating Ara h 6 as a marker of peanut protein absorption in tolerant and allergic humans following ingestion of peanut-containing foods

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SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table S1: Peanut-based foods used in this study

	Ingredients	Protein	Fat	Carbohydrate
Water-continuous dessert matrix	Defatted peanut flour, starch, cocoa powder, sugar, vegetable oil, oat flour, emulsifier, flavouring	3.4%	8.7%	20.6%
Peanut butter*	Roasted peanuts (85%), vegetable oil,sugar, salt.	23.8%	51.5%	13.2%
Cookie	Defatted peanut flour, flour, biscuit fat, caster sugar, water, cocoa powder, salt, sodium bicarbonate, ammonium bicarbonate, flavouring	13.2%	20.5%	45.7%
Oil-roasted, whole peanut kernel (shelled)	N/A	30%	51%	5.6%

* To aid consumption, the 3000mg dose of peanut butter was consumed as a sandwich, prepared using 1 slice of commercially-sourced brown bread

Supplementary Table S2: Recipe used for water-continuous dessert base. The matrix was prepared as described previously.[1,2]

Ingredient	**Ingredient in dessert base % (w/w)**		**Ingredient Source**
	Dessert alone	**Peanut containing dessert**	
Peanut flour	0	13.3	Byrd Mill (Byrd Mill Co., Ashland, VA23005, USA).
Starch	19.53	13.47	National Starch and Chemical, Manchester, UK (sourced direct).
Toasted, ground oatmeal	6.67	3.34	The Oatmeal of Altford, Laurencekirk, UK
Cocoa	18.33	18.33	Birchalls Foodservice, Hapton, UK.
Sugar	30.74	26.6	Birchalls Foodservice, Hapton, UK.
Oil	23.33	23.33	Birchalls Foodservice, Hapton, UK.
Tween60	0.6	0.6	Seppic AS, Paris, France, obtainedfrom Macphie of Glenbervie Ltd,Stonehaven, UK.
Orange oil	0.8	1 .00	Lionel Hitchin (Essential Oils) Ltd(sourced direct)
Total	100	100	

Supplementary Table S3: Recipe used for cookies containing peanut. The palm oil, orange oil and sugar were mixed to a smooth paste. Separately, sodium bicarbonate, ammonium bicarbonate and salt were mixed with water and added to the fat and sugar mix. Peanut flour was blended with ~10% by weight of the biscuit flour before adding to the remainder of the flour, cocoa powder added and then incorporated slowly to form a dough which was rolled out to a thickness of 1.5mm and cut into 1" x 1" square biscuits. Biscuits were placed on greaseproof paper and baked in Lincat oven for 9mins at 150°C and 10% humidity.

Ingredients	**% (w/w)**	**Source**
Peanut Flour	20.41	Byrd Mill (Byrd Mill Co., Ashland, VA23005, USA).
Wheat flour	29.59	Heygates Flour Mills (DM8-UT Biscuit Flour)
Palm Oil	16.857	AAK(Palm Oil CAT-0050)
Caster sugar	15.443	British Sugar
Salt	0.55	Univar(Cas No:7647-14-5)
Sodium Bicarbonate	0.183	TATA Chemicals Europe Ltd.
Ammonium bicarbonate	0.105	Univar
Orange oil	0.09	Lionel Hitchin (Essential Oils) Ltd(sourced direct)
Cocoa powder	3.177	Birchalls Foodservice, Hapton, UK.
Water	13.595	
Total	100	

Supplementary Table S4: Characteristics of the immunometric assay for Ara h 6

Ara h 6	
LOD (Mean of all blank values+3 x Standard deviation) (ng/ml)	0.006
LOQ (Mean of all blank values+10 x Standard deviation) (ng/ml)	0.008
Working range (ng/ml)*	0.008-1
WPPE	
LOD (Mean of all blank values+3 x Standard deviation) (ng/ml)	0.1
LOQ (Mean of all blank values+10 x Standard deviation) (ng/ml)	0.16
Working range (ng/ml)*	0.16-25
Cross-reactivity (%)**	
Ara h 6 purified from raw peanut***	105
Proteolytic product of Ara h 6 isoforms***	90-95
Ara h 2 isoforms from raw /roasted peanut***	<0.05
Ara h 1 from raw peanut***	<0.05
Ara h 3 from roasted peanut***	0.1
Protein extracts from Hazelnut, Sunflower seed	<0.05

*The working range was defined as the range with an intra-assay CV <10%.

