New UHPLC coupled to mass spectrometry approaches for screening of non-volatile compounds as potential migrants from adhesives used in food packaging materials

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Abstract

The objective of this study was to identify the non-volatile compounds as potential migrants from adhesives used in food packaging. A number of the current acrylic adhesive formulations were extracted and prepared for analysis. The extracts were screened using ultra-high-performance liquid chromatography coupled to a time of flight mass spectrometer detector (UHPLC-TOF-MS). This approach allowed the identification of several components by a combination of exact mass and in-source collision induced dissociation (CID). Due to the lack of freely available information on adhesive formulations further analyses were undertaken using ultra-high-performance liquid chromatography coupled to high definition mass spectrometry (UHPLC-HDMS). Using the MassFragment™ tool to interrogate fragmentation data, a wide series of compounds were identified, demonstrating the usefulness and importance of these tools for difficult problems. Moreover, using several packaging materials containing adhesives, qualitative migration tests were performed with Tenax® as a food simulant. Several non-volatile compounds were identified as well in the Tenax® which emphasizes the importance of this work and demonstrates that even the non-volatile compounds have the potential to migrate into food which is in contact with packaging materials. The main characteristics of the screening study and the results obtained are shown and discussed.

Keywords: Food contact material, adhesive composition, Screening analysis, UHPLC-TOF-MS, UHPLC-HDMS, migration, Tenax, non volatile migrants.

1. Introduction

Adhesives are used worldwide to manufacture food packages and food contact materials since packaging rarely comprise a single component. They are used in packages to form multilayer laminates by combination of several substrates (paper, cardboard, polymers, metal foil, metalized films…), to form the geometric shape of the package and the multilayer structures as well as to form labels to stick on the packages[1].

Like all components of food contact materials, adhesives have to compliant Article 3 of EU Regulation 1935/2004 which means no transfer of substances on/into the food in amounts which could endanger human health. In contrast to plastics that are regulated by positive lists of authorized ingredients, adhesives have not yet a specific regulation [2]. Although adhesives are often applied between two surfaces, they are not considered as a layer of material, even though most of adhesive formulations contain resins and polymers in their formulation. However, they take part of the whole packaging material and can seriously affect the final migration performance. Taking into account this approach, hundreds of raw materials would be out of scope and human health could be under threat.

Modern-day adhesives are often fairly complex formulations of components that perform specialized functions. Very few polymers are used without the addition of some modifying substance. The various components that can constitute an adhesive formulation include: base resin or binder, catalyst or hardeners, accelerators, inhibitors, retarders, solvents, thickners, diluents, extenders, fillers, carriers, plasticizers, flexibilizers, tackifiers, film formers, antioxidants, antihydrolisis, antifungals, soaps, surfactants and wetting agents [3]. This list of components gives an idea of the complexity of the adhesives formulation. However, the real formulations are not known and that is why powerful analytical techniques are required for screening, to identify as much as possible the compounds likely related to the adhesives in a packaging material and finally in migration analysis.

Nowadays, the identification of non-volatile compounds in complex mixtures is a significant analytical challenge. Screening procedures are not very common in analytical developments, probably because of the intrinsic difficulties related to them. To obtain an unbiased dataset, full-spectrum acquisition techniques are required. Single- and triple-quadrupole instrumentation does not provide adequate sensitivity in full-scan mode when applied to samples of this nature. All quadrupole and linear ion-trap instruments normally have higher sensitivity in selected ion, precursor or neutral loss scan modes. However, their selectivity is low because they can only acquire nominal mass spectra, a drawback that also applies to single-quadrupole and triple-quadrupole mass analyzers.

In contrast, the time-of-flight (TOF) mass analyzer provides the selectivity and sensitivity required for efficient and wide-range screening, as it combines high, full-spectral sensitivity with high mass resolution so as to accurately measure the mass of any ionizable components in the sample. Elemental compositions can be confidently proposed with low mass errors. TOF-MS can provide a notable amount of chemical information in a single experiment, so this technique is very attractive for performing non-targeted analyses. It has been used broadly in many areas, such as environmental and food safety analysis [4-19] but it has not been applied to adhesives yet.

