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First report on the presence of Alloxan in bleached flour by LC-MS/MS method

Vita Giaccone [a, *], Gaetano Cammilleri [a], Vita Di Stefano [b], Rosa Pitonzo [c], Antonio Vella [a], Andrea Pulvirenti [d], Gianluigi Maria Lo Dico [a], Vincenzo Ferrantelli [a], Andrea Macaluso [a]

[a] Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri", via Gino Marinuzzi 3, 90129 Palermo, Italy
[b] Department of Biological, Chemical and Pharmaceutical Science and Technology (STEBICEF), Università degli studi di Palermo, via Archirafi 32, 90123 Palermo, Italy
[c] CGA, UNINETLAB, Università degli studi di Palermo, Via Filippo Marini 14, 90128 Palermo, Italy
[d] Dipartimento di Scienze della Vita, Università degli studi di Modena e Reggio Emilia, Via Università 4, 41121 Modena, Italy

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ABSTRACT

In this work the presence of Alloxan in bread, pastry and cake bleached flour was investigate in order to verify possible risk for consumers related to the use of chemicals for flour bleaching. A selective UHPLC–MS/MS method has been developed and validated for the purpose. Alloxan is one of the possible minor side products of oxidation after chemical bleaching of wheat flours, when several chemical agents are used. One hundred and seventy-five flour samples were analyzed for Alloxan determination. The validation of the method was performed in accordance with the ISO/IEC/EN 17025 for linearity, detection limit, quantification limit, accuracy, precision and ruggedness determination. Satisfactory performances were obtained for the analyte, with a Limit of Detection (LOD) of 0.73 mg kg^{-1}, a Limit of Quantification (LOQ) of 0.85 mg kg^{-1} and recovery values between 94% and 102%. The present work report for the first time the presence of trace levels of Alloxan in 24% of the analyzed samples, with mean values of 0,95 ± 0,04 mg kg^{-1}. The presence of Alloxan was detected only in cake flour samples. Further studies on toxicological levels of Alloxan are needed in order to evaluate possible risks for consumers linked to the consumption of bakery products.

1. Introduction

Alloxan (2,4,5,6–tetraoxypyrimidine or 2,4,5,6-pyrimidinetetrone) is an oxygenated pyrimidine synthesized by uric acid oxidation that can be found in hydrated form. Since 1943, it was found to have toxicological effects on pancreatic β-cells leading to diabetogenic action, therefore (Jacobs, 1937; Pincus, 2013) is commonly employee for the development of Type-I Diabetes Mellitus in animal models. (Carvalho et al., 2003; Dunn et al., 1943; Goldner and Gomori, 1943; Lenzen and Panten, 1988; Saadia et al., 2005; Saleem Mir et al., 2013; Seifu et al., 2017; Shaw Dunn and Mcletchie, 1943; Szkudelski, 2001; Webb, 1966).

As an analogous of the glucose, this molecule has two distinct effects on the pathology of diabetes: selective inhibition of glucose-induced insulin secretion by specific inactivation of glucokinase, (Dhanabal et al., 2007; Weaver et al., 1978; Webb, 1966; Zhou et al., 2017) and inducer of the formation of Reactive Oxygen Species (ROS), which can damage different cellular components through the oxidation of proteins, lipids and nucleic acids (Kim et al., 1994; Lenzen, 2008).

Alloxan also demonstrated to have a carcinogenic action in rats and fishes; furthermore, it can induce adenohypophysis cancer in mices (Suganuma et al., 1993). Mrozikiewicz et al. (1994) have found elevated levels of Alloxan in the blood of diabetic children with insulin-dependent diabetes mellitus, correlated to the onset of insulin-dependent diabetes mellitus (Mrozikiewicz et al., 1994). Alloxan is a minor product of the proteins oxidation, so, it may be produced during the bleaching processes of the alimentary flour for dough and colour improvement, becoming possible toxicant (Banu Shakila and Sasikala, 2012).

