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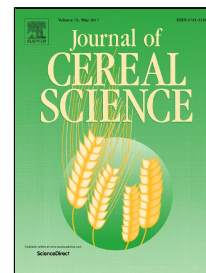
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First report on the presence of Alloxan in bleached flour by Lc-Ms/Ms method

Vita Giaccone, Gaetano Cammilleri, Vita Di Stefano, Rosa Pitonzo, Antonio Vella, Andrea Pulvirenti, Gianluigi Maria Lo Dico, Vincenzo Ferrantelli, Andrea Macaluso



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ABSTRACT

In this work the presence of Alloxan in bread, pastry and cake bleached flour was investigated 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 reports 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.

Keywords: *Flour; Alloxan; LC–ESI-MS/MS; validation procedure; bakery products*

1. INTRODUCTION

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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 employed 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 analogue 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 mice (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 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.,

68 1986). In other Countries, the chlorine dioxide and the chlorine gases have been banished because of
69 their possible toxic effects. (Joye et al., 2009). These compounds, in fact, destroy the conjugated
70 double bonds and oxidise the thiol groups of the gluten proteins; the chlorine processing involves the
71 breakage of hydrogen bonds and the cleavage of peptide bonds; it degrade also aromatic amino acids.
72 (Joye et al., 2009; Kulp et al., 1985); these series of oxidation reactions may modify many flour
73 components (Thomasson et al., 1995) and lead to the formation of toxic products, such as Alloxan.
74 (Fukayama et al., 1986; “Idaho Observer: Bleaching agent in flour linked to diabetes,” 2005).
75 The aim of this work is to prove the presence of Alloxan in bleached flour, given the absence of data in
76 literature on the relative toxicity of this molecule in bakery products. A selective UHPLC–MS/MS
77 method using precolumn derivatization was developed for the determination of Alloxan in flours. The
78 method was validated by an in-house validation protocol according to ISO/IEC/EN 17025.

79 80 2. MATERIAL AND METHODS

81 82 *2.1 Sample collection*

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

89 90 *2.2 Chemicals and reagents*

91 Acetonitrile and formic acid 99.9% (LC-MS grade), water (HPLC gradient grade), were supplied from
92 VWR (VWR International PBI Srl Milan, Italy). Alloxan, Alloxazine, o-phenylenediamine,
93 hydrochloride acid 1M and sodium hydroxide 1M were purchased from Sigma (Sigma-Aldrich, Milan,
94 Italy). Standard stock solutions of Alloxan and o-phenylenediamine were prepared in hydrochloride
95 acid 0.1M (concentration of 1 mg mL⁻¹). Standard stock solution of Alloxazine was prepared in
96 sodium hydroxide 0.1M at the concentration of 1 mg mL⁻¹. These solutions were stored at 2 to 8 °C up
97 to 3 months. Working stock solutions of Alloxan and Alloxazine were prepared in formic acid 0.1%
98 (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
99 0.45 µm were purchased from Chromacol LTD (Thermo Fisher, Waltham, Massachusetts, USA).

100

101 *2.3 Sample preparation*

102 2 g of the homogenized flour samples were weighed in a polypropylene centrifuge tube and spiked
103 with 200 μl of Alloxan working solution at $10 \mu\text{g mL}^{-1}$ in HCl 0.1 M to obtain a concentration of 1 mg
104 kg^{-1} . Every sample was mixed and allowed to rest for 15 min. Subsequently, 10 mL of hydrochloride
105 acid 0.1M were added in the tube and then mixed for 1 min.

106 The tube was vigorously centrifuged for 10 min at 3500 rpm; the supernatant was collected in a 50 mL
107 polypropylene centrifuge tube that was filtered with filters of $0.45 \mu\text{m}$.

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

112

113 *2.4 Chromatographic conditions*

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

123

124 *2.5 MS conditions*

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

129 ESI parameters optimized were as follows: capillary voltage, 4.5kV; capillary temperature, 310°C ;
130 vaporizer temperature, 150°C ; sheath and auxiliary were fixed at 30 and 15 (arbitrary unit),
131 respectively. The collision gas was argon at 1.5 mTorr and peak resolution of 0.7 FWHM was used on
132 Q1 and Q3. The scan time for each monitored transition was 0.05 s and the scan width was 0.05 m/z.

