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Fate of free and modified *Alternaria* mycotoxins during the production of apple concentrates

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ABSTRACT

Mouldy core is a frequent apple fungal disease, mainly caused by toxigenic *Alternaria* species. Mouldy core is hardly detected in pre-selection procedures when the apples are destined for industrialization, and to date no information is available on the fate of *Alternaria* toxins during apple concentrate production. Therefore, we evaluated the effect of this process on the natural contamination levels of 10 *Alternaria* metabolites: alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tenuazonic acid (TeA), tentoxin (TEN), altertoxin-I (ATX-I), alternariol 3-sulfate (AOH-3-S), alternariol 3-glucoside (AOH-3-G), alternariol monomethyl ether 3-sulfate (AME-3-S), and alternariol monomethyl ether 3-glucoside (AME-3-G). Six stages (grinding, turbos, decanter muds, pre-concentration, concentrate and rejection) of five independent Red Delicious and one of Granny Smith apple concentrate processes were sampled. Four out of the six processes included clarification, while two did not. The Granny Smith raw material was the least contaminated one, both in quality and quantity of *Alternaria* mycotoxins. Quantifiable levels of AOH, AME, TeA and TEN, were observed in the ground apples of the Red Delicious processes. Regarding the modified mycotoxins, only AME-3-S was present in the raw material; nevertheless, AOH-3-S and AOH-3-G were detected along the process. ALT, ATX-I, and AME-3-G were not detected at any stage. Clear and cloudy processes showed similar variations on mycotoxin quantities until the clarification step, in which all the mycotoxins analysed underwent a significant reduction to non-quantifiable levels. Only TeA remained at detectable levels in one of the clarified final products. The concentration in the final cloudy product increased with respect to the raw material for AOH (301%), AME (221%), TEN (872%) and TeA (1024%). This is the first report of AOH-3-S and AME-3-S in apple-by-products. The clarification stage in apple concentrate production has a relevant role in reducing *Alternaria* toxins to safe levels in the final products. A major risk might be associated with cloudy apple-by-products.

1. Introduction

Apple juice is the second most consumed juice worldwide (Sulaiman, Farid, & Silva, 2017) and can be produced either from the apple concentrate or directly from the fruit (not from concentrate juice) (Gou, Tian, Yang, Sun, & Guo, 2019). There are two types of juices: the "clarified" or "clear" juice, in which a clarification treatment is included, and the so-called "with pulp" or "cloudy" that contains natural colloidal suspensions. In the past, consumers on the global level preferred the clear conventional apple juices; nevertheless, a shift to less processed and organic products was observed as these are considered healthier. Cloudy apple juices, besides being perceived by consumers as a more natural, minimal processed product, have a higher antioxidant activity due to a higher polyphenolic content (Oszmianski, Wolniak, Wojdylo, & Wawer, 2007; Teleszko, Nowicka, & Wojdyło, 2016).

Argentina is one of the main apple producing countries in the world,

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with 510 thousand tons of fruit harvested in 2018 (FAOSTAT, 2020). In Northern Patagonia, in the same apple producing area an important apple concentrate industry developed, producing concentrates from conventional and organic crops. Their production supplies both Argentinean apple by-product industries, as well as the demand from international markets. Fruits that do not comply with quality standards for fresh consume are usually derived to these industries for concentrate production (Idigoras, 2014).

Apples might suffer a number of fungal diseases in the field and during postharvest with the blue mould caused by *Penicillium expansum* as one of the most spread (Logrieco, Bottalico, Mulé, Moretti, & Perrone, 2003). Food processing industries need to monitor this disease, since this fungus produces patulin, a mycotoxin which is regulated by several international food safety authorities in apple products. A maximum concentration of 50 μg/l in apple juices is recommended by the World Health Organization and its levels are considered a food quality standard in the apple concentrate industry (Welke, Hoeltz, Dottori, & Noll, 2009).

Mouldy core, another frequent fungal disease of apples, is mainly caused by *Alternaria* species (Gao et al., 2013; Ntasiou, Myresiotis, Konstantinou, Papadopoulou-Mourkidou, & Karaoglanidis, 2015). The mould develops in the centre of the fruit without causing visual external symptoms or lesions. This phenomenon hinders its detection, and its incidence is worsened by long-term storage of fruit (Pavicich, Cárdenas, Pose, Fernández Pinto, & Patriarca, 2020). In addition, *Alternaria* species produce a wide variety of secondary metabolites (López et al., 2016) that include the dibenzopyrone derivatives alternariol (AOH), alternariol monomethyl ether (AME) and altenuene (ALT), the tetramic acid derivative tenuazonic acid (TeA), perylenequinone derivatives called altertoxins (ATX) and miscellaneous compounds such as tentoxin (TEN) amongst others. Furthermore, these secondary metabolites can be modified by conjugation producing phase-II derivatives that can be reconverted to their native form during the production process of contaminated food commodities or by the human metabolism, contributing to the intake of the native form of the mycotoxin (Puntscher, Aichinger, et al., 2019; Puntscher, Cobankovic, Marko, & Warth, 2019).

Currently, the only *Alternaria* toxin legislated in a food commodity is TeA, having a limit of 500 μg/kg in sorghum/millet infant food in Bavaria, Germany (Solfrizzo, 2017). For the remaining mycotoxins produced by this genus, threshold of toxicological concern (TTC) values have been established (Arcella, Eskola, & Gómez Ruiz, 2016), but further information is imperative to implement safe limits for consumers around the globe. The TTC of 2.5 ng/kg of body weight estimated for both AOH and AME does not consider the possible additive effect of conjugated forms, such as their sulfates or glycosides. Data of natural contamination of foods with *Alternaria* toxins showed that the TTC levels for AOH and AME were exceeded in some European countries (Hickert, Bergmann, Ersen, Cramer, & Humpf, 2016; Walravens et al., 2016).

