Food Anal. Methods (2012) 5:480–486
DOI 10.1007/s12161-011-9270-9

Validation by a Collaborative Interlaboratory Study of an ELISA Method for the Detection of Caseinate Used as a Fining Agent in Wine

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Received: 4 March 2011 / Accepted: 30 June 2011 / Published online: 16 July 2011
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Abstract The clarification or fining of wine removes undesirable substances such as proteins, phenols, and tannin compounds that would cloud the wine and cause bitterness and astringency. Caseinates have useful fining properties, but their residues could present a risk for allergic subjects. A commercial kit that was developed to detect caseinates in food has been examined for its applicability to a wine matrix; it is sensitive to caseinates at concentrations as low as 1 ppm. The general characteristics of the caseinate assay, described below in detail, are as follows. It is a sandwich enzyme-linked immunosorbent assay (ELISA): the microplate is first coated with a specific anti-casein antibody; and after incubation with the wine sample, a secondary anti-casein antibody conjugated with horseradish peroxidase is added to form a sandwich. The antibodies have been tested for their immunoreactivity and the reproducibility of antigen recognition has been measured. An interlaboratory collaborative trial was organized to evaluate the performance of this ELISA method and its ease of use by laboratories routinely dealing with wine/food analyses. The results satisfy the criteria established by the International Organisation of Vine and Wine in the Compendium of International Methods of Analysis MA-EAS1-07-ETCOL.

Keywords Fining agents · Caseinates · Milk allergens · ELISA

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Introduction

It is a common practice to add organic or inorganic substances to the must or wine to interact with elements responsible for turbidity and organoleptic imbalances Harbertson 2008; Morris and Main 1995). Unlike filtration, which can only remove particles, the clarification process removes soluble substances such as polymerized tannins, phenols, and undesirable proteins. Protein-clarifying agents have a positive charge at the pH of wine and generally react with polyphenols by forming hydrogen bonds between phenolic radicals and the peptide bonds of proteins. The complexes can then be filtered off.

Included among the organic substances used in clarifying are some animal proteins known to have allergenic potential. Milk allergy, though rare in adults, can lead to severe, even dangerous illness (Sackesen et al. 2011).

Potassium caseinate is a water-soluble clarifying agent used mainly for white wines (Braga et al. 2007). It flocculates when added to wine because of the acidic pH and binds compounds responsible for turbidity. Any residues left in the wine could represent a risk for subjects with milk allergy.

The aim of Directive 2000/13/EC (Directive 2000) was to harmonize the laws of member states relating to the labeling, presentation, and advertising of foodstuffs. The rules for allergen labeling were detailed in Directives 2003/89/EC (Directive 2003) and 2006/142/EC (Commission Directive 2006). Annex IIIa of Directive 2003/89/EC (Directive 2003) appended a list of food ingredients (later including lupines and molluscs) that are likely to cause adverse reactions in susceptible individuals (Sicherer and Sampson 2006; Boyce et al. 2010), whereas Directive 2005/26/EC (Commission Directive 2005) listed some food ingredients provisionally excluded from labeling until 25 November 2007 [extended by the Commission Directive 2007/68/EC (Commission Directive 2007) to 31 May 2009 and furtherly to June 2012] to allow for further research; this directive included clarifying agents used in wine production,

The aim of this study was to validate an enzyme-linked immunosorbent assay (ELISA) test for the detection of allergenic residues in wines fined with caseinates; such a test is particularly important as the EU is considering exempting caseinates from wine-labeling requirements, as it has done for isinglass (EFSA NDA Panel 2007).

Materials and Methods

ELISA Plates

A commercial sandwich ELISA kit for the measurement of caseinates in foods supplied by Euroclone SpA was used and modified for the analysis of wine in terms of sample buffer composition and dilution (see "Results" section).

Ring Trial Samples

Fourteen different white wine samples were prepared at the Department of Pharmacological Sciences, Università degli Studi di Milano and sent to the participants in the interlaboratory testing.