**The cross-reactivity coefficients were determined as the ratio of the Ara h 6 purified from roasted peanut concentration divided by cross-reactant concentration providing the same signal in the two-site immunometric assay

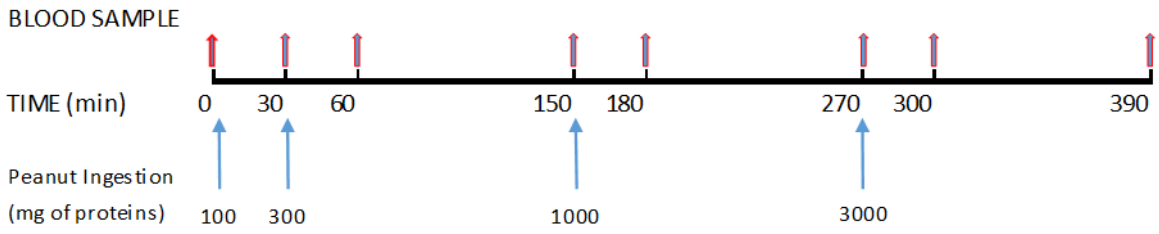
***Peanut proteins and processed Ara h 6 were purified as previously described. [3–6]

Supplementary Table S5: Concentrations of anti-Ara h 6 antibodies in blood samples from either purchased plasma or healthy volunteers sera.

