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Determination of Haloanisols in White Wine by Immunosorbent Solid-Phase Extraction Followed by Enzyme-Linked Immunosorbent Assay

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A high through-put screening immunochemical method to control the presence of 2,4,6-trichloroanisol (TCA) and 2,4,6-tribromoanisol (TBA), the main agents responsible for the musty odor in wine samples, has been developed. The method involves a selective (antibody−antigen) solid-phase extraction (SPE), followed by enzyme-linked immunosorbent assay (ELISA) analysis. The sample preparation method established uses for immunosorbents (ISs) prepared by covalently coupling antibodies developed for TCA on a sepharose support. At present, about 200−400 ng L^{-1} of TBA and TCA can be detected in white wine samples by the IS-SPE-ELISA method described here without any preconcentration step. Simultaneous analyses of many samples are possible with this method. Related chloroanisoles (2,3- and 2,6-dichloroanisols and 2,3,4,5-tetrachloroanisol) and chlorophenols (2,3,4,6-tetrachlorophenol and pentachlorophenol) usually present in contaminated wine samples are also effectively retained by the IS, although only 2,4,6-TCA and 2,4,6-TBA are detected by the ELISA used. The immunopurification procedure developed could also be useful as a selective cleanup method prior to chromatographic analysis.

KEYWORDS: Immunoaffinity purification; ELISA; 2,4,6-trichloroanisole (TCA); 2,4,6-tribromoanisole (TBA); wine; musty odor; cork; immunosorbent

INTRODUCTION

The use of cork stoppers as closures of wine bottles dates back to the XVII century, when Dom Perignon (1638−1715) introduced them for the first time; an idea taken from the Spanish pilgrims who used to close their pumpkins with this material. Since then, cork has been employed because of its excellent influence on the flavor of wine and other spirits. Despite the benefits of cork, in past decades some defects in drink taste have been associated with the use of cork stoppers due to the migration of contaminants from cork stoppers to the bottled wine. One of the most serious is the so-called “cork taint”. Wines affected by “cork taint” are characterized with a musty, moldy, earthy taint, and it has been estimated that this problem generates economical losses of 1 billion dollars per year in the wine industry (*1*). Tanner and co-workers identified for the first time 2,4,6-trichloroanisole (TCA) as the principal compound responsible for “cork taint” (*2*). Recently, 2,4,6-tribromoanisole (TBA) has also been identified as a potent odor agent related to cork taint (*3*). The findings have concluded that TBA can cause an intense musty odor in wines where the chloroanisole content is below its detection threshold. This compound is also perceived at low concentration level, especially in water samples, and can provide unpleasant aromas in many other food products (*4*−*6*).

Although the perception levels of TCA and TBA vary within persons and depend on the concentration of the anisole in the wine, it has been reported that the olfactory sensorial threshold of TCA in red and white wine is around 50 ng L^{-1} and 4 ng L^{-1}, respectively (*7*, *8*). Similarly, on a perception threshold study a “musty” off-odor was perceptible on smelling red wine containing as little as 4 ng L^{-1} TBA. Gas chromatography (GC) is the most suitable analytical technique coupled to either a mass spectrometer detector (MSD) or an electron capture detector (ECD). However, due to the low level at which trihaloanisoles contaminate wine, a preconcentration step is always necessary after wine or cork extraction, which limits the speed and efficiency of this sequential analytical method. Moreover, as a result of the high lipophilicity, often the extraction recovery is not always satisfactory, particularly in the case of the cork stoppers, where it has been described that only a small

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percentage of the overall TCA content is released to the wine (*9*, *10*). Some of the most frequently used extraction methods include reverse-phase solid-phase extraction (SPE) (*11*, *12*) and liquid−liquid extraction with organic solvents, such as pentane (*13*), dichloromethane, or ethyl acetate (*14*, *15*). More recently, solid-phase microextraction (SPME) (*10*, *16*−*18*) or supercritical fluid extraction (*19*) has provided better extraction efficiencies, allowing very low detection limits to be reached (*9*). Despite the large number of protocols available, no agreement has been achieved regarding the most effective extraction procedure. In this context, Riu et al. have recently established a SPME/GC-ECD method able to quantify the total amount of chloroanisoles in cork stoppers. As a result of the high cost of some of the equipment and the lack of suitable personnel to run them, small and medium companies, often working in this industrial sector, potentially affected by the "cork taint" problem still continue to perform sensorial analyses on routine bases. However, only trained personal may perform these types of analyses and, moreover, the sensorial threshold that can be reached is dramatically reduced after smelling or tasting a few samples (*7*), preventing a proper quality control of their products. Thus, simple and low-cost quality control systems, avoiding complex instrumental techniques and expert personnel, would increase the control efficiency, significantly reducing economical losses caused by the musty aroma.