Three types of white wine were used:

1. Pinot Bianco del Veneto
2. Bianco di Sicilia
3. Garganega

The physicochemical characteristics of these wines were determined before spiking (Table 1) according to the International Organisation of Vine and Wine (OIV) Compendium of International Methods of Wine and Must Analysis (OIV Compendium of International Methods of Wine and Must Analysis 2009). As Table 1 shows, the three wines are quite different and constituted a good test that the ELISA method is not affected by different wine matrices.

A mixture of caseinates (sodium salt) from bovine milk (Sigma Aldrich Italia) was used to prepare a standard solution (1 mg/mL) in water.

Fourteen samples were prepared at the following concentrations:

Sample ID	Wine	Spiked amounts (ppm)
1	Pinot bianco	3.0
2	Pinot bianco	6.4
3	Pinot bianco	0
4	Pinot bianco	1.6
5	Pinot bianco	1.6
6	Bianco di Sicilia	1.0
7	Bianco di Sicilia	0
8	Bianco di Sicilia	2.2
9	Bianco di Sicilia	7
10	Bianco di Sicilia	7
11	Garganega	0
12	Garganega	1.0
13	Garganega	6.5
14	Garganega	2.5

Samples were stirred to achieve homogeneity. Stabilizing agents (protein buffer+sodium azide 0.01%) were added to prevent precipitation or flocculation. The range of amounts of added caseinate was chosen to cover the whole range of residues to be expected in wine processing.

The samples were numbered (blind samples) and sent to each participating laboratory. Samples 5 and 10 were duplicates of 4 and 9, respectively. The samples were stored and shipped at 4°C.

Organization of the Interlaboratory Trial

The spiked wine samples contained caseinates in the range of 0–7 ppm.

Repeatability This is the value below which the absolute difference between two single test results, obtained using the same method on identical test material, under the same conditions (same operator, same apparatus, same laboratory, and over a short period of time) lies within a specified probability (usually 95%).

Reproducibility This is the value below which the absolute difference between two single test results, obtained using the same method on identical test material, under different conditions (different operators, different apparatus and/or different laboratories, and/or different time) lies within a specified probability (95%).

Table 1 Analysis of three white wines used for the preparation of test samples

Description	Bianco Sicilia	Garganega	Pinot Bianco del Veneto
Alcoholic strength by volume (mL/100 mL)	13.46	11.87	11.90
Total alcoholic strength (mL/100 mL)	13.52	11.95	11.97
Reducing sugars (g/L)	1.0	1.4	1.2
Density	0.99289	0.99321	0.99209
pH	3.46	3.30	3.26
Total dry extract (g/L)	27.2	22.6	19.8
Reduced extract (g/L)	27.2	22.2	19.6
Total acidity (g/L)	6.4	6.3	5.3
Volatile acidity—SO2 (g/L)	0.33	0.34	0.21
Ash (g/L)	2.73	1.93	1.95
Total phenolic compounds (as gallic acid) (mg/L)	295	224	180

The term "individual result" is the value obtained when the standardized trial method is applied, once and fully, to a single sample. Unless otherwise stated, the probability is 95%.

General Principles

The test was performed according to the characteristics listed below:

1. The protocol was clear and precise.
2. The samples used in the trials were taken from homogeneous batches of material.
3. The concentrations of analyte to be determined covered the concentrations generally encountered.
4. All laboratories taking part had good experience in the technique employed.
5. For each participant, all analyses were conducted within the same laboratory by the same analyst.
6. The method was well described and had to be strictly followed. Any change from the method described had to be documented.
7. The experimental values were determined under identical conditions that were described in the protocol given to all participants.
8. The experimental values were determined independently of each other and immediately after each other.
9. The results were expressed by all laboratories in the same units (parts per million) to the same number of decimal places.

Ten laboratories participated in the collaborative study. A Lab-Code number was given to each laboratory. The participating laboratories, listed below, had proven experience in ELISA analysis.