Flours obtained from freshly ground wheat have a pale yellow colour due to their carotenoid content. This flour produces sticky dough not easy to work and cook. During storage, the natural aging of the flour is due to a series of oxidative reactions involving carotenoids and sulfhydryl groups of proteins systems. The result is a

* Corresponding author.
E-mail address: vita.giaccone@gmail.com (V. Giaccone).

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white, soft and bulky flour, more suitable for the preparation of bakery products (Fennema, 1985).

To accelerate these natural processes, the food industry uses chemical methods (Joye et al., 2009) able to improve both the colour and the pasting properties. Bleaching agent commonly used are benzoyl peroxide, chlorine gas, chlorine dioxide, nitrosyl chloride and nitrogen oxides (Chittrakorn et al., 2014).

The chlorine dioxide improves the properties of dough and makes the flour less yellow. In the United States, chlorine and hypochlorites are considered safe compounds for food processing, in particular chlorine falls within the food additive list of the Food and Drug Administration (Fukayama et al., 1986). In other Countries, the chlorine dioxide and the chlorine gases have been banished because of their possible toxic effects. (Joye et al., 2009). These compounds, in fact, destroy the conjugated double bonds and oxidise the thiol groups of the gluten proteins; the chlorine processing involves the breakage of hydrogen bonds and the cleavage of peptide bonds; it degrade also aromatic amino acids. (Joye et al., 2009; Kulp et al., 1985); these series of oxidation reactions may modify many flour components (Thomasson et al., 1995) and lead to the formation of toxic products, such as Alloxan. (Fukayama et al., 1986; "Idaho Observer: Bleaching agent in flour linked to diabetes," 2005).

The aim of this work is to prove the presence of Alloxan in bleached flour, given the absence of data in literature on the relative toxicity of this molecule in bakery products. A selective UHPLC–MS/MS method using precolumn derivatization was developed for the determination of Alloxan in flours. The method was validated by an in-house validation protocol according to ISO/IEC/EN 17025.

2. Material and methods

2.1. Sample collection

A total of 175 bleached flour samples were collected from manufacturers and local market of Sicily (Southern Italy). All the samples considered in this study were randomly collected by choosing different texture and size of granulation: 62 bread flour (slightly coarse), 55 pastry flour and 58 cake flour (smooth and fine), respectively. The flours used for the validation have been collected from a farm that produces flour for personal use with ancient means of production. Flour samples that were not subjected to bleaching process were used as blank samples.

2.2. Chemicals and reagents

Acetonitrile and formic acid 99.9% (LC-MS grade), water (HPLC gradient grade), were supplied from VWR (VWR International PBI Srl Milan, Italy). Alloxan, Alloxazine, o-phenylenediamine, hydrochloride acid 1M and sodium hydroxide 1M were purchased from Sigma (Sigma-Aldrich, Milan, Italy). Standard stock solutions of Alloxan and o-phenylenediamine were prepared in hydrochloride acid 0.1M (concentration of 1 mg mL^{-1}). Standard stock solution of Alloxazine was prepared in sodium hydroxide 0.1M at the concentration of 1 mg mL^{-1}. These solutions were stored at 2 to 8 °C up to 3 months. Working stock solutions of Alloxan and Alloxazine were prepared in formic acid 0.1% (v/v) in water at a concentration of 10 μg mL^{-1} and stored at 2 to 8 °C up to 30 days. PTFE filters of 0.45 μm were purchased from Chromacol LTD (Thermo Fisher, Waltham, Massachusetts, USA).

2.3. Sample preparation

2 g of the homogenized flour samples were weighed in a polypropylene centrifuge tube and spiked with 200 μl of Alloxan working solution at 10 μg mL^{-1} in HCl 0.1 M to obtain a concentration of 1 mg kg^{-1}. Every sample was mixed and allowed to rest for 15 min. Subsequently, 10 mL of hydrochloride acid 0.1M were added in the tube and then mixed for 1 min.