Previously, we reported the production and characterization of polyclonal antibodies for the analysis of 2,4,6-TCA by enzyme-linked immunosorbert assay (ELISA) (*20*, *21*). In buffered media containing 7% ethanol, the assay showed a good accuracy and an excellent detectability for both TCA and TBA (limit of detection of 0.04 and 0.02 μg L^{-1}, respectively). However, cork extracts and wine samples caused important interferences, placing the detection limit around 4 and 2 μg L^{-1} for red and white wine samples, respectively. These results raised the need to develop sample treatment methods compatible with the immunochemical protocol. Immunoaffinity procedures combine the advantages of SPE methods with the specificity of the antibody−antigen interaction (*22*−*26*). This technique allows the selective extraction of an analyte, which is specifically retained in the stationary phase, from a complex matrix. Moreover, usually rend aqueous extracts that can directly be analyzed by immunochemical methods. Although immunoaffinity extraction procedures have been mainly used in areas such as medicine and biology, already in the year 1986, Van Emon and co-workers described the application of the immunoaffinity principle for the extraction of the pesticide paraquat from environmental samples (*27*). Several publications have reported immunoextraction of drugs (*28*), pesticides (*29*, *30*), and toxins (*31*−*33*) from complex real matrices. Thus, immunoaffinity columns have been reported as an alternative extraction method for the determination of ocratoxin A in wine samples previously to liquid chromatography (LC) analysis (*34*, *35*), and recently we have reported a HTS-IS-ELISA method for the analysis of 2,4,6-trichlorophenol (2,4,6-TCP) in urine samples (*36*, *37*). In the present work, we describe the development of an immunoextraction procedure, followed by ELISA analysis for the determination of haloanisoles in wine samples.

MATERIALS AND METHODS

Chemicals and Immunochemicals. Phenols and anisoles were purchased from Aldrich Chemical Co. (Milwaukee, WI). Standard solutions and spiked samples were prepared from stock solutions of 2,4,6-TCA and other chemicals (800 mM in DMSO). White wine was kindly provided by Rich Xiberta S.A. (Girona, Spain). The production of the antiserum As90 and As78, used for the immunosorbent (IS) preparation and the ELISA protocol, respectively, has already been described elsewhere (*20*). Briefly, As78 and As90 were obtained by immunization of two New Zealand white rabbits with 5-(2,4,6-trichlorophenoxy)-pentanoic acid and 3-(3,5-dichloro-4-methoxyphenyl)-propanoic acid, respectively, and conjugated to keyhole limpet hemocyanin protein by the active ester method. The ELISA protocol also employed the 3-(2-metoxy-3,5,6-trichlorophenyl)-2-propenoic acid conjugated to ovalbumin by the mixed anhydride as coating antigen (*21*).

Buffers. PBS is 10 mM phosphate buffer, 0.8% saline solution, and, unless otherwise indicated, the pH is 7.5. EtOH/PBS (7%) buffer is PBS containing 7% ethanol. Borate buffer is 0.2 M boric acid−sodium borate, pH 8.7. Coating buffer is 50 mM carbonate−bicarbonate buffer, pH 9.6. PBST is PBS with 0.05% Tween 20. PBST (2×) is PBST double concentrated. Citrate buffer is a 40 mM solution of sodium citrate, pH 5.5. The substrate solution contains 0.01% tetramethylbenzidine and 0.004% H_2O_2 in citrate buffer. The coupling buffer employed to immobilize the immunoglobulins to the sepharose support was 0.2 M $NaHCO_3$ (0.5 M NaCl, pH = 8.3). Capping buffer A was 0.5 M ethanolamine (0.5 M NaCl, pH = 8.3). Capping buffer B was 0.1 M acetate (0.5 NaCl, pH = 4). Gly−HCl buffer was 0.05 M glycine−HCl at pH = 3. Gly−NaOH/NaCl is Gly−HCl with the pH adjusted to pH 8.6 with NaOH and the conductivity adjusted to 15 mS cm^{-1} with NaCl.

Materials. *N*-Hydroxysuccinimide (NHS)-activated Sepharose columns (Hi-Trap R NHS-activated) were purchased from Pharmacia Biotech (Uppsala, Sweden). Briefly, the columns are made of medical-grade polypropylene, and their gel consists of highly cross-linked agarose beads activated by NHS. Desalting columns PD10 (Sephadex G-25) were also purchased from Pharmacia Biotech. Polystyrene microtiter plates used for the ELISA analysis were purchased from Nunc Maxisorb (Roskilde, Denmark).

Instruments. The pH and the conductivity of the buffers and the samples were adjusted using a pH meter pH 540 GLP and a conductimeter LF 340, respectively, both purchased from WLT (Weilheim, Germany). For the analysis of 2,4,6-TCA by ELISA, washing steps were carried out using a SLY96 PW microplate washer SLT Labinstruments (Salzburg, Austria). Absorbances were read with a Multiskan Plus MK II microplate reader Labsystems (Helsinki, Finland). GC-ECD was performed on a HP 5890A gas chromatograph with a HP 7673A automatic injector and an ECD ($^{63}{}_{28}Ni$) and a HP3396 Series II integrator. A capillary column BPX35 (35% phenyl(equiv)-polysilphenylen-siloxane) (SGE Europe Ltd, U.K.) of 25 m × 0.22 mm id × 0.25 μm (film thickness) was used. He (130 kPa; 20 cm seg^{-1}) was the carrier gas employed.