1. Laboratorio Chimico Camera Commercio Torino-I-10127 Torino
2. Veneto Agricoltura-Istituto per la Qualità e le Tecnologie Agroalimentari-I-36016 Thiene (VI)
3. IASMA—Istituto Agrario San Michele all'Adige-I-38010 S. Michele all'Adige (TN)
4. Unione Italiana Vini soc. Coop.-I-37135—Verona
5. Unione Italiana Vini soc. Coop.-I-53100—Siena
6. Di.C.T.F.A. University of Genoa-I-16147—Genoa
7. Department of Pharmacological Sciences, Università degli Studi di Milano-Lab Toxicology-I-20133 Milano
8. ISS-Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare-I-00161 Roma
9. Dal Cin Gildo S.p.A.-I-20049 Concorezzo (MB)
10. ISPA-CNR-I-70126 Bari—Italy

The 14 blind-spiked samples were sent to all laboratories and named from 1 to 14. To monitor internally the assays, a negative (untreated wine) and a positive control (a wine containing >1 ppm of caseinate) were included in the kit. The ELISA kit (enough for the number of analyses described in the ring trial protocol) was sent together with the samples and a detailed protocol.

The protocol described:

1. Samples and kit storage
2. Protocol for the ELISA reader check
3. Samples preparation
4. ELISA test protocol
5. Interpretation of results (a specific Excel file was prepared and sent by e-mail)

Specific critical points were highlighted in the protocol in order to avoid any possibility of mistakes during the analyses. Protocol details are summarized below:

1. Dilute all samples (1–14), and negative and positive controls, 1:5 (*v*/*v*) with the buffer provided with the kit.
2. Dilute the caseinate standards (supplied with the kit) with the same buffer to give a standard curve ranging from 0.061 to 2 ppm.

3. Load samples 1–14, the negative and positive controls, and each dilution of the standard curve in duplicate onto the microplate. Table 2 shows the required pattern in the microplates.
4. Incubate the plate for 30 min at room temperature.
5. Apply a washing step using the solution supplied with the kit, consisting of PBS buffer containing Tween, add the specific antibody HRP conjugate and incubate for further 30 min at room temperature.
6. After an additional washing step, incubate with the chromogenic agent (tetramethylbenzidine). Apply the stop solution (sulfuric acid) and measure the absorbances at 450 nm with a specific ELISA reader.
7. Report the raw data in the Excel file provided (which does the calculation automatically) and send it to the ring trial coordinator (Università degli Studi di Milano).

Results

One of the critical points for immunochemical detections is the specificity of the antibody used. The immunospecificity of the antibody provided to all laboratories was verified by immunoblotting (Restani et al. 2009). Each casein fraction was found to be well identified and with similar affinity and specificity (α-casein 100%; β and κ-caseins >70%). Even polypeptides with low molecular weight, such as γ-caseins (fragments of beta-casein), were bound. This reactivity is particularly important since even if caseinates are partially hydrolyzed during clarification, any remaining allergen must be detectable by the method. The antibody was also challenged with the different enological caseinates that are available, and a suitable immunoreactivity was obtained (Restani et al. 2009).

Development of an ELISA Method for a Wine Matrix

Also of vital importance in the development of an ELISA method is its applicability to a specific food/beverage matrix. The ELISA method we examined had already been developed to detect traces of milk in foods; it was modified to optimize the detection of caseinates in wine samples. In particular, the protocol of sample preparation was changed in terms of buffer composition in order to avoid possible interference of wine matrix. The wine sample buffer contained 0.5 M sodium carbonate, pH 9.5, in order to bring wine samples to a neutral pH. Several kinds of sample dilutions were tested and finally the dilution factor was also changed from ten- to sixfold (1+5). Moreover, the whole procedure was internally controlled using negative and positive standard solutions in wine.

Internal Standards and Wine Sample Preparation

Preparation of the wine samples is extremely easy: it requires only a dilution with specific buffer. To improve the quality of the test, negative and positive "wine matrix" standard solutions were prepared, containing no or known quantities (parts per million) of caseinate. Internal positive and negative controls were treated like all other wine samples (dilution with the buffer).

The Assay

The sandwich ELISA test described takes 90 min to complete. The 450-nm absorbance value is compared with the standard curve and the value obtained is multiplied by the dilution factor.

The range and mean of absorbances in an ELISA test are shown in Fig. 1 as an example, together with the related standard curve. The range of values is from 0.06 to 2 ppm (corresponding to 0.36–12 ppm in the wine sample because samples were diluted sixfold).

Some experimental wine samples were prepared by adding caseinate to an untreated white wine and included in the ELISA test; the internal "wine sample" control was analyzed in parallel. The results are reported in Table 3.