133 The collision energy parameters associated with the precursor and the product ions are given in Table

134 2. Acquisition data were recorded and elaborated using Xcalibur TM version 2.1.0.1139 software from
135 Thermo Fisher Scientific (California, U.S.A.). The tune of the MS conditions for Alloxazine standard
136 was performed by direct infusion of 1 $\mu\text{g mL}^{-1}$ solution with the built-in syringe pump. It was be found
137 that the precursor ion with the most abundant signal are composed by the adduct $[\text{M}+\text{H}^+]$ in
138 electrospray positive mode. After, the chromatographic conditions were optimized by several
139 injections of Alloxazine standard solution at the concentration of 0.1 $\mu\text{g mL}^{-1}$ in order to test different
140 combinations of mobile phases. Then we found the best gradient condition, reported in the
141 experimental section of this paper, for the best symmetry and resolution of the peak. The spectrometric
142 determination was performed in MRM mode in order to obtain a better selectivity and sensitivity.

143

144 *2.6 Validation procedure*

145 The method was validate by an in-house model, including determination of linearity, specificity,
146 recovery, limit of detection (LOD), limit of quantification (LOQ), precision (repeatability and the
147 within-laboratory reproducibility), accuracy and ruggedness. The validation was performed according
148 to ISO/IEC/EN 17025 (2000). For the estimation of the validation parameters, we chose flour samples
149 from companies operating in Sicily (Italy).

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

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

167 repeatability values calculated for each level (Table 3). The average recovery was estimated using
168 these matrix results.

169 The detection limit (LOD) was estimated on the basis of the results of ten replicates of flour sample
170 spiked at 0.5 mg kg⁻¹.

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

175

176 *2.7 Data collection*

177

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

181

182 **3. RESULTS AND DISCUSSION**

183

184 *3.1 Chromatography and derivatization studies*

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

192 The comparison between Alloxazine standard and derivatization product gave a full matching, for each
193 the four identification points monitored with MRM conditions. An alloxan solution was admixed with
194 an excess of derivatizing agent (o-phenylenediamine), considering the stoichiometry as known and the
195 yield as unknown. The experiments were carried out evaluating the temperature and the reaction times.
196 The kinetic of reaction was studied at three different incubation temperatures: 4 - 25 and 50°C.
197 Furthermore, the instrumental results was collected at different time intervals: 5min – 15min – 30min –
198 45min – 90min - 12h - 24h - 30h and 48h, in order to evaluating the reaction times. It was found an
199 increasing trend of the yield percentage, up to a maximum value in the 24h, following by a plateau in

200 the next hours. The effect of the temperature on the yield was 4% at 4°C, 100% at 25 °C and 29% at 50
201 °C, respectively. All the results are shown in Figure 2.

202 203 *3.2 Validation of the method*

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

206 The blank samples were fortified at three different concentrations: 1 - 5 - 10 mg kg⁻¹ with standard
207 solution of Alloxan. Ten spiked samples, at each of the three levels, were analysed. The analysis of
208 replicates (ten for each level) were repeated once a week, for a total of three weeks. Representative
209 chromatogram of fortified sample is shown in Figure 3b. For the linearity, the calibration curve
210 obtained for Alloxazine, constructed by plotting the peak area (y) versus concentration (x) of the
211 analyte, giving a correlation coefficient (r²) of 0.9991. The areas used for the quantification are
212 generated by the base peak signal only.

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

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

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

226 227 *3.3 Samples analysis*

228 Alloxan trace levels were found in 42 (24%) of the analyzed samples, with mean values of 0,95±0,04
229 mg kg⁻¹ and a range between 0.88 and 1.02 mg kg⁻¹. The positive samples were confirmed by repeated
230 analysis. The identification of the target compound was based on RT with a window of ± 3 times the
231 SD value of the RT, and MS² productions of the characteristic ion. The quantification was performed
232 using the signal of base peak only. The chromatograms of a positive flour sample and spiked sample

233 are shown in Figure 3a and 3b, respectively. The presence of Alloxan was found only in cake flour,
234 suggesting possible correlations with the use of chemical substances such as chlorine gas and chlorine
235 dioxide for smooth and fine cake flour production. Bleaching compounds tightens the protein
236 molecules in the flour, enabling it to carry more than its weight in sugar and fat. Therefore, most of the
237 cake flour are bleached in order to improve their baking performance and responding to a wide request
238 for production (about 140 million pounds of flour each day in USA). The use of chemical oxidizing
239 agents and bleaches were developed to produce quick aging of wheat flour (48 hours), instead the
240 natural conditions that require several months (Fennema, 1985). Chlorine gas used as bleaching agent
241 may reacts with some proteins in the flour (including the gluten) producing Alloxan as a by-product
242 (Cohen, 2010). High-gluten flours, such as the cake flour examined in this work, may contain 5 to 8 g
243 of gluten per 100 g of flour, suggesting a possible presence of Alloxan due to reaction with chlorine gas
244 and other chemicals.