The tube was vigorously centrifuged for 10 min at 3500 rpm; the supernatant was collected in a 50 mL polypropylene centrifuge tube that was filtered with filters of 0.45 μm.

A 0.5 mL aliquot was added to 1.5 mL of 0.1% aqueous formic acid solution. This solution was spiked with 2 mL of o-phenylenediamine at 1 mg mL^{-1}. After a gentle stirring, an aliquot of 1 mL was transferred into vials and set at the appropriate temperature of 25 °C for 24 h, prior to LC–MS/MS analysis.

2.4. Chromatographic conditions

LC analysis was carried out through a Thermo Fischer UHPLC system (Thermo Fisher Scientific, California, U.S.A.) composed by an ACCELA 1250 LC pump and an ACCELA autosampler (Thermo Fisher Scientific, California, U.S.A.). Chromatographic separation was obtained using a Thermo Scientific Hypersil Gold PFP - UHPLC column (100 mm × 2.1 mm; 1.9 μm). The LC eluents were water (A) and acetonitrile (B) everyone containing 0.1% (v/v) of formic acid. The gradient (Table 1) was initiated with 85% eluent A for 0.5 min, continued with linear variation to 5% A at 2 min; this condition was maintained for 1 min. The system returned to 85% A in 0.5 min and was re-equilibrated for 3.5 min. The column temperature was 30 °C and the sample temperature was kept at 25 °C. The flow rate was 0.3 mL min^{-1} and the injection volume 5 μL.

2.5. MS conditions

The mass spectrometer was a triple quadrupole TSQ Vantage (Thermo Fisher Scientific, California, U.S.A.) in positive electrospray ionization mode (ESI+). Product ion scans of analyte was performed by direct infusion (10 μl min^{-1}) of 1 μg mL^{-1} Alloxazine standard solutions with the built-in syringe pump through a T-junction, mixing with the blank column eluate (200 μl min^{-1}).

ESI parameters optimized were as follows: capillary voltage, 4.5 kV; capillary temperature, 310 °C; vaporizer temperature, 150 °C; sheath and auxiliary were fixed at 30 and 15 (arbitrary unit), respectively. The collision gas was argon at 1.5 mTorr and peak resolution of 0.7 FWHM was used on Q1 and Q3. The scan time for each monitored transition was 0.05 s and the scan width was 0.05 m/z. The collision energy parameters associated with the precursor and the product ions are given in Table 2. Acquisition data were recorded and elaborated using Xcalibur TM version 2.1.0.1139 software from Thermo Fisher Scientific (California, U.S.A.). The tune of the MS conditions for Alloxazine standard was performed by direct infusion of 1 μg mL^{-1} solution with the built-in syringe pump. It was be found that the precursor ion with the most abundant signal are composed by the adduct [M + H+] in electrospray positive mode. After, the chromatographic conditions were optimized by several injections of Alloxazine standard solution at the concentration of 0.1 μg mL^{-1} in order to test different

Table 1
Gradient profile for the determination of Alloxan.

Time (min.)	Component A (%)	Component B (%)
0.0	85	15
0.5	85	15
2.0	5	95
3.0	5	95
3.5	85	15
7.0	85	15

Table 2
Mass spectrometry parameters for detection and confirmation of Alloxazine.

Analyte	Parent ion	Transition 1	Collision energy (eV)
Alloxazine	215.1	169.9[a]	19
		Transition 2	**Collision energy** (eV)
		144.1	22

[a] The most abundant product ion.

combinations of mobile phases. Then we found the best gradient condition, reported in the experimental section of this paper, for the best symmetry and resolution of the peak. The spectrometric determination was performed in MRM mode in order to obtain a better selectivity and sensitivity.

2.6. Validation procedure

The method was validate by an in-house model, including determination of linearity, specificity, recovery, limit of detection (LOD), limit of quantification (LOQ), precision (repeatability and the within-laboratory reproducibility), accuracy and ruggedness. The validation was performed according to ISO/IEC/EN 17025 (2005). For the estimation of the validation parameters, we chose flour samples from companies operating in Sicily (Italy).