Table 2 ELISA microplate scheme supplied to all participants

	1	2	3	4	5	6
A	Blank	Blank	Neg ctrl	Neg ctrl	Sample 7	Sample 7
B	Std 0	Std 0	Pos ctrl	Pos ctrl	Sample 8	Sample 8
C	Std 0.061	Std 0.061	Sample 1	Sample 1	Sample 9	Sample 9
D	Std 0.125	Std 0.125	Sample 2	Sample 2	Sample 10	Sample 10
E	Std 0.25	Std 0.25	Sample 3	Sample 3	Sample 11	Sample 11
F	Std 0.5	Std 0.5	Sample 4	Sample 4	Sample 12	Sample 12
G	Std 1	Std 1	Sample 5	Sample 5	Sample 13	Sample 13
H	Std 2	Std 2	Sample 6	Sample 6	Sample 14	Sample 14

Ctrl control, *pos* positive, *neg* negative

ppm	absorbance at 450nm			Mean±SD
0	-0.003	0.003	-0.002	-0.001±0.00
0.061	0.285	0.297	0.270	0.284±0.014
0.125	0.459	0.432	0.429	0.440±0.017
0.25	0.756	0.786	0.849	0.797±0.047
0.5	1.222	1.113	1.167	1.167±0.055
1.0	1.535	1.432	1.430	1.466±0.060
2.0	2.109	2.132	2.150	2.130±0.021

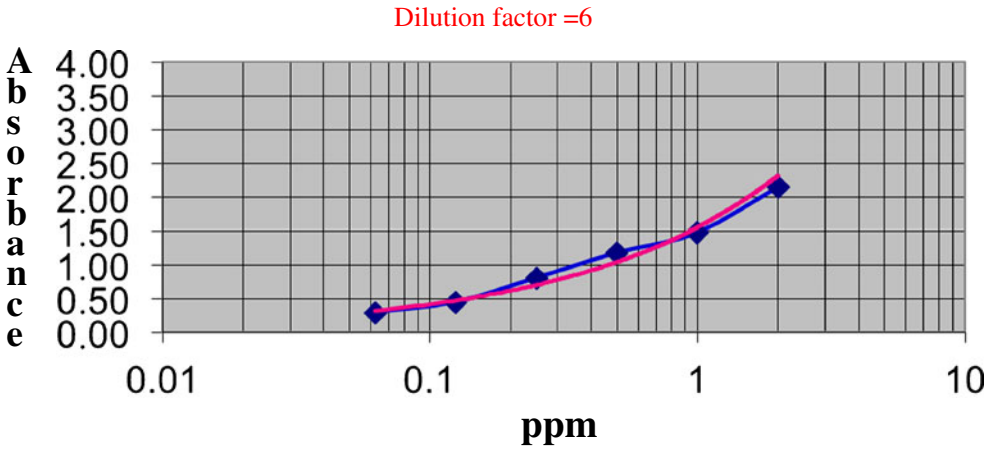


Fig. 1 An example of standard curve obtained with the developed ELISA test (r=0.9906)

Validation of the ELISA Test by a Collaborative Interlaboratory Study

Statistical analysis was performed according to the norm UNI ISO 5725–2:2004 and to the OIV "Compendium of International Methods of Analysis" MA-EAS1-07-ETCOL (OIV Compendium of International Methods of Wine and Must Analysis 2009). It consists of three steps:

1. To compare variances among laboratories, outlier data were assessed by the Cochrane test.
2. To compare variances inside a laboratory, Grubb's test was performed.
3. Statistical analysis was performed to obtain repeatability and reproducibility data.
4. Horrat values were calculated.

The Horrat value gives a comparison of actual measured precision with the precision predicted by the Horwitz formula for the method and at that particular level of analyte concentration. It is calculated as follows: Horrat value for reproducibility (HoR)=RSDR (measured)/RSDR (Horwitz), where RSDR is the relative estimate of a standard deviation of reproducibility. See footnote to Table 4). A Horrat value between 1 and 2 usually indicates satisfactory interlaboratory precision for most analytical purposes.