Samples. Spiked white wine samples were prepared using a 2,4,6-TCA stock solution (800 mM) prepared in DMF. Prior analyses of the pH and the conductivity were adjusted to 7.5 and 15 mS cm^{-1}, respectively.

IS Preparation: *Antibody Purification (Ab90).* Antiserum As90 (*20*) was purified by 35% $(NH_4)_2SO_4$ precipitation (*38*). The precipitate was restored with 10 mM phosphate-buffered saline (PBS, pH 7.5) solution and dialyzed against 0.5 mM PBS (5 L × 4) and Milli-Q water (5 L × 1). The aqueous solution was finally freeze-dried and stored at 4 °C until use.

Antibody Immobilization on the Support. Antibodies were covalently bound to the NHS-activated Sepharose column, following the procedure recommended by the supplier. Briefly, a 10 mg mL^{-1} solution (1 mL) of the purified antibodies was prepared in coupling buffer (0.2 M $NaHCO_3$, pH 8.3, 0.5 M NaCl) and passed through the high-trap column at a flow-rate of 1 mL min^{-1}. The column was then left to stand for 30 min at room temperature (RT) and then washed with three column volumes of coupling buffer. Capping of the not-coupled active groups was then accomplished by alternate (6 mL each) and repetitive (3 cycles) injections of 0.5 M ethanolamine (pH 8.3, 0.5 M NaCl) and 0.1 M acetate (pH 4, 0.5 M NaCl) buffers. Finally, the column was washed with 10 mM PBS buffer (2 mL) and stored at 4 °C in the presence of 0.02% NaN_3 until used. The coupling efficiency was estimated by comparing the UV absorption at 280 nm of the antibody solution initially injected onto the column and the one eluted with the coupling buffer. The eluted fraction was desalted before measurements were

performed in order to remove any interference due to the NHS groups, which absorbance is the same as the IgG.

Evaluation of the IS: *Determination of Antibody Coupling Efficiency.* The coupling efficiency of the IgG molecules to the HiTrap column was estimated as the difference between the total amount of IgG initially loaded onto the column and the amount of IgG found in the eluted buffer after the coupling took place. The IgG concentration in both solutions was determined by UV measurements at $\lambda = 280$ nm. Previously, the collected solution after coupling of IgG to the column was purified from the coeluting NHS groups, absorbing at the same wavelength as the proteins, using a HiTrap desalting column prepacked with SephadexG-25 superfine.

Analyte Binding Capacity of the IS. The experimental capacity of the column was determined by loading PBS samples (10 mL) spiked at different levels (130, 325, 390, 650, and 1170 ng) with 2,4,6-TCA. After a washing step to remove the unbound fraction, the bound fraction was eluted following the general IS-SPE procedure (see below). The 2,4,6-TCA content in the collected loading, washing, and eluted fractions were measured by ELISA as described below.

Specificity Studies: *GC-ECD Analysis.* Samples were prepared at 13 ppb in 10 mM PBS (10 mL) and passed through the IS. Haloanisols were extracted from the aqueous fractions with toluene (2,3-DCA, 3,5-DCA, and 2,6-DCA) or hexane (2,4,6-TCA, 2,3,4,5-TeCA, and 2,4,6-TBA) shaking manually for 30 s. Chlorophenols were extracted with toluene and analyzed by GC-ECD after derivatization with *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide, as described (*36*). GC conditions for the analysis of the phenolic compounds 2,4,5-TCP, 2,4,6-TCP, 2,3,4,6-TeCP, and PCP were as follows: 100 °C (1 min) to 250 °C (5 °C/min), 250−300 °C (15 °C/min). For the chlorophenols 3-CP, 4-CP, and 2,4-CP, the temperature program was: 50 °C (1 min) to 250 °C (5 °C/min), 250−300 °C (15 °C/min). For the anisoles 2,4,6-TCA, 2,3,4,5-TeCA, and 2,4,6-TBA, the temperature varied from 100 °C (1 min) to 250 °C (7 °C/min), 250−300 °C (15 °C/min) and for 2,3-DCA, 3,5-DCA, and 2,6-DCA from 50 °C (1 min) to 200 °C (4 °C/min), 200−300 °C (15 °C/min). In all cases studied, the injector and the detector temperature were kept at 250 °C and 300 °C, respectively.