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

253 Although there are different studies on the acute effects of Alloxan in rats and other organisms at
254 concentrations higher than what was found in this study (50-100 mg kg⁻¹) (Bakirel et al., 2008; Lenzen,
255 2008), very little is known about the chronic effects of Alloxan to specific concentrations. Vadlamudi
256 et al. (1982) showed depressed left ventricular pressure and positive and negative dP/dt development in
257 Wistar rats after 100, 180 and 360 days of treatment with Alloxan (Vadlamudi et al., 1982), while a
258 preliminary study conducted by de Olivera et al. (2005) showed blood and tissue alterations after 90
259 days of Alloxan treatment (De Oliveira et al., 2005). So further studies are needed in order to evaluate
260 the chronic effects of this molecule, the timing of dose and the toxic levels for human health. In United
261 States, wheat flour is normally bleached with chlorine gas prior to its use in baking cakes. Chemical
262 treatments and chemical additives have become suspect and alternative methods to avoid such
263 treatments are needed. Many European countries ban the use of chemical bleaching and oxidation
264 chemicals and other additives in bread completely. The Environmental Protection Agency (EPA)
265 identifies chlorine gas as a flour-bleaching, aging and oxidizing agent that is a powerful irritant,

266 dangerous to inhale, and lethal (Cohen, 2010). An alternative to the chlorination method is to subject
267 the flour to specified temperatures for limited periods of time, this process does not pose a potential
268 hazard to the health of those who consume the products (Hanamoto and Bean, 2005).

269

270 **4. CONCLUSIONS**

271

272 According to our knowledge, this work represents the first report on the presence of Alloxan in cake
273 bleached flour, suggesting a potential risk for consumers due to the application of chlorine gas and
274 other chemicals for baking cakes. The reported UHPLC–MS/MS method was found very sensitive and
275 accurate for the determination of Alloxan in wheat flour starting from 0.85 mg kg⁻¹. The method was
276 successfully applied to the analysis of 175 real samples, with the aim of verify the presence of this
277 contaminant. The results obtained show that the flour bleached with chlorine dioxide and chlorine gas
278 may contain Alloxan as a minor product of a series of oxidation reactions. As a pilot study, further
279 studies are needed with a larger number of flour samples, in order to understand the real risk for the
280 consumers.

281

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378

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 1. Gradient profile for the determination of Alloxan.

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

Table 2. Mass spectrometry parameters for detection and confirmation of Alloxazine. ^aThe most abundant product ion.

Fortification level (mg kg ⁻¹)	Precision		Trueness	Uncertainty (mg kg ⁻¹)
	Intra-day analysis RSD (%) (n=10)	Inter-day analysis RSD (%) (n=30)	Recovery (%)	
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

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

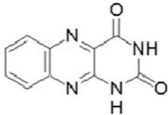
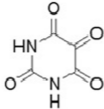
387 **Fig.1.** Chemical Formula of Alloxan (2,4,5,6- tetraoxypyrimidine) and Alloxazine (Benzo[g]pteridine-
388 2,4, (1H,3H)-dione): Schematic representation of derivatization with 1,2-Phenylenediamine.
389
390 **Fig. 2** Graphic representation of the yield percentage of the derivatization reaction: kinetic of reaction
391 studied as a function of the time at three different temperatures.
392
393 **Fig. 3.** Chromatograms of a real positive wheat flour sample (a) and spiked wheat flour sample (b)
394 with relative m/z ratios.
395
396

- In this work the presence of Alloxan in bleached flour was reported for the first time;
- A reliable UHPLC–MS/MS method was carried out according to EN/ISO 17025;
- Alloxan trace levels were found in 24% of the cake flour analyzed samples;
- Bleached flour may contain Alloxan as a minor product of a series of oxidation reactions.

ACCEPTED MANUSCRIPT



+



+ 2 H₂O

1,2-Phenylenediamine

Alloxan

Alloxazine