Table 3 Intra-assay repeatability of caseinate measurement in spiked wine samples

Spiked amounts (mg/L)	1st measure (mg/L)	2nd measure (mg/L)	3rd measure (mg/L)	Mean±SD (mg/L)
1[a]	0.94	1.21	0.98	1.04±0.15
22	AV	AV	AV	AV
10	8.94	7.99	8.72	8.55±0.50
5.50	5.84	5.23	5.03	5.37±0.42
2.76	2.73	2.75	2.44	2.64±0.17
1.37	0.99	1.08	1.17	1.08±0.09

AV exceeded the highest standard value

[a] Positive control

Table 4 Reproducibility and repeatability assessment

Sample	mu	Mean[a]	Total data[b]	Valid data[c]	Reproducibility[d]	sR	RSDR	HoR[e]	%*R*	Repeatability[f]	sr	RSDr	Hor	%*r*
1	mg/L	2.9844	10	10	1.7159	0.6067	20.3282	1.50	57.5	0.0680	0.0240	0.8052	0.09	2.28
2	mg/L	6.4924	10	10	2.7205	0.9618	14.8150	1.23	41.9	0.0659	0.0233	0.3591	0.05	1.02
3	mg/L	0.0574	10	10	0.1011	0.0358	62.2341	2.53	176.0	0.0464	0.0164	28.5452	1.76	80.74
4	mg/L	1.6953	10	10	1.3858	0.4900	28.9007	1.96	81.7	0.1160	0.0410	2.4198	0.25	6.84
5	mg/L	1.4392	10	10	1.0163	0.3593	24.9668	1.65	70.6	0.0497	0.0176	1.2218	0.12	3.46
6	mg/L	0.7827	10	10	0.7767	0.2746	35.0876	2.00	99.2	0.0351	0.0124	1.5874	0.14	4.49
7	mg/L	0.0719	10	10	0.1832	0.0648	90.0161	3.79	254.6	0.0376	0.0133	18.4615	1.18	52.22
8	mg/L	1.7323	10	10	1.1784	0.4166	24.0503	1.63	68.0	0.0593	0.0210	1.2105	0.12	3.42
9	mg/L	6.6366	10	10	3.1438	1.1115	16.7480	1.39	47.4	0.0682	0.0241	0.3635	0.05	1.03
10	mg/L	6.7704	10	10	2.3155	0.8186	12.0915	1.01	34.2	0.1381	0.0481	0.7106	0.09	2.01
11	mg/L	0.0597	10	10	0.1439	0.0509	85.2622	3.49	241.2	0.0261	0.0092	15.4748	0.96	43.77
12	mg/L	0.6877	10	10	0.6529	0.2309	33.5664	1.98	94.9	0.0393	0.0139	2.0223	0.18	5.72
13	mg/L	6.3385	10	10	3.8321	1.3549	21.3751	1.76	60.5	0.0759	0.0268	0.4233	0.05	1.20
14	mg/L	2.2471	10	10	1.8684	0.6606	29.3967	2.00	83.2	0.1168	0.0413	1.8381	0.20	5.20

sR Estimate of a standard deviation of reproducibility, *RSDR* relative estimate of a standard deviation of reproducibility, *HoR* Horrat value for reproducibility, *%R* relative reproducibility, *sr* estimate of a standard deviation of repeatability, *RSDr* relative estimate of a standard deviation of repeatability, *Hor* Horrat value for repeatability, *%r* relative repeatability

[a] Mean value after elimination of outliers

[b] Total number of test results used for calculation

[c] Number of test results used for calculation after elimination of outliers

[d] Reproducibility limit

[e] Note: samples 3, 7, and 11 have a Horrat value greater than 2, but the mean value is below the calculated quantification limit

[f] Repeatability limit

Reproducibility and repeatability (precision) data are summarized in Table 4. All statistical requirements for the validation of the ELISA method were satisfied, as demonstrated by Horrat values ranging between 1 and 2. Samples 3, 7, and 11 had a Horrat value greater than 2, but the mean value is below the calculated quantification limit (LOQ).