IS-SPE-ELISA: *Immunoaffinity Procedure.* The columns were first conditioned by adding of 50:50 ethanol−water (5 mL), followed by 10 mM PBS (10 mL pH 7.5). Then the wine sample (3 mL), diluted 10 times with PBS (total volume 30 mL), was loaded through the column. Immediately after the sample percolation, the column was washed by passing 1:9 ethanol/PBS (10 mL; the immunosorbent was only washed with PBS if just haloanisol or chlorophenol standard solutions were loaded into the column) and the 2,4,6-TCA specifically bound to the attached antibodies was eluted with 7:3 ethanol−PBS (3 mL). Finally, the column was regenerated by passing additional 7:3 ethanol/PBS (10 mL), followed by PBS (10 mL) and stored like this at 4 °C until next use. For long periods, a 0.02% NaN_3 was added to the buffer. All the steps were carried out at RT and at a flow rate of 0.5 mL min^{-1}.

ELISA Analysis of 2,4,6-TCA. Fractions eluted from the immunoaffinity chromatography column were diluted ten times with 10 mM PBST (PBS−Tween, pH 7.5) buffer and analyzed by ELISA using the protocol already reported (*21*). The standard curve was fitted to a four-parameter equation using the software GraphPad Prism (Graph Pad Software, San Diego, CA). Unless otherwise indicted, analyses were carried out in triplicate.

RESULTS

ISs allow extraction of an analyte from a complex matrix by its interaction with a specific antibody (Ab). It has been reported that it is advisable to prepare ISs using an Ab with an affinity high enough for the analyte, but at the same time, low enough to prevent using stringent conditions to elute the analyte (*39*, *40*). Attending to this criteria, As90 was selected from the different antisera developed for the immunochemical determination of 2,4,6-TCA (*20*). The isolated immunoglobulin G (IgG) fraction was immobilized onto the sepharose gel support by means of the amino groups of the Ab through a stable amide bond. High and comparable coupling efficiencies (92−96%) were achieved in all batches, suggesting comparable properties among different immunoaffinity columns (see **Table 1**). A *theoretical binding capacity* of 1.28 ± 0.02 μg ($N = 3$) 2,4,6-TCA was estimated based on the amount of IgG coupled, the bivalent binding properties of the IgG molecules, and the assumptions that 10% of the polyclonal IgG are specific and that about 50% of the immobilized IgG may not be accessible due to steric hindrance or to a random antibody orientation.

Table 1. Characteristics of the ISs Prepared for 2,4,6-TCA Extraction

column[a]	IgG,[b] mg	coupling efficiency, %	theoretical capacity,[c] μg
IS90 I	10	95%	1.29
IS90 II	10	96%	1.30
IS90 III	10	92%	1.25

[a] The columns were prepared by covalently coupling purified IgGs from As90 to 1 mL of bed Sepharose support functionalized with NHS esters. [b] Mass of the specific IgG used for each 1 mL bed column. [c] Theoretical capacity in μg of TCA, considering that Abs are bivalent and that only 10% of the total IgG fractions are specific to 2,4,6-TCA and only 50% of them have the right orientation to interact with the analyte.

Selection of the most appropriate *eluting conditions* was made attending to (*i*) the recovery of the analyte, (*ii*) the volume needed for an acceptable recovery, (*iii*) the potential damage of the ISs after several cycles, and (*iv*) the compatibility with the immunochemical analytical method. Unfortunately, full desorption of low molecular weight analytes from immunoaffinity columns has very seldom been achieved with only aqueous buffers (*29*, *41*). Although it has been described the use of acidic, basic buffers or solutions with high ionic strength (*26*, *42*) as well as detergents or chaotropic agents (*43*, *44*), organic solvent−water mixtures such as methanol−water (*43*, *45*−*47*) or ethanol−water (*29*, *36*, *48*) have often provided the best recoveries for these type of molecules. In this case, a 7:3 ethanol/PBS released effectively 2,4,6-TCA from the column, although the fractions had to be diluted 10 times before measuring them with the ELISA. As previously reported, due to the low water solubility of haloanisols, the presence of 7% ethanol in the buffer significantly improved immunoassay features. However, at 14%, the IC_{50} value decreased and percentages greater than 35% of the organic solvent completely inhibited the assay (*21*). TCA started to elute from the IS with 7:14 ethanol/PBS, but the elution profile was very wide and there was necessary several fractions to accomplish an acceptable recovery.