Since adhesives are unknown complex mixtures, the most advanced techniques are necessary for screening purposes to elucidate the composition. Synapt HDMS developed by Waters is a technique capable to provide much structural information. This equipment provides an expanded range of fragmentation protocols for structural characterization studies because it provides first- and second-generation product ions from a precursor in one experiment. The first generation of fragments are separated by ion mobility that is a gas-phase electrophoretic technique that gives rapid separations of gas-phase ions on the milliseconds timescale. The theory and applications of ion mobility have been presented in many reviews [20-22]. The ions are accelerated through a drift tube, under the influence of a weak electric field gradient and in the presence of a neutral buffer gas (typically nitrogen, helium, or air), resulting in the separation of ions on the basis of different mobilities. This technique has been mainly developed to elucidate protein structure [23-26] and could be very useful in many other applications such as the identification of non-target compounds in complex samples, which is one of the objectives of this paper.

This work deals with the study carried out by Ultra-high-performance liquid chromatography-time of flight-mass spectrometry (UHPLC-ESI-TOF-MS) and UHPLC-ESI-high definition mass spectrometry (HDMS) to identify the non-volatile compounds extracted from four different acrylic adhesives. Once the likely migrants were identified, migration tests were performed and the migrants identified again in the solid food simulant used for the test.The comparison of both MS approaches for the identification of compounds as well as the results obtained are shown and discussed.

2. Experimental

2.1. Materials and reagents

Acetonitrile, water and methanol were from J.T. Baker (Deventer, The Netherlands). Three different water based acrylic adhesives (adhesive 1, 2 and 3) were taken for the analysis. The origin and main characteristics are confidential and cannot be explained here. These adhesives are commonly used to make laminates consisting of two or more substrates such as plastics, paper, cardboard or aluminium, glued with the adhesive Several laminates commonly used in food packaging were made in the laboratory according to the directions of the adhesive companies. The laminates were manufactured as follows: a) a label prepared with adhesive 1 applied on aluminum and polyethylene; b) a laminate made with polypropylene and polyethylene using adhesive 2 and c) a laminate made with polypropylene and paper using adhesive 3. The standard 4-nonyl phenol ethoxilated (4-NPEO) (technical) 99.0% was from Dr Ehrenstorfer GmbH (Germany).

2.2. UHPLC separation

Chromatography was carried out in an Acquity system using an Acquity UPLC BEH C18 column of 1.7 μm particle size (2.1 x 100 mm), both from Waters (Milford, MA, USA).

UPLC conditions were optimized in order to achieve a good chromatographic resolution and sensitivity. Several parameters were tested such as the mobile phase composition. Different mobile phases and different pHs ranging between acidic to basic conditions were tested. Finally, acetonitrile and water were used as mobile phases. Chromatography was carried out at 0.4 mL/min column flow and the column temperature was 30ºC. The gradient used here was 5-95% acetonitrile (0-8.5 min) and the volume of sample injected was 2 μl.

2.3. Mass spectrometry detection

126 Firstly, it was used a time of flight mass spectrometer (TOF) LCT Premier XE from
127 Waters (Milford, MA, USA) with an electrospray probe in positive mode (ESI+) and in
128 positive mode (ESI-) in W mode. Cone voltages were optimized between 20 and 50 V,
129 finally 30 V was selected for the screening because more peaks were detected. The tune
130 MS parameters, desolvation gas temperature was 450ºC and desolvation gas flow was
131 900 L h^{-1}. The MS range adquired was 100-1000 Da.

132 Secondly, a high definition mass spectrometer (HDMS) Waters SYNAPT HDMS™
133 (Waters, Milford MA, USA) was used with an electrospray probe in positive mode
134 (ESI+) and in positive mode (ESI-) in W mode. Tune parameters were the same used
135 for TOF spectrometer. Masses fragmented with this equipment were selected from the
136 spectra of the peaks obtained by UHPLC-TOF-MS. Collision energies used to fragment
137 the masses are shown in table 1.

138 MassLynx v 4.1 software (Waters, Milford MA, USA) was used to analyse the samples
139 and ChromaLynx (Waters, Milford MA, USA) was used to deconvolve the spectra.

140 **2.4. Dilution of the adhesive samples**

141 1 gram of pure adhesive sample (non cured) was extracted with 10 grams of acetonitrile.
142 When acetonitrile was added the acrylic polymer precipitated. The solution containing
143 the additives from the adhesive was filtered using a 0.22 µm pore filter. Then, samples
144 were diluted 1/100 with acetonitrile. A blank of acetonitrile and these solutions were
145 analyzed by UHPLC-TOF-MS and UHPLC-HDMS in order to identify the compounds
146 present in the adhesive.