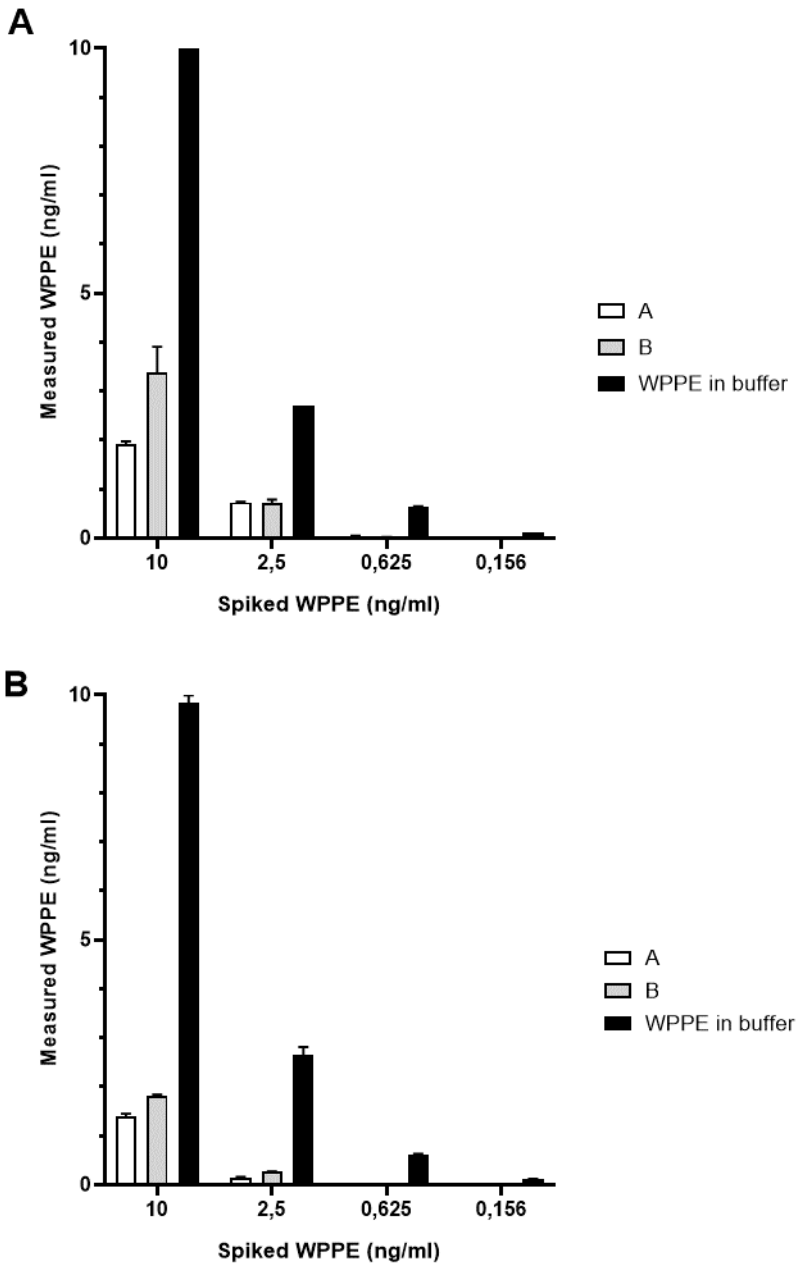
		IgE (IU/mL)	IgG1 (ng/mL)	IgG2 (ng/mL)	IgG4 (ng/mL)
Pooled commercial plasma from peanut-allergic individuals		80	3500	300	300
Non-allergic volunteers	A	ND	160	60	ND
	B	ND	260	ND	ND
	C	ND	250	ND	ND
	D	ND	140	ND	ND
	E	ND	160	60	ND
	F	ND	130	80	15
Peanut-allergic patients	1	16.4	660	-	ND
	2	1.9	1620	-	ND
	3	29.2	920	-	100
	4	48.5	3380	-	150
	5	4.6	820	-	240
	6	26.4	1970	-	ND
	7	14.3	3090	-	ND
	8	4.6	1450	-	ND
	9	1.1	280	-	250
	10	21.5	290	-	300
	11	0.6	310	-	240
	12	2.4	460	-	ND
	13	0.8	3220	-	820
	14	5.0	890	-	420