To determine the detection limit and the LOQ, we followed the protocol described in the OIV "Compendium of International Methods of Analysis" E-AS1-10-LIMDET (OIV Compendium of International Methods of Wine and Must Analysis 2009). The limits were calculated using the procedure "determination on blank" using the data from all laboratories. According to the mean of all values of blanks from the calibration curve and its standard deviation ($m \pm s$, 0.0812 ± 0.0677), the limit of detection ($m+3$ s) was 0.28 ppm and limit of quantification ($m \pm 10$ s) was 0.76 ppm.

Conclusions

The ELISA test is one of the most frequently used analytical methods for the detection of allergens in food (Besler et al. 2001). The specificity and sensitivity of immunological procedures are well known. Because the equipment needed is readily available, the ELISA test can be applied in most laboratories involved in quality control. The critical point in ELISA methods is the effect, if any, of the particular matrix in which it is applied. This is the reason why a specific protocol must be developed for any food or beverage. In this paper, we describe the data obtained applying an ELISA test to the detection of allergenic residues in wine clarified with caseinates. The assay is internally controlled by using negative and positive control wine samples.

The collaborative trial showed that the ELISA technique had good reproducibility, repeatability, and robustness. The detection limit and quantification limit were in line with the requirements fixed for the trial and the Horrat values obtained indicate a satisfactory interlaboratory precision. The ELISA method described here therefore provides a simple but reliable system for the detection of caseinates in wines.

Acknowledgment We acknowledge all laboratories involved in the collaborative study for their contribution and in particular the following persons: Marco Ciarrocchi (Istituto Superiore di Sanità, Rome), Luigi Falzarano (Dal Cin Gildo S.p.A), Linda Monaci (ISPA-CNR), Raffaella Boggia (Di.C.T.F.A. University of Genoa), and Laura Lucchi (Department of Pharmacological Sciences, University of Milan).

References

Besler M, Kasel U, Wichmann G (2001) Internet Symposium on Food Allergens 4, 1. http://www.food-allergens.de/password-2002/symposium-4-1/besler-4-1/besler-text-4-1.html

Braga A, Cosme F, Da Silva JR, Lauretano O (2007) Int J Vine Wine Sciences 41:203

Commission Directive 2005/26/EC of 21 March 2005 establishing a list of food ingredients or substances provisionally excluded from Annex IIIa of the Directive 2000/13/EC of the European Parliament and of the Council. Official Journal of the European Union L 75/33, 23.3.2005

Commission Directive 2006/142/EC of 22 December 2006 amending Annex IIIa of Directive 2000/13/EC of the European Parliament and of the Council listing the ingredients which must under all circumstances appear on the labelling of foodstuffs. Official Journal of the European Union L 368/110, 23.12.2006

Commission Directive 2007/68/EC of 27 November 2007 amending Annex IIIa of the Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients. Official Journal of the European Union L 310/11, 28.11.2007

Directive 2000/13/EC of the European Parliament and of the Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs. Official Journal of the European Union L 109/29, 6.5.2000

Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. Official Journal of the European Union L 308/15, 25.11.2003

EFSA NDA Panel (2007) The EFSA Journal 533:1

Harbertson JF, at the Washington State University (2008) http://cru.cahe.wsu.edu/CEPublications/em016/em016.pdf

Morris JR, G.L. Main. Proc. 14th NM Conf. (1995) http://www.uark.edu/depts/ifse/grapeprog/articles/nmc14wg.pdf

Boyce et al. (2010) Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-sponsored expert panel J Allergy Clin Immunol 126:S1. http://download.journals.elsevierhealth.com/pdfs/journals/0091-6749/PIIS0091674910015666.pdf

OIV Compendium of International Methods of Wine and Must Analysis (2009) http://news.reseau-concept.net/images/oiv_uk/Client/VOL1.pdf ; http://news.reseau-concept.net/images/oiv_uk/Client/VOL2.pdf

Restani P, Ballabio C, Mottini M, Tarantino C (2009) Bulletin de l'OIV 82:387

Sackesen C, Assa'ad A, Baena-Cagnani C, Ebisawa M, Fiocchi A, Heine RG, Von Berg A, Kalayci O (2011) Curr Opin Allergy Clin Immunol 11: 243

Sicherer SH, Sampson HA (2006) J Allergy Clin Immunol 117, S470