147 **2.5. Qualitative migration tests**

148 1.7 cm^2 of each laminate were placed in a 7 ml vial and covered with a monolayer of
149 Tenax (approximately 20 mg). The migration from both sides of each laminate was
150 measured with Tenax in independent tests. For adhesive 1 Tenax was applied only on
151 the PE side because aluminum was considered inert and high barrier to migration. In the
152 case of adhesives 2 and 3, both sides of the laminate were studied, so that two assays
153 were done for each laminate (PP and PE for adhesive 2 and PP and paper for adhesive
154 3). Therefore, a total of 5 different migration experiments were done. Vials were closed

and placed in the oven at 70ºC for 2 hours. Then the laminates were removed from the vial and Tenax was extracted with 1.5 ml of methanol in an ultrasonic bath for 10 minutes. The extract was concentrated under nitrogen stream to 0.5 ml gravimetrically controlled and finally this extract was analyzed by UHPLC-TOF-MS with the same method used for the screening and identification of the compounds.

Once the identification was finished, a standard of 10 µg/g of NPEO in acetonitrile was prepared and analyzed.

3. Results and discussion

3.1. UHPLC-ESI-TOF-MS

An AQUITY UPLC BEH C18 column was selected for this work as it was considered as a universal column of choice for most UHPLC separations and very appropriate for screening purposes. Chromatographic separation was optimized until resolution of peaks didn´t improve significantly. Acidic and basic mobile phases were used in order to identify more compounds but new peaks were not found, so the work was continued with the optimized conditions explained in section 2.2.

Figure 1 shows that there was not a common profile of compounds for the pure adhesives, even though all the adhesives theoretically belong to the acrylic class. The chromatograms corresponding to the extracts of adhesive 1 and adhesive 2 showed a series of peaks (1-19 for adhesive 1 and 1-11 for adhesive 2) that could be polymers. Moreover, some wide peaks are observed in the figure. These peaks could correspond to more than one compound but it was almost impossible to separate them using this column. UHPLC offers much narrower peaks and better separation of compounds in complex mixtures than standard HPLC. However even after optimizing both the mobile phase and the chromatographic conditions, the samples under study were so complex that resolution of individual compounds was complicated. The use of high resolution MS maintained the possibility to identify individual compounds on the spectra in the absence of perfect chromatographic separation. In this case, TOF-MS was essential for gaining the necessary information about the compounds of interest. ESI probe was selected because the compounds were supposed to be polar compounds as they were in the water base acrylic adhesives that was considered a polar medium. Both positive and

185 negative modes were used but no compounds were identified with ESI negative mode.
186 The MS range acquired was 100-1000 Da because it was considered that it is unlikely
187 that masses bigger than 1000 Da would migrate from the adhesive to the food.

188 Each adhesive was compared to the blank with the help of ChromaLynx software. This
189 software deconvolved the spectra and provided a list with the retention times, accurate
190 mass and abundance of each mass detected by the equipment. Then the comparison of
191 the data to the blank in each case provided the list of masses that were only present in
192 the adhesives. Once the list of accurate masses of each adhesive were known, different
193 possibilities for elemental composition were established considering that the molecules
194 were formed with the most common element (C,H,O,N,Cl,S,Br,Na). Na, which was
195 present in the mobile phase, was also considered as often the molecules form adducts
196 with it.

197 Once different options for elemental composition of each accurate mass were known it
198 was necessary to know the typical composition of an acrylic adhesive in order to
199 elucidate the possible compounds that could be present. Acrylic adhesive formulation
200 may include the following additives: monomer, catalyzers, plasticizers, surfactants,
201 fillers, stickers, adhesion promotors and toughеners among others. The most common
202 monomer is methyl methacrylate and the most common catalyst is a tertiary amine.
203 Plasticizers include the less volatile phthalates, adipates, sebacates, phosphates, and
204 other ester types. There are many types of surfactants that can be ionic and non-ionic.
205 Common fillers are wood flour, silica, alumina, titanium oxide, metal powders, glass
206 fibers…Toughеners can be chlorosulphonated polyethylene, acrylonitrile elastomeric
207 copolymers and polyurethane elastomers. This description of additives used in the
208 formulation of adhesives is very general and brief but it is worth to point out that there
209 are thousands of additives that can be used in adhesive systems and new formulations
210 bring out constantly in the market. The choice depends on the composition of the
211 adhesive, on how it will be used, the substrates on which they will be applied to, the
212 cost, and the properties that need to be obtained[3].