To verify the presence of Alloxan in the spiked sample, after extraction and derivatization, we compared the retention time and the relative abundance of the fragment with the signal of Alloxazine standard. The interpolation of the signal generated by the base peak in the calibration curve was used for the quantitative determination.

The instrumental linearity was calculated constructing calibration curve using Alloxazine commercial standard; it represents the final derivatization product with o-phenylenediamine, which occurs at the end of extraction. The calibration curve for the standard solution of Alloxazine was made with the concentration levels of 0, 0.005, 0.010, 0.025, 0.050 and 0.100 μg mL^{-1} (including zero point). These solutions were prepared in 0.1% aqueous formic acid solution. The calibration curve was built by representing concentrations of Alloxazine against the corresponding peak area. The selectivity/specificity was analyzed by testing 20 representative blank flour samples of different origin in order to verify the absence of potential interfering compounds at retention time of the analyte. The precision (repeatability and the within-laboratory reproducibility) and the trueness were calculated by the determination of samples fortified at three levels (1–5–10 mg kg^{-1}), at beginning of the extraction procedure. Ten aliquots were analyzed for each level, for three batches successively in a 3-week period, giving a total of 90 replicates. The concentration of each replicate was determined using the calibration curve prepared on the same batch. The precision was expressed as the RSD and repeatability values calculated for each level (Table 3). The average recovery was estimated using these matrix results.

The detection limit (LOD) was estimated on the basis of the results of ten replicates of flour sample spiked at 0.5 mg kg^{-1}.

Five operating factors were chosen for ruggedness study (performed at 5 mg kg^{-1}): adsorption time, centrifugation time and speed, concentration of hydrochloric acid in working solution, vortex time. The different factors and their levels were mixed in the Youden experimental plan (Youden and Steiner, 1975).

2.7. Data collection

All the data obtained from the LC-MS/MS analysis were collected on an Excel datasheet and then sorted by flour type in order to evaluate possible differences in Alloxan presence between bread, pastry and cake flour.

3. Results and discussion

3.1. Chromatography and derivatization studies

Alloxan is a ketone with a low molecular weight and neutral functional groups. For these compounds, the fragmentation is difficult due to unstable transitions, so the ionization efficiency in ESI is low (Santa, 2011). The pre-column derivatization with o-phenylenediamine, which produces Alloxazine, has provided a better way to detect the analyte in question, due to a greater stability (Raghavamenon et al., 2009). Alloxazine is the result of reaction between a primary amine and carbonyl groups with formation of a product containing carbon nitrogen double bonds (Fig. 1). The reaction is acid catalyzed by hydrochloric acid with elimination of two molecules of water.

The comparison between Alloxazine standard and derivatization product gave a full matching, for each the four identification points monitored with MRM conditions. An alloxan solution was admixed with an excess of derivatizing agent (o-phenylenediamine), considering the stoichiometry as known and the yield as unknown. The experiments were carried out evaluating the temperature and the reaction times. The kinetic of reaction was studied at three different incubation temperatures: 4–25 and 50 °C. Furthermore, the instrumental results was collected at different time intervals: 5min–15min–30min–45min–90min–12 h–24 h–30 h and 48 h, in order to evaluating the reaction times. It was found an increasing trend of the yield percentage, up to a maximum value in the 24 h, following by a plateau in the next hours. The effect of the temperature on the yield was 4% at 4 °C, 100% at 25 °C and 29% at 50 °C, respectively. All the results are shown in Fig. 2.

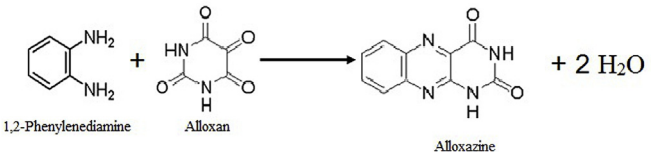

Fig. 1. Chemical Formula of Alloxan (2,4,5,6- tetraoxypyrimidine) and Alloxazine (Benzo[g]pteridine-2,4, (1H,3H)-dione): Schematic representation of derivatization with 1,2-Phenylenediamine.