The effect of the apple concentrate process on patulin has been studied and it is believed that safe levels of this toxin in the final product are achievable (Pinton, Suman, Barilla, Fratelli, & Parma, 2019;

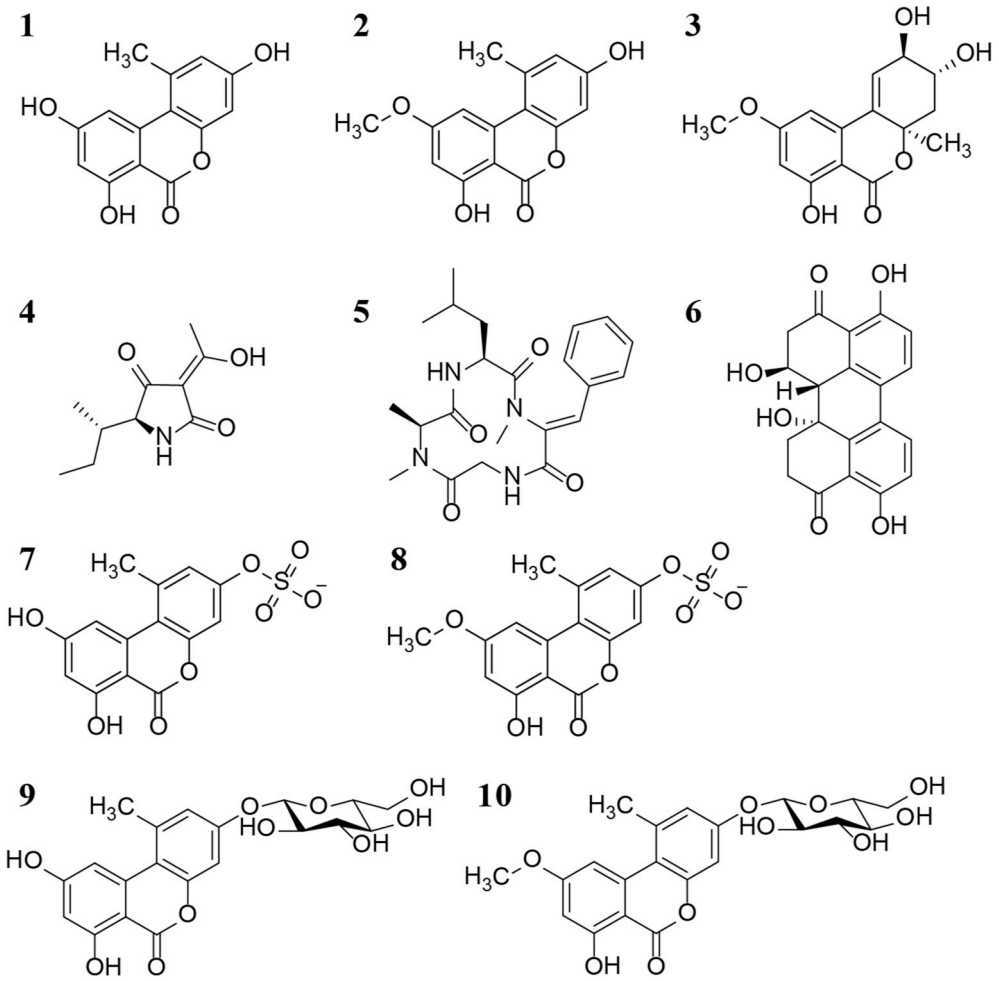

Fig. 1. Chemical structures of the analysed mycotoxins, 1, alternariol (AOH); 2, alternariol monomethyl ether (AME); 3, altenuene (ALT); 4, tenuazonic acid (TeA); 5, tentoxin (TEN); 6, altertoxin-I (ATX-I); 7, alternariol-3-sulfate (AOH-3-S); 8, alternariol monomethyl ether-3-sulfate (AME-3-S); 9, alternariol-3-glucoside (AOH-3-G); 10, alternariol monomethyl ether-3-glucoside (AME-3-G).

Welke et al., 2009). However, to date no information is available on the fate of *Alternaria* toxins during apple concentrate production. A recent study in our group demonstrated that apples destined for industry were contaminated with the *Alternaria* species mainly causing mouldy core, and that secondary toxic metabolites of this genus were present in the fruit (Pavicich et al., 2020). It is therefore likely that contaminated raw material is incorporated into the process line. Therefore, the objective of this study was to evaluate the effect of the apple concentrate process on the natural contamination levels of 6 key *Alternaria* mycotoxins, namely AOH, AME, ALT, TeA, TEN, and altertoxin-I (ATX-I), and two modified forms of AOH, alternariol 3-sulfate (AOH-3-S) and alternariol 3-glucoside (AOH-3-G) and two of AME, alternariol monomethyl ether 3-sulfate (AME-3-S) and alternariol monomethyl ether 3-glucoside (AME-3-G) (Fig. 1).

2. Materials and methods

2.1. Standards and reagents

AOH, AME (1 mg standard each), ATX-I and ALT (0.1 mg standard each) were obtained from Fermentek (Jerusalem, Israel) and dissolved in 1 ml of methanol (MeOH). Certified reference standards of TeA and TEN (101.3 and 100.5 mg respectively, dried down) were obtained from Romer Laboratories Diagnostic GmbH (Tulln, Austria) and dissolved in 1 ml of acetonitrile (AcN). Reference standards of conjugated *Alternaria* toxins (AOH3-S, AOH-3-G, AME-3-S, AME-3-G) were synthesized as previously described (Mikula et al., 2013) and stock solutions were prepared at a concentration of 10 μg/ml in MeOH. The internal standard urolithin A (UR-A) (5 mg) was purchased from Sigma-Aldrich (Bornem, Belgium) and dissolved in 5 ml of dimethyl sulfoxide (DMSO). Internal standard tenuazonic acid ^{2}H-13 (1 mg) was bought from Toronto Research Chemical (Toronto, Canada) and dissolved in MeOH. Ultra-pure water was obtained from an Arium® pro system (Sartorius, Goettingen, Germany). ACN (absolute, LC-MS grade) and acetic acid (UPLC/MS) were obtained from BioSolve BV (Valkenswaard, The Netherlands), and ACN (HiPerSolv Chromanorm HPLC grade) was acquired from VWR International (Leuven, Belgium). Sodium chloride (NaCl) was purchased from Merck (Darmstadt, Germany), whereas magnesium sulfate ($MgSO_4$, anhydrous) from Sigma-Aldrich (Bornem, Belgium).

2.2. Samples

A total of 34 samples corresponding to five independent apple concentrate processes from an Argentinian company from the Alto Valle of Río Negro, Patagonia were sampled at different stages and analysed for *Alternaria* mycotoxins. The samples were collected between February and May 2018. The five processes used Red Delicious apples; 3/5 included a clarification step, yielding a clear final product, while 2/5 omitted this step resulting in a cloudy final product. From the three clear processes, two used conventionally grown fruit and one organic apples. Meanwhile, of the two cloudy processes, one was made with conventional and the other with organic fruit. Additionally, a sixth process using Granny Smith apples was sampled for comparison; this used conventionally grown fruit and included the clarification step (clear product). Six stages of concentrate production were sampled, namely grinding (1), turbos (2), decanter muds (3), pre-concentration (4), concentrate (5) and rejection (6); the latter was only applied in the clear concentrate process since the cloudy process does not include this step (Fig. 2). Table 1 provides a description of the different processes and stages sampled.