ND: Not Detectable

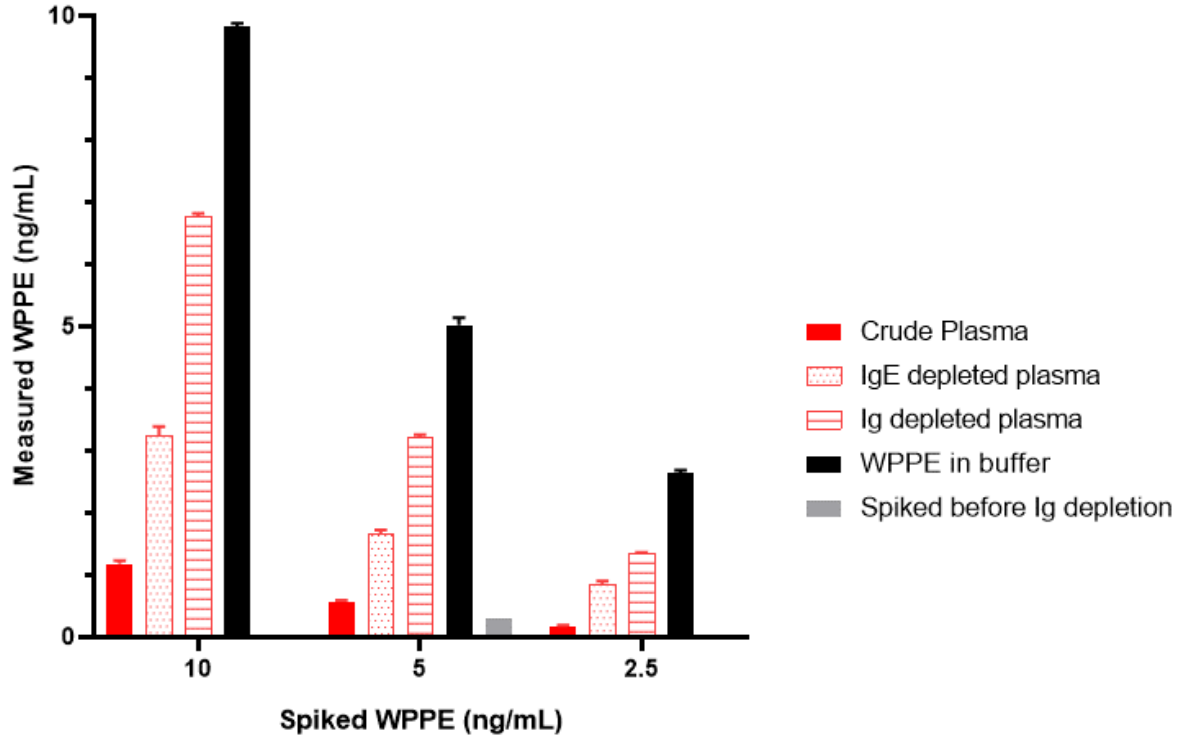
Supplementary Figures



Supplementary Figure 1: Feeding trial with peanut proteins included in various matrices in human healthy volunteers. Blood samples were taken prior to, and 30mins after 100mg peanut protein, and then 30mins and 120mins after ingestion of 300, 1000 and 3000mg peanut protein incurred in various matrices. Peanut protein ingestions are shown by blue arrows, also indicating peanut protein levels. The 8 timepoints for blood sampling are indicated with the red arrows : T0 baseline ; T30 : 30 min post 100mg peanut (PN) proteins ; T60 min : 30min post 300mg PN proteins ; T150 min : 2hr post 300mg PN proteins ; T180 min : 30min post 1000mg PN proteins ; T 270 min : 2hr post 1000mg PN protein ; T300 min : 30min post 3000mg PN proteins ; T 390 min : 2hr post 3000mg PN proteins.