In order to determine the *experimental capacity* of the ISs, PBS solutions (10 mL) spiked at different concentrations with TCA (130, 325, 390, 650 and 1170 ng, corresponding to 10, 25, 30, 50, and 90% of the theoretical capacity) were loaded into the column. After a washing step with PBS, the TCA retained was removed from the column using a 7:3 ethanol/PBS solution. As it can be observed in **Figure 1A**, the immunoaffinity column could effectively retain amounts up to 325 ng of TCA under these conditions. When higher loads were passed through the column, a fraction of the analyte was detected in the loading and washing fractions, indicating that the real capacity of the column was lower than the theoretically estimated (see **Table 1**). While some of the immunoaffinity columns prepared in our group have shown very close theoretical and real capacities, others have exhibited a big disparity between both values. Thus, the theoretical capacity of an IS developed for the selective extraction of 2,4,6-TCP from urine samples was in the same range as the theoretical capacity (*36*). In contrast, a column prepared with specific antibodies against

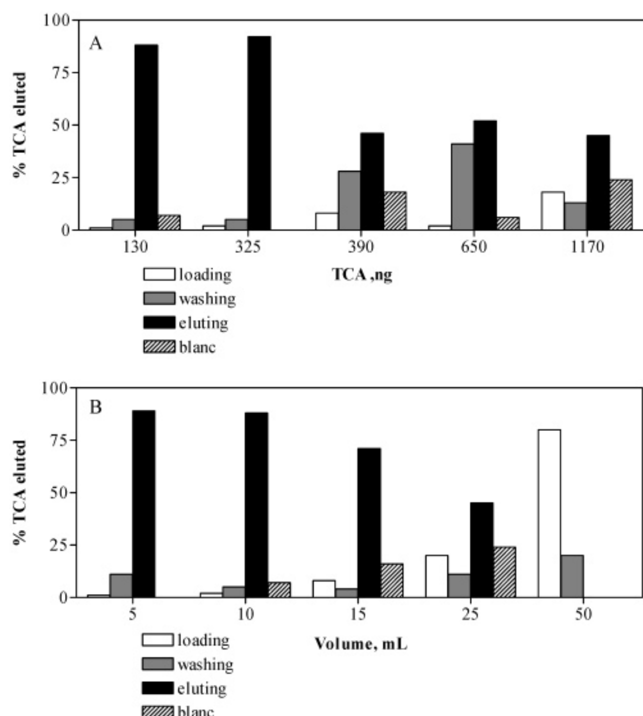

Figure 1. Recovery of 2,4,6-TCA as a function of the (A) concentration and (B) volume loaded. For the case of the effect of the concentration of PBS samples (10 mL) spiked at different levels loaded into the column, different sample volumes (5–50 mL) of PBS spiked with 130 ng of TCA were percolated through the column. Washing was performed in both studies with 10 mL of PBS buffer. A mixture of ethanol/PBS (70:30) was used to elute TCA from the column. The fractions named "blanks" correspond to the fractions collected after each cycle by passing through the column 10 mL PBS. All the eluted fractions were diluted 10 times in PBST buffer and analyzed in triplicate by ELISA.

Irgarol 1051 showed a capacity that was only 0.54 μg when the theoretical capacity was 3 μg (*29*). The disparity between the theoretical and the real capacity of the ISs may be related to the antiserum avidity. In this case, in two-dimensional checkerboard experiments, As90 showed a moderate avidity compared to the As78 used in the ELISA. We selected this antiserum to avoid a too-tight retention of the analyte in the IS that would prevent its effective elution. Nevertheless, this capacity is high enough to retain TCA from real samples considering the low concentration of this substance in spoiled wines (i.e., considering that the volume loaded 300 ng corresponds to a concentration of 30 ng mL^{-1}).

Additional studies were performed to determine the *effect of the volume loaded* by passing through the IS samples spiked with 130 ng of 2,4,6-TCA (10% of the theoretical capacity) at different PBS volumes (5, 10, 15, 25, and 50 mL). **Figure 1B** illustrates a clear dependence of the capacity of the column on the volume of sample passed through it. Volumes higher than 15 mL significantly reduced the amount of TCA specifically retained by the IS.