Table 3
Precision and trueness study at the three validation levels. *n*: number of determinations.

Fortification level (mg kg^{-1})	Precision		Trueness	
	Intra-day analysis RSD (%) (n = 10)	Inter-day analysis RSD (%) (n = 30)	Recovery (%)	Uncertainty (mg kg^{-1})
1.0	6.0	9.9	94	0.2
5.0	4.6	8.9	102	0.5
10.0	3.1	7.0	99	1.1

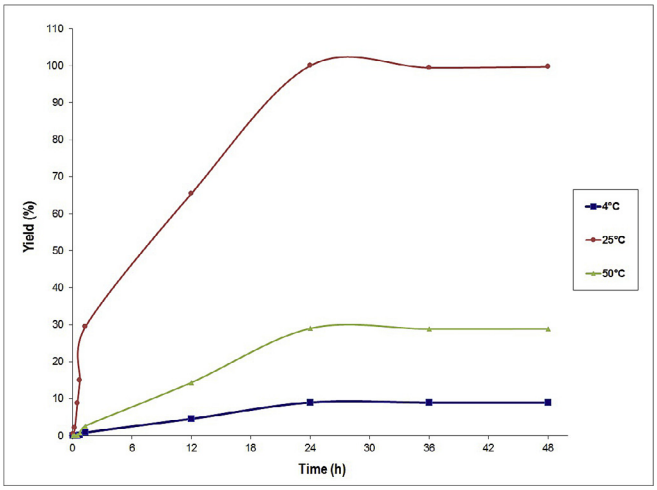

Fig. 2. Graphic representation of the yield percentage of the derivatization reaction: kinetic of reaction studied as a function of the time at three different temperatures.

3.2. Validation of the method

Good validation parameters were obtained (linearity, selectivity/specificity, precision and accuracy, ruggedness, recovery, LOD and LOQ), according to ISO/IEC/EN 17025/2005.

The blank samples were fortified at three different concentrations: 1–5–10 mg kg^{-1} with standard solution of Alloxan. Ten spiked samples, at each of the three levels, were analysed. The analysis of replicates (ten for each level) were repeated once a week, for a total of three weeks. Representative chromatogram of fortified sample is shown in Fig. 3b. For the linearity, the calibration curve obtained for Alloxazine, constructed by plotting the peak area (y) versus concentration (x) of the analyte, giving a correlation coefficient (r^2) of 0.9991. The areas used for the quantification are generated by the base peak signal only.

The Selectivity/Specificity test verified no interfering peaks near the retention time of the analyte. The retention time was 2.1 min for Alloxazine and the obtained peak is symmetrical.

The results of intraday and interday reproducibility are listed in Table 3. The overall precision of the assay expressed as RSD was less than 10% in the flour samples. Trueness was expressed as recovery rate and ranged from 94% to 102%. The recovery data for the low, medium and high levels are shown in Table 3. A detection limit (LOD) of 0.73 mg kg^{-1} and a quantification limit (LOQ) of 0.85 mg kg^{-1} were achieved by the method proposed.

The measuring range, studied in this work, is included between 0.85 and 10.0 mg kg^{-1}; representing the range of concentration between the lowest limit, the LOQ, and the highest limit (the validation point with the highest concentration). The expanded uncertainty of the method was estimated having regard to every contributions of the important elements (weights, volumes, repeatability, standards, calibration uncertainty). The obtained values are shown in Table 3. The ruggedness study, performed at 5 mg kg^{-1}, confirmed that the tested factors are not critical.