2.3. Extraction

For each step, blank samples were made mimicking the apple concentrate process at laboratory scale with apple fruits free from fungal spoilage for the construction of matrix-matched calibration (MMC) curves. The extraction procedure was made according to Walravens et al. (2016). Briefly, samples and blanks were homogenized by vortexing for 30 s and an aliquot of 2.000 ± 0.0020 g was weighed in an extraction tube. Five blanks per step were fortified with the studied mycotoxins at concentration levels ranging from 5 to 100 μg/kg and soaked for 15 min. Internal standards UR-A, a dibenzopirone from the transformation of ellegitannins by the gut bacteria (Garcia-Muñoz & Vaillant, 2014), and TeA D-13 were added in concentrations of 10 μg/kg. After 10 s of vortex-mixing, samples were kept in the dark for 15 min. Subsequently, 10 ml of ACN (HPLC grade) were added and the tubes were shaken in an overhead shaker for 30 min. Sample extracts were briefly centrifuged (1 min, 3200 g), and $MgSO_4$ anhydrous salt (2.00 ± 0.05 g) and NaCl (0.50 ± 0.05 g) were added. Afterwards, the tubes were vigorously shaken for 30 s, placed in an overhead shaker for 15 min, and centrifuged (10 min, 3200 g). Six (6.00) ml of the supernatant were transferred to a tube and evaporated to dryness using a

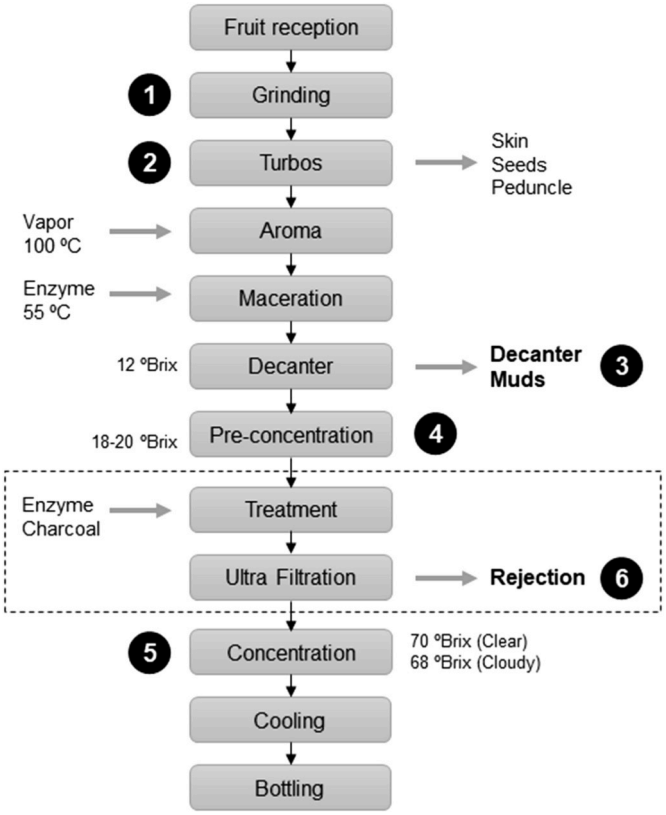

Fig. 2. Flow diagram of the apple concentrate production. The production of the clear apple concentrate includes all steps as shown in the diagram. The cloudy apple concentrate production does not include stages inside the dashed line box. Circles with numbers represent the sampling points.

Table 1
Description of the six apple concentrate processes sampled, including the process number, the type of final product, the type of crop used, the apple variety and the stages sampled in each process. Stages 1: grinding; 2: turbos; 3: decanter muds; 4: pre-concentrate; 5: concentrate; 6: rejection.

Process Number	Product Type	Crop	Apple Variety	Stages sampled
1	Cloudy	Conventional	Red Delicious	1–5
2	Cloudy	Organic	Red Delicious	1–5
3	Clear	Organic	Red Delicious	1–6
4	Clear	Conventional	Red Delicious	1–6
5	Clear	Conventional	Red Delicious	1–6
6	Clear	Conventional	Granny Smith	1–6

Table 2
Concentration of *Alternaria* metabolites in each stage of the six independent apple concentrate processes.

Step		Process Number	Process Type	Crop	Metabolite concentration (μg/kg)									
					AOH	AME	ALT	TeA	TEN	ATX-I	AOH-3-S	AME-3-S	AOH-3-G	AME-3-G
GRINDING	1	1	Cloudy	Conventional	7.6	4.5	n.d.	7.1	8.0	n.d.	n.d.	n.d.	n.d.	n.d.
	1	2	Cloudy	Organic	11.1	8.9	n.d.	30.6	n.d.	n.d.	n.d.	4.8	n.d.	n.d.
	1	3	Clear	Organic	13.6	9.6	n.d.	119.0	18.4	n.d.	n.d.	2.5 [a]	n.d.	n.d.
	1	4	Clear	Conventional	14.8	6.4	n.d.	50.7	17.7	n.d.	n.d.	5.8	n.d.	n.d.
	1	5	Clear	Conventional	11.4	9.1	n.d.	15.9	7.0	n.d.	n.d.	n.d.	n.d.	n.d.
	1	6[b]	Clear	Conventional	n.d.	4.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TURBOS	2	1	Cloudy	Conventional	n.d.	2.6	n.d.	2.8 [a]	n.d.	n.d.	n.d.	3.0 [a]	n.d.	n.d.
	2	2	Cloudy	Organic	2.9 [a]	6.3	n.d.	13.6	n.d.	n.d.	n.d.	3.9 [a]	n.d.	n.d.
	2	3	Clear	Organic	8.0	7.9	n.d.	89.6	1.4 [a]	n.d.	n.d.	3.9 [a]	n.d.	n.d.
	2	4	Clear	Conventional	7.9	5.2	n.d.	70.5	12.1	n.d.	n.d.	4.4 [a]	n.d.	n.d.
	2	5	Clear	Conventional	4.0 [a]	4.6	n.d.	26.9	7.6	n.d.	n.d.	3.6 [a]	n.d.	n.d.
	2	6[b]	Clear	Conventional	n.d.	1.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
DECANTER MUDS	3	1	Cloudy	Conventional	42.1	8.0	n.d.	31.1	n.d.	n.d.	2.4	7.3	4.0	n.d.
	3	2	Cloudy	Organic	19.9	11.6	n.d.	18.6	n.d.	n.d.	n.d.	4.8	2.6	n.d.
	3	3	Clear	Organic	44.9	10.6	n.d.	29.9	7.4	n.d.	n.d.	9.7	3.6	n.d.
	3	4	Clear	Conventional	28.5	6.8	n.d.	41.8	9.2	n.d.	1.4	6.0	n.d.	n.d.
	3	5	Clear	Conventional	29.1	5.9	n.d.	33.1	13.8	n.d.	n.d	4.3 [a]	2.2	n.d.
	3	6[b]	Clear	Conventional	n.d.	2.7	n.d.	n.d.	n.d.	n.d.	n.d.	4.5 [a]	n.d.	n.d.
PRE-CONCENTRATE	4	1	Cloudy	Conventional	6.7	2.5	n.d.	61.9	n.d.	n.d.	2.3	4.8	n.d.	n.d.
	4	2	Cloudy	Organic	1.5 [a]	1.1	n.d.	20.9	3.1[a]	n.d.	1.8	2.4 [a]	n.d.	n.d.
	4	3	Clear	Organic	9.5	3.5	n.d.	103.8	13.3	n.d.	3.2	5.6	n.d.	n.d.
	4	4	Clear	Conventional	3.4 [a]	1.2	n.d.	63.0	8.5	n.d.	2.0	2.3 [a]	n.d.	n.d.
	4	5	Clear	Conventional	n.d.	0.9[a]	n.d.	52.9	8.8	n.d.	1.5	1.7 [a]	n.d.	n.d.
	4	6[b]	Clear	Conventional	n.d.	n.d.	n.d.	1.4[a]	n.d.	n.d.	1.6	2.0 [a]	n.d.	n.d.
CONCENTRATE	5	1	Cloudy	Conventional	46.4	18.5	n.d.	135.8	10.7	n.d.	6.0	10.0	n.d.	n.d.
	5	2	Cloudy	Organic	21.1	20.1	n.d.	102.6	18.1	n.d.	4.4	9.7	n.d.	n.d.
	5	3	Clear	Organic	n.d.	n.d.	n.d.	1.9 [a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	5	4	Clear	Conventional	n.d.	n.d.	n.d.	19.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	5	5	Clear	Conventional	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	5	6[b]	Clear	Conventional	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
REJECTION	6	3	Clear	Organic	n.d.	n.d.	n.d.	30.3	8.3	n.d.	n.d.	n.d.	n.d.	n.d.
	6	4	Clear	Conventional	n.d.	n.d.	n.d.	37.2	14.8	n.d.	n.d.	n.d.	n.d.	n.d.
	6	5	Clear	Conventional	n.d.	n.d.	n.d.	46.2	26.2	n.d.	10.8	n.d.	18.7	n.d.
	6	6[b]	Clear	Conventional	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene, TeA: tenuazonic acid, TEN: tentoxin, ATX-I: altertoxin-I, AOH-3-S: alternariol-3-sulfate, AME-3-S: alternariol monomethyl ether-3-sulfate, AOH-3-G: alternariol-3-glucoside, AME-3-G: alternariol monomethyl ether-3-glucoside.
n.d.: not detected.
[a] Values between LOD and LOQ.
[b] Process using Granny Smith variety apples.