Specificity of the IS. Although 2,4,6-TCA has been identified as the main responsible agent of the cork taint problem, other structurally related compounds, such as chloro/bromophenols and chloro/bromoanisoles, are also present in contaminated samples (*8*, *49*−*51*). Moreover, some of them can also significantly contribute to the musty odor. Thus, as mentioned in the introduction, 2,4,6-TBA has been identified as the second important source for this problem. With the aim to know how these substances were retained by the IS, we evaluated the specificity of the immunoaffinity procedure. With this purpose, PBS solutions (10 mL) of seven chlorophenols, five chloroanisoles, and a bromoanisole (see **Table 2** for chemical structures) were individually extracted with the ISs. In this case, the eluted fractions were analyzed by GC-ECD after appropriate extraction of the eluted fractions with organic solvent (toluene for chlorophenols (*36*) and hexane for chloroanisoles). By applying a standard protocol (PBS washing and 7:3 ethanol/PBS for the elution), many chloroanisoles evaluated were retained significantly by the IS (see **Table 2**). This result illustrates that the IS prepared for TCA specifically retains those compounds structurally related with the analyte. The differences observed within the substances evaluated can be related to the hapten chemical structure used to raise these antibodies (see hapten C in **Figure 2**). Thus, 3,5-DCA (3,5-dichloroanisol), with the chlorine atoms in completely different positions, showed a recovery of 31%, 2,3-DCA, with a chlorine atom in the *ortho* position to the methoxy group, showed a 60% recovery, and the recovery of 2,6-DCA increased to 84% because both chlorine atoms are placed in *ortho* and *para* position as in TCA. In contrast, most of the chlorophenols were poorly retained (see **Table 2**); the only exceptions were 2,3,4,6-TeCP and PCP that were retained with recoveries greater than 70%. Finally, 2,4,6-TBA was also very well retained, in spite of the lack of bromine atoms of the immunizing hapten. As mentioned above, an increasing interest in the detection of brominated anisoles has arisen in the last few years on certain samples (water, wood, and so on) because the sensory threshold has shown to be even lower than that of the 2,4,6-TCA, being also associated to similar and undesirable musty and moldy aromas (*5*, *52*). The presence of these brominated organic compounds has become a matter of concern in the last few years as a result of the increasing detection frequency in contaminated environmental and food samples. On a recent study published by our group, it was evidenced the high frequency of exposure of the human population to these kind of substances (*37*). The origin of these residues has been related to their use as a flame retardant in plastics, treated woods, or textiles. In this context, we should notice that the brominated derivatives are also highly recognized by the TCA ELISA (*21*). The greater recognition of brominated analytes by antibodies raised against the corresponding chlorinated analogues has already been reported in our group for the 2,4,6-TCP indirect and direct ELISAs (*53*, *54*). The role of the hydrophobic interactions in the stabilization of the immunocomplex when considering nonpolar analytes has been regarded as a reasonable explanation for this behavior (*54*, *55*).

Evaluation of the IS-SPE Procedure for Wine Samples. Establishment of an IS-SPE-ELISA Analytical Protocol. The effect of the ethanol content of the wine on the retention capability of the IS was investigated in a first step. In this manner, PBS samples (10 mL) containing 130 ng of analyte (13 μg L^{-1}) were prepared at ethanol percentages varying from 1 to 10% and passed through the column. **Figure 3** summarizes the results of this study. As it can be observed, increasing percentages of ethanol had a strong influence in the capacity of the column. The presence of 10% ethanol in the samples reduced the ability of the IS to retain TCA from 95% recovery, in the absence of solvent, to 23%, while at 1% recovery values around 80% were observed. However, as expected, recovery improved if lower TCA concentrations were loaded. Thus, as can be observed in **Figure 3**, if samples contained a concentration of 6 μg L^{-1}, the recovery increased 61% even if 10% ethanol was present in the sample. These results were promising considering that the concentration of haloanisoles in spoiled wines is usually 2 orders of magnitude lower.

Table 2. Recoveries of Different Anisoles and Chlorophenols Passed through the TCA IS[a]

Compound	Recovery (%)	Structure	Compound	Recovery (%)	Structure
2,4,6-TCA (analyte)	100		2,3-DCA	60	
3-CP	0		3,5-DCA	31	
4-CP	0		2,6-DCA	84	
2,4-DCP	0		2,4,6-TBA	79	
2,4,5-TCP	2		2,3,4,5-TeCA	100	
2,4,6-TCP	3		PCP	73	
2,3,4,6-TeCP	81				

[a] Results were obtained by passing the compounds through the IS at 13 ppb in 10 mL PBS buffer. The washing step was made with PBS.

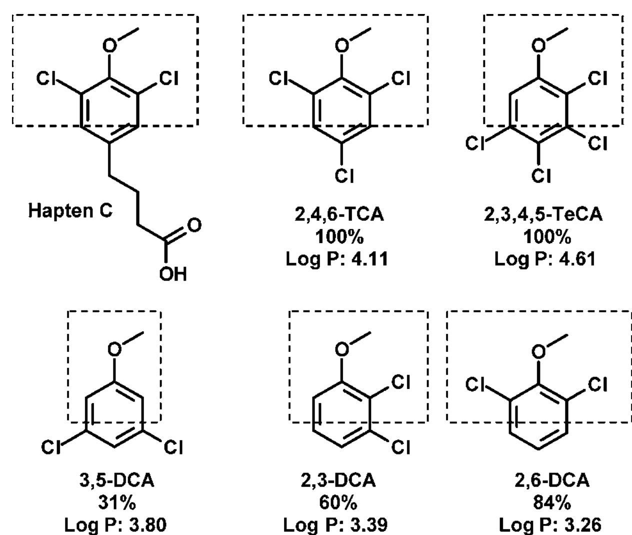

Figure 2. Chemical structures of the immunizing hapten and the main cross-reactants. The boxes show the recognition area directed by immunizing hapten C used to raise As90. A close relationship with the recovery values obtained can be observed.