213 Since the composition was unknown, the identification of single compounds was a
214 difficult task. Nevertheless, for the masses that were supposed to be polymers, the mass
215 difference between each polymer masses was calculated and the comparison of

elemental composition for each polymer was done. This way the identification of some polymers present in the adhesive was achieved (table 2). For instance, in adhesive 1 from mass 173.0790 Da (peak 1) to mass 965.5523 Da (peak 19) it was found that the elemental composition ranged from $C_6H_{14}O_4Na$ to $C_{42}H_{86}O_{22}Na$, with a difference between the measured accurate mass of each polymer of the series of 44.0262 Da corresponding to one unit of C_2H_4O. Then, a bibliographic search was done among the possible additives in the adhesive that could be of polymeric nature with this elemental composition and with this mass difference between each polymer of the series. This series of polymers corresponds to Polyethylene glycol (PEG). PEG with a molecular weight of about 200-800 Da is usually employed as plasticizer in pressure sensitive adhesives, like the acrylic adhesives that were studied in this work [27].

This procedure was applied to all the polymers detected in the adhesive and most of them were identified. In adhesive 2, the elemental composition of peaks from 1 to 11 ranged from $C_{12}H_{24}O_4Na$ to $C_{42}H_{84}O_{14}$ with a measured mass difference of 58.0419 corresponding to one unit of C_3H_6O between each polymer of the series. This accurate mass difference corresponds to propylene oxide that is the repeating unit in poly (propylene oxide). This polymer is used in the production of polyurethanes[28] that could be used as toughеners in acrylic adhesives, as a plasticizer in pressure-sensitive adhesives [27] and also as a non-ionic polymerisable surfactant in combination with ethylene oxide[29].

Octyl phenol ethoxylate (OPEO) and nonylphenol ethoxylate (NPEO) that are non-ionic surfactants [30] were among them but in this case, each polymer could not be separated by liquid chromatography using this method. They were identified through their spectra (Figure 2). This figure shows NPEO spectrum, where the typical shape of a polymer spectrum is observed. The study of the difference in the accurate masses and the knowledge of adhesives that could contain surfactants like these, drove to the identification of NPEO. This compound, used as nonionic surfactant, is suspected to have endocrine disrupting properties and cause harmful effects, including feminization and carcinogenesis on various organisms [31]. As a result, the migration study of this compound from the packaging that contain the adhesive to the food is necessary according to the European Commission [32].

3.2. UHPLC-ESI-HDMS

As has been above explained, the identification of single compounds was a difficult task using UHPLC-ESI-TOF-MS, since adhesive composition is unknown and it is difficult to assure the identity of a compound with only the information of accurate mass. For this reason, UHPLC-ESI-HDMS was used. This analytical technique facilitates acquisition of accurate mass, selected ion fragmentation and matrix interference reduction using ion mobility. It provides the first and second generation product ions from a precursor in one experiment, so maximizes the information content from each sample. It uses a quadrupole to select the masses and then three T-Wave™ ion guides trap ions, separate them by their ion mobility, and transfer them to the orthogonal acceleration time-of-flight analyzer for high-resolution mass analysis. The trap and transfer regions can also be used for fragmentation analyses. Firstly, the selected precursor ions are fragmented in the trap region, these fragments are separated by ion mobility spectrometry and then, a second generation of fragments are produced in the transfer region. Finally, first and second generation of ions are time aligned. Data interpretation is facilitated with the help of DriftScope™ Mobility Environment Software (v2.0) (Waters, Milford MA, USA).

In this work, UHPLC and MS conditions optimized for UHPLC-TOF-MS were applied. Then, optimization of the first fragmentation was done. The trap region allowed working with collision energy ramps, optimum values are shown in table 1. Once first fragmentation was optimized, optimization was done for the second fragmentation in the transfer region; collision energies are shown in table 1.