3.3. Samples analysis

Alloxan trace levels were found in 42 (24%) of the analyzed samples, with mean values of 0,95 ± 0,04 mg kg^{-1} and a range between 0.88 and 1.02 mg kg^{-1}. The positive samples were confirmed by repeated analysis. The identification of the target compound was based on RT with a window of ±3 times the SD value of the RT, and MS^2 productions of the characteristic ion. The quantification was performed using the signal of base peak only. The chromatograms of a positive flour sample and spiked sample are shown in Fig. 3a and b, respectively. The presence of Alloxan was found only in cake flour, suggesting possible correlations with the use of chemical substances such as chlorine gas and chlorine dioxide for smooth and fine cake flour production. Bleaching compounds tightens the protein molecules in the flour, enabling it to carry more than its weight in sugar and fat. Therefore, most of the cake flour are bleached in order to improve their baking performance and responding to a wide request for production (about 140 million pounds of flour each day in USA). The use of chemical oxidizing agents and bleaches were developed to produce quick aging of wheat flour (48 h), instead the natural conditions that require several months (Fennema, 1985). Chlorine gas used as bleaching agent may reacts with some proteins in the flour (including the gluten) producing Alloxan as a by-product (Cohen, 2010). High-gluten flours, such as the cake flour examined in this work, may contain 5 to 8 g of gluten per 100 g of flour, suggesting a possible presence of Alloxan due to reaction with chlorine gas and other chemicals.

Furthermore, the presence of these compounds could lead to a series of oxidation reactions that may modify many flour components such as the pterins (Socaciu, 2007). Pterins share with flavins properties such as radical formation, participation in redox chains, photosensitizing capacity and absorption of near-ultraviolet light (Galland and Senger, 1988). The total amount of pterins depends on the plant species, on the developmental stage, and on external factors; good sources of these compounds are legumes and wheat germ (Rébeillé et al., 2006). Several of these compounds on oxidation with chlorine and methanol yield 5-methoxyuramil-7-oxalic acid methyl ester together with glycol monomethyl ether, and the ester on hydrolysis gives oxalic acid and Alloxan (Engineers, 2005).

Although there are different studies on the acute effects of Alloxan in rats and other organisms at concentrations higher than what was found in this study (50–100 mg kg^{-1}) (Bakırel et al., 2008; Lenzen, 2008), very little is known about the chronic effects of Alloxan to specific concentrations. Vadlamudi et al. (1982) showed depressed left ventricular pressure and positive and negative dP/dt development in Witsar rats after 100, 180 and 360 days of treatment with Alloxan (Vadlamudi et al., 1982), while a preliminary study conducted by de Olivera et al. (2005) showed blood and tissue alterations after 90 days of Alloxan treatment (De Oliveira et al., 2005). So further studies are needed in order to evaluate the chronic effects of this molecule, the timing of dose and the toxic levels for human health. In United States, wheat flour is normally bleached with chlorine gas prior to its use in baking cakes. Chemical treatments and chemical additives have become suspect and alternative methods to avoid such treatments are needed. Many European countries ban the use of chemical bleaching and oxidation chemicals and other additives in bread completely. The Environmental Protection Agency (EPA) identifies chlorine gas as a flour-bleaching, aging and oxidizing agent that is a powerful irritant, dangerous to inhale, and lethal (Cohen, 2010). An alternative to the chlorination method is to subject the flour to specified temperatures for limited periods of time, this process does not pose a potential hazard to the health of those who consume the products (Hanamoto and Bean, 2005).

4. Conclusions

According to our knowledge, this work represents the first report on the presence of Alloxan in cake bleached flour, suggesting a potential risk for consumers due to the application of chlorine gas and other chemicals for baking cakes. The reported UHPLC–MS/MS