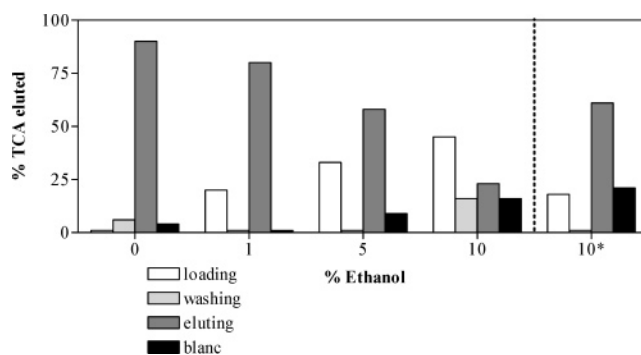

Figure 3. Recovery of 2,4,6-TCA in the eluted fractions of the immunoaffinity column when loading samples with different ethanol percentages. Experiments were done introducing ethanol/PBS solutions (10 mL) spiked with 130 ng of 2,4,6-TCA (13 μg L^{-1}). Sample 10* contained 10% ethanol and was spiked with 65 ng of analyte (6 μg L^{-1}). Washing was performed with PBS buffer (10 mL). A mixture of ethanol/PBS (70:30) was used to elute TCA from the column. The fractions named "blanks" correspond to the fractions collected after each cycle by passing through the column 10 mL PBS. All the eluted fractions were diluted 10 times in PBST buffer and analyzed in triplicate by ELISA.

Although similar results were obtained with white wine samples, we also observed that when wine samples were spiked with high TCA concentrations, dilution of the wine samples prior to loading them into the column improved recovery of TCA in the elution fractions. **Table 3** shows IS performance at very extreme conditions if wine samples with TCA at 130 μg L^{-1} were passed through the immunoaffinity column at different dilution factors. Thus, for unknown samples, a 1:10 dilution of the wine samples would be perhaps advisable before the immunoaffinity extraction. Nevertheless, the overestimation encountered when wine was introduced after just 1:5 and 1:2 dilution factors was interpreted as a coelution of matrix components that interfered with the immunochemical analysis (see **Table 3A**). For this reason, we intended to increase the strength of the washing step to accomplish a more efficient cleanup of these samples. Because previous experiments

Table 3. Recovery of the IS-SPE-ELISA Method Analyzing White Wine Samples[a]

wine, mL	TCA, μg L^{-1}	dil. factor[c]	TCA, recovery[b] ng	TCA, recovery[b] %
1	13	1:2	291	224
1	13	1:5	205	158
1	13	1:10	136	105
1	1.3	1:10	14	108
1	0.13	1:10	<LOD[d]	<LOD
3	4.30	1:10	17.4	134[e]
3	2.15	1:10	8.64	133[e]
3	0.433	1:10	1.83	140[e]
5	1.30	1:10	10.8	167[e]

[a] A volume of 1 mL of white wine was spiked with 130 ng of TCA in every case, conveniently diluted, and introduced in the column. The analyses were done in triplicate. [b] TCA recovered in the first 3 mL when a 7:3 ethanol/PBS solution was passed after a washing step with PBS. [c] Wine samples were diluted with PBS. [d] Limit of detection of the TCA ELISA used to measure the eluted fractions. [e] The washing step was a mixture of ethanol–PBS (10:90).

demonstrated that the elution of 2,4,6-TCA did not start until a solution with 35% ethanol was used, a 10% ethanol was introduced in the washing buffer.

Moreover, as it can be observed in **Table 3**, the IS-SPE-ELISA detectability was limited by the dilution factor that had to be applied after immunopurification to decrease the ethanol content of the fractions before the ELISA analytes. Thus, with the present protocol, the TCA content of 1 mL of wine is finally diluted up to 30 times before immunochemical analysis (three collected fractions at 70% ethanol are diluted 10 times to place ethanol content to 7%). Thus, with the aim to increase detectability, high sample volumes were loaded into the column and processed using 10% ethanol in the washing steps. Results showed that a systematic overestimation was produced if higher volumes were introduced into the column. Therefore, using wine volumes greater than 3 mL was rejected for the IS-SPE-ELIS method reported here.

In light of the above results, the following IS-ELISA protocol was finally established to analyze trihaloanisoles in wine samples. Wine samples (1−3 mL) are diluted 1:10 times in PBS before loading them in the column. Next, 10 mL of 1:9 ethanol/PBS is passed through the column to wash out the interferences, and. finally, the TCA is eluted by passing 10 mL of 7:3 ethanol/PBS. Under these conditions, TCA eluted within the first 3 mL. The combined fractions were diluted 10 times with PBS and analyzed directly by ELISA. The whole dilution factor applied to the wine samples using this protocol is 10 times. Considering that the LOD of the ELISA method is 0.044 μg L^{-1}, the overall detectability of this IS-ELISA protocol is 440 ng L^{-1}. Regarding the potential presence of 2,4,6-TBA, the IS-SPE-ELISA method presented here could detect this substance down to 200 ng L^{-1} due to its high cross reactivity in the ELISA (CR = 200%) (*21*). The column is regenerated by passing 10 mL more of PBS and stored until the next use at 4 °C. For longer periods without use, the column is stored in the presence of a 0.02% of NaN_3.