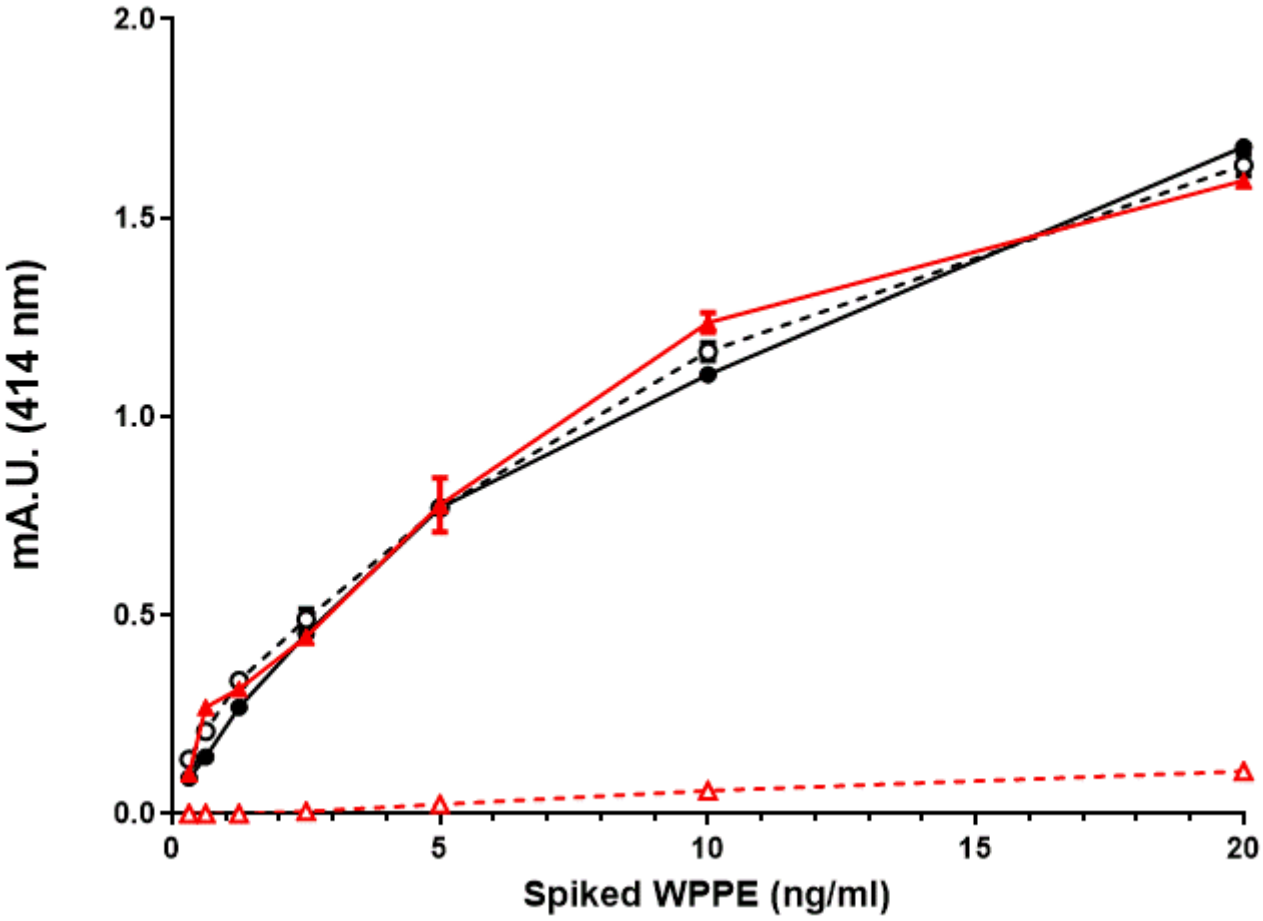


Supplementary Figure 2: Ara h 6 detection in untreated serum from two healthy volunteers, A (white bar) and B (grey bar), and in control buffer (black bar) spiked with WPPE. Aliquots of serum and buffer were spiked with 10, 2.5, 0.625 and 0.156 ng/mL and further diluted to 1/4 before Ara h 6 detection was performed using Immunoassay described in the study (**A**); Immunoassay developed with a mix of three anti-Ara h 6 mAbs as capture and three anti-Ara h 6 complementary mAbs as revelation antibodies (**B**). Quantification was performed with WPPE used for spiking.



Supplementary Figure 3: Ara h 6 detection in non-depleted (red full bar), IgE-depleted (red dotted bar), Ig-depleted (red hatched bar) pooled plasma from peanut allergic patient and in control buffer (black bar), spiked with WPPE.

IgG and IgE depletions of a pooled plasma samples (250µl, diluted 2-fold in 0.1M sodium phosphate buffer pH 7.4, 0.3M NaCl) from peanut allergic patients were performed twice on a column containing 500 µl of Protein A resin (ProSep®-vA ultra from Merckmillipore) equilibrated with PBS, or using a column containing 500 µl of Anti-human IgE Sepharose prepared as previously described.[5] Flowthrough was collected and aliquots of each depleted sample were immediately spiked with 10, 5, 2.5 ng/mL of WPPE. Additional spiking experiment with 5 ng/mL of WPPE was performed on two fold diluted plasma, before Ig depletion (grey bars). Each spiked sample was further diluted to 1/4 before Ara h 6 detection. Quantification was performed with WPPE used for spiking as a reference.



Supplementary Figure 4: Ara h 6 detection in samples spiked with whole peanut protein extract (WPPE). Aliquots of buffer (black symbols) or plasma (red symbols) were spiked with 20, 10, 5, 2.5, 1.25, 0.625 and 0.16 ng/mL, then treated (closed symbols) or not (open symbols) using simultaneous acidic shock (citrate, pH 3) and heating (60°C, 30 minutes). Results are reported as milli-absorbance Units at 414nm (mAU414nm).

Supplementary References

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