Once the samples were acquired, many data were obtained so it was a very tedious task to interpret the spectra. Therefore, MassFragment was used to help in fragment ion assignment. This is a MassLynx tool that works in combination with online chemical databases and consists of comparing the fragmented accurate masses with the fragments expected in a molecule proposed as a candidate. Measured accurate mass of each compound were searched in a chemical database [www.chemspider.com] and different candidates for each mass were found. Then, using the MassFragment tool, a comparison of the accurate masses of the most probable fragments that each candidate generates and the accurate mass of fragments obtained experimentally coming from each mass was

done. Finally, the candidate that gave the same accurate mass fragments than the measured experimental mass was assigned. Figure 3 shows an example of one compound identified using this tool. Several candidates were found for accurate mass 327.2505(peak number 6, table 3) in the database and 3 accurate mass fragments (figure 3) were compared with the accurate mass fragments that the candidates most likely generate. Finally, this mass was assigned to 2-[2-(2-undecoxyethoxy)ethoxy]ethanol. Table 3 shows the new compounds identified using these techniques. It was possible to identify 13 new compounds in the adhesive 3 most of them were esters. As was above explained plasticizers could be esters, so these compounds identified with UHPLC-HDMS could be plasticizers added to acrylic adhesive formulation, which would match with the expected composition.

3.3. Qualitative migration studies

Preliminary qualitative migration tests were carried out using Tenax in contact with the laminates prepared from the adhesives under study and using different materials, as was described in the experimental section. Tenax was selected because some of the laminates were made of paper so liquid simulants cannot be used with it. The methanol extract of Tenax was analyzed by UHPLC-MS-TOF with the same UHPLC conditions used in the identification and the results obtained are listed in Table 4. As can be seen, 26 compounds were detected. For adhesive 1, 6 NPEO polymers were detected; for adhesive 2, 6 poly (propylene oxide) polymers were detected and for adhesive 3, 14 different compounds were detected. The most important finding here was to demonstrate that even the non volatile compounds could migrate from the laminate to Tenax. Although the migration tests are performed nowadays in any food contact material, almost always the effort is focused on the volatile compounds, as their migration kinetics are likely faster and thus a higher concentration values of migrants are expected in the food. However, in this case the real migration confirms that not only the volatiles but also the non volatile compounds can migrate throughout the laminate to the solid food simulant.

307 The comparison of the NPEO spectra (Figure 2) obtained analyzing the pure adhesives
308 extracts and NPEO spectra obtained in the methanol extract of Tenax after the migration
309 test (Figure 4) shows that only the small polymers corresponding to low masses (331 Da
310 to 551 Da) migrated to the Tenax. This fact confirms the general idea that the migration
311 is mainly governed by the molecular mass and that the smaller molecules can easily
312 cross the materials and be dissolved or adsorbed on the food. Among the NPEO
313 polymers, NPEO2 was found. This compound is considered the most persistent and
314 toxic NPEO metabolite together with NPEO1 and NP [33, 34]. The toxic effect of these
315 compounds is associated to their ability to mimic natural estrogens and disrupt the
316 endocrine systems of living organisms.

317 NPEO is not listed in the annexes of the plastics Directive and its amendments [2]
318 therefore the maximum concentration in a food simulant must be 10 µg/Kg (ppb) [32].
319 In order to have an idea of the NPEO concentration in Tenax, a NPEO standard was
320 analyzed. The result of this semiquantitative experiment provided a concentration value
321 in Tenax close to the limit value of 10 ppb. This work emphasizes even more the
322 importance of removing NPEO from adhesive formulations, as if they are present, they
323 migrate and could appear in the food in contact with the laminate.

324

325 **Conclusion**

326 UHPLC-TOF-MS and UHPLC-HDMS have demonstrated a strong potential for
327 screening and identification of adhesive compounds and proved to be one of the most
328 powerful tools for elucidating unknown compounds present in complex samples. The
329 availability of full scan mass spectra, the possibility of fragmentation of each single
330 mass and accurate mass measurements provided qualitative information that could be
331 used to ascertain many compounds that were present in samples. This is extremely
332 important in food contact materials, as the legislation establishes that even the unknown
333 compounds and those non intentionally added to the packaging materials should be
334 identified. In this case, concentrations around 10 ppb could be expected and those
335 compounds providing higher level of concentration in the food should be deeply
336 investigated and removed from the formulation. In the case of adhesives, where slightly

information is known about the formulations, the availability of powerful analytical tools is of paramount importance to ensure the safety in use when being applied to the food packaging materials.

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