Finally, blind wine samples were prepared by spiking them with TCA, and they were measured using the above-described IS-SPE-ELISA protocol. The results are shown in **Figure 4**. The results match very well the spiked values, which demonstrate performance of the method as a screening tool for TCA determination.

DISCUSSION

A selective SPE method has been developed and evaluated with the final aim of establishing a reliable immunochemical method to assess TCA contamination of wine products. Using the previously developed 2,4,6-TCA microplate-based ELISA, the necessary detectability was compromised by the nondesired matrix effects produced by the wine samples. Samples had to be diluted between 50 and 100 times to get rid of these nonspecific interferences. The results of this study have demonstrated that the IS-SPE-ELISA procedure allows the significant increase in detectability of the immunochemical analytical method reported before. After immunoaffinity extraction, wine samples could be measured after just 10 dilutions of the sample. A standard IS-SPE-ELISA established protocol has been evaluated measuring several spiked white wine samples, showing that although the method overestimates the real concentration, there is quite a close correlation at the different spiked levels assayed. Although the TCA detectability achieved at this moment is still over the sensorial threshold, the IS-SPE-ELISA developed can be an excellent screening method for the quality control laboratories of wine and cork producers, considering the actual limitations of the actual sensorial analysis usually employed by many small companies. At present, detectability is still limited by the necessary dilution of the eluted fractions prior to immunochemical analysis. Evaporation of the organic solvent to avoid this step was not contemplated due to the high volatility of the analyte in this case. In this context, it is worth noting the existence of solid evidence that points to the fact that the concentration of haloanisoles of the cork stoppers of contaminated wine bottles is several orders of magnitude greater than the levels found in the wine. Previous studies performed in our group using SPME-GC-ECD analysis revealed that the amount of 2,4,6-TCA released by cork stoppers to the wine is below 2% of its content (unpublished results). For example, cork stoppers contaminated with 1500 ng of TCA (average weight of the cork stopper is 3 g, 0.5 ppb) only released to the wine 30 ng (30 μg L^{-1}). Thus, the IS-SPE-ELISA method presented here could provide sufficient detectability to analyze this matrix if an efficient extraction procedure from the stopper is finally established. A complete TCA extraction from the cork has often been contemplated as a challenge, although recently, Riu et al. have reported a procedure to quantify the total amount of chloranisoles in a cork stopper. Moreover, the IS-SPE procedure presented here could also be a powerful procedure for selective extraction prior to chromatographic analysis.

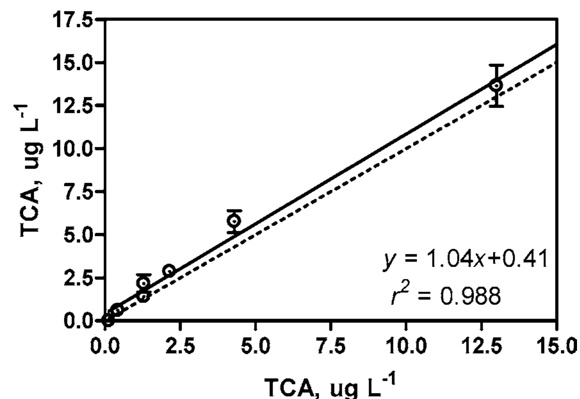

Figure 4. Correlation between the spiked concentrations and the corresponding values measured by IS-SPE-ELISA. Blind samples were prepared in white wine, diluted 10 times with PBS, and loaded into the IS. After a washing step with 1:9 ethanol/PBS, TCA was eluted with 7:3 ethanol/PBS. The first 3 mL were combined, diluted 10 times with PBS, and measured by ELISA. The dotted line corresponds to a perfect correlation (slope = 1). Each point is the average and standard deviation of analyses made on three different days.

Further studies will be oriented toward proving this fact and also toward improving the speed of analysis through its adaptation to a parallel high throughput 96-SPE format.

ABBREVIATIONS USED

Ab, antibody; As, antiserum; ELISA, enzyme-linked immunosorbert assay; GC-ECD, gas chromatography with electron capture detection; IS, immunosorbent; LC, liquid chromatography; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; SPE, solid-phase extraction; SPME, solid-phase microextraction; 3-CP, 3-chlorophenol; 4-CP, 4-chlorophenol; 2,4-DCP, 2,4-dichlorophenol; 2,3-DCA, 2,3-dichloroanisole; 2,6-DCA, 2,6-dichloroanisole; 3,5-DCA, 3,5-dichloroanisole; 2,4,5-TCP, 2,4,5-trichlorophenol; 2,4,6-TCP, 2,4,6-trichlorophenol; 2,4,6-TCA, 2,4,6-trichloroanisole; 2,4,6-TBA, 2,4,6-tribromoanisole; 2,3,4,5-TeCA, 2,3,4,5-tetrachloroanisole; 2,3,4,6-TeCP, 2,3,4,6-tetrachlorophenol; PCP, pentachloroanisole.

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