Turbovap LV module (Biotage AB, Uppsala, Sweden) maintained at 40 °C. Finally, the residue was redissolved in 100 μl of injection solvent (ultrapure water/AcN (LCMS grade), 70/30, v/v), vortex-mixed for 30 s, and centrifuged (Ultrafree-MC PVDF centrifugal filter units, 0.22 μm; Merck Millipore, Darmstadt, Germany) for 10 min at 10,000 g prior to analysis.

2.4. Determination and quantification: LC-MS/MS analysis

Determination and quantification of the studied mycotoxins was done on a Waters Acquity UPLC coupled to a XEVO TQ-S mass spectrometer (Waters, Milford, MA, USA). To achieve compound separation, an Acquity UPLC High Strength Silica trifunctional C18 Alkyl phase (HSS T3, 1.8 μm, 2.1 × 100 mm) (Waters, Milford, MA) column was used. The instrument was used in the negative electrospray ionisation (ESI^-) mode. The temperature of the column was 35 °C, and the mobile phases were A: ultra-pure water/acetic acid (AA) (99/1, v/v) and B: ACN/AA (99/1, v/v). The flow rate was 0.4 ml/min and the total run time 7 min following the gradient described by Walravens et al. (2014). The capillary voltage was 30 kV, and nitrogen was applied as spray gas. The source and desolvation temperatures were set at 150 °C and 200 °C, respectively. The argon collision gas pressure was 9×10^{-6} bar, the cone gas flow 50 l/h and the desolvation gas flow 500 l/h. Two selected reaction monitoring (SRM) transitions with a specific dwell-time were optimised for each analyte, in order to increase the sensitivity and the selectivity of the mass spectrometric condition. The validation details of this method are described in detail by Walravens et al. (2014), as well as the limits of detection (LOD) and quantification (LOQ) that are also informed in Supplementary Table 1. For data acquisition and processing, the MassLynx and QuanLynx® version 4.1. software (Micromass, Manchester, UK) were used.

3. Results and discussion

Table 2 shows the concentration of each of the six *Alternaria* toxins and their modified forms by step and process. ALT and ATX-I were not detected in any of the processes studied at any stage, as well as AME-3-G, a modified form of AME. The levels of AOH, AME, TeA and TEN and their changes throughout the stages of the six processes studied are represented in Fig. 3.

3.1. Apple variety

The process using Granny Smith apples differed from those based on Red Delicious apples both in qualitative as quantitative terms of *Alternaria* metabolites detected (Table 2). The batch made with Granny Smith apples was contaminated with low levels of AME and its modified form, AME-3-S, at some stages of the production, although these compounds were not detected in the final product. Low levels of AOH-3-S and non-quantifiable but detectable levels of TeA were also observed, but only in the pre-concentrate step. The other five processes showed higher levels of contamination, and most of the studied metabolites