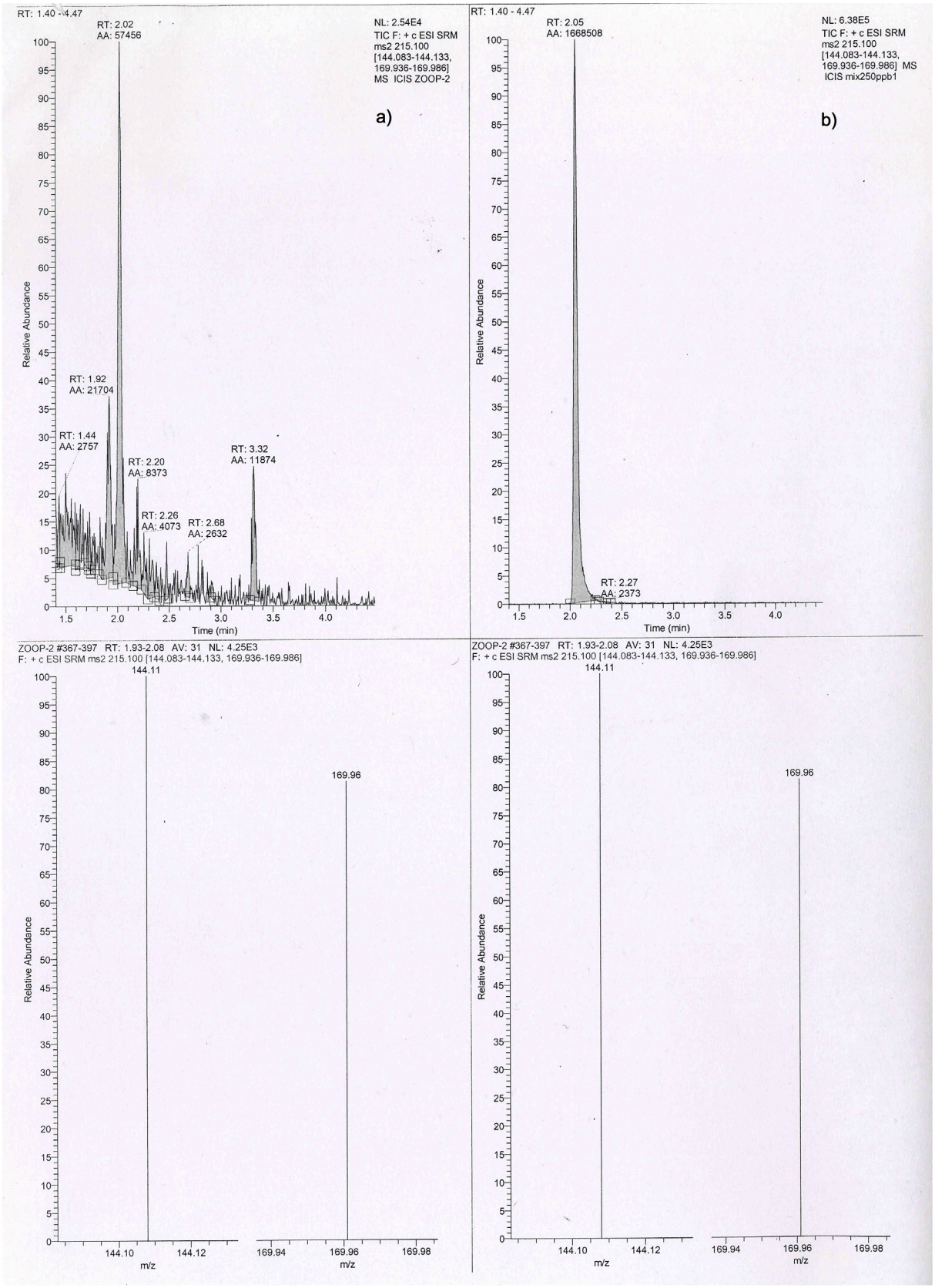

Fig. 3. Chromatograms of a real positive wheat flour sample (a) and spiked wheat flour sample (b) with relative m/z ratios.

method was found very sensitive and accurate for the determination of Alloxan in wheat flour starting from 0.85 mg kg^{-1}. The method was successfully applied to the analysis of 175 real samples, with the aim of verify the presence of this contaminant. The results obtained show that the flour bleached with chlorine dioxide and chlorine gas may contain Alloxan as a minor product of a series of oxidation reactions. As a pilot study, further studies are needed with a larger number of flour samples, in order to understand the real risk for the consumers.

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