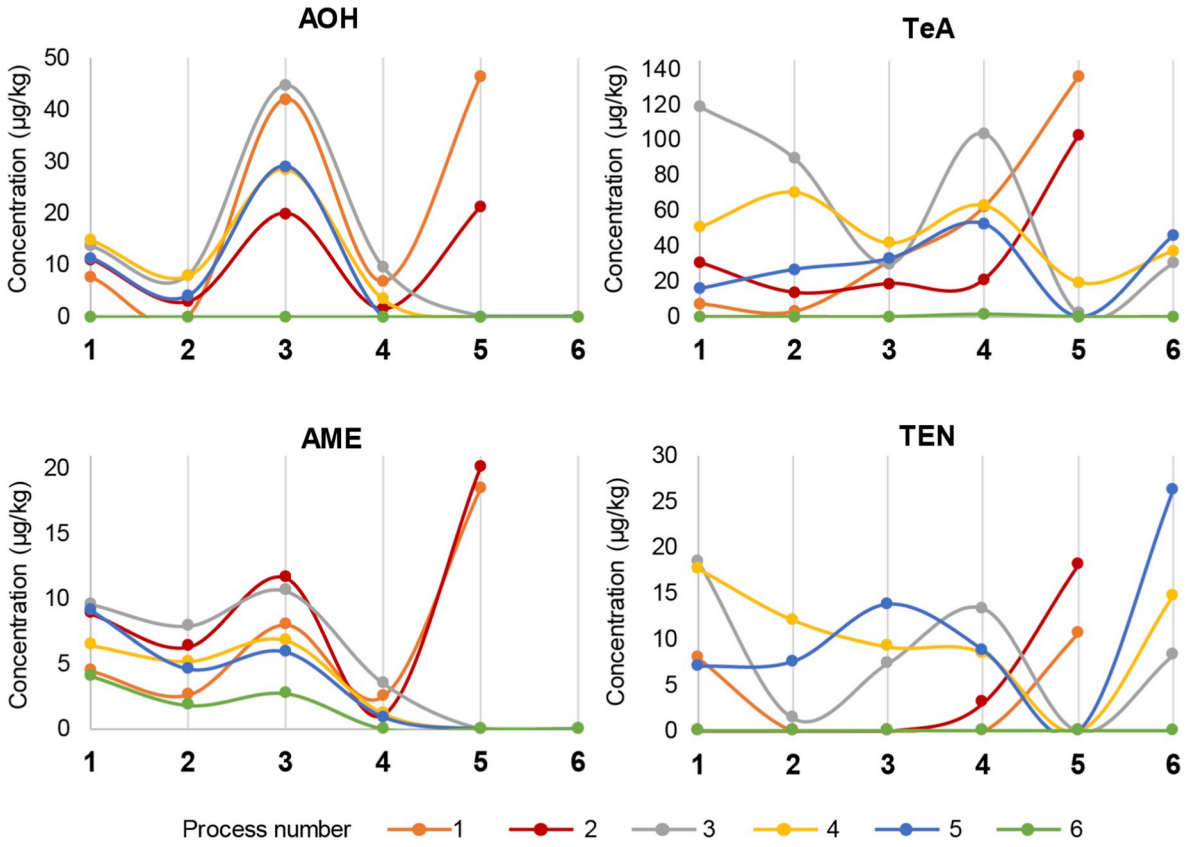

Fig. 3. Concentration of alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA) and tentoxin (TEN) in different stages of six independent apple concentrate processes. Stages 1: grinding; 2: turbos; 3: decanter muds; 4: pre-concentrate; 5: concentrate; 6: rejection (only present in clear processes).

were found in any of the stages sampled. The prevalence of each metabolite, average, median, and range for the five Red Delicious processes, detailed by stage, are listed in Table 3.

Although more samples should be analysed to confirm that Granny Smith apples are less prone to mycotoxin accumulation, a lower susceptibility to *Alternaria* infection has been reported for this variety. In a study in Greece, the frequency of recovery of *Alternaria tenuissima* from Granny Smith apples was lower than from others (Konstantinou, Karaoglanidis, Bardas, Pathology, & Minas, 2011). It was attributed to the fact that *Alternaria* species mainly contaminate the centre of this fruit, causing mouldy core, and this variety of apple does not have an open sinus which consequently affect the postharvest non-contamination by this genus. In the same study, significantly lower levels of patulin were synthetized on Granny Smith apples than on other varieties, showing a correlation with the acidity of this cultivar. The lower incidence of *Alternaria* toxins in this variety is in accordance with Tournas and Uppal Memon (2009), who indicated that intact Granny Smith apples are less susceptible to fungal contamination due to their high acidity.

3.2. Type of field handling

No significant differences were observed between organic and conventionally grown apples with respect to AOH, AME and TEN contamination ($p > 0.1$). Only one of the organic processes used raw material highly contaminated with TeA (process number 3), but the other toxins were in similar levels than those detected in the rest of the raw material sampled. da Cruz Cabral, Delgado, Patriarca, and Rodríguez (2019) showed that the application of fungicides in a synthetic culture media partially reduced the production of TeA by *A. tenuissima*, while it had no impact on the production of the alternariol-derivatives. This could explain the higher levels of TeA found in the organic apples with respect to the other processes and the rest of the metabolites. Another possible explanation is that since stronger fungal competition occurs in the organic grown apples, the biosynthesis of TeA could be used as a virulence factor, favouring competition with other fungal species (Kang et al., 2017).

3.3. The effect of processing steps

For a better understanding of the effect of the different process stages on *Alternaria* toxins, the percentage of variation of the concentration of AOH, AME, TeA, and TEN with respect to the initial contamination (grinding step) was calculated for the five Red Delicious processes. The behaviour of the toxins was similar for all processes involving a clarification step (clear process) and differed from those which omitted this step (cloudy process). The average percentage of variation of these toxins for cloudy and clear processes is shown in Fig. 4.

3.3.1. Step 1. Grinding

Quantifiable levels of AOH, AME, TeA and TEN, were observed in the ground raw material (step 1) from the five Red Delicious processes, except for process 2, in which TEN levels were < LOD. Regarding the modified forms of these mycotoxins, only AME-3-S was present in quantifiable levels in the raw material of three processes.

The natural presence of these toxins in the analysed ground apples indicates that the raw material used in these batches was contaminated with toxigenic species of *Alternaria*. Since most of the fungal contamination is located in the inner centre of the fruit, their presence is not detected by apple concentrate industries when they perform visual inspection of raw material. The *Alternaria* mycotoxin concentration in ground apples may vary due to the quality of the fruit destined to industrialization or the season in which the fruit is processed. The

Table 3
Number of positive samples, average (μg/kg), median (μg/kg), and range (μg/kg) per step of production in five Red Delicious apple concentrate processes.

	AOH	AME	ALT	TeA	TEN	ATX-I	AOH-3-S	AME-3-S	AOH-3-G	AME-3-G
Grinding										
N° of positive samples	5/5	5/5	0/5	5/5	4/5	0/5	0/5	3/5	0/5	0/5
Average (μg/kg)	11.7	7.7	–	44.7	12.8	–	–	4.4 [a]	–	–
Median (μg/kg)	11.4	8.9	–	30.6	12.9	–	–	4.8	–	–
Range (μg/kg)	7.6–14.8	4.5–9.6	< LOD	7.1–119.0	7.0–18.4	< LOD	< LOD	2.5[a]-5.8	< LOD	< LOD
Turbos										
N° of positive samples	4/5	5/5	0/5	5/5	3/5	0/5	0/5	5/5	0/5	0/5
Average (μg/kg)	5.7	5.3	–	33.9	7.0	–	–	3.8	–	–
Median (μg/kg)	6.0	5.2	–	23.3	9.2	–	–	3.9	–	–
Range (μg/kg)	2.9[a]-8.00	2.6–7.9	< LOD	2.8[a]-89.6	1.4[a]-12.1	< LOD	< LOD	3.0[a]-4.4[a]	< LOD	< LOD
Decanter muds										
N° of positive samples	5/5	5/5	0/5	5/5	3/5	0/5	2/5	5/5	4/5	0/5
Average (μg/kg)	32.9	8.6	–	30.9	10.1	–	1.9	6.4	3.1	–
Median (μg/kg)	29.1	8.0	–	31.1	9.2	–	1.9	6.0	3.1	–
Range (μg/kg)	19.9–44.9	5.9–11.6	< LOD	18.6–41.8	7.4–13.8	< LOD	1.4–2.4	4.3[a]-9.7	2.2–4.0	< LOD
Pre-concentration										
N° of positive samples	4/5	5/5	0/5	5/5	4/5	0/5	5/5	5/5	0/5	0/5
Average (μg/kg)	5.3	1.8	–	60.5	8.4	–	2.2	3.4 [a]	–	–
Median (μg/kg)	5.1	1.2	–	61.9	8.7	–	2.0	2.4 [a]	–	–
Range (μg/kg)	1.5[a]-9.5	0.9[a]-3.5	< LOD	20.9–103.8	3.1[a]-13.3	< LOD	1.5–3.2	1.7[a]-5.6	< LOD	< LOD
Clear Concentrate										
N° of positive samples	0/3	0/3	0/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3
Average (μg/kg)	–	–	–	10.7	–	–	–	–	–	–
Median (μg/kg)	–	–	–	10.7	–	–	–	–	–	–
Range (μg/kg)	< LOD	< LOD	< LOD	1.9[a]-19.5	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Cloudy Concentrate										
N° of positive samples	2/2	2/2	0/2	2/2	2/2	0/2	2/2	2/2	0/2	0/2
Average (μg/kg)	33.8	19.3	–	119.2	14.4	–	5.2	9.9	–	–
Median (μg/kg)	33.8	19.3	–	119.2	14.4	–	5.2	9.9	–	–
Range (μg/kg)	21.1–46.4	18.5–20.1	< LOD	102.6–135.8	10.7–18.1	< LOD	4.4–6.0	9.7–10.0	< LOD	< LOD
Rejection										
N° of positive samples	0/3	0/3	0/3	3/3	3/3	0/3	1/3	0/3	1/3	0/3
Average (μg/kg)	–	–	–	37.9	16.4	–	10.8	–	18.7	–
Median (μg/kg)	–	–	–	37.2	14.8	–	10.8	–	18.7	–
Range (μg/kg)	< LOD	< LOD	< LOD	30.3–46.2	8.3–26.1	< LOD	< LOD-10.8	< LOD	< LOD-18.7	< LOD

AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene, TeA: tenuazonic acid, TEN: tentoxin, ATX-I: altertoxin-I, AOH-3-S: alternariol-3-sulfate, AME-3-S: alternariol monomethyl ether-3-sulfate, AOH-3-G: alternariol-3-glucoside, AME-3-G: alternariol monomethyl ether-3-glucoside.
Average, median and range do not include negative samples.
[a] Values between LOD and LOQ.

grinding step does not reduce the mycotoxins concentrations, however, grinding as milling step could redistribute the mycotoxin concentration.

The finding of AME-3-S in ground raw material could mean this modified form of AME is present in the fruit itself as a phase-II metabolite. Recent studies reported the presence of this metabolite in tomato products as well as in wheat flour (Puntscher et al., 2019) and contaminated apple fruit stored professionally for 6 months (Puntscher, Marko, & Warth, 2020). Consistently, Walravens et al. (2016) observed this metabolite in 50%, 32% and 78% of tomato juice, sauce and concentrate, respectively. Its presence should be considered in surveys of incidence of *Alternaria* mycotoxins in apple or apple-by-products since it might contribute to the total ingestion of its parent form.

3.3.2. Step 2. Turbos

After grinding the complete fruit, the resulting paste is centrifuged in a turbo, where peels, seeds and other solid parts of the fruit are eliminated from the flow line. The concentration of the neutral parent toxins (AOH, AME, TEN) decreased in the flow line after solids were eliminated. On the contrary, the acidic toxin TeA, with higher affinity for the aqueous phase, showed a slight average increment (27%) in the clear processes after the solid removal stage. Presumably, this step did not indicate a mycotoxin degradation, but redistributed the mycotoxin content.

The use of the waste, generated at this stage, from fruit processing industries for compost, cookies and other by-products has been suggested (Maldonado, Agüero, Iturmendi, & Buglione, 2019; Quiles, Campbell, Struck, Rohm, & Hernando, 2018; Rocha Parra, Sahagún, Ribotta, Ferrero, & Gómez, 2019). However, since the concentration of neutral toxins (AOH, AME, TEN) decreased in the flow line after eliminating solids such as skin, seeds, peduncle, it is likely that they are concentrated in toxins. Thus, this waste should be analysed for their presence prior to its use for food or feed.

3.3.3. Step 3. Decanter muds

After the elimination of the solid parts, the product from the turbos is treated with water vapor at 100 °C for pasteurization, enzyme inactivation, protein denaturalization and starch gelatinization. In this part of the process, the aroma is extracted. The resulting paste is subjected to an enzymatic treatment at 55 °C and a maceration occurs to maximize the yield of the process. After these thermal treatments, a separation takes place and decanter muds are obtained as waste (step 3).

The decanter muds showed high contamination with AOH. The concentration detected in this waste was in average 55% and 159% higher than the original contamination for the cloudy and clear processes, respectively. AME and TeA concentrations only were higher than those in the raw material in cloudy processes; AME in 55% and TeA in 149%, while TEN was in lower amounts than in the ground apples.

The thermal treatment did not seem to have a degradation effect on the analysed mycotoxins since they were detected in further steps in the process line. Similar results are presented by Estiarte, Crespo-Sempere, Marín, Ramos, and Worobo (2018) where they concluded that AOH and AME are relatively stable in the food process chain, the latter showing

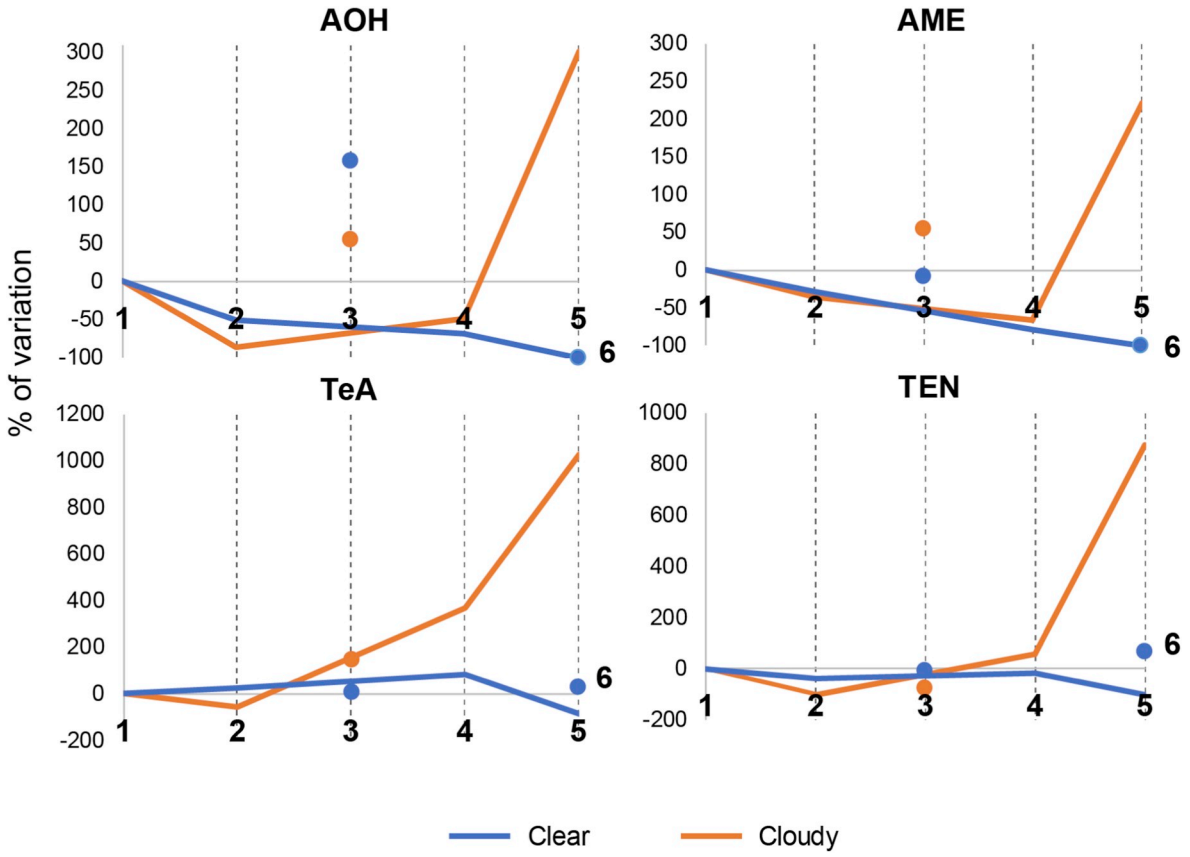

Fig. 4. Percentage of variation of alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA) and tentoxin (TEN) throughout the apple concentrate production process with respect to the initial contamination. 1: grinding; 2: turbos; 3: decanter muds; 4: pre-concentrate; 5: concentrate; 6: rejection (only present in clear process). Circles represent out-of-process steps.

the highest stability. Scott and Kanhere (2001) also reported that AOH and AME were stable at 80 °C up to 20 min and 2 weeks at room temperature.

3.3.4. Step 4. Pre-concentrate

Following the separation of decanter muds, a pre-concentration to 18 to 20 °Brix occurs (step 4). Both AOH and AME suffered further reductions in this step, except for AOH in the cloudy processes, where its concentration was slightly higher than in the previous step but still 49% lower than that from the raw material (Fig. 4). The concentration of TEN continued reducing in the clear process, but increased in the cloudy one, reaching an average level of 55% higher than the initial value, although there were no significant differences between the processes. TeA, on the other hand, increased in both types of process, although the increment was higher in the cloudy one, with average levels reaching 370% the initial contamination.

Mycotoxin reduction has been previously observed in the flow line of apple concentrate after the enzymatic treatment; Welke et al. (2009) reported that patulin was reduced in 28% due to the pectinase treatment. Nevertheless, the observed decline in the concentration of the alternariol-derivatives can be attributed to their adsorption in the decanter muds, causing the decanter juice, which has less free water than the initial phases of this process, resulting in a minor average concentration of these toxins. This effect was not observed for TEN, which agrees with its low adsorption in the muds. On the other hand, the average concentration of TeA in the decanter juice increased with respect to previous stages. Given the acidic nature of the molecule, this toxin represents a higher water solubility, thus its retention in the solids is expected to be lower. Even in cloudy processes, in which the average retention of TeA in the decanter muds was higher, the concentration of the toxin rose after the enzymatic treatment. Our results showed that while for the alternariol-derivatives the retention in the solids was proportional to the initial contamination, for the TeA the decanter muds seem to saturate with 30–40 μg/kg of this fungal metabolite. Consequently, if the raw material shows low contamination with this toxin, a big proportion is transferred to the flow line in the muds. On the other hand, with high initial contamination, the muds get saturated and this mycotoxin remains in the flow line causing a concentration in step 4 of the process.

3.3.5. Step 5. Concentrate

After the pre-concentration step, the cloudy process continues with further concentration by water evaporation, while the clear one includes a clarification treatment with enzymes and activated charcoal. Then, this product is ultra-filtrated to eliminate fine particles, generating a retentate as a waste (step 6, rejection). The final clear product is obtained by concentration to 68–72 °Brix.

The biggest difference in mycotoxin concentration between both type of processes was observed in this final step. For the clear processes, after enzymatic treatment and ultra-filtration (clarification step) all the mycotoxins analysed underwent a significant reduction to non-detectable levels. Only TeA remained in the final product of 2 out of the 3 clear processes, but, in average, it was reduced with 86% with respect to the original contamination of the fruit.

On the contrary, cloudy processes showed much higher final levels of the four mycotoxins. The concentration in the final product increased 301% for AOH and 221% for AME, with respect to the raw material, TEN was concentrated 872% and TeA 1024%.

This cloudy final product can either be clarified once the market demand grows again, in which case some of the toxins would diminish to non-quantifiable levels, or can be destined to by-products other than juice, in which case the final destination of the toxins should be further

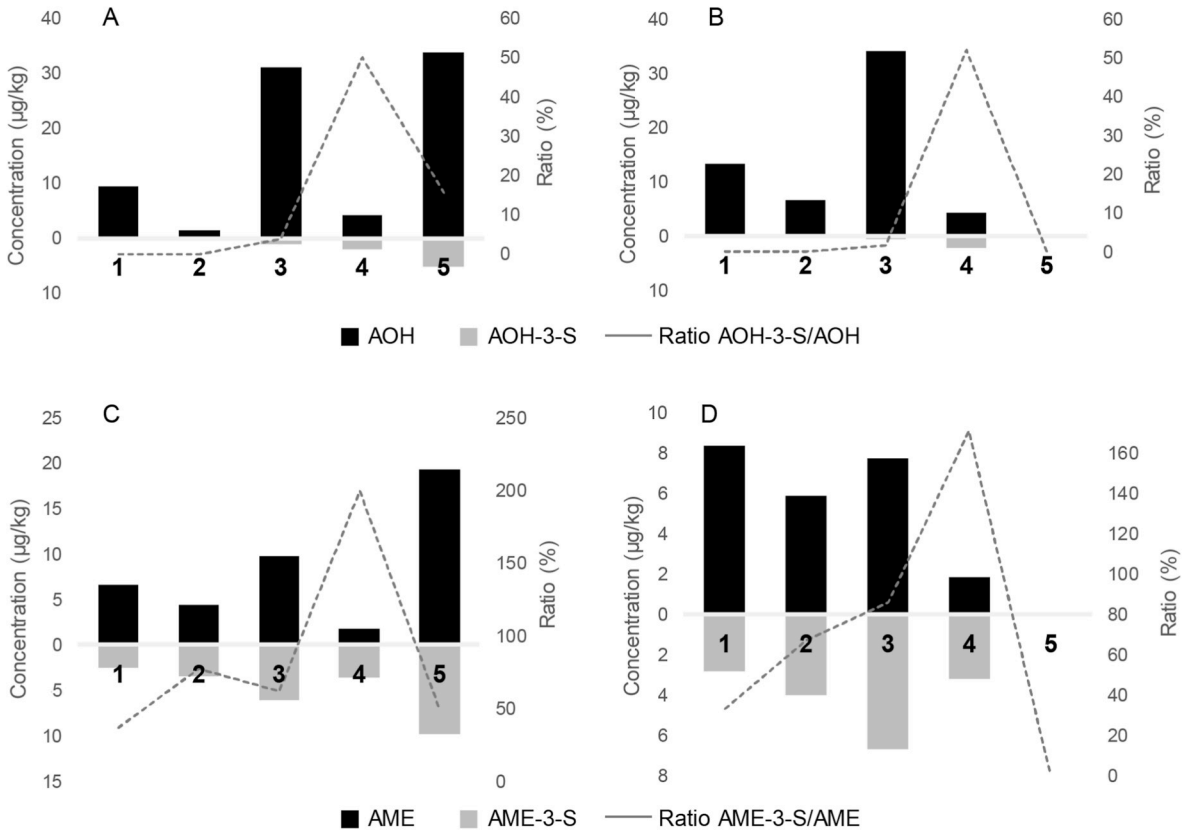

Fig. 5. Average concentration (bars) and ratio modified/native form mycotoxin (dash line) in each step of the five Red Delicious apple concentrate processes for alternariol (AOH) *vs.* Alternariol 3-sulfate (AOH-3-S) in A) cloudy and B) clear processes, and alternariol monomethyl ether (AME) *vs.* alternariol monomethyl ether-3-glucoside (AME-3-S) in C) cloudy and D) clear processes.

investigated in each of them. TeA should be of special concern since it was quantified in both cloudy and clarified products, implying that the clarification is not able to reduce this toxin concentration to non-detectable levels, and moreover it is considered the most acutely toxic *Alternaria* mycotoxin (Asam & Rychlik, 2013). Based on the recent report of the presence of AOH-9-G (alternariol-9-glucoside), altertoxin-II and alterperylenol in apples contaminated with *Alternaria* fruit spot, the screening of these *Alternaria* toxins should also be evaluated in apple by-products (Puntscher et al., 2020).

3.3.6. Step 6. Rejection

The analysed retentates generated in clear processes were contaminated with TEN and TeA, besides the modified forms of AOH, AOH-3-G and AOH-3-S. The levels of TeA were similar to those present in the raw material, but TEN was found in higher amounts. Similarly, Kadakal, Sebahattin, and Poyrazoglu (2002) reported that a percentage of patulin was adsorbed in the charcoal, in the clarification step of the apple concentrate process.

This fact should be taken into account, since many uses have been proposed for this waste for being a source of free sugars, protein, polysaccharides, amino acids, fatty acids, sterols, triacylglycerides, and procyanidins (Cruz et al., 2018). Nevertheless, the presence of *Alternaria* toxins should be determined if it is intended for food or animal feed.

3.4. Modified toxins

The average concentration of AOH-3-S and AME-3-S and their respective parent forms, as well as the ratios modified/free form throughout the cloudy and clear processes is represented in Fig. 5. AOH-3-S was not detected in the early stages of the process, while AME-3-S was found in the raw material but in lower levels than its parent form. Both metabolites increased their concentration throughout the process with respect to the initial contamination. The ratio of modified to parent form showed an increasing transformation of both alternariol-derivatives into their sulfate conjugates, which reached its peak in the pre-concentrate. After this step, a difference was observed between both type of processes; while the ratio of modified to parent form was reduced in the cloudy process for both metabolites, the sulfate conjugates were reduced to non-detectable levels in the clear one. This could be due to the enzymatic treatment applied in the latter, which might be responsible for the reversion of conjugates to free forms. Another possibility is that the modified forms can be more easily adsorbed in the solids that are removed after this step, thus being eliminated from the final clear product. Nevertheless, both metabolites were concentrated to quantifiable levels in the final cloudy product. It is noteworthy that the process using the Granny Smith variety also showed contamination with the sulfate conjugates in the pre-concentrate step, and AME-3-S was detected in the decanter muds obtained from this process (Table 2).

With respect to the alternariol glucosides, even though AOH-3-G was not detected in the raw material or consistently along the process, it was present in quantifiable levels in the decanter muds and in one of the retentates. Contrarily, AME-3-G was not detected in any step of the process.

To our knowledge, this is the first report of these modified *Alternaria* toxins in apple by-products. Both AOH-3-S and AME-3-S have been reported in tomato products before (Puntscher, Cobankovic, et al., 2019b; Walravens et al., 2016). No reports of quantifiable levels for AOH-3-G in foods are available, although AOH-9-G has been detected in an organic tomato sauce from Italy (Puntscher et al., 2018). Considering that the presence of modified alternariol-derivatives has been

confirmed in apple concentrate, their concentration should be taken into account in the quantification of alternariol-derivatives in apple juices or other by-products made by the dilution of cloudy concentrates. Moreover, and due to the presence of multiple *Alternaria* metabolites in the apple concentrate, the combined toxic effects of them should be evaluated to perform an adequate risk assessment.

4. Conclusions

To our knowledge, this is the first report stating the fate of free and modified forms of *Alternaria* toxins in the apple concentrate production and the first report of AOH-3-S and AME-3-S in apple by-products. These results indicate that the clarification stage in the apple concentrate process is of crucial importance to significantly reduce *Alternaria* toxins to safe levels in the final products. The major risk could be associated with cloudy apple by-products, especially if those are intended for infant foods. Although *Alternaria* mycotoxins are relatively stable, their contamination levels can be reduced to some extent during apple concentrate processing.

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CRediT authorship contribution statement

María Agustina Pavicich: Investigation, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Marthe De Boevre:** Methodology, Writing - review & editing, Project administration. **Arnau Vidal:** Investigation, Writing - review & editing. **Facundo Iturmendi:** Resources. **Hannes Mikula:** Resources, Writing - review & editing. **Benedikt Warth:** Resources, Writing - review & editing. **Doris Marko:** Data curation. **Sarah De Saeger:** Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **Andrea Patriarca:** Conceptualization, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2020.